frontiers | Research Topics

Development of COVID-19 therapies: Lessons learnt and ongoing efforts, 2nd Edition

Edited by
Bruno Villoutreix, Juan Fernandez-Recio and Claudio N. Cavasotto

Published in
Frontiers in Drug Discovery

ISSN 1664-8714
ISBN 978-2-8325-3757-2
DOI 10.3389/978-2-8325-3757-2

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Development of COVID-19 therapies: Lessons learnt and ongoing efforts, 2nd Edition

Topic editors

Bruno Villoutreix — Institut National de la Santé et de la Recherche Médicale (INSERM), France

Juan Fernandez-Recio — Institute of Vine and Wine Sciences, Spanish National Research Council (CSIC), Spain

Claudio N. Cavasotto — Universidad Austral, Argentina

Citation

Villoutreix, B., Fernandez-Recio, J., Cavasotto, C. N., eds. (2023). *Development of COVID-19 therapies: Lessons learnt and ongoing efforts, 2nd Edition*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3757-2

Publisher's note: In this 2nd edition, the following article has been updated:

Bastolla U, Chambers P, Abia D, Garcia-Bermejo M-L and Fresno M (2022) Is Covid-19 Severity Associated With ACE2 Degradation?. Front. Drug. Discov. 1:789710. doi: 10.3389/fddsv.2021.789710

Table of contents

04 Editorial: Development of COVID-19 therapies: Lessons learnt and ongoing efforts
Bruno O. Villoutreix, Claudio N. Cavasotto and Juan Fernandez-Recio

06 Is Covid-19 Severity Associated With ACE2 Degradation?
Ugo Bastolla, Patrick Chambers, David Abia, Maria-Laura Garcia-Bermejo and Manuel Fresno

19 Review of Clinical Trials of COVID-19 Vaccination Booster in SARS-CoV-2 Variants Era: To Take It or Not To Take It
Michael Zhipeng Yan, Ming Yang and Ching-Lung Lai

37 The Need for Speed and Efficiency: A Brief Review of Small Molecule Antivirals for COVID-19
Ana C. Puhl, Thomas R. Lane, Fabio Urbina and Sean Ekins

55 COVID-19 Therapies: Protease Inhibitions and Novel Degrader Strategies
Michèle Reboud-Ravaux and Chahrazade El Amri

64 Developing Small-Molecule Inhibitors of Protein-Protein Interactions Involved in Viral Entry as Potential Antivirals for COVID-19
Peter Buchwald

80 Advances in Modelling COVID-19 in Animals
Petr Nickl, Miles Joseph Raishbrook, Linn Amanda Syding and Radislav Sedlacek

95 Furin and COVID-19: Structure, Function and Chemoinformatic Analysis of Representative Active Site Inhibitors
Bruno O. Villoutreix, Iker Badiola and Abdel-Majid Khatib

102 Peptide-Based Strategies Against SARS-CoV-2 Attack: An Updated *In Silico* Perspective
G. Moroy and P. Tuffery

114 Overcoming Market Failures in Pandemic Drug Discovery Through Open Science: A Canadian Solution
E. Richard Gold and Aled M. Edwards

119 Nanobodies: COVID-19 and Future Perspectives
Guillermo Valenzuela-Nieto, Zaray Miranda-Chacon, Constanza Salinas-Rebolledo, Ronald Jara, Alexei Cuevas, Anne Berking and Alejandro Rojas-Fernandez

130 The challenges and opportunities for the development of COVID-19 therapeutics and preparing for the next pandemic
Emmanuel O. Ogbadoyi and Ndagi Umar

147 Bismuth subsalicylate as potential treatment for Covid-19 pneumonia: A case series report
Chris Kahlenborn, Walter B. Severs and Khalid Nawab

frontiers | Frontiers in Drug Discovery

TYPE Editorial
PUBLISHED 08 September 2022
DOI 10.3389/fddsv.2022.1019705

OPEN ACCESS

EDITED AND REVIEWED BY
José L Medina-Franco,
National Autonomous University of Mexico, Mexico

*CORRESPONDENCE
Bruno O. Villoutreix,
bruno.villoutreix@inserm.fr

SPECIALTY SECTION
This article was submitted to Neurological Drugs, a section of the journal Frontiers in Drug Discovery

RECEIVED 15 August 2022
ACCEPTED 17 August 2022
PUBLISHED 08 September 2022

CITATION
Villoutreix BO, Cavasotto CN and Fernandez-Recio J (2022), Editorial: Development of COVID-19 therapies: Lessons learnt and ongoing efforts. *Front. Drug. Discov.* 2:1019705. doi: 10.3389/fddsv.2022.1019705

Editorial: Development of COVID-19 therapies: Lessons learnt and ongoing efforts

Bruno O. Villoutreix[1]*, Claudio N. Cavasotto[2,3,4] and Juan Fernandez-Recio[5]

[1]INSERM Unit 1141, Hospital Robert Debré, University of Paris, Paris, France, [2]Computational Drug Design and Biomedical Informatics Laboratory, Instituto de Investigaciones en Medicina Traslacional (IIMT), Universidad Austral-CONICET, Pilar, Argentina, [3]Facultad de Ciencias Biomédicasand Facultad de Ingeniería, Universidad Austral, Pilar, Argentina, [4]Austral Institute for Applied Artificial Intelligence, Universidad Austral, Pilar, Argentina, [5]Institute of Vine and Wine Sciences, Spanish National Research Council (CSIC), Logroño, Spain

KEYWORDS

small molecules, peptides, vaccines, nanobodies, animal model, drug discovery

Editorial on the Research Topic
Development of COVID-19 Therapies: Lessons learnt and ongoing efforts

The COVID-19 pandemic has led to a dramatic loss of human life worldwide, presented an unprecedented challenge to public health, and caused economic and social disruption at a global scale. The reviews and articles reported in this Research Topics are examples of studies dedicated to small chemical compounds, peptides, nanobodies, and vaccines that could interfere with COVID-19 while one review focuses more specifically on research strategies to that could help facing future health crises.

Research about molecular mechanisms at play and development of appropriate animal models got started soon after the pandemic began. Bastolla et al. investigate the importance of ACE2 in reversing inflammation and its functional role in COVID-19 infection. They propose that degradation of ACE2 by SARS-CoV-2 may cause failing the termination of the inflammatory process, which is ultimately related to both severe COVID-19 infection and its many post-infection manifestations, including the multi-inflammatory syndrome of children (MIS-C). This hypothesis brings potential therapeutic perspectives that might alleviate severe complications of COVID-19 by inhibiting the processes that prevent the reversion of the inflammatory state. Nickl et al. discuss the different SARS-CoV-2 animal models that were developed to assist COVID-19 research and facilitate drug discovery. They highlight the importance of such models during the pandemic, how some could help study long-COVID and comment about future directions. The challenges posed by new SARS-CoV-2 variants to existing vaccines, the partial protection and vaccine refusal, among others, are fueling the development of alternative anti-COVID therapies based on small molecules inhibitors (SMI) or small peptides. Puhl et al. analyzed 25 drugs which are either approved, in the

process of approval, or in the pipeline against COVID-19 for which both *in vitro* and *in vivo* data are available. They concluded that those drugs are structurally diverse, spanning a wide chemical space. Their characterization may help in identifying the fingerprints of a successful treatment for COVID-19, and provide insights about how to discover novel antivirals more efficiently in case of future pandemics. Ogbadoyi and Umar review the potential for development of therapeutics against COVID-19 based on different strategies, such as multicomponent and multi-target pan-viral therapies, multivalent or edible vaccines, and the use of natural products and phytopharming, as well as new methodologies like bioinformatics, computational biology and artificial intelligence methods, or nanomedicine. Villoutreix et al. report a mini review on furin, a critical host enzyme involved in several diseases that was shown to cleave and activate the SARS-CoV-2 spike protein. They extracted over 600 small compounds known to act on furin from open databases and analyzed these small molecules. Most of these compounds display structural alerts or have poor ADME-T properties and thus would need to be optimized in order to become drug candidates. Reboud-Ravaux and El Amri discuss the potential therapeutic power of induced degradations of viral proteins by PROTACs and of RNA by RIBOTACs for the treatment of COVID-19. They note that these approaches seem beneficial in the field of oncology or for autoimmune disorders and could definitively be valuable for infectious diseases. Buchwald review drug design strategies towards the identification of SMIs targeting protein-protein interactions (PPIs), such as the SARS-CoV-2 spike protein and the host ACE2, critical to virus attachment and entry into the cells. A summary of the progress in developing such PPI inhibitors is presented. Kahlenborn et al. report an observational study about the possible beneficial use of bismuth subsalicylate to Covid-19 patients suffering from gastric problems. They suggest that the preliminary positive results call for a detailed evaluation of this molecule as an adjunct treatment of Covid-19. Moroy and Tuffery discuss the rational design of peptides to assist the development of vaccines or as possible drugs, for instance to block protein-protein interactions or to act on enzyme active sites. Valenzuela-Nieto et al. review the use of nanobodies for the diagnosis and treatment of Covid-19 infection. Nanobodies are different from conventional monoclonal antibodies and their specific characteristics provide new possibilities for the generation of effective neutralizing antibodies. The authors discuss the current strategies for the production of nanobodies, and their application to help fighting SARS-CoV-2 infection, as well as other viral pathogens. Yan et al. note that vaccine efficacy has been greatly reduced by the advent of variants. This is of concern for the general population and even more so for vulnerable subjects. They comment about the benefits and risks of receiving booster vaccinations. Gold and Edwards present alternatives to traditional drug development models based, primarily, on patents. Having in mind the next pandemic, they state that open science partnerships (OSPs), combining the activity of the academic, philanthropic, and governmental sectors, and private incentives, will be instrumental to share knowledge and develop and evaluated SMIs. An example of this is the Viral Interruption Medicines Initiative, a not-for-profit Canadian OSP.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Acknowledgments

The Topic Editors express their gratefulness to all the contributors for submitting their work to this Research Topic, to the Review Editors, and external Reviewers who participated in the review process, and to the Editorial and Production teams of Frontiers for their valuable assistance through the various stages of the publication process.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Drug Discovery

REVIEW
published: 25 January 2022
doi: 10.3389/fddsv.2021.789710

Is Covid-19 Severity Associated With ACE2 Degradation?

Ugo Bastolla[1]*, *Patrick Chambers*[2], *David Abia*[1], *Maria-Laura Garcia-Bermejo*[3] and *Manuel Fresno*[1]

[1]Centro de Biologia Molecular Severo Ochoa CSIC-UAM Cantoblanco, Madrid, Spain, [2]Department of Pathology, Torrance Memorial Medical Center, Torrance, CA, United States, [3]Instituto Ramón y Cajal de Investigaciones Sanitarias, Madrid, Spain

Covid-19 is particularly mild with children, and its severity escalates with age. Several theories have been proposed to explain these facts. In particular, it was proposed that the lower expression of the viral receptor ACE2 in children protects them from severe Covid-19. However, other works suggested an inverse relationship between ACE2 expression and disease severity. Here we review the seemingly contradicting observations on ACE2 expression at the levels of mRNA, membrane protein and serum protein in humans and rodents and try to reconcile them at the light of the Renin-Angiotensin system (RAS) and bradykinin system, which constitute an integrated inflammatory system connected by common peptidases and interacting receptors. We find that ACE2 level is not monotonically related with age but it reaches a maximum at a young age that depends on the cell type and then decreases, consistently with almost all existing data. The increase with age of the protease Tumor necrosis factor alpha (TNF-α) converting enzyme (TACE), also known as ADAM17 (a disintegrin and metalloproteinase 17) that sheds ACE2 from the cell membrane to the serum predicts that the decrease occurs before and is steeper for ACE2 cell protein than for its mRNA. This negative relation between ACE2 level and Covid-19 severity at old age is not paradoxical but it is consistent with a mathematical model that predicts that higher viral receptor does not necessarily favour virus propagation and it can even slow it down. More importantly, the angiotensin-bradykinin system is characterized by a powerful positive feedback loop that enhances inflammation through the Angiotensin and Bradykinin receptors that upregulate ADAM17, which in turn downregulates ACE2 and upregulates TNF-α and the pro-inflammatory receptor of the cytokine interleukin 6 (IL6). Here we propose that ACE2 contributes essentially to reverse this inflammatory state by downregulating the pro-inflammatory peptides of the angiotensin-bradykinin system, and that failure to do this, possibly induced by the degradation of ACE2 by SARS-COV-2, may underlie both severe CoViD-19 infection and its many post-infection manifestations, including the multi-inflammatory syndrome of children (MIS-C). Within this view, lower severity in children despite lower ACE2 expression may be consistent with their higher expression of the alternative angiotensin II receptor ATR2 and in general of the anti-inflammatory arm of the RAS at young age.

Keywords: COVID-19, SARS-CoV-2, ACE2, renin angiotensin system, bradykinin, TACE/ADAM17, angiotensin receptors

OPEN ACCESS

Edited by:
Juan Fernandez-Recio, Spanish National Research Council (CSIC), Spain

Reviewed by:
Rupesh Gautam, Maharishi Markandeshwar University, India
TingTing Hong, University of Utah, United States

***Correspondence:**
Ugo Bastolla
ubastolla@cbm.csic.es

Specialty section:
This article was submitted to In silico Methods and Artificial Intelligence for Drug Discovery, a section of the journal Frontiers in Drug Discovery

Received: *05 October 2021*
Accepted: *30 November 2021*
Published: *25 January 2022*

Citation:
Bastolla U, Chambers P, Abia D, Garcia-Bermejo M-L and Fresno M (2022) Is Covid-19 Severity Associated With ACE2 Degradation? Front. Drug. Discov. 1:789710. doi: 10.3389/fddsv.2021.789710

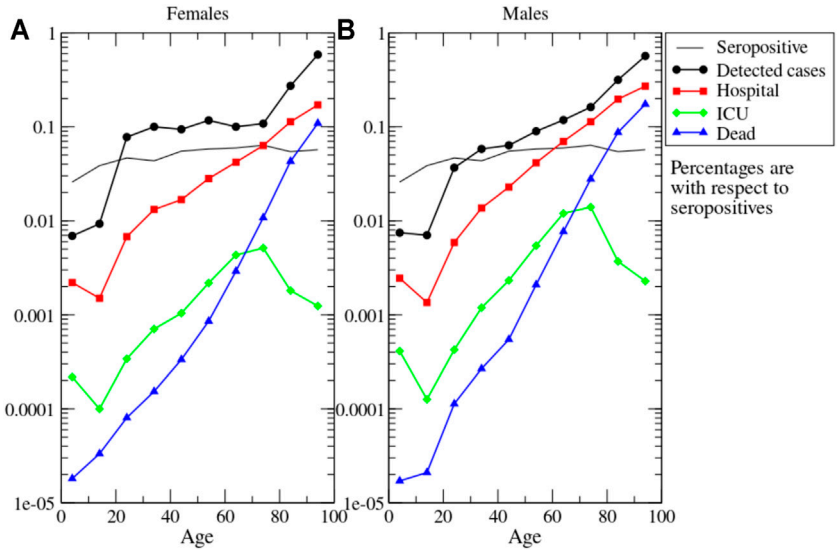


FIGURE 1 | Covid-19 severity increases exponentially with age. Black: Cases detected through positive PCR. Red: PCR-positive patients that required hospitalization. Green: PCR-positive patients in ICU. The drop at high age probably reflects the saturation of the health system during the first wave of the pandemics. Blue: Deceased patients with positive PCR (the real toll estimated with excess deaths is unfortunately much higher). The thin black line reports the fraction of seropositive for each age class, which is used as the normalization for the fractions reported in the figures. Panels **(A)** and **(B)** refer to female and male patients, respectively. Data are from Spain but they are qualitatively similar in other countries.

INTRODUCTION

The Covid-19 pandemics (Zhou et al., 2020) has overcome four million confirmed deaths (Dong et al., 2020), and excess death indicates that the real number may be substantially higher. However, in contrast with the high mortality of the elderlies, children predominantly develop a mild form of Covid-19 and their mortality is very low, as part of a general trend of increasing Covid-19 severity with age. Age-stratified mortality rates with respect to detected cases increase very steeply with age, and even more steeply if we consider that most infections are asymptomatic at young age and they tend to go undetected. **Figure 1** shows the approximately exponential increase with age of detected cases, hospitalizations, ICU and deaths with respect to the number of seropositives detected in population-wide antibody surveys in Spain at May 10, 2020. One can see that the increase with age is apparent at all levels of severity, except for a drop of ICU at high age that unfortunately is most likely explained by the saturation of the health system during the first pandemic wave.

Several theories have been proposed to explain why Covid-19 is so mild with children, recently reviewed by Zimmerman and Curtis (Zimmerman and Curtis, 2020). One group of theories postulates different immune response in children. Cristiani et al. (Cristiani et al., 2020) invoke the strength of the innate immunity of children, which is further reinforced by the frequent infections and the vaccines that they are exposed to, which enhance their trained immunity. However, although the immune response to SARS-CoV-2 of children and adults is markedly different, Pierce et al. argued that the greater severity of hospitalized adults compared to children could not be attributed to their less efficient immune responses (Pierce et al., 2020). A second group of theories is based on factors that put aged adults at increased risk, including co-morbidities and, above all, differences in the endothelial system. The uncompromised state of the endothelial system of children may protect them from the most severe complications of Covid-19 that originate from endothelial inflammation and dysfunction, as reviewed in a Nature news article (Cyranoski, 2020). However, the theory that captured most attention is probably the one based on the variation of the viral receptor ACE2 with age. It was recently observed that ACE2 mRNA and serum protein is lower in children than in adults (Bunyavanich et al., 2020; Muus et al., 2020; Saheb Sharif-Askari et al., 2020; Pavel et al., 2021), and it was proposed that lower receptor levels protect children from severe SARS-CoV-2 infection. Nevertheless, data on ACE2 expression across age are contradictory. Some works found that ACE2 protein in cells is lower in children than in adults (Inde et al., 2020) and others reached the opposite conclusion (Ortiz et al., 2020; Zhang et al., 2021).

Here we go beyond the dichotomy between children and adults and propose an alternative interpretation of the data, supported by observations that indicate that ACE2 mRNA and protein expression are not monotonic with age. ACE2 mRNA starts from zero during foetal life and reaches a maximum at young age, either adolescence or twenties depending on the examined organ (Inde et al., 2020; Muus et al., 2020), after which they decay with age (Chen et al., 2020), as also observed in mice (Booeshaghi and Pachter, 2020). At the protein level, decay of ACE2 was observed in adult rats and mice (Xie et al., 2006; Yoon et al., 2016) and humans (Zhang et al., 2021), with strong inter-individual variation and cell-type dependent maximum age (Inde et al., 2020; Ortiz et al., 2020).

ACE2 cell protein level behaves differently from ACE2 mRNA, due to the shedding of ACE2 from the cell membrane to the serum produced by the metalloprotease ADAM17 (Lambert et al.,

2005) (a disintegrin and metalloproteinase 17), also known as Tumor necrosis factor alpha (TNF-α) Converting enzyme (TACE) since it activates this crucial cytokine. The expression of ADAM17 increases through age (Dou et al., 2017; Liu et al., 2019), which predicts increasing ACE2 shedding and implies that the maximum expression is achieved at earlier age for ACE2 protein than for ACE2 mRNA (see below).

Whereas the comparison between children and adults supports a positive correlation between ACE2 level and disease severity, the comparison between young and old adults supports their negative correlation. That lower receptor level is not necessarily a protective factor is predicted by a mathematical model according to which, in some circumstances, viruses propagate in the infected organism more slowly with higher receptor level (Ortega-Cejas et al., 2004) (see section Effect of Receptor Level on Viral Propagation). Most importantly, low ACE2 levels expose the lungs to acute inflammation (Imai et al., 2005), and ACE2 is low in most common chronic pathologies including hypertension, angiocardiopathy, type 2 diabetes, chronic renal failure, pulmonary diseases and liver diseases (Li et al., 2020a; Pagliaro and Penna, 2020). For these reasons, several authors proposed that the severity of Covid-19 is exacerbated by the degradation of ACE2 by the virus (Annweiler et al., 2020; Sun et al., 2020a; Ciaglia et al., 2020; Gurwitz, 2020; Offringa et al., 2020; Verdecchia et al., 2020).

HYPOTHESES

In this paper, we propose the following hypotheses.

1. One of the main functions of ACE2 consists in contributing to reverse the inflammatory state produced by the peptides of the Angiotensin and Bradykinin system and their receptors (we advocate for considering it an integrated system).
2. The activity of this molecular system increases through age at multiple levels, including chromatin remodelling, gene regulation and protein degradation. This hypothesis is highly consistent with the "inflammaging" theory. Key molecular events that contribute to this trend are the decrease of ACE2 at old age, the decrease through age of the alternative Angiotensin receptor ATR2 that is highly expressed in children, and the increase through age of the protease ADAM17/TACE that degrades ACE2 and activates TNF-α. We illustrate how the current data on ACE2 expression are consistent with this hypothesis.
3. The angiotensin-bradykinin system is characterized by a powerful positive feedback loop that amplifies the inflammation by downregulating its negative regulator ACE2 both at the transcription level and at the protein level, the latter through the protease ADAM17/TACE. At the same time, ADAM17 activates inflammatory cytokines such as TNF-α and Interleukin 6 (IL6).
4. The degradation of ACE2 by SARS-CoV-2 may strengthen the positive feedback loop described above. We propose that this is one of the main triggers of the acute inflammation observed in severe Covid-19.
5. The differential activity of the inflammatory system through age and sex may provide an explanation for the extreme differences in severity between groups of Covid-19 patients.

Figure 2 illustrates graphically the pathways of the angiotensin/bradykinin peptides, their inflammatory receptors ATR1, BKR1 and BKR2, their downregulator ACE2 and the negative effect that SARS-Cov-2 has on it.

Angiotensin Converting Enzyme 2 in the Context of the Angiotensin and Bradykinin System

The Angiotensin converting enzyme 2 (ACE2), the receptor of SARS, SARS-CoV-2 and other coronaviruses, plays a regulatory role in the RAS and in the Kallikrenin-kinin system (KKS), whose signalling peptides are the family of angiotensin for the RAS and bradykinin for the KKS. These two systems are strongly coupled, since they share some of their main functions (control of blood pressure, blood coagulation, control of inflammatory processes, regulation of the immune system response after infection or traumatic events) and two key enzymes, the carboxy-peptidases ACE and ACE2 that regulate the signal peptides of both systems. Their receptors, which belong to the large family of G-protein coupled receptors (GPCR), may act in a synergistic manner, forming complexes that mutually enhance each other signalling through allosteric interactions (Rukavina Mikusic et al., 2020). Thus, it can be considered that RAS and KKS form an integrated system.

In the RAS, ACE produces the pro-inflammatory and vasoconstrictor peptide Angiotensin II (Ang-II, which we also denote as Ang1-8 since it consists of the first 8 amino acids of the peptide Angiotensin I or Ang1-10), while ACE2 downregulates it, transforming it into the form Ang1-7 that mediates vasodilator and anti-inflammatory effects through the Mas receptor (a GPCR that forms complexes with others of the same system). Moreover, ACE2 transforms Ang1-10, the precursor of Ang1-8, into the form Ang1-9 that is later transformed into Ang1-7 by ACE.

Ang II signals through the pro-inflammatory receptor ATR1, whose action is counteracted by the alternative receptor of AngII, ATR2, which has anti-inflammatory effects. Therefore, the functioning of the RAS arises from a fine dynamical balance between ACE and ACE2 at the level of the production of the signalling peptides, and between ATR1 and ATR2 at the level of the transmission of the signal.

In the KKS, ACE2 degrades Des-Arg-Bradykinin and Lys-Des-Arg-Bradykinin (BK1-8 and LysBK0-8), two peptides that signal through the receptor BKR1 that is expressed upon inflammation, and ACE degrades BK1-9 and LysBK0-9, so that the action of ACE in the KKS is anti-inflammatory, in contrast with its action in the RAS, while it increases blood pressure in both systems. Bradykinin signalling produces vasodilation, decreases blood pressure, increases vascular permeability and induces capillary leakage. These actions can produce the edemas suffered by severe Covid-19 patients. Moreover, bradykinin enhances the inflammation locally by recruiting neutrophils and leukocytes.

The involvement of the KKS in Covid-19 cases has been discussed in recent publications (Garvin et al., 2020; Nicolau et al., 2020; van de Veerdonk et al., 2020). It is consistent with the observation that a fraction of Covid-19 patients present

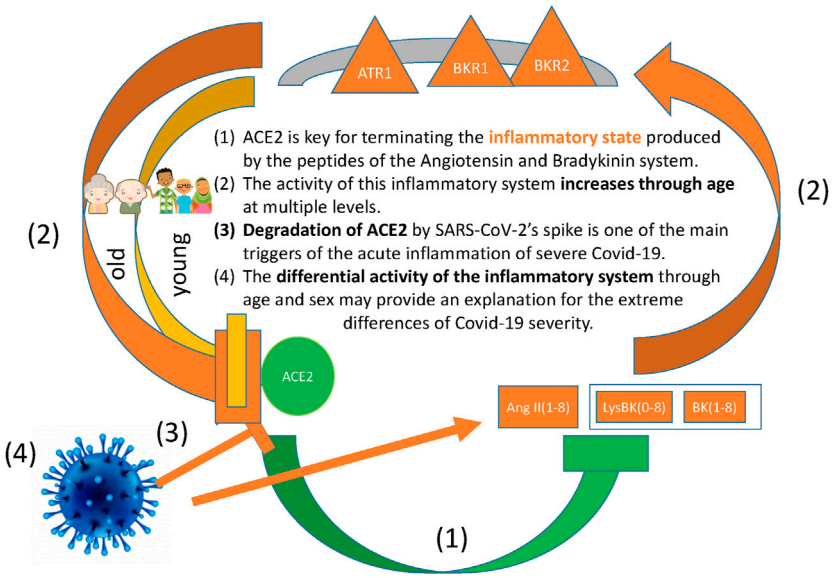


FIGURE 2 | The figure illustrates the positive feedback loop of inflammation, namely: The inflammatrory peptides AngII(1–8), BK(1–8) and LysBK(0–8) signal through the inflammatory receptors ATR1, BKR1 and BKR2, which in turn downregulate the negative regulator ACE2. Our hypotheses are the following: **(A)** One of the main functions of ACE2 consists in terminating the inflammatory state produced by the peptides of the Angiotensin and Bradykinin system and their receptors. **(B)** The activity of this inflammatory system increases through age at multiple levels, including chromatin remodelling, gene regulation and protein degradation, and this has the effect of downregulating ACE2. **(C)** The degradation of ACE2 by SARS-CoV-2 strenghens the positive feedback loop of inflammation, enhancing the acute inflammation observed in severe Covid-19. **(D)** The differential activity of the inflammatory system through age and sex may provide an explanation for the extreme differences in severity between groups of Covid-19 patients.

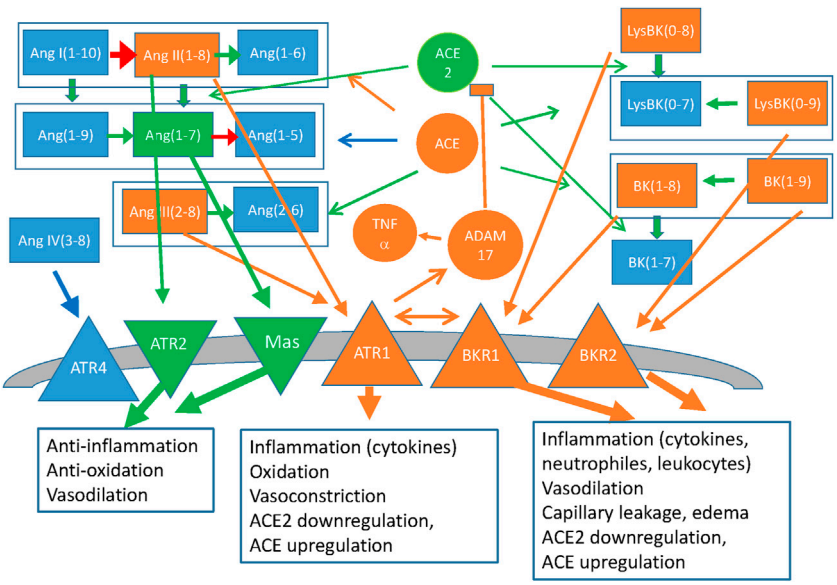


FIGURE 3 | Simplified representation of the Renin-Angiotensin system and the Kallikrein-Bradykinin system, coupled through the peptidases ACE and ACE2 that act on both families of peptides and through the allosteric interactions between the G-protein coupled receptors of both families. Peptides of the angiotensin (Ang) and bradykinin (BK) family are represented as rectangles, coloured arrows indicate their transformations and circles indicate the peptidases that catalyse them (black arrows). Membrane receptors are indicated as triangles and the boxes indicate the main effects of their signalling. The colour code represents inflammatory effects (orange: pro-inflammatory; green: anti-inflammatory, blue: Neutral or not clear at present). Also represented as ellipses are Vitamin D, which downregulates Renin and the downstream effectors NFkB and NOX, and Interferons α and β, both of which upregulate ACE2 with anti-inflammatory effects, and γ, which downregulates ACE2.

hypotension: 66% of critical patients (Michard and Vieillard-Baron, 2020), and 8% of hospitalized patients compared with 39% with hypertension (Lala et al., 2020).

Both bradykinin and Angiotensin II upregulate the protease ADAM17/TACE that removes ACE2 from the cell membrane, activates TNF-α and transforms the receptor of the cytokine IL6 to a soluble form that enhances inflammation (Matthews et al., 2003; Rose-John, 2012). These processes cause a synergistic positive feedback loop that amplifies inflammation.

We represent in **Figure 3** the reduced molecular network of the angiotensin/bradykinin system. The figure represents different peptide species, their transformations through the peptidases ACE and ACE2, the anti-inflammatory receptors ATR2 and Mas, and the metalloprotease ADAM17/TACE.

Furthermore, ACE2 has been recently shown through evolutionary rate correlation analysis to possess strong biological interactions with proteins in the coagulation pathway such as Clusterin and Fibrinogen alpha, beta and gamma chains and with proteins involved in cytokine signaling such as X-C Motif Chemokine Receptor 1 (XCR1), Interferon alpha/beta receptor 2 (IFNAR2) and toll-like receptor 8 (TLR8) (Varela et al., 2021).

The Main Functional Role of Angiotensin Converting Enzyme 2 May Consist in Reversing the Inflammation Process.

ACE2 counteracts high blood pressure in the RAS and low blood pressure in the KKS, but it has anti-inflammatory effects in both systems. Interestingly, several studies have shown that ACE2 knockout mice do not present significant hypertension or cardiac anomalies, but they show enhanced response to Ang-II stimulation (Alghamri et al., 2013) and they develop lung edema after acute-inflammation (Imai et al., 2005). Edema is not the mechanical consequence of increased blood pressure, which does not happen in ACE2 KO mice, but it may derive from increased vascular permeability due to high bradykinin levels (van de Veerdonk et al., 2020). This is supported by the observation that attenuation of pulmonary ACE2 activity impairs inactivation of the DABK/BK1R axis and facilitates neutrophil infiltration (Sodhi et al., 2018). These observations connect ACE2 with the termination of the inflammatory process, as further discussed below.

A crucial link between ACE2 and the inflammatory process goes through ADAM17. This metallo-protease sheds ACE2 from the cellular membrane to the serum (Lambert et al., 2005), from which it is rapidly eliminated through urine, and it is triggered by Angiotensin II via AT1Rs (Deshotels et al., 2014; Xu et al., 2017). In addition, bradykinin triggers BKR1s, which also upregulate ADAM17 (Parekh and Sriramula, 2020), establishing a synergistic mechanism that enhances ACE2 degradation. At the same time, ADAM17 activates the cytokine TNF-α and it transforms the receptor of the cytokine IL6 into its soluble form that is pro-inflammatory (Matthews et al., 2003; Rose-John, 2012), initiating a cascade process that, through the transcription factor nuclear factor kB (NFkB), promotes the activation of the inflammatory response.

Here we summarize the main steps with which the RAS and the KKS participate in the inflammation process.

1. The infection activates the RAS, enhancing Ang1-8 production
2. High Ang-II/ATR1 generates a positive feedback by upregulating the expression of ACE, downregulating ACE2 (Koka et al., 2008), and upregulating ADAM17/TACE. This positive feedback loop leads to lower ACE2 and higher local Ang-II production.
3. At the same time, Ang-II/ATR1 enhances the inflammation:
   - Ang-II/ATR1 activates the KKS, which induces vasodilation and possible drop of blood pressure, increased capillary leakage and recruitment of neutrophils.
   - The Ang-II/ATR1 axis has different effects on the two receptors of the KKS, BKR1 and BKR2. Through IL1 and TNF-α, ATR1 upregulates the BKR1 receptor of DABK, whose level increase through the decrease of ACE2, activating the DABK/BKR1 axis. BKR1 contributes to upregulating ADAM17, producing another positive feedback loop.
   - At the same time, the other bradykinin receptor BKR2 is sensitized by ATR1, with which it forms a dimer (Rukavina Mikusic et al., 2020). However, increased ACE downregulates the ligand of BKR2, so the effect on BKR2 signalling is unclear.
   - Ang-II/ATR1 upregulates the Vascular Endothelial Growth Factor (VEGF) through TGF-beta and Angiopoietin-2, thus amplifying capillary leakage. At the same time, VEGF may be also activated by BKR2.
   - Ang-II/ATR1 and DABK/BKR1 recruit macrophages and neutrophils to the infection.
4. At some point, ACE2 is upregulated and it contributes to reversing the inflammation in a cascade process: ACE2 degrades Ang-II and Ang1-10, downregulating the Ang-II/ATR1 axis, produces Ang1-7, upregulating the anti-inflammatory Ang1-7/Mas, and degrades DABK, diminishing vascular leakage. Downregulation of ATR1 desensitives the BKR2 receptor and reduces the level of the BKR1 receptor (whose ligand is also decreased), and, through them, ADAM17-TACE, which in turn decreases TNF-α and the pro-inflammatory IL6 soluble receptor. Moreover, the lower activity of ATR1 reduces ACE, further reducing AngII, and reduces the VEGF.

Consistent with this view, ACE2 is upregulated during inflammation (Hanafy et al., 2011), in part through interferon I stimulation (Ziegler et al., 2020). ACE2 upregulation is also observed in a recent analysis of mRNA expression of bronchoalveolar lavage fluid cells (Garvin et al., 2020) and lung cells (Wu et al., 2020) of Covid-19 patients. However, the ACE2/ACE ratio is decreased in most chronic inflammatory diseases (Li et al., 2020a; Pagliaro and Penna, 2020). As discussed below, we hypothesize that epigenetic mechanisms have a role in the discrepancy between short term activation and chronic reduction of ACE2.

Severe Covid-19 as a Failure of Angiotensin Converting Enzyme 2 to Revert Inflammation

As discussed above, ACE2 plays a key role in reverting the inflammatory process in the context of the RAS and the KKS, and its downregulation favour endothelial damage, capillary leakage and angioedema, as shown by Imai et al. and Kuba et al. among others (Imai et al., 2005; Kuba et al., 2005). It has been shown that SARS-CoV (Haga et al., 2008) and SARS-CoV-2 degrade ACE2. We hypothesize that the degradation of ACE2, especially in presence of low initial levels, or the failure to upregulate ACE2 may prevent the termination of the inflammatory process and perpetuate the propagation of inflammation through the affected organs, leading to organ damage and severe manifestations that can be life threatening.

In a transcriptomic study of lung tissue obtained from patients who died of Covid-19 in China it was found that the viral load was low in all samples. According to the authors, this "suggests that the patient deaths may be related to the host response rather than an active fulminant infection" (Wu et al., 2020).

A retrospective study of almost 48,000 Covid-19 patients discharged from English hospitals (and, accordingly, negative to the virus in their large majority) found rates of hospital readmission and death 3.5 and 7.7 times greater, respectively, than in age- and comorbidities-matched controls, and higher rates of respiratory, diabetes and cardiovascular events, evidencing elevated rates of multi-organ dysfunction in individuals discharged from hospital (Ayoubkhani et al., 2021). These observations indicate that many severe consequences of Covid-19 arise from the immune-inflammatory response rather than being a direct consequence of SARS-COV-2 infection.

Increase of ADAM17 in Severe Covid-19 Patients

A key role in the activation of the inflammatory process is played by ADAM17/TACE, which eliminates ACE2 from the cell membranes preventing its anti-inflammatory function (Lambert et al., 2005), it transforms the pro-inflammatory cytokine TNF-α into the active form and it transforms the receptor of the cytokine IL6 to its soluble form, which is pro-inflammatory, contrasting the anti-inflammatory effect of the membrane bound IL6R (Matthews et al., 2003; Rose-John, 2012).

A recent study on a cohort of 102 Covid-19 patients from Mexico found that ADAM17 is increased in the serum of Covid-19 patients with respect to healthy donors, and in severe patients with respect to mild ones. Possibly as a consequence of this increase, some of the proteins shed in the serum by ADAM17, including TNF-α, its receptors TNFR1 and TNFR2 and T-cell immunoglobulin and mucin domain 3 (TIM3), but not transforming growth factor beta (TGF-β), are increased in the serum of these patients at the protein level while their mRNA is unchanged, which suggests increased ADAM17 activity (Palacios et al., 2021). Moreover, increased level of ADAM17 and its substrates TNFR1 and TIM3 was higher in patients that died of Covid-19 compared with survivors.

These results support the proposed central role that ADAM17 may play in severe Covid-19, and suggest that the dangerous positive feedback loop of ACE2 downregulation through the action of ADAM17 is active in Covid-19 patients, in particular severe ones.

Variation of Angiotensin Converting Enzyme 2 Across Age

Several studies based on RNA-seq experiments found that the mRNA of ACE2 is absent in the early foetus and it is lower in children than adults (Bunyavanich et al., 2020; Inde et al., 2020; Muus et al., 2020; Saheb Sharif-Askari et al., 2020). A similar observation applies to the ACE2 protein in serum (Pavel et al., 2021). Therefore, it was proposed that the low expression of the virus receptor ACE2 hinders the propagation of SARS-CoV-2 in children organs. However, several studies, including also some of those cited above, indicate that the expression of ACE2 with age is not monotonic, since ACE2 expression decreases at advanced age in several organs after reaching a maximum.

At the mRNA level, decrease of ACE2 mRNA at advanced age was observed in an analysis of several human tissues collected in the GTeX database (Chen et al., 2020) and in mouse lung samples (Booeshaghi and Pachter, 2020). Single cell data of human respiratory cells reported in **Figure 3G** of Muus et al. (Muus et al., 2020) are also consistent with decrease or stationarity of ACE2 mRNA after a maximum reached at 10–25 years in multiciliated cells and 25–40 years in alveolar type 2 (AT2) cells. A recent preprint by Inde et al. (Inde et al., 2020) also found a maximum of ACE2 mRNA in mouse lungs at few days after birth, evidenced a minimum at approximately 10 days and an increase at least until 9–11 months, which in mice is middle age. For human data, the same preprint found a maximum of ACE2 mRNA short after birth in the hearth and at about 10 years in the testis (Fig. 6C and 6D of (Inde et al., 2020)). Inde et al. also present data of ACE2 mRNA in human lungs in their **Figure 3G**, which does not show the existence of a maximum but cannot rule it out due to the sparsity of data at advanced age.

Concerning ACE2 protein in cells, which is the relevant molecular species for virus propagation, western blot analysis of rat lungs (Xie et al., 2006) and mouse aorta (Yoon et al., 2016) indicate that ACE2 membrane protein levels decrease with age in adult rodents. These studies did not include juvenile animals and examined three age classes: 2 (3 for rats), 12 and 24 months, corresponding to young adults, middle age and old. In mouse thoracic aorta, the whole anti-inflammatory arm of the RAS to which ACE2 belongs decreases with age, while the pro-inflammatory arm of the RAS increases with age (Yoon et al., 2016).

In humans, a study found that children below 10 years have on the average higher ACE2 protein in AT2 lung cells than adults, although these values vary considerably from cell to cell and from individual to individual (Ortiz et al., 2020). However, this comparison depended on the discretization of ACE2 expression with an arbitrary threshold of more than 1% of positive cells, and it included adults with asthma that downregulates ACE2, whose exclusion would have raised the

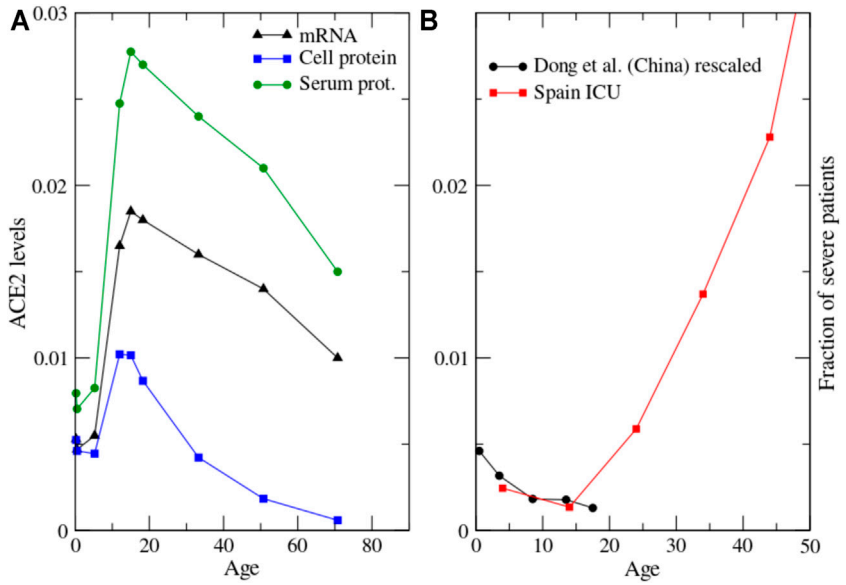


FIGURE 4 | (A): Proposed model of the dependence of ACE2 with age at the three levels of mRNA, protein in the cellular membrane and protein in serum. The mRNA data are inspired by human multiciliated cells reported in **Figure 3G** of Muus et al. (2020). The cell protein levels result from time-independent mRNA translation and shedding through ADAM17 that we assume to increase with age as exp (age/25y); other functional forms such as power law yield qualitatively similar results. **(B)**: The severity of pediatric cases of Covid-19 in China (Dong, 2020), rescaled to take into account undetected cases, and put in the context of severe Covid-19 cases of children and adults from **Figure 1**.

p-value. Moreover, other cell types did not confirm the same trend as AT2 lung cells. Zhang, Guo et al. (Zhang et al., 2021) found that ACE2 positive cells in lung biopsy samples from 26 children and 24 adults were significantly decreased in patients older than 50 with respect to children. This reduction was observed in bronchial cells but it was not significant in pulmonary alveolar cells, and it was also observed at the mRNA level. The study by Inde et al. (Inde et al., 2020) also found extreme intra- and inter-individual heterogeneity, but it reached the opposite conclusion that ACE2 protein in human lung epithelial cells increases with age; however, also this conclusion was reached measuring the percentage of cells that express ACE2 above a threshold. Inde et al. also plot ACE2 intensities versus the donor age in their **Figures 1C,G**, but these figures do not suggest a clear increase of ACE2 with age. Furthermore, they did not observe increase of ACE2 with age in AT2 cells, which are the cell type with maximum ACE2 expression in the lungs (Li et al., 2020b).

We conclude that most existing data are consistent with a decrease across age of ACE2 mRNA and protein levels in rodents and several human cell types. Accordingly, we plot in **Figure 4A** a model in which ACE2 expression starts in the last stage of foetal life, reaches a maximum at young age and then decreases at old age, with data inspired on the data of Muus et al. (Muus et al., 2020) in multiciliated cells. The position of the maximum depends on the cell type and it is influenced by the strong inter- and intra-individual variability evidenced by several studies (Inde et al., 2020; Ortiz et al., 2020).

For understanding the relation between ACE2 mRNA and protein expression it is necessary to consider the expression of the metalloprotease ADAM17/TACE that sheds ACE2 from the cell membrane to the serum, from which it is rapidly lost through urine. The expression of ADAM17 increases with age (Dou et al., 2017; Liu et al., 2019), consistent with the increase with age of Ang-II and its receptor ATR1 (Yoon et al., 2016) that upregulates ADAM17 (Deshotels et al., 2014; Xu et al., 2017) together with the bradykinin receptor BKR1 (Parekh and Sriramula, 2020).

As a result of mRNA expression and shedding through ADAM17, the stationary concentration of ACE2 cell protein is proportional to the ratio ACE2mRNA/ADAM17. We plot this function in **Figure 4A**, where we assume that ADAM17 increases exponentially with age as exp (age/25years). Other functional forms such as power law with exponent 1/3 yield qualitatively similar plots. One can see that the maximum of ACE2 cell protein is reached at earlier age than the maximum of ACE2 mRNA, as the consequence of the increase of ADAM17 with age. ACE2 cell protein decreases in rats and mice from 3 to 12 months (Xie et al., 2006; Yoon et al., 2016) while ACE2 mRNA increases in mice of the same age (Inde et al., 2020). Although this comparison involves different species (rats versus mice) or different cell types (aorta versus lungs), it is consistent with the above prediction. ACE2 protein in the serum is proportional to the product of the cell protein times the shedding rate, i.e. it is proportional to ACE2 mRNA averaged over the different cell types. We depict in **Figure 4A** the proposed behaviour of ACE2 versus age at the levels of mRNA, cell protein and serum protein.

Covid-19 Severity Across Age Presents a Minimum

A paediatric study in China observed that the severity of Covid-19 in children (not including the MIS-C syndrome) decreases with age (Dong, 2020): the proportion of severe and critical paediatric cases was 10.6, 7.3, 4.2, 4.1, and 3.0% for the age groups<1, 1–5, 6–10, 11–15 and 16–18 years, respectively (**Figure 4B**). This decrease of severity from young children to teen-agers is consistent with the drop in the hospitalization rate in Spain from the age class 0–10 to 10–20 that one can see in **Figure 1**. Covid-19 severity increase with age in adults (**Figure 1**), therefore severity versus age has a minimum (**Figure 4B**). Suggestively, the shape of this curve appears inversely related to the ACE2 protein level across age (**Figure 4A**), although the position of the maximum of ACE2 depends on the cell type. Another recent paper analysed similar data but proposed a direct instead of inverse relationship between Covid-19 severity and ACE2 expression (Inde et al., 2020). However, most existing data suggest that ACE2 expression decreases at high age, although this point is still debated, and the increase of severity with age is a very clear property of Covid-19.

Comorbidities Support a Negative Relation Between Angiotensin Converting Enzyme 2 and Covid-19 Severity

As discussed in more detail in Ref. (Bastolla, 2021), this inverse relationship between ACE2 levels and Covid-19 severity might rationalize not only the influence of age and sex but also most of the other known risk factors of Covid-19 examined in the OpenSAFELY study (Williamson et al., 2020), including diabetes, hypertension, obesity, vitamin D deficit (that may explain at least part of the risk factors connected with ethnicity) and perhaps even the curious protecting effect of smoke that increases ACE2 expression (Saheb Sharif-Askari et al., 2020).

The ATR2 Receptor

The proposed inverse relationship between ACE2 expression and Covid-19 severity has an exception in children, who experience mild Covid-19 but express less ACE2 than adults. However, children express more than adults the Ang-II receptor ATR2, which competes with ATR1 and counteracts its inflammatory effect (Kaschina et al., 2017).

The decrease of ATR2 with age has been disputed (Gao et al., 2012), but most works support it, and they also concluded that the receptor binding activity of ATR2, which is most relevant in this context, decreases with age. Both ATR1 and ATR2 are GPCR, and they can form complexes between themselves and with other receptors, including BKR2 (Rukavina Mikusic et al., 2020), underscoring complex regulatory interactions and downregulation of ATR1 by ATR2. We propose that the high level of the ATR2 receptor in children may compensate their lower ACE2 level.

This proposal is consistent with the protective influence on Covid-19 severity of angiotensin receptor blockers that block the signaling of ATR1, as evidenced by a meta-analysis of several clinical trials (Guo et al., 2020), and of anti-ATR1 auto-antibodies (Papola et al., 2021), which are known to induce the increase of ATR2 expression (Sun et al., 2020b).

Epigenetic Enhancement of the Inflammatory Process

An increasing amount of evidence indicates that inflammation related genes in general, and ACE2 in particular, are subject to epigenetic control through chromatin modifications. Transcriptional memory provides faster and enhanced transcription upon repeated stimulations, and it is a common hallmark of interferon-stimulated genes, mediated by histone variants such as H3.3 and histone modifications that favour transcription (Kamada et al., 2018) and by de-methylation of CpG islands at active promoters, associated with sustained stimulation by the proinflammatory cytokine TNF-α (Zhao et al., 2020) that is activated by ADAM17.

The ACE2 gene has been shown to be subject to transcriptional memory through CpG island demethylation that increases through age (Corley and Ndhlovu, 2020), and ACE2 transcription in samples of patients with comorbidities associated with severe COVID-19 was correlated with the expression of genes related to histone modifications (Pinto et al., 2020). Similarly, genome-wide CRISPR screens identified several chromatin modifiers that facilitate SARS-COV-2 infection (Wei et al., 2020), likely by upregulating ACE2.

The transcriptional memory of the ACE2 gene has been interpreted as evidence of a positive correlation between ACE2 expression and Covid-19 severity (Pruimboom, 2020). However, it is important to note that the transcriptional memory also acts on pro-inflammatory genes (Kamada et al., 2018; Zhao et al., 2020). The reported decrease of the ACE2/ACE ratio in chronic inflammatory diseases associated with severe Covid-19 (Li et al., 2020a; Pagliaro and Penna, 2020) suggests that the pro-inflammatory side prevails in this balance, with the final result to decrease the ACE2/ACE ratio through the transcriptional downregulation of ACE2, which is also consistent with the theory of inflammaging, which postulates that the increase through age of inflammatory processes contributes to the phenomenology of aging and senescence (Franceschi et al., 2007).

Future work should address the complex regulation of ACE2 and other inflammatory and anti-inflammatory genes from a system perspective, considering multiple levels of regulation (epigenetic, transcriptional, post-translational).

Effect of Receptor Level on Viral Propagation

The theory according to which lower ACE2 level may protect from severe disease is based on the premise that lower receptor level hinders the propagation of the virus. But is this assumption warranted? Mathematical models of virus propagation, tested with experiments with bacteriophages, suggest that there is an optimal receptor density at which the virus propagates fastest, with viral velocity declining both for lower and for higher receptor density (Ortega-Cejas et al., 2004) (see **Figure 5**). A mathematical model of the dependence of Covid-19 mortality on

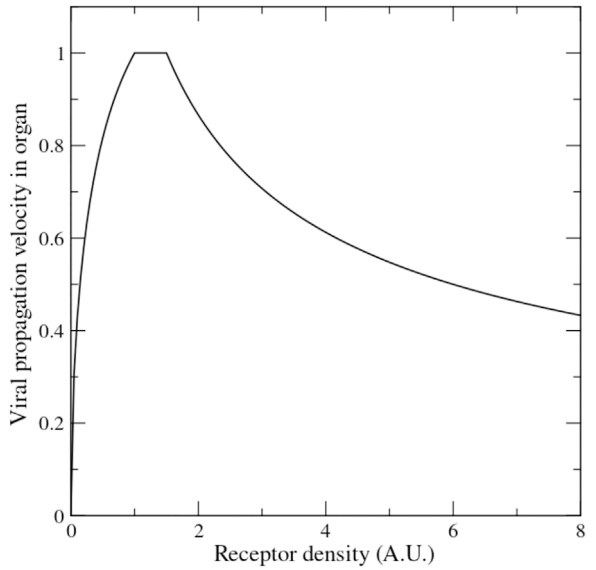


FIGURE 5 | Mathematical model of the velocity of viral propagation in an organ as a function of the receptor density (adapted from the formulas presented in Ref. (Ortega-Cejas et al., 2004) tested with experiments on bacteriophage propagation).

receptor density fitted to both Covid-19 and SARS 2003 data of lethality across age and sex suggests that both viruses propagate in a regime in which increasing receptor density slows down their propagation (Bastolla, 2021). Thus, the inverse relation between ACE2 expression and Covid-19 severity is not implausible.

The Role of Interferons

It has been recently shown that type-I interferons (IFN-I = IFN-α, IFN-β) upregulate ACE2 expression (Ziegler et al., 2020) and ACE2 has been shown to coevolve with interferon alpha/beta receptor 2 (IFNAR2) throughout mammalian evolution, in the sense that their evolutionary rates are strongly correlated (Varela et al., 2021). Interestingly, IFN-β are used as anti-inflammatory therapy in multiple sclerosis (Kieseier, 2011), supporting their anti-inflammatory role. We conjecture that the upregulation of ACE2 by IFN-I may contribute to stop the inflammatory cascade. This conjecture is consistent with the recent finding that impaired type-I IFN response (Hadjadj et al., 2020), auto-antibodies against IFN-α (Bastard et al., 2020) and genetic variants that hinder interferon activation (Zhang et al., 2020) are associated with more severe Covid-19 cases. Moreover, mutations of the IFN-I receptor have been found to be risk factors of severe Covid-19 through genome wide association studies (GWAS) analysis (Pairo-Castineira et al., 2020). Finally, SARS-COV-2 infection is characterized by low levels of IFN-I (Blanco-Melo et al., 2020), partly because the virus dysregulates IFN-I (Konno et al., 2020). Therefore, we suggest that IFN downregulation cooperates with ACE2 dysregulation by SARS-COV-2 in preventing the arrest of the inflammatory process in severe Covid-19 cases. However, interferon therapy did not provide any reduction of mortality or hospitalization in the Solidarity clinical trial (olidarity Trial Cons, 2020).

Therapeutic Perspectives

Our hypothesis supports several candidate drugs that might alleviate severe complications of CoViD-19 and even its post infection consequences (MIS-C and long Covid-19) by inhibiting the processes that prevent the reversion of the inflammatory state. Luckily for several of these drugs clinical trials are already on-going.

1. Human recombinant ACE2 has been suggested as a potential therapeutic agent against SARS-CoV-2. It can have a double protective effect. On one hand, it can alleviate the inflammatory process, and in the other one it can sequester the virus and difficult its entry in the cells (Batlle et al., 2020; Huang et al., 2021). A recent case report presents very promising results, and clinical trials are ongoing (Zoufaly et al., 2020).
2. Angiotensin receptor blockers (ARB) that block the ATR1 receptor, downregulating the inflammatory process and preventing the positive feedback loop of Ang-II through upregulation of ACE and downregulation of ACE2. They are often discussed together with ACE inhibitors (ACE-I) that reduce the formation of Ang-II. However, ACE has anti-inflammatory effect on the KKS, where it downregulates bradykinin, so that one of the known adverse effects of ACE-I is the upregulation of KKS eventually leading to edema. Moreover, Ang-II can be also formed through other peptidases. The possible therapeutic role of ARB and ACE-I was suggested by several groups already at the time of SARS 2003 and later proposed for SARS-COV-2 (Annweiler et al., 2020; Sun et al., 2020a; Ciaglia et al., 2020; Gurwitz, 2020; Offringa et al., 2020; Verdecchia et al., 2020). Although there was the concern that, by upregulating ACE2, ARB and ACE-I might favour the virus, meta-analysis of several clinical studies suggested that they are associated with lower mortality of COVID-19 in patients with hypertension, with odds ratio 0.57 (95% CI, 0.38–0.84) (Guo et al., 2020). The clinical trials NCT04312009 and NCT04311177 are ongoing to test possible protective effects, although caution is necessary if blood pressure becomes too low, which is a frequent consequence of severe Covid-19 (Lala et al., 2020; Michard and Vieillard-Baron, 2020), probably through the activation of the bradykinin axis.
3. Drugs that enhance the ATR2 receptor. Their effect may be in principle similar to ARB.
4. The product of ACE2, Ang1-7, which exerts anti-inflammatory and anti-fibrotic effects (Chappell and Al Zayadneh, 2017) through the receptor Mas. However, its mechanism of action is still not fully known, it is rapidly degraded by ACE, and it has a moderate affinity for ATR1, thus it might produce effects contrary to the intentions.
5. IFN-I that upregulate ACE2. Although interferon may promote inflammation, IFN-β (type I) is used as anti-inflammatory drug to treat multiple sclerosis (Kieseier, 2011). However, the Solidarity clinical trial did not observe any reduction of Covid-19 mortality upon IFN-I treatment (olidarity Trial Cons, 2020).
6. Other drugs that upregulate ACE2.
7. Inhibitors of TACE/ADAM17, which may prevent the degradation of ACE2 and the increase of TNF-α.
8. Inhibitors of IL-1 and TNF-α, which are successfully used to treat KD and MIS-C, might also be useful to treat Covid-19 for preventing excessive activation of the KKS.

9. Vitamin D, which exerts a protective role by downregulating ATR1 and ADAM17, and in this way limits the inflammatory process and protects ACE2 from excessive shedding. A preprint reporting a retrospective study of almost 5 million people found an association between vitamin D deficiency and Covid-19 severity (Israel et al., 2020), and a clinical study with 76 patients found that a high dose of Calcifediol, a metabolite of vitamin D, significantly reduced the ICU treatment for hospitalized Covid-19 patients (Entrenas Castillo et al., 2020).

Of course, experimental verifications of the above hypothesis are necessary, and clinical trials are urgently needed to test these therapeutic indications.

CONCLUSION

As reviewed above by us and elsewhere by many other authors, the peptidase ACE2, besides being the cellular receptor of SARS-COV-2 and other coronavirus, exerts a central regulatory role in inflammatory processes by downregulating the main pro-inflammatory peptides of the Renin-angiotensin system (RAS) and the Kinin kallikrein system (KKS) (**Figure 3**). These two highly integrated signalling systems, whose receptors act synergistically and strengthen each other, activate the inflammatory response, activate cytokines starting from a central controller of inflammation such as TNF-α, which is activated by the same protease TACE/ADAM17 that degrades ACE2 and recruits macrophages and neutrophils. We propose that the main role of ACE2 consists in terminating this inflammatory process. Failure to do so, because of ACE2 degradation or failure to activate it, may exacerbate the inflammatory response leading to organ damage, including edema, and it may be responsible of the most severe consequences of Covid-19 even after the infection has been resolved, as it happens in the Multi-inflammatory syndrome of children (MIS-C).

Because of the central role of ACE2 both for SARS-COV-2 propagation and for controlling the inflammatory process, and because of the very marked increase of Covid-19 with age (**Figure 1**), a strong effort has been dedicated to elucidate how ACE2 expression changes with age. However, these works have produced conflicting results, that have been interpreted either as support of the theory that the receptor expression favours SARS-COV-2 infection or as support of the opposite theory that high ACE2 expression favours milder disease because of its anti-inflammatory role.

We have shown that these contrasting results on ACE2 expression across age can be reconciled going beyond the dichotomy between adults and children and recognizing that ACE2 expression does not change monotonically with age but it starts in late foetal life, reaches a maximum at a young age that depends on the cell type and exhibits strong inter-individual and inter-cellular variation, and decreases at advanced age. At the protein level, this decrease precedes that of mRNA, Remove starts before and it is more accentuated than at the mRNA level, due to the increase with age of the activity of the protease TACE/ADAM17 that sheds ACE2 from the cell membrane to the serum. This proposed behaviour, represented in **Figures 4A**, is consistent with essentially all existing data on the ACE2 expression across age in rodents and humans, except some of the figures of the preprint by Inde et al. (Inde et al., 2020).

The complexity and the intricacies of ACE2 regulation will require future approaches that integrate multiple regulatory levels, from chromatin remodelling that plays a key role in transcriptional memory to regulation through transcription factors and post-translational regulation through ADAM17 and other proteases that degrade ACE2. This is an important subject, since the decrease of ACE2 with age, and the simultaneous increase of the pro-inflammatory components of the RAS may play an important role in the theory of inflammaging, which postulates that the increase through age of inflammatory processes contributes to the phenomenology of aging and senescence (Franceschi et al., 2007).

The proposed decrease of ACE2 across age and the observed decrease of the ACE2/ACE ratio in chronic inflammatory diseases often associated with severe Covid-19 (Pagliaro and Penna, 2020; Li et al., 2020a) support a negative relationship between Covid-19 severity and ACE2 expression (**Figure 4B**). This is not as paradoxical as it seems, since a mathematical model of virus propagation across cells predicts that an increase of the receptor level does not necessarily accelerate viral propagation and it may even slow it down (Ortega-Cejas et al., 2004) (see **Figure 5** for illustration). Furthermore, ACE2 is located in the X chromosome and its protein expression is higher in females than in males in murine models (Xie et al., 2006), which may extend the proposed negative correlation also to the comparison between sexs. Interestingly, in a recent analysis of human lung transcriptomic data it was found that the proinflammatory angiotensin receptor ATR1 is significantly more expressed in male tissues than in female ones, while the anti-inflammatory ATR2 has similar expression in both sexes (Hachim Ibrahim et al., 2021), which is also consistent with the hypothesis presented here.

The exception to this proposed negative relationship is constituted by children, who express less ACE2 than adults and experience much milder disease. However, in children the RAS is much less dis-balanced towards the pro-inflammatory side than in adults. In particular children express more ATR2, which is anti-inflammatory.

Of course, experimental work is needed for supporting the hypothesis presented in this review, but we hope that they may help rationalizing apparently conflicting observations.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.mscbs.gob.es/profesionales/saludPublica/ccayes/alertasActual/nCov/documentos/Actualizacion_101_COVID-19.pdf.

AUTHOR CONTRIBUTIONS

UB conceived this study, developed the model of ACE2 expression across age, computed Covid-19 across age and

made the figure of the RAS and bradykinin system. All authors provided references and participated in the discussion of the RAS and bradykinin system.

FUNDING

This review paper is related with our experimental study of ACE2 in the serum of Covid-19 patients funded by the Spanish Research Council (CSIC) under the grant CSIC-COV19-108.

ACKNOWLEDGMENTS

We gratefully acknowledge financial support from the Spanish Research Council (CSIC) under the grant CSIC-COV19-108. We thank Juan Carlos Alonso for interesting discussions.

REFERENCES

Alghamri, M. S., Weir, N. M., Anstadt, M. P., Elased, K. M., Gurley, S. B., and Morris, M. (2013). Enhanced Angiotensin II-Induced Cardiac and Aortic Remodeling in ACE2 Knockout Mice. *J. Cardiovasc. Pharmacol. Ther.* 18, 138–151. doi:10.1177/1074248412460124

Annweiler, C., Cao, Z., Wu, Y., Faucon, E., Mouhat, S., Kovacic, H., et al. (2020). Counter-regulatory 'Renin-Angiotensin' System-Based Candidate Drugs to Treat COVID-19 Diseases in SARS-CoV-2-Infected Patients. *Iddt* 20, 407–408. doi:10.2174/1871526520666200518073329

Ayoubkhani, D., Khunti, K., Nafilyan, V., Maddox, T., Humberstone, B., Diamond, S. I., et al. (2021). Epidemiology of post-COVID Syndrome Following Hospitalisation with Coronavirus: a Retrospective Cohort Study. *Medrxiv Jan*. doi:10.1101/2021.01.15.21249885

Bastard, P., Rosen, L. B., Zhang, Q., Michailidis, E., Hoffmann, H-H., Zhang, Y., et al. (2020). Auto-antibodies against Type I IFNs in Patients with Life-Threatening COVID-19. *Science*, eabd4585. doi:10.1126/science.abd4585

Bastolla, U. (2021). Mathematical Model of SARS-Cov-2 Propagation versus ACE2 Fits COVID-19 Lethality across Age and Sex and Predicts that of SARS. *Front. Mol. Biosci.* 8, 706122. doi:10.3389/fmolb.2021.706122

Batlle, D., Wysocki, J., and Satchell, K. (2020). Soluble Angiotensin-Converting Enzyme 2: a Potential Approach for Coronavirus Infection Therapy? *Clin. Sci.* 134, 543–545. doi:10.1042/cs20200163

Blanco-Melo, D., Nilsson-Payant, B. E., Liu, W.-C., Uhl, S., Hoagland, D., Møller, R., et al. (2020). Imbalanced Host Response to SARS-CoV-2 Drives Development of COVID-19. *Cell* 181, 1036–1045. doi:10.1016/j.cell.2020.04.026

Booeshaghi, A. S., and Pachter, L. Decrease inACE2mRNA Expression in Aged Mouse Lung. Preprint 2020. doi:10.1101/2020.04.02.021451

Bunyavanich, S., Do, A., and Vicencio, A. (2020). Nasal Gene Expression of Angiotensin-Converting Enzyme 2 in Children and Adults. *JAMA* 323, 2427–2429. doi:10.1001/jama.2020.8707

Chappell, M. C., and Al Zayadneh, E. M. (2017). Angiotensin-(1-7) and the Regulation of Anti-fibrotic Signaling Pathways. *J. Cel Signal* 2, 134. doi:10.4172/2576-1471.1000134

Chen, J., Jiang, Q., Xia, X., Liu, K., Yu, Z., Tao, W., et al. (2020). Individual Variation of the SARS-CoV-2 Receptor ACE2 Gene Expression and Regulation. *Aging Cell* 19, e13168. doi:10.1111/acel.13168

Ciaglia, E., Vecchione, C., and Puca, A. A. (2020). COVID-19 Infection and Circulating ACE2 Levels: Protective Role in Women and Children. *Front. Pediatr.* 8, 206. doi:10.3389/fped.2020.00206

Corley, M. J., and Ndhlovu, L. C. DNA Methylation Analysis of the COVID-19 Host Cell Receptor, Angiotensin I Converting Enzyme 2 Gene (ACE2) in the Respiratory System Reveal Age and Gender Differences. *Preprints* 2020, 2020030295. doi:10.20944/preprints202003.0295.v1

Cristiani, L., Mancino, E., Matera, L., Nenna, R., Pierangeli, A., Scagnolari, C., et al. (2020). Will Children Reveal Their Secret? the Coronavirus Dilemma. *Eur. Respir. J.* 55 (4), 2000749. doi:10.1183/13993003.00749-2020

Cyranoski, D. (2020). Why Children Avoid the Worst Coronavirus Complications Might Lie in Their Arteries. *Nature* 582, 324–325. doi:10.1038/d41586-020-01692-z

Deshotels, M. R., Xia, H., Sriramula, S., Lazartigues, E., and Filipeanu, C. M. (2014). Angiotensin II Mediates Angiotensin Converting Enzyme Type 2 Internalization and Degradation through an Angiotensin II Type I Receptor-dependent Mechanism. *Hypertension* 64, 1368–1375. doi:10.1161/hypertensionaha.114.03743

Dong, E., Du, H., and Gardner, L. (2020). An Interactive Web-Based Dashboard to Track COVID-19 in Real Time. *Lancet Infect. Dis.* 20, 533–534. doi:10.1016/s1473-3099(20)30120-1

Dong, Y. (2020). Epidemiology of COVID-19 Among Children in China. *Pediatrics* 145, e20200702.

Dou, H., Feher, A., Davila, A. C., Romero, M. J., Patel, V. S., Kamath, V. M., et al. (2017). Role of Adipose Tissue Endothelial ADAM17 in Age-Related Coronary Microvascular Dysfunction. *Atvb* 37, 1180–1193. doi:10.1161/atvbaha.117.309430

Entrenas Castillo, M., Entrenas Costa, L. M., Vaquero Barrios, J. M., Alcalá Díaz, J. F., López Miranda, J., Bouillon, R., et al. (2020). "Effect of Calcifediol Treatment and Best Available Therapy versus Best Available Therapy on Intensive Care Unit Admission and Mortality Among Patients Hospitalized for COVID-19: A Pilot Randomized Clinical Study". *J. Steroid Biochem. Mol. Biol.* 203, 105751. doi:10.1016/j.jsbmb.2020.105751

Franceschi, C., Capri, M., Monti, D., Giunta, S., Olivieri, F., Sevini, F., et al. (2007). Inflammaging and Anti-inflammaging: a Systemic Perspective on Aging and Longevity Emerged from Studies in Humans. *Mech. Ageing Dev.* 128, 92–105. doi:10.1016/j.mad.2006.11.016

Gao, J., Chao, J., Parbhu, K.-J. K., Yu, L., Xiao, L., Gao, F., et al. (2012). Ontogeny of Angiotensin Type 2 and Type 1 Receptor Expression in Mice. *J. Renin Angiotensin Aldosterone Syst.* 13, 341–352. doi:10.1177/1470320312443720

Garvin, M. R., Alvarez, C., Miller, J. I., Prates, E. T., Walker, A. M., Amos, B. K., et al. (2020). A Mechanistic Model and Therapeutic Interventions for COVID-19 Involving a RAS-Mediated Bradykinin Storm. *Elife* 9, e59177. doi:10.7554/eLife.59177

Guo, X., Zhu, Y., and Hong, Y. (2020). Decreased Mortality of COVID-19 with Renin-Angiotensin-Aldosterone System Inhibitors Therapy in Patients with Hypertension: A Meta-Analysis. *Hypertension* 76, e13–e14. doi:10.1161/HYPERTENSIONAHA.120.15572

Gurwitz, D. (2020). Angiotensin Receptor Blockers as Tentative SARS-CoV-2 Therapeutics. *Drug Dev. Res.* 81, 537–540. doi:10.1002/ddr.21656

Hachim Ibrahim, Y., Hachim Mahmood, Y., Talaat Iman Mamdouh, López-Ozuna Vanessa, M., Saheb Sharif-Askari, N., Al Heialy, S., Rabih, Halwani., et al. (2021). The Molecular Basis of Gender Variations in Mortality Rates Associated with the Novel Coronavirus (COVID-19) Outbreak. *Front. Mol. Biosci.* 8, 894. doi:10.3389/fmolb.2021.728409

Hadjadj, J., Yatim, N., Barnabei, L., Corneau, A., Boussier, J., Smith, N., et al. (2020). Impaired Type I Interferon Activity and Inflammatory Responses in Severe COVID-19 Patients. *Science* 369, 718–724. doi:10.1126/science.abc6027

Haga, S., Yamamoto, N., Nakai-Murakami, C., Osawa, Y., Tokunaga, K., Sata, T., et al. (2008). Modulation of TNF- -converting Enzyme by the Spike Protein of SARS-CoV and ACE2 Induces TNF- Production and Facilitates Viral Entry. *Proc. Natl. Acad. Sci.* 105, 7809–7814. doi:10.1073/pnas.0711241105

Hanafy, S., Tavasoli, M., and Jamali, F. (2011). Inflammation Alters Angiotensin Converting Enzymes (ACE and ACE-2) Balance in Rat Heart. *Inflammation* 34, 609–613. doi:10.1007/s10753-010-9269-1

Huang, K. Y., Lin, M. S., Kuo, T. C., Chen, C. L., Lin, C. C., Chou, Y. C., et al. (2021). Humanized COVID-19 Decoy Antibody Effectively Blocks Viral Entry and Prevents SARS-CoV-2 Infection. *EMBO Mol. Med.* 13, e12828. doi:10.15252/emmm.202012828

Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., et al. (2005). Angiotensin-converting Enzyme 2 Protects from Severe Acute Lung Failure. *Nature* 436, 112–116. doi:10.1038/nature03712

Inde, Z., Yapp, C., Joshi, G. N., Spetz, J., Fraser, C., Deskin, B., et al. Age-dependent Regulation of SARS-CoV-2 Cell Entry Genes and Cell Death Programs Correlates with COVID-19 Disease Severity. *Preprint. Biorxiv.* Published 2020. doi:10.1101/2020.09.13.276923

Israel, A., Cicurel, A. A., Feldhamer, I., Dror, Y., Giveon, S. M., Gillis, D., et al. (2020). The Link between Vitamin D Deficiency and Covid-19 in a Large Population. *medRxiv*, 20188268, 2020 . 09.04. doi:10.1101/2020.09.04.20188268

Kamada, R., Yang, W., Zhang, Y., Patel, M. C., Yang, Y., Ouda, R., et al. (2018). Interferon Stimulation Creates Chromatin marks and Establishes Transcriptional Memory. *Proc. Natl. Acad. Sci. USA* 115, E9162–E9171. doi:10.1073/pnas.1720930115

Kaschina, E., Namsolleck, P., and Unger, T. (2017). AT2 Receptors in Cardiovascular and Renal Diseases. *Pharmacol. Res.* 125, 39–47. doi:10.1016/j.phrs.2017.07.008

Kieseier, B. C. (2011). The Mechanism of Action of Interferon-β in Relapsing Multiple Sclerosis. *CNS Drugs* 25, 491–502. doi:10.2165/11591110-000000000-00000

Koka, V., Huang, X. R., Chung, A. C. K., Wang, W., Truong, L. D., and Lan, H. Y. (2008). Angiotensin II Up-Regulates Angiotensin I-Converting Enzyme (ACE), but Down-Regulates ACE2 via the AT1-ERK/p38 MAP Kinase Pathway. *Am. J. Pathol.* 172, 1174–1183. doi:10.2353/ajpath.2008.070762

Konno, Y., Kimura, I., Uriu, K., Fukushi, M., Irie, T., Koyanagi, Y., et al. (2020). SARS-CoV-2 ORF3b Is a Potent Interferon Antagonist Whose Activity Is Increased by a Naturally Occurring Elongation Variant. *Cel Rep.* 32, 108185. doi:10.1016/j.celrep.2020.108185

Kuba, K., Imai, Y., Rao, S., Gao, H., Guo, F., Guan, B., et al. (2005). A Crucial Role of Angiotensin Converting Enzyme 2 (ACE2) in SARS Coronavirus-Induced Lung Injury. *Nat. Med.* 11, 875–879. doi:10.1038/nm1267

Lala, A., Johnson, K. W., Januzzi, J. L., Russak, A. J., Paranjpe, I., Richter, F., et al. (2020). Prevalence and Impact of Myocardial Injury in Patients Hospitalized with COVID-19 Infection. *J. Am. Coll. Cardiol.* 76, 533–546. doi:10.1016/j.jacc.2020.06.007

Lambert, D. W., Yarski, M., Warner, F. J., Thornhill, P., Parkin, E. T., Smith, A. I., et al. (2005). Tumor Necrosis Factor-α Convertase (ADAM17) Mediates Regulated Ectodomain Shedding of the Severe-Acute Respiratory Syndrome-Coronavirus (SARS-CoV) Receptor, Angiotensin-Converting Enzyme-2 (ACE2). *J. Biol. Chem.* 280, 30113–30119. doi:10.1074/jbc.m505111200

Li, Y., Xu, Q., Ma, L., Wu, D., Gao, J., Chen, G., et al. (2020). Systematic Profiling of ACE2 Expression in Diverse Physiological and Pathological Conditions for COVID-19/SARS-CoV-2. *J. Cel Mol Med* 24, 9478–9482. doi:10.1111/jcmm.15607

Li, Y., Zhou, W., Yang, L., and You, R. (2020). Physiological and Pathological Regulation of ACE2, the SARS-CoV-2 Receptor. *Pharmacol. Res.* 157, 104833. doi:10.1016/j.phrs.2020.104833

Liu, H., Wang, H., Cheng, D., Wang, Q., Pei, Z., Zhu, N., et al. (2019). Potential Role of a Disintegrin and Metalloproteinase-17 (ADAM17) in Age-Associated Ventricular Remodeling of Rats. *RSC Adv.* 9, 14321–14330. doi:10.1039/c9ra01190k

Matthews, V., Schuster, B., Schütze, S., Kallen, K-J., and Rose-John, S. (2003). Cholesterol Depletion of the Plasma Membrane Triggers Shedding of the Human Interleukin-6 Receptor by TACE and Independently of PKC. *J. Biol. Chem.* 278, 3882939. doi:10.1074/jbc.m210584200

Michard, F., and Vieillard-Baron, A. (2020). Critically Ill Patients with COVID-19: Are They Hemodynamically Unstable and Do We Know Why? *Intensive Care Med.*

Muus, C., Luecken, M. D., Eraslan, G., Waghray, A., Heimberg, G., Sikkema, L., et al. (2020). Integrated Analyses of Single-Cell Atlases Reveal Age, Sex, and Smoking Status Associations with Cell Type-specific Expression of Mediators of SARS-CoV-2 Viral Entry and Highlights Inflammatory Programs in Putative Target Cells. *Preprint bioRxiv*, 049254, 2020 04.19. doi:10.1101/2020.04.19.049254

Nicolau, L. A. D., Magalhães, P. J. C., and Vale, M. L. (2020). What Would Sérgio Ferreira Say to Your Physician in This War against COVID-19: How about Kallikrein/kinin System? *Med. Hypotheses* 143, 109886. doi:10.1016/j.mehy.2020.109886

Offringa, A., Montijn, R., Singh, S., Paul, M., Pinto, Y. M., and Pinto-Sietsma, S-J. (2020). The Mechanistic Overview of SARS-CoV-2 Using Angiotensin-Converting Enzyme 2 to Enter the Cell for Replication: Possible Treatment Options Related to the Renin-Angiotensin System. *Eur. Heart J. Cardiovasc. Pharmacother.* 6, 317–325. doi:10.1093/ehjcvp/pvaa053

Ortega-Cejas, V., Fort, J., Méndez, V., and Campos, D. (2004). Approximate Solution to the Speed of Spreading Viruses. *Phys. Rev. E Stat. Nonlin Soft Matter Phys.* 69, 031909. doi:10.1103/PhysRevE.69.031909

Ortiz, M. E., Thurman, A., Pezzulo, A. A., Leidinger, M. R., Klesney-Tait, J. A., Karp, P. H., et al. (2020). Heterogeneous Expression of the SARS-Coronavirus-2 Receptor ACE2 in the Human Respiratory Tract. *EBioMedicine* 60, 102976. doi:10.1016/j.ebiom.2020.102976

Pagliaro, P., and Penna, C. (2020). ACE/ACE2 Ratio: A Key Also in 2019 Coronavirus Disease (Covid-19)? *Front. Med.* 7, 335. doi:10.3389/fmed.2020.00335

Pairo-Castineira, E., Clohisey, S., Klaric, L., Bretherick, A. D., Rawlik, K., Pasko, D., et al. (2020). Genetic Mechanisms of Critical Illness in Covid-19. *Nature* 591 (7848), 92–98. doi:10.1038/s41586-020-03065-y

Palacios, Y., Ruiz, A., Ramón-Luing, L. A., Ocaña-Guzman, R., Barreto-Rodriguez, O., Sánchez-Monciváis, A., et al. (2021). Severe COVID-19 Patients Show an Increase in Soluble TNFR1 and ADAM17, with a Relationship to Mortality. *Ijms* 22, 8423. doi:10.3390/ijms22168423

Papola, F., Biancofiore, V., Angeletti, C., Grimaldi, A., Carucci, A. C., Cofini, V., et al. (2021). Anti-AT1R Autoantibodies and Prediction of the Severity of Covid-19. *Hum. Immunol.* S0198-8859 (21), 00251–00252. doi:10.1016/j.humimm.2021.10.006

Parekh, R. U., and Sriramula, S. (2020). Activation of Kinin B1R Upregulates ADAM17 and Results in ACE2 Shedding in Neurons. *Ijms* 22, 145. doi:10.3390/ijms22010145

Pavel, A. B., Wu, J., Renert-Yuval, Y., Del Duca, E., Glickman, J. W., Miller, R. L., et al. (2021). SARS-CoV-2 Receptor ACE2 Protein Expression in Serum Is Significantly Associated with Age. *Allergy* 76, 875–878. doi:10.1111/all.14522

Pierce, C. A., Preston-Hurlburt, P., Dai, Y., Aschner, C. B., Cheshenko, N., Galen, B., et al. (2020). Immune Responses to SARS-CoV-2 Infection in Hospitalized Pediatric and Adult Patients. *Sci. Transl. Med.* 12, eabd5487. doi:10.1126/scitranslmed.abd5487

Pinto, B. G., Oliveira, A. E. R., Singh, Y., Jimenez, L., Gonçalves, A. N. A., Ogava, R. L. T., et al. (2020). ACE2 Expression Is Increased in the Lungs of Patients with Comorbidities Associated with Severe COVID-19. *J. Inf. Dis.* 222 (4), 556–563. doi:10.1093/infdis/jiaa332

Pruimboom, L. (2020). Methylation Pathways and SARS-CoV-2 Lung Infiltration and Cell Membrane-Virus Fusion Are Both Subject to Epigenetics. *Front. Cel. Infect. Microbiol.* 10, 290. doi:10.3389/fcimb.2020.00290

Rose-John, S. (2012). IL-6 Trans-signaling via the Soluble IL-6 Receptor: Importance for the Pro-inflammatory Activities of IL-6. *Int. J. Biol. Sci.* 8, 1237–1247. doi:10.7150/ijbs.4989

Rukavina Mikusic, N. L., Silva, M. G., Pineda, A. M., and Gironacci, M. M. (2020). Angiotensin Receptors Heterodimerization and Trafficking: How Much Do They Influence Their Biological Function? *Front. Pharmacol.* 11, 1179. doi:10.3389/fphar.2020.01179

Saheb Sharif-Askari, N., Saheb Sharif-Askari, F., Alabed, M., Temsah, M.-H., Al Heialy, S., Hamid, Q., et al. (2020). Airways Expression of SARS-CoV-2 Receptor, ACE2, and TMPRSS2 Is Lower in Children Than Adults and Increases with Smoking and COPD. *Mol. Ther. - Methods Clin. Dev.* 18, 1–6. doi:10.1016/j.omtm.2020.05.013

Sodhi, C. P., Wohlford-Lenane, C., Yamaguchi, Y., Prindle, T., Fulton, W. B., Wang, S., et al. (2018). Attenuation of Pulmonary ACE2 Activity Impairs Inactivation of Des-Arg9 bradykinin/BKB1R axis and Facilitates LPS-Induced Neutrophil Infiltration. *Am. J. Physiology-Lung Cell Mol. Physiol.* 314, L17–L31. doi:10.1152/ajplung.00498.2016

Sun, M. L., Yang, J. M., Sun, Y. P., and Su, G. H. (2020). Inhibitors of RAS Might Be a Good Choice for the Therapy of COVID-19 Pneumonia. *Zhonghua Jie He He Hu Xi Za Zhi* 43, 219–222. doi:10.3760/cma.j.issn.1001-0939.2020.03.016

Sun, Y., Li, Y., Wang, M., Yue, M., Bai, L., Bian, J., et al. (2020). Increased AT2R Expression Is Induced by AT1R Autoantibody via Two Axes, Klf-5/IRF-1 and circErbB4/miR-29a-5p, to Promote VSMC Migration. *Cell Death Dis* 11, 432. doi:10.1038/s41419-020-2643-5

van de Veerdonk, F. L., Netea, M. G., van Deuren, M., van der Meer, J. W., de Mast, Q., Brüggemann, R. J., et al. (2020). Kallikrein-kinin Blockade in Patients with COVID-19 to Prevent Acute Respiratory Distress Syndrome. *Elife* 9, e57555. doi:10.7554/eLife.57555

Varela, A. A., Cheng, S., and Werren, J. H. (2021). Novel ACE2 Protein Interactions Relevant to COVID-19 Predicted by Evolutionary Rate Correlations. *PeerJ* 9, e12159. doi:10.7717/peerj.12159

Verdecchia, P., Cavallini, C., Spanevello, A., and Angeli, F. (2020). The Pivotal Link between ACE2 Deficiency and SARS-CoV-2 Infection. *Eur. J. Intern. Med.* 76, 14–20. doi:10.1016/j.ejim.2020.04.037

Wei, J., Alfajaro, M. M., DeWeirdt, P. C., Hanna, R. E., Lu-Culligan, W. J., Cai, W. L., et al. (2021). Genome-wide CRISPR Screens Reveal Host Factors Critical for SARS-CoV-2 Infection. *Cell* 184, 76–91.e13. doi:10.1016/j.cell.2020.10.028

WHO Solidarity Trial Consortium (2021). WHO Solidarity Trial Consortium. Repurposed Antiviral Drugs for Covid-19 - Interim WHO Solidarity Trial Results. *N Engl J Med.* 384, 497–511. doi:10.1016/j.cell.2020.10.028

Williamson, E. J., Walker, A. J., Bhaskaran, K., Bacon, S., Bates, C., Morton, C. E., et al. (2020). Factors Associated with COVID-19-Related Death Using OpenSAFELY. *Nature* 584, 430–436. doi:10.1038/s41586-020-2521-4

Wu, M., Chen, Y., Xia, H., Wang, C., Tan, C. Y., Cai, X., et al. (2020). Transcriptional and Proteomic Insights into the Host Response in Fatal COVID-19 Cases. *Proc. Natl. Acad. Sci. U S A.* 117, 28336–28343. doi:10.1073/pnas.2018030117

Xie, X., Xudong, X., Chen, J., Junzhu, C., Wang, X., Xingxiang, W., et al. (2006). Age- and Gender-Related Difference of ACE2 Expression in Rat Lung. *Life Sci.* 78, 2166–2171. doi:10.1016/j.lfs.2005.09.038

Xu, J., Sriramula, S., Xia, H., Moreno-Walton, L., Culicchia, F., Domenig, O., et al. (2017). Clinical Relevance and Role of Neuronal at 1 Receptors in ADAM17-Mediated ACE2 Shedding in Neurogenic Hypertension. *Circ. Res.* 121, 43–55. doi:10.1161/circresaha.116.310509

Yoon, H. E., Kim, E. N., Kim, M. Y., Lim, J. H., Jang, I. A., Ban, T. H., et al. (2016). Age-associated Changes in the Vascular Renin-Angiotensin System in Mice. *Oxid. Med. Cel. Longev.* 2016, 6731093. doi:10.1155/2016/6731093

Zhang, Q., Bastard, P., Liu, Z., Le Pen, J., Moncada-Velez, M., Chen, J., et al. (2020). Inborn Errors of Type I IFN Immunity in Patients with Life-Threatening COVID-19. *Science* 370, eabd4570. doi:10.1126/science.abd4570

Zhang, Z., Guo, L., Huang, L., Zhang, C., Luo, R., Zeng, L., et al. (2021). Distinct Disease Severity between Children and Older Adults with COVID-19: Impacts of ACE2 Expression, Distribution, and Lung Progenitor Cells. *Clin. Infect. Dis.* 2021, ciaa1911. doi:10.1093/cid/ciaa1911

Zhao, Z., Zhang, Z., Li, J., Dong, Q., Xiong, J., Li, Y., et al. (2020). Sustained TNF-α Stimulation Leads to Transcriptional Memory that Greatly Enhances Signal Sensitivity and Robustness. *eLife* 9, e61965. doi:10.7554/eLife.61965

Zhou, P., Yang, X.-L., Wang, X.-G., Hu, B., Zhang, L., Zhang, W., et al. (2020). A Pneumonia Outbreak Associated with a New Coronavirus of Probable Bat Origin. *Nature* 579, 270–273. doi:10.1038/s41586-020-2012-7

Ziegler, C. G. K., Allon, S. J., Nyquist, S. K., Mbano, I. M., Miao, V. N., Tzouanas, C. N., et al. (2020). SARS-CoV-2 Receptor ACE2 Is an Interferon-Stimulated Gene in Human Airway Epithelial Cells and Is Detected in Specific Cell Subsets across Tissues. *Cell* 181, 1016–e19. doi:10.1016/j.cell.2020.04.035

Zimmerman, P., and Curtis, N. (2020). Why Is COVID-19 Less Severe in Children? A Review of the Proposed Mechanisms Underlying the Age-Related Difference in Severity of SARS-CoV-2 Infections. *Arch. Dis. Child.* 106, 429–439. doi:10.1136/archdischild-2020-320338

Zoufaly, A., Poglitsch, M., Aberle, J. H., Hoepler, W., Seitz, T., Traugott, M., et al. (2020). Human Recombinant Soluble ACE2 in Severe COVID-19. *Lancet Respir. Med.* 8, 1154–1158. doi:10.1016/s2213-2600(20)30418-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Drug Discovery

REVIEW
published: 22 March 2022
doi: 10.3389/fddsv.2022.858006

Review of Clinical Trials of COVID-19 Vaccination Booster in SARS-CoV-2 Variants Era: To Take It or Not To Take It

Michael Zhipeng Yan[1*†], *Ming Yang*[2†] *and Ching-Lung Lai*[1*]

[1]*Department of Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong Special Administrative Region, Pokfulam, China,* [2]*Department of Ophthalmology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, Pokfulam, China*

Since the COVID-19 outbreak in China in 2019, the pandemic has spread globally. There is no definitive cure, but vaccines have greatly protected humans from symptomatic infections and severe complications. However, vaccine efficacy has been greatly reduced by the advent of SARS-CoV-2 variants worldwide. The World Health Organization has classified the variants into two groups: variants of concern (Alpha, Beta, Gamma, Delta, Omicron) and variants of interest (Lambda, Mu). Clinical trials and modifications of vaccines are currently undertaken to improve their clinical efficacies. This is particularly worrying in immunocompromised patients since breakthrough infections with multiple lineages of variants can pose a continuous threat of severe diseases in these vulnerable subjects, though there is no evidence showing immunocompromised patients are at a higher risk of vaccine-associated adverse events. However, there is no consensus on the schedule, benefits, and risks as well as contraindications (both absolute and relative) of receiving booster vaccinations. This review looks into the efficacy and safety of COVID-19 vaccination booster to guide clinical decisions on when and who to receive booster vaccination.

Keywords: COVID-19, vaccinations, booster, risks analysis, review

OPEN ACCESS

Edited by:
Bruno Villoutreix, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Om Prakash Choudhary, Central Agricultural University, India
Obasanjo Afolabi Bolarinwa, University of KwaZulu Natal, South Africa

****Correspondence:***
Michael Zhipeng Yan
u3537821@connect.hku.hk
Ching-Lung Lai
hrmelcl@hku.hk

†These authors share first authorship

Specialty section:
This article was submitted to Anti-Infective Agents, a section of the journal Frontiers in Drug Discovery

Received: *19 January 2022*
Accepted: *22 February 2022*
Published: *22 March 2022*

Citation:
Yan MZ, Yang M and Lai C-L (2022) Review of Clinical Trials of COVID-19 Vaccination Booster in SARS-CoV-2 Variants Era: To Take It or Not To Take It. Front. Drug. Discov. 2:858006. doi: 10.3389/fddsv.2022.858006

INTRODUCTION

Since the outbreak of COVID-19 infection in late 2019 in China, it has spread globally causing massive morbidity and mortality. It spreads through contact, droplets, and aerosol transmission (PriyankaChoudhary et al., 2020). The morbidity and mortality rates in developed countries slowed down because COVID-19 vaccines provided immune protection against SARS-CoV-2 (Arbel et al., 2021; Yan et al., 2021a). In spite of the high efficacy and safety profile of vaccines as reported in various studies, some patient groups are still undecided whether to receive vaccinations (Abu-Farha et al., 2021; Yigit et al., 2021a; Choudhary et al., 2021; Zhou et al., 2021). Previous studies and guidelines have documented the special considerations for different clinical special populations, and medical staff should assist in the risk–benefit analysis to make a proper clinical decision (Yan et al., 2021b; Furer et al., 2021; Powers et al., 2021; Soiza et al., 2021).

Worldwide, a significant portion of people in developed countries has completed COVID-19 vaccination schedule, while some in developing and under-developed countries have not completed basic vaccination schedules due to resources scarcity and income disparity (The Lancet Infectious

TABLE 1 | Summary of characteristics of SARS-CoV-2 variants by the WHO Classification (WHO, 2021a). a) Variants of concern.

WHO label	Pango lineage	GISAID clade	Nextstrain clade	Additional amino acid changes monitored	Earliest documented samples	Date of designation
Alpha	B.1.1.7	GRY	20I (V1)	+S: 484K +S: 452R	United Kingdom, September 2020	December 18, 2020
Beta	B.1.351	GH/501Y.V2	20H (V2)	+S: L18F	South Africa, May 2020	December 18, 2020
Gamma	P.1	GR/501Y.V3	20J (V3)	+S: 681H	Brazil, November 2020	January 11, 2021
Delta	B.1.617.2	G/478K.V1	21A	+S: 417N	India, October 2020	VOI: April 4, 2021 VOC: May 11, 2021
Omicron	B.1.1.529	GR/484A	21K	-	Multiple countries, November 2021	VUM: November 24, 2021 VOC: November 26, 2021

WHO label	Pango lineage	GISAID clade	Nextstrain clade	Earliest documented samples	Date of designation
Lambda	C.37	GR/452Q.V1	21G	Peru, December 2020	June 14, 2021
Mu	B.1.621	GH	21H	Colombia, January 2021	Aug 30, 2021

Pango lineage	GISAID clade	Nextstrain clade	Earliest documented samples	Date of designation
AZ.5	GR	-	Multiple countries, January 2021	VUM: June 2, 2021
C.1.2	GR	-	South Africa, May 2021	September 1, 2021
B.1.617.1	G/452R.V3	21B	India, October 2020	VOI: April 4, 2021 VUM: September 20, 2021
B.1.526	GH/253G.V1	21F	United States, November 2020	VOI: March 24, 2021 VUM: September 20, 2021
B.1.525	G/484K.V3	21D	Multiple countries, December 2020	VOI: March 17, 2021 VUM September 20, 2021
B.1.630	GH	-	Dominic Republic, March 2021	October 12, 2021
B.1.640	GH/490R	-	Republic of Congo, September 2021	November 22, 2021

VOC, variants of concern; VOI, variants of interest; VUM, variants under monitoring.
VOI, variants of interest; VUM, variants under monitoring.

Diseases, 2021; Van De Pas et al., 2022). Vaccination does not confer sufficient lifelong protection: post-vaccination humoral responses decrease after 3–6 months (Chemaitelly et al., 2021; Levin et al., 2021; Erice et al., 2022). Early studies on booster vaccination show that the infection rate in the booster cohort is lower compared with those without receiving a booster (Bar-On et al., 2021). Of even greater concern, vaccine efficacy dwindles when it comes to Delta- and Omicron-related variant infections, which is associated with higher viral load and transmissibility (Campbell et al., 2021; Torjesen, 2021). Patients without sufficient protective efficacy run a higher risk of symptomatic infection, severe hospitalization, mortality, and long COVID-19 syndrome (Yan et al., 2021c).

This prompts a key question: who should receive a booster 6 months after the last dose since vaccination per se may be associated with risks? This paper evaluates the risk and benefits of booster vaccination to assist clinical decision making. Literature data were retrieved from electronic databases (PubMed, Medline, Scopus, Cochrane, Google Scholar) on December 14, 2021, with the following keywords: COVID-19, Vaccine, Booster, and Variants. Related articles are reviewed to address the key question.

COVID-19 Variants

Since the outbreak in 2019, various variants have been identified worldwide. The transmissibility and mortality of SARS-CoV-2 have been changing with the new mutations. Various reports have shown the reduced efficacy of vaccines to neutralize SARS-CoV-2 variants. WHO has proposed the classification of SARS-CoV-2 variants into two major types: variants of concern (VOC) and variants of interest (VOI) (WHO, 2021a). Their characteristics are listed in **Table 1** (WHO, 2021a). In view of the emerging variants globally, vaccine efficacy against different variant strains are recorded in **Table 2**.

BENEFITS OF BOOSTER VACCINATION

Waning humoral responses have been reported worldwide 6 months after completion of vaccination (one to three doses, depending on vaccination design) (Levin et al., 2021; Chemaitelly et al., 2021; Shrotri et al., 2021). This is particularly evident in men, participants older than 65 years old, or people with immunosuppression (Levin et al., 2021) for BNT162b2 (Pfizer-BioNTech) recipients. In spite of the waning antibody titer after 6 months of completion of BNT162b2 (Pfizer-BioNTech) vaccination, the protection against hospitalization and death persists at a robust level. The estimated effectiveness of BNT162b2 (Pfizer-BioNTech) against SARs-CoV-2 infection peaked at 77.5% (95% CI, 76.4–78.6) in the first month after the second dose, but it progressively dropped to 20% 5 months after the second dose (Chemaitelly et al., 2021). Thus, reinfection by SARS-CoV-2 is possible (Cromer et al., 2021). Vaccine

TABLE 2 | COVID-19 vaccine efficacy against symptomatic infection 14 days after complete vaccination schedule (without booster) against variant strains 2 weeks after administration, stratified by region.

	BNT162b2 (Pfizer), 2-dose	mRNA-1273 (Moderna), 2-dose	ChAdOx1 nCoV-19 (AZD 1222), 2-doses	Ad26.COV2.S (Janssen), 1-dose	Coronavac (Sinovac), 2-dose
Alpha (B.1.1.7)	United Kingdom: 93.7% (Lopez Bernal et al., 2021) (95% CI, 91.6–95.3) Qatar: 89.5% (Abu-Raddad et al., 2021) (95% CI, 85.9–92.3) Canada: 89% (Nasreen et al., 2021) (95% CI, 87–90)	Canada: 92% (Nasreen et al., 2021) (95% CI, 88–95) France: 86% (Charmet et al., 2021) (95% CI, 81–90)	United Kingdom: 74.5% (Lopez Bernal et al., 2021) (95% CI, 68.4–79.4) Canada: 91% (Nasreen et al., 2021) (95% CI, 62–98)	Multinational: 69.7% (Sadoff et al., 2022) (95% CI, 60.7–76.9)	Not reported
Beta (B.1.351)	Qatar:75% (Abu-Raddad et al., 2021) (95% CI, 70.5–78.9) Canada: 82% (Nasreen et al., 2021) (95% CI, 65–91) France: 49% (elderly) (Lefevre et al., 2021) (95% CI, 14–69)	Canada: 89% (Nasreen et al., 2021) (95% CI, 21–98) France: 77% (Charmet et al., 2021) (95% CI, 63–86)	Canada: 41% (Nasreen et al., 2021) (95% CI, 12–60) US: 21.9% (Madhi et al., 2021) (95% CI, −49.9 to 59.8)	South Africa: 52% (Sadoff et al., 2021) (95% CI, 30.3–67.4) Multinational: 51.9% (Sadoff et al., 2022) (95% CI, 19.1–72.2)	Not reported
Gamma (P.1)	Canada: 82% (Nasreen et al., 2021) (95% CI, 65–91)	Canada: 89% (Nasreen et al., 2021) (95% CI, 21–98) France: 77% (Charmet et al., 2021) (95% CI, 63–86)	Canada: 41% (Nasreen et al., 2021) (95% CI, 12–60)	Multinational: 36.4 (Sadoff et al., 2022) (95% CI, 13.9–53.2)	Brazil:50.7% (Palacios et al., 2020) (95% CI, 35.6–62.2) Brazil:47% (Ranzani et al., 2021) (95% CI, 39–54) Turkey: 84% (Tanriover et al., 2021) (95% CI, 65.0–92.0) Chile: 66.6% (Jara et al., 2021) (95% CI, 65.4–67.8)
Delta (B.1.617.2 and AY lineages)	United Kingdom: 88% (Lopez Bernal et al., 2021) (95% CI, 85.3–90.1) Canada: 92% (Nasreen et al., 2021) (95% CI, 90–94) Israel: 88% (Reis et al., 2021) (95% CI, 85–90)	Canada: 95% (Nasreen et al., 2021) (95% CI, 1–97) Qatar:51.9% (Tang et al., 2021b) (95% CI, 47.0–56.4) US: 86.7% (Bruxvoort et al., 2021) (95% CI, 84.3–88.7)	United Kingdom: 67.0% (Lopez Bernal et al., 2021) (95% CI, 61.3–71.8) Canada: 87% (Nasreen et al., 2021) (95% CI, 69–95)	Multinational: 6.0 (Sadoff et al., 2022) (95% CI, −178.3 to 59.2)	Not reported
Omicron	South Africa: 70% (Collie et al., 2022) (95% CI, 62–76)	US: 30.4% (Tseng et al., 2022) (95% CI, 5–49)	Not reported	South Africa[a]: 63% (Gray et al., 2021) (95% CI, 31–81)	Not reported
Lambda (C.37)	Brazil: 66.5% (Zuckerman et al., 2021) (95% CI, 42.8–103.4)	Not reported	Not reported	Multinational: 10.0 (Sadoff et al., 2022) (95% CI, −39.5 to 42.0)	Not reported
Mu (B.1621; B.1.621.1)	Not reported	US: 90.4% (Bruxvoort et al., 2021) (95% CI, 73.9–96.5)	Not reported	Multinational: 35.8 (Sadoff et al., 2022) (95% CI, 1.5–58.6)	Not reported

[a]*Vaccine efficacy of two doses of Ad26.COV2.S (Janssen), 2 weeks after vaccination.*

protection against major variants of SARS-CoV-2 is summarized in **Tables 2–8**. One of the major concerns is that all vaccines express the ancestral SARS-CoV-2 spike protein, whereas currently circulating variants such as Delta variant possess several mutations to evade the response, resulting in a 4-fold lower neutralizing antibody response to Delta-variant infections (Kent and Juno, 2021).

Vaccination Efficacy Against Variants of Concern

VOC include Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) variants. They have been associated with higher transmissibility and reduced vaccination efficacy. Despite being infected with B.1.1.7 (Alpha) and B.1.351

TABLE 3 | BNT162-b2 (Pfizer-BioNTech) vaccine efficacy (without booster) against different clinical severity in variant strain infection 2 weeks after administration.

Doses	Severity of illness	Alpha	Beta	Gamma	Delta	Omicron
1	Asymptomatic	38% (95% CI, 29–45) (Sheikh et al., 2021)	17% (95% CI, 10–23) (Abu-Raddad et al., 2021)	Not reported	30% (95% CI, 17–41) (Sheikh et al., 2021)	Not reported
	Symptomatic	27% (95% CI, 13–39) (Sheikh et al., 2021)	43% (95% CI, 22–59) (Chung et al., 2021)	43% (95% CI, 22–59) (Chung et al., 2021)	33% (95% CI, 15–47) (Sheikh et al., 2021)	Not reported
	Hospitalization	83% (95% CI, 62–93) (England, 2021; Stowe et al., 2021)	0% (95% CI, 0–19) (Abu-Raddad et al., 2021)	56% (95% CI, −9 to 82) (Chung et al., 2021)	94% (95% CI, 46–99) (England, 2021; Stowe et al., 2021)	Not reported
2	Asymptomatic	92% (95% CI, 90–93) (Sheikh et al., 2021)	75% (95% CI, 71–79) (Abu-Raddad et al., 2021)	Not reported	79% (95% CI, 75–82)	Not reported
	Symptomatic	92% (95% CI, 88–94) (Sheikh et al., 2021)	88% (95% CI, 61–96) (Chung et al., 2021)	88% (95% CI, 61–96) (Chung et al., 2021)	83% (95% CI, 78–87)	Not reported
	Hospitalization	95% (95% CI, 78–99) (England, 2021; Stowe et al., 2021)	100% (95% CI, 74–100) (Abu-Raddad et al., 2021)	100% (Chung et al., 2021) (95% CI not reported)	96% (95% CI, 86–99) (England, 2021; Stowe et al., 2021)	Not reported

TABLE 4 | mRNA-1273 (Moderna) vaccine efficacy (without booster) against different clinical severity in variant strain infection 2 weeks after administration.

Doses	Severity of illness	Alpha	Beta	Gamma	Delta	Omicron
1	Asymptomatic	Not reported	Not reported	Not reported	Not reported	
	Symptomatic	61% (95% CI, 56–66) (Chung et al., 2021)	56% (Chung et al., 2021) (95% CI, −9 to 82)		77.0% (Bruxvoort et al., 2021) (95% CI, 60.7–86.5)	Not reported
	Hospitalization	59% (95% CI, 39–73) (Chung et al., 2021)				Not reported
2	Asymptomatic	98.4% (Bruxvoort et al., 2021) (95% CI, 96.9–99.1%)	Not reported	Not reported	86.7% (Bruxvoort et al., 2021) (95% CI, 84.3–88.7)	Not reported
	Symptomatic	90% (95% CI, 85–94) (Chung et al., 2021)	88% (Chung et al., 2021) (95% CI, 61–96)		94.1% (Bruxvoort et al., 2021) (95% CI, 90.5–96.3)	30.4% (Tseng et al., 2022) (95% CI, 5–49)
	Hospitalization	94% (95% CI, 59–99) (Chung et al., 2021)	100% (Chung et al., 2021) (95% CI not reported)		97.5% (Bruxvoort et al., 2021) (95% CI, 92.7–99.2)	100%[a] (Tseng et al., 2022)

[a]*No hospitalization case after receiving two doses of vaccine.*

TABLE 5 | ChAdOx1 nCoV-19 (AZD 1222) vaccine efficacy (without booster) against different clinical severity in variant strain infection 2 weeks after administration.

Doses	Severity of illness	Alpha	Beta	Gamma	Delta	Omicron
1	Asymptomatic	37% (95% CI, 32–42) (Sheikh et al., 2021)	10.4% (95% CI, −76.8 to 54.8) (Madhi et al., 2021)	33% (95% CI, 32–34) (Cerqueira-Silva et al., 2021)	18% (95% CI, 9–25) (Sheikh et al., 2021)	Not reported
	Symptomatic	39% (95% CI, 32–45) (Sheikh et al., 2021)	21.9% (95% CI, −49.9 to 59.8) (Madhi et al., 2021)	33% (95% CI, 26–40) (Hitchings et al., 2021)	33% (95% CI, 23–41) (Sheikh et al., 2021)	Not reported
	Hospitalization	76% (95% CI, 61–85) (England, 2021; Stowe et al., 2021)	61% (95% CI,−64 to 91) (Nasreen et al., 2021)	52% (95% CI, 50–53) (Cerqueira-Silva et al., 2021)	71% (95% CI, 51–83) (England, 2021; Stowe et al., 2021)	Not reported
2	Asymptomatic	73% (95% CI, 66–78) (Sheikh et al., 2021)	Not reported	70% (95% CI, 69–71) (Cerqueira-Silva et al., 2021)	60% (95% CI, 53–66) (Sheikh et al., 2021)	Not reported
	Symptomatic	81% (95% CI, 72–87) (Sheikh et al., 2021)	10% (95% CI, −77 to 50) (Madhi et al., 2021)	78% (95% CI, 69–84) (Hitchings et al., 2021)	61% (95% CI, 51–70) (Sheikh et al., 2021)	Not reported
	Hospitalization	86% (95% CI, 53–96) (England, 2021; Stowe et al., 2021)	Not reported	87% (95% CI, 85–88) (Cerqueira-Silva et al., 2021)	92% (95% CI, 75–97) (England, 2021; Stowe et al., 2021)	Not reported

(Beta) variants after mRNA-based vaccination, the protection against severe, critical, or fatal COVID-19 cases remains 96% in Qatar (Chemaitelly et al., 2021). The vaccine efficacy against symptomatic infection quickly drops from over 90% before the spread of Delta variant to 42–80% after the spread of the variant (Tang et al., 2021a; Pouwels et al., 2021; Sheikh et al., 2021). This

TABLE 6 | Ad26.COV2.S (Janssen) vaccine efficacy (without booster) against different clinical severity in variant strain infection 2 weeks after administration.

Doses	Severity of illness	Alpha	Beta	Gamma	Delta	Omicron	Lineage B.1
1	Mild	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported
	Moderate to severe	70.2 (95% CI, 35.3–87.6) (Sadoff et al., 2022)	64% (95% CI, 41–79) (Alter et al., 2021) 51.9% (95% CI, 19.1–72.2) (Sadoff et al., 2022)	36.5% (95% CI, 14.1–53.3) (Sadoff et al., 2022)	−5.7% (95% CI, −177.7 to 59.2) (Sadoff et al., 2022)	Not reported	72% (95% CI, 58–82) (Zhukova et al., 2020)
	Severe to critical		64% (95% CI, 46–95) (Alter et al., 2021) 51.9% (95% CI, 19.1–72.2) (Sadoff et al., 2022)		71% (95% CI, 56–81) (CDC, 2021b)	Not reported	86% (95% CI, −9 to 100) (Zhukova et al., 2020)

TABLE 7 | Sputnik V COVID-19 Vaccine (Gam-COVID-Vac) vaccine efficacy (without booster) against different clinical severity in variant strain infection 2 weeks after administration.

Doses	Severity of illness	Alpha	Beta	Gamma	Delta	Omicron
1	Asymptomatic	Not reported	Not reported	Not reported	Not reported	Not reported
	Symptomatic	Not reported	Not reported	Not reported	Not reported	Not reported
	Hospitalization	Not reported	Not reported	Not reported	35% (95% CI, −21 to 65) (Barchuk et al., 2021)	Not reported
2	Asymptomatic	Not reported	Not reported	Not reported	Not reported	Not reported
	Symptomatic	Not reported	Not reported	Not reported	Not reported	Not reported
	Hospitalization	Not reported	Not reported	Not reported	81% (95% CI, 68–88) (Barchuk et al., 2021)	Not reported

TABLE 8 | Coronavac (Sinovac) COVID-19 vaccine efficacy (without booster) against different clinical severity in variant strain infection 2 weeks after administration.

Doses	Severity of illness	Alpha	Beta	Gamma	Delta	Omicron
1	Asymptomatic	Not reported	Not reported	16% (95% CI, 15–17) (Cerqueira-Silva et al., 2021)	Not reported	Not reported
	Symptomatic	Not reported	Not reported	Not reported	14% (95% CI, −60 to 55) (Li et al., 2021)	Not reported
	Hospitalization	Not reported	Not reported	27% (95% CI, 25–28) (Cerqueira-Silva et al., 2021)	Not reported	Not reported
2	Asymptomatic	Not reported	Not reported	54% (95% CI, 53–55) (Cerqueira-Silva et al., 2021)	59% (95% CI, 16–82) (Li et al., 2021)	Not reported
	Symptomatic	Not reported	Not reported	Not reported	Not reported	Not reported
	Hospitalization	Not reported	Not reported	73% (95% CI, 72–74) (Cerqueira-Silva et al., 2021)	100% (Kang et al., 2021) (95% CI not reported)	Not reported

efficacy also dwindles as time elapses (Barouch et al., 2021; Ciabattini et al., 2021; Naaber et al., 2021; Pegu et al., 2021).

However, recent studies have shown that the mRNA-based booster vaccine still induces a robust immune response to variants, though weaker when compared with its prototype. The mRNA-based booster vaccination is safe and well tolerated (Ebinger et al., 2021). It boosts antibody production to neutralize variant strains especially wild-type D614G ($p < 0.0001$). The neutralization titers against B.1.351 (Beta), P.1 (Gamma), and B.1.617.2 (Delta) are either low or undetectable 1 month after vaccination (Ebinger et al., 2021). The vaccine-mediated protection against variants in the respiratory tract is durable but delayed (Gagne et al., 2021). It is dependent on anamnestic antibody responses which can be maximized by a booster dose (Gagne et al., 2021).

Upon receiving BNT162b2 (Pfizer-BioNTech) booster vaccination, the infection and severe illness rates are lower, compared with those without booster vaccination in Israel (Bar-On et al., 2021). A third-dose booster vaccine in Israel

showed a significant reduction of confirmed infection and severe illness by a factor of 11.3 and 19.9, respectively (Bar-On et al., 2021). This finding is consistent with another serological study which showed that the occurrence of breakthrough infection with SARS-CoV-2 correlated with neutralizing antibody titers during the peri-infection period (Bergwerk et al., 2021). Therefore, an elevated titer is protective against breakthrough infections. This is particularly important in ambulatory and inpatient settings in less developed countries where the resources are scarce and the hospital wards are usually crowded to favor transmission. A similar booster recommendation is recommended in recipients of CoronaVac since over one-fifth of recipients become seronegative 2 months after the second dose of vaccination (Yigit et al., 2021b). Vaccination protects patients in ambulatory and inpatient care settings (Thompson et al., 2021). A full-dose mRNA-based vaccination (even without booster) is associated with 90% (95% CI, 86–93) effectiveness against intensive care unit (ICU) admission and 91% (95% CI, 89–93) effectiveness against emergency care visits. The effectiveness of Ad26.COV2.S is 68% (95% CI, 50–79) against hospitalization and 73% (95% CI, 59–82) against emergency care visits.

Various studies show a close association between infection rate and serological titer of circulating antibody levels after vaccination (Bergwerk et al., 2021; Khoury et al., 2021). The vaccination regimen of ChAdOx1 nCoV-19 also recommends a booster regime since it induces multifunctional antibody responses, including antibody-dependent neutrophil/monocyte phagocytosis, complement activation, and natural killer activation (Barrett et al., 2021).

Some special populations are at high risks of breakthrough infections. The incidence risk ratio in the immunocompromised patient is 1.66 (95% CI, 1.17–2.35) even after full-dose mRNA-based vaccination. An increased incidence is observed over time, showing the decreasing protective efficacy as time elapsed. However, among those with COVID-19 infection, vaccination significantly reduces the risk of death (hazard ratio 0.20, 95% CI, 0.08–0.49) (Liu et al., 2021a).

Vaccination Efficacy Against Variant of Interest

VOI include Lambda (C.37) and Mu (B.1.621) variants. A study using a micro-neutralization assay following mRNA vaccine demonstrated a 1.6-fold reduction of neutralizing titers compared with the wild-type virus, increasing the likelihood of infection and disease transmission after vaccination (Zuckerman et al., 2021). The Lambda variant harbors two key mutations in the receptor-binding domain (RBD), L452Q and F490S, changing the antigenicity and infectivity. Cell line studies show that the convalescent and vaccine-based sera recorded 1.3- to 2.5-fold lower neutralizing antibody titers (Wang et al., 2021). The reason of partial escape from neutralizing antibodies in vaccinated individuals behind is closely related to the increased affinity between RBD and angiotensin-converting enzyme 2 (ACE2) binding, leading to increased processing of spike protein to yield a higher fusion activity and syncytium formation in these variants (Moghaddar et al., 2021). The antibody titer drop after 6 months against Lambda variant is 3-fold, leading to breakthrough infections (Liu et al., 2021b). The rapidly dropping antibody titers against Lambda and Mu infections can be countered by a third-dose booster to cope with the surge of variants transmission.

The Mu variant demonstrates a remarkable resistance to antibodies by convalescent plasma and vaccine-induced protection (Uriu et al., 2021). Pseudovirus model serological assay was performed on Mu variant infection. It was 9.1 times as resistant as the parenteral virus in response to mRNA-based vaccination (BioNTech), 2.0 times as resistant to neutralization by convalescent serum, and 1.5 times as resistant to neutralization by vaccine serum as the Beta variant. Similar resistance has been reported in inactivated vaccine-elicited antibodies (Xie et al., 2021).

RISKS OF BOOSTER VACCINATION

A booster vaccination is not without risks. Myocarditis has been reported worldwide, particularly in young male recipients after receiving the second dosage of BNT162b2 (Pfizer-Biotech) vaccine (Mevorach et al., 2021). The risk ratio 30 days after the second dose in fully vaccinated recipients (without booster) is 2.35 (95% CI, 1.10–5.02), while the risk ratio rises to 8.96 (95% CI, 4.50–17.83) in male subjects between 16 and 19 years old. It is uncertain whether the third dose will trigger further myocardial damage. However, severe complications such as myocarditis or allergy from the first two doses of BNT162b2 are relatively rare (Barda et al., 2021). Guillain–Barre syndrome has also been reported in adenovirus-vectored COVID-19 vaccines (WHO, 2021b). For the majority of fully vaccinated recipients who have no significant associated complications, a booster vaccination may be safer.

The booster may greatly safeguard the effect offered by the first two dosages. When serum antibody titer drops below the protective threshold, a "start over" vaccination may still be necessary, which may also trigger complications. Studies also reveal that antibody levels in the aged population are relatively lower after full vaccination, though no severe complications are observed (Wei et al., 2021). Therefore, a booster vaccination may be necessary for this population.

Overall, vaccination is very safe, though common transient side effects happen, and a booster is a good option that significantly consolidates the protective effect to fight against this long-term pandemic. It is recommended that the earliest time to receive a booster is 6 months after the initial vaccination (Chemaitelly et al., 2021; Levin et al., 2021). A future study evaluating long-term serum antibody titer and booster clinical trials are needed for guidance on booster vaccination.

WHETHER TO ADVOCATE BOOSTER VACCINATION

Though booster vaccination is associated with both benefits and risks, overall it is safe and effective. The BNT162b2 third-dose booster vaccination in Israel showed that the side effects were

mild and self-limiting, including immunocompromised patients and senior citizens (Shapiro Ben David et al., 2021). The most common side effects were fatigue (19.6%), myalgia (9.2%), and fever (8.1%) in immunocompromised patients and fatigue (21.3%), myalgia (9.9%), and fever (9.2%) in senior citizens. Over two-thirds of the recipients developed a better or similar response compared to the second dose. This is consistent with the study in the United Kingdom which showed that although there were numerous local or systemic side effects in the short term, the vaccine efficacy exceeded 60% within 2–3 weeks (Menni et al., 2021).

One concern of advocating booster vaccination is related to equity (The Lancet Infectious Diseases, 2021). The income disparity between developed countries and developing countries results in a competition for gaining access to vaccinations. Up to August 9, 2021, over 80% of the vaccines were distributed to high-income countries, while only 20% were in low-income countries with only 3% of the African population fully vaccinated (without a booster) (Kherabi et al., 2021; The Lancet Infectious Diseases, 2021). This results in increasing reporting of variants in different countries, in particular from low-income countries (see **Supplementary Appendix** with reports of different variants reported internationally). Vaccines thus should be made available to other countries before offering domestic booster vaccinations to reduce variants transmission and new variants from evolving (Dyer, 2021; Schaefer et al., 2021).

The vaccination schedule should be individualized for different populations, such as healthy individuals without previous SARS-CoV-2 infection and COVID-19 survivors with prior SARS-CoV-2 infection. The underlying reason is related to the waning trajectory of antibody titers in different populations. After a two-dose schedule of mRNA-based vaccination, healthy subjects developed similar antibody levels to COVID-19 survivors who recover from the infection for 1 year and receive single-dose mRNA-based vaccination (Gluck et al., 2021). None of the study participants experienced reinfection. The half-life of anti-SARS-CoV-2 IgG antibody ranges between 85 and 158 days (Lumley et al., 2021; Dan et al., 2021; den Hartog et al., 2021), while some studies also show the immunity may last over 300 days (Gluck et al., 2021; Lee et al., 2021). There is uncertainty about the booster schedule for different populations, and this may create a burden on healthcare service and spark controversy on vaccine equity between developing countries and developed countries (The Lancet Infectious Diseases, 2021). Overall, vaccine boosters quickly increase antibody levels since the large number of memory B cells and plasma cells derived from previous immunity drives rapid antibody productions after booster vaccination (Ebinger et al., 2021; Gobbi et al., 2021; Liao et al., 2021; Turner et al., 2021). Booster vaccination thus is protective against SARS-CoV-2 infection, but the booster schedule requires further studies.

Though vaccine recipients may have more persistent nasopharynx-homing SARS-CoV-2-specific T cells compared to infection-naïve subjects (Neidleman et al., 2021), a further booster vaccination still induces a stronger immune response while giving manageable side effects (Ebinger et al., 2021).

CURRENT RECOMMENDATION ON BOOSTER VACCINATION

The US Center for disease Control and Prevention and French National Authority of Health have recommended prioritization of booster vaccination for high-risk groups, subjects aged over 65 years, subjects between 50 and 64 years with underlying medical conditions, healthcare workers, and residents of long-term care facilities (Burki, 2021; Kherabi et al., 2021). The United Kingdom is currently considering a third-shot booster vaccination for all adults (Burki, 2021).

Healthcare workers are at high risk of infection. The British study of BNT162b2 vaccine showed vaccine effectiveness of 70% (95% CI 55–85) 21 days after the first dose and 85% (95% CI 74–96) 7 days after two doses in healthcare staff when the dominant variant was Alpha (B.1.1.7) (Hall et al., 2021).

The timing to receive booster vaccination is subject to debate. A study focusing on the immunogenicity and efficacy of ChAdOx1 nCoV-19 (AZD1222) recommends a 3-month interval to receive a booster since a high protective efficacy is maintained until 3 months (Voysey et al., 2021). Efficacy was higher with a 12-week prime–boost interval (vaccine efficacy 81.3%, 95% CI 60.3–91.2), compared with a 6-week interval (vaccine efficacy 55.1%, 95% CI 33.0–69.9). Meanwhile, BNT162b2 (Pfizer) vaccine recommends a 6-month interval to receive a booster (CDC, 2021a).

Whether subjects with previous SARS-CoV-2 should receive a three-dose vaccination schedule and when they should receive the booster vaccination as antibody titers drop are still under debate. The antibody responses to the first vaccine dose in individuals with pre-existing immunity are equal to or exceed the titers found in naïve individuals after the second dose of BNT162b2 (Pfizer-BioNTech). The antibody titers against VOC, Delta variant (1.617.2), in COVID-19 recoverers (at least 11 months after complete resolution of the previous infection) receiving one-dose ChAdOx1 nCoV-19 vaccine are similar to or higher than the counterparts receiving two-dose BNT162b2 (Pfizer-BioNTech) (Havervall et al., 2021). BBV152 (Bharat Biotech) vaccine of India also induces a similar response in COVID-19 recoverers, suggesting one-dose vaccinations can give similar antibody levels as two-dose vaccinations (Kumar et al., 2021). Their adverse reaction to vaccination is higher than those of healthy adults (Krammer et al., 2021). COVID-19 recoverers receiving a second-dose vaccination show no additional benefits since they have reached the peak of their immunity after the first dose (Lozano-Ojalvo et al., 2021). These studies suggest that one-dose vaccinations in COVID-19 recoverers is sufficient to confer protection non-inferior to healthy individuals with two-dose vaccinations (Cavalcanti et al., 2021; Vicenti et al., 2021), and they should be put lower on the vaccination priority list (Saadat et al., 2021). Despite the similar efficacy, the post-vaccine symptoms are more prominent for those with prior infection after the first dose (Ebinger et al., 2021).

TABLE 9 | Absolute contraindications of vaccinations.

Absolute contraindications	Type of vaccine	Recommended actions
Severe allergic reaction, e.g., anaphylaxis	All (USCDC, 2021a)	1. Do not vaccinate 2. Referral to allergy immunologist 3. Consider other vaccine alternatives
Immediate allergic reaction	All (USCDC, 2021a)	1. Risk assessment 2. Referral to allergy immunologist 3. Prolong observation period after vaccination (e.g., 30 min)

TABLE 10 | Common relative contraindications of vaccinations reported in literatures and guidelines.

Relative contraindications	Type of vaccine	Recommended actions
Acute PCR-confirmed COVID-19 infection	All	Delay vaccination schedule until recovered from acute illness and the criteria for ending isolation have been met (WHO, 2021c)
With fever more than 38.5°C	All	Postpone vaccination until fever subsided (WHO, 2021c)
High thrombosis and thrombocytopenia risk	AstraZeneca/COVISHIELD and Janssen	Cautious for patients with history of heparin-induced thrombocytopenia, antiphospholipid syndrome, or major venous or arterial thrombosis with thrombocytopenia after viral vector COVID-19 vaccine (EMA, 2021a; USCDC, 2021b)
Capillary leak syndrome (CLS)	AstraZeneca COVISHIELD	Patients with history of CLS should not receive AstraZeneca vaccine. Vaccination with alternative vaccine is recommended (NACI, 2021)
Myocarditis and pericarditis	Pfizer and Moderna	Defer the second dose schedule if patients developed myocarditis or pericarditis after the first dose. Choice of alternative vaccine or continue with mRNA vaccine should be discussed with medical workers (cardiologist if possible) (WHO, 2021d; NACI, 2021)
Pregnancy, planning for pregnancy or breastfeeding	Viral-vector vaccines	USCDC recommends safe administration of viral-vector vaccine in all trimesters of pregnancy and breastfeeding. (as of August 11, 2021) (USCDC, 2021c) Canadian NACI recommends viral-vector vaccines should be avoided in pregnancy due to elevated risk of VITT (NACI, 2021). Vaccination is safe during breastfeeding (NACI, 2021)

NACI, National Advisory Committee on Immunization; USCDC, United States Center for disease Control and Prevention; VITT, vaccine-induced immune thrombotic thrombocytopenia.

CONTRAINDICATIONS FOR VACCINATION

The absolute and relative contraindications of vaccination are documented in **Tables 9** and **10**. Absolute contraindications include mainly severe allergic reactions to its constituents shown in **Table 9** (USCDC, 2021a). Relative contraindications and recommended actions are listed in **Table 10**. Subjects with relative contraindications are recommended to discuss individual risk profiles to plan their vaccination decision. Counseling should include risk factors, relative contraindications, benefits and risks of vaccinations, alternative vaccines, and risks of without vaccinations. **Table 11** documents the ingredients of 24 COVID-19 vaccines with emergency use authorizations by national regulatory authorities (as of October 26, 2021).

RECOMMENDATIONS FOR BOOSTER VACCINATION

Heterologous vaccination (Com-COV study) is safe and induces robust immunity without serious adverse events (Borobia et al., 2021; Liu et al., 2021c; Moghnieh et al., 2021). This has been performed in subjects receiving "BNT162b2 (Pfizer/BioNTech) plus ChAdOx1 nCoV-19 (AZD1222)" and "BNT162b2 (Pfizer/BioNTech) plus BBIBP-Cor-V (Sinopharm)." The interim analysis documents that heterologous ChAdOx/BNT immunization regimen with 10–12 weeks vaccination interval is well tolerated and slightly more immunogenic compared to homologous BNT/BNT vaccination with 3-week intervals. A recent randomized controlled trial showed that heterologous vaccination with other vaccines after the initial two doses (either two-dose ChAdOx-1 or BioNTech) yields a higher SARS-CoV-2 anti-spike IgG titer and stronger cellular response (Munro et al., 2021). This may be a viable choice for countries without a stable source of vaccines or in immunocompromised patients who could not produce sufficient protective antibodies (Borobia et al., 2021; Liu et al., 2021c; Hillus et al., 2021; Moghnieh et al., 2021). Initial SARS-CoV-2 vaccination response can predict booster response; thus, reassessment of antibody response may be a viable choice whether to receive homologous or heterologous vaccination (Perkmann et al., 2021).

Timing of vaccinations is vital in certain patients after specific treatments. Suboptimal immunological response has been found in patients receiving BNT162b2 (Pfizer-BioNTech) after rituximab administration (Kant and Geetha, 2021). Delaying vaccination for 6 months after rituximab administration or B-cell reconstitution has been suggested

TABLE 11 | Components of 24 COVID-19 vaccines with emergency use authorizations by national regulatory authorities (as at October 26, 2021).

Type of vaccine	Active ingredient	Inactive ingredients
Pfizer (mRNA) (USFDA, 2021a), the United States	Nucleoside-modified mRNA encoding the viral spike (S) glycoprotein of SARS-CoV-2	1. 2-Polyethylene glycol (PEG)-2000-N, N-ditetradecyclacetamide 2. Cholesterol 3. 1,2-Distearoyl-sn-glycero-3-phosphocholine 4. (4-Hydroxybutyl) azanediyl)bis (hexane-6,1-diyl)bis (2-hexyldecanoate) 5. Sodium chloride 6. Monobasic potassium phosphate 7. Potassium chloride 8. Dibasic sodium phosphate dihydrate 9. Sucrose
Moderna (mRNA) (USFDA, 2021b), the United States	Nucleoside-modified mRNA encoding the viral spike (S) glycoprotein of SARS-CoV-2	1. PEG2000-DMG: 1,2-dimyristoyl-rac-glycerol,methoxypolyethylene glycol 2. 1,2-Distearoyl-sn-glycero-3-phosphocholine 3. Cholesterol 4. SM102: heptadecane-9-yl 8- ((2-hydroxyethyl) (6-oxo-6 (undecyloxyl)hexyl)amino) octanoate 5. Tromethamine 6. Tromethamine hydrochloride 7. Acetic acid 8. Sodium acetate 9. Sucrose
Janssen (viral vector) (USFDA, 2021c), the United States	Recombinant, replication incompetent Ad26 vector encoding a stabilized variant of the SARS-CoV-2 spike (S) protein	1. Polysorbate-80 2. 2-Hydroxypropyl-beta-cyclodextrin 3. Citric acid monohydrate 4. Trisodium citrate dihydrate 5. Sodium chloride 6. Ethanol
Sinovac/Coronavac (Vero cell) (Ltd SLSC, 2021), China	Inactivated SARS-CoV-2 virus (CZ02 strain)	1. Aluminum hydroxide 2. Disodium hydrogen dodecahydrate 3. Sodium dihydrogen phosphate monohydrate 4. Sodium chloride
Oxford-AstraZeneca Vaxzevria (EMA, 2021b), the United Kingdom	Chimpanzee adenovirus encoding the SARS-CoV-2 spike (S) protein ChAdOx1-S	1. L-Histidine 2. L-Histidine hydrochloride monohydrate 3. Magnesium chloride hexahydrate 4. Polysorbate 80 (E 433) 5. Sucrose 6. Disodium edetate (dihydrate)
Serum Institute of India Covishield (Oxford-AstraZeneca formulation) (Jeewandara et al., 2021); Ramasamy et al., 2021), India	Recombinant, replication-deficient chimpanzee adenovirus vector encoding the SARS-CoV-2 spike (S) protein in genetically modified human embryonic kidney 293 cells	1. L-Histidine 2. L-Histidine hydrochloride monohydrate 3. Magnesium chloride hexahydrate 4. Polysorbate 80 (E 433) 5. Sucrose 6. Ethanol 7. Sodium chloride 8. Disodium edetate dihydrate (EDTA)
Sinopharm-BBIBP (inactivated virus in Vero cells) (Wang et al., 2020a), China	Inactivated SARS-CoV-2 virus (HB02 strain) in Vero cells culture	1. Aluminum hydroxide adjuvant 2. Beta-propiolactone 3. Disodium hydrogen phosphate 4. Sodium dihydrogen phosphate 5. Sodium chloride
Sputnik V (viral vector) (Logunov et al., 2021), Russia	Modified replication-deficient Ad26 and Ad5 encoding the SARS-CoV-2 spike (S) protein	1. Tris-(hydroxymethyl)-aminomethane 2. Sodium chloride 3. Sucrose 4. Magnesium chloride hexahydrate 5. Disodium EDTA dihydrate 6. Polysorbate 80 7. Ethanol

(Continued on following page)

TABLE 11 | (*Continued*) Components of 24 COVID-19 vaccines with emergency use authorizations by national regulatory authorities (as at October 26, 2021).

Type of vaccine	Active ingredient	Inactive ingredients
Abdala (Reuters, 2021; RPCEC, 2021; Vie Pce, 2021), Cuba	Protein subunit vaccine containing COVID-19-derived proteins	No clinical results and information on ingredients found on electronic databases (PubMed, Google Scholar, Medline, Scopus, embase)
Chinese Academy of Medical Sciences Covidful (ClinicalTrials.Gov, 2021a; ClinicalTrials.Gov, 2021b), China	Inactivated virus vaccine	No clinical results and information on ingredients found on electronic databases (PubMed, Google Scholar, Medline, Scopus, embase)
Cansino Convidecia (Wang et al., 2020b; Wu et al., 2021), China	Recombinant replication-deficient adenovirustype 5-vectored vaccine expressing full-length spike gene based on Wuhan-Hu-1 (Genebank accession number YP_009,724,390)	Detailed inactive components were not listed
Covaxin (BIOTECH, 2021; Sapkal et al., 2021), India	Whole-virion inactivated SARS-CoV-2 antigen (strain: NIV-2020770)	1. Aluminum hydroxide 2. Imidazoquinolinone 3. 2-Phenoxyethonol 4. Phosphate buffer saline
COVIran Barakat (Asghar Abdoli et al., 2021; Mallapaty, 2021), Iran	Inactivated SARS-CoV-2 virus with Vero cell culture	1. Aluminum hydroxide 2. Modified Egg's medium 3. Fetal bovine serum
CoviVac (ClinicalTrials.Gov, 2021c; Kozlovskaya et al., 2021; EMA, 2021b), Russia	Inactivated SARS-CoV-2 virus (strain:AYDAR-1) with Vero cell culture	1. Beta-propiolactone 2. Aluminum hydroxide 3. Disodium phosphate dihydrate 4. Sodium dihydrogen phosphate dihydrate 5. Sodium chloride
EpiVacCorona (Ryzhikov et al., 2021; Рыжиков ЕАР et al., 2021), Russia	Chemically synthesized peptides (short fragments of viral spike protein) conjugating to a carrier protein containing nucleocapsid proteins and maltose-binding proteins	1. L-Histidine 2. Aluminum hydroxide
FAKHRAVAC (IRCT, 2021a; IRCT, 2021b), Iran	Inactivated SARS-CoV-2 virus-based with cell culture	Detailed ingredients not published
Medigen (ClinicalTrials.Gov, 2021d; ClinicalTrials.Gov, 2021e; Hsieh et al., 2021), Taiwan	Recombinant S-2P spike protein adjuvanted with CpG 1,018	1. CpG 1,018 2. Aluminum hydroxide 3. Phosphate buffer solution
Minhai (ClinicalTrials.Gov, 2021f; ClinicalTrials.Gov, 2021g; ClinicalTrials.Gov, 2021h), China	Inactivated SARS-CoV-2 virus-based with Vero cell culture	Detailed ingredients not published
QazCovid-in (ClinicalTrials.Gov, 2021i; ClinicalTrials.Gov, 2021j), Kazakhstan	Inactivated SARS-CoV-2 virus-based with cell culture	Detailed ingredients not published
Sinopharm-WIBP (Xia et al., 2020; Al Kaabi et al., 2021; Xia et al., 2021), China	Inactivated SARS-CoV-2 virus (strain WIV-04) in Vero cell culture	1. Aluminum hydroxide 2. Disodium hydrogen phosphate 3. Sodium dihydrogen phosphate 4. Sodium chloride
Soberana (Malik et al., 2021; Mega, 2021; Valdes-Balbin et al., 2021), Cuba	Receptor binding domain of SARS-CoV-2 spike protein conjugated chemically to tetanus toxoid	Detailed ingredients not published
Sputnik light (ClinicalTrials.Gov, 2021k; ClinicalTrials.Gov, 2021l), Russia	Recombinant replication-deficient Ad26 encoding the SARS-CoV-2 spike (S) protein	1. Tris-(hydroxymethyl)-aminomethane 2. Sodium chloride 3. Sucrose 4. Magnesium chloride hexahydrate 5. Disodium EDTA dihydrate 6. Polysorbate 80 7. Ethanol
Zifivax (Yang et al., 2021; Zhao et al., 2021), China	Recombinant tandem repeat dimeric receptor binding domain-based protein subunit vaccine	1. Aluminum hydroxide Detailed ingredients not published
ZyCoV-D (Dey et al., 2021; Momin et al., 2021) (DNA plasmid vactor), India	DNA plasmid vector carrying the gene encoding the spike protein (S) of the SARS-CoV-2 virus	Detailed ingredients not published

The first seven vaccines in the table have been approved for emergency or full use by at least one WHO-recognized stringent regulatory authority (Pfizer, Moderna, Janssen, Sinovac, Oxford-AstraZeneca, Serum Insitute of India Covishield, and Sinopharm-BBIBP). The remaining vaccine candidates are arranged in alphabetical order.

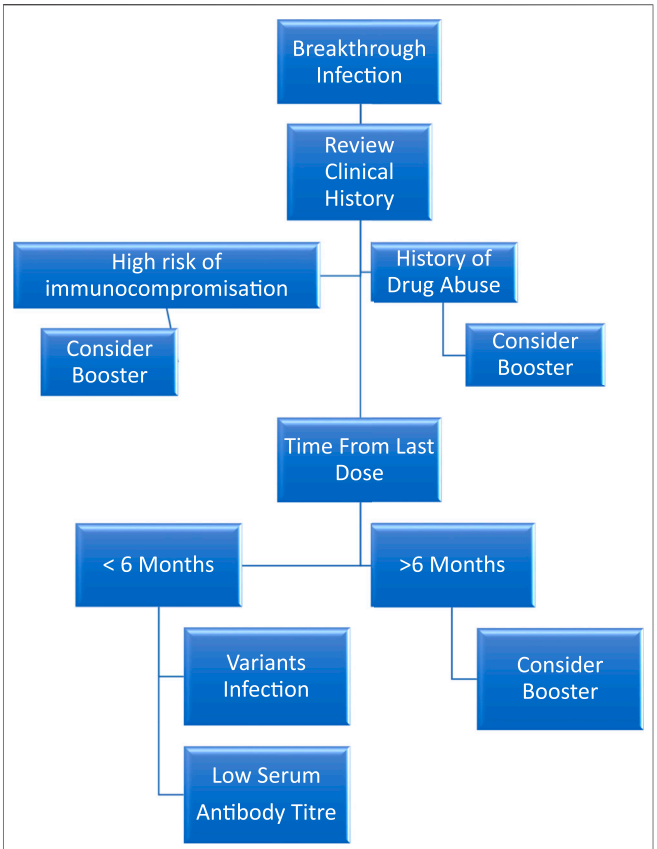


FIGURE 1 | Management algorithm for COVID-19 breakthrough infection. Common reasons include the following: **(A)** Abuse of drugs. **(B)** Baseline multiple comorbidities. **(C)** Changing strains. **(D)** Decreasing antibody level. **(E)** Elderly.

in previous studies (Kant et al., 2021). Vaccine booster schedule should be individualized according to the half-life of immunity decline. A British study shows that an extended interval before the second dose of ChAdOx1 nCoV-19 leads to increased antibody titers, while a third dose of ChAdOx1 nCoV-19 induces antibody that correlates with higher efficacy after second dose due to robust T-cell responses (Flaxman et al., 2021).

Patients receiving immunosuppressants or with chronic kidney impairment receiving renal replacement therapy also have suboptimal anti-SARS-CoV-2 antibodies after the second dose (Boyarsky et al., 2021a; Boyarsky et al., 2021b; Bensouna et al., 2021; Kamar et al., 2021; Peled et al., 2021; Werbel et al., 2021). A booster dose of mRNA-1273 vaccine induces serological response in 49% of renal recipients who are refractory to produce antibodies after two doses (Benotmane et al., 2021). This is similar in patients receiving solid organ transplants on immunosuppressants and with negative antibody titers before the third dose: 25% of them develop high-positive antibodies after a third dose, while over two-thirds of them remain negative (Werbel et al., 2021). A significant proportion of patients who fail to develop immunity after a third-dose booster is on triple immunosuppressants (Benotmane et al., 2021). The SENCOVAC study shows that an absence of antibody protection is associated with kidney transplant recipients due to their immunosuppression therapy (odds ratio 20.56, $p < 0.01$), while receiving BNT162b2 increases the chance of antibody response (odds ratio 6.03, $p = 0.02$) (Quiroga et al., 2021). These patients are advised to adopt persistent isolation measures and consider booster vaccines to optimize protection against COVID-19 infection (Quiroga et al., 2021). Use of rituximab is common in cancer or autoimmune disease treatments leading to failure of immunological response (Yahav et al., 2021). Heterologous vaccination leads to stronger induction of antibodies and CD4 T cells in immunocompromised patients: SARS-CoV-2-specific antibodies and T-cells response after second vaccination were induced 100% and 70.6% in transplant recipients (Baker et al., 2021; Schmidt et al., 2021).

A recent systematic review compared the relative likelihood of non-responders (Galmiche et al., 2021). The proportion of non-responders is higher among solid organ transplant recipients (range 18–100%), hematological malignancy (range 14–61%), cancers (2–36%), and dialysis usage (2–30%). Risk factors of failure of antibody induction include older age, use of corticosteroids, immunosuppressants, and anti-CD20 agents.

Currently, a new approach is consideration of immunosuppressant dosage adjustment or additional booster to maximize immunological response induction (Albach et al., 2021; Yan et al., 2021b; Connolly et al., 2021; Mackintosh et al., 2021; Yahav et al., 2021). With vaccination-refractory in immunocompromised subjects (solid organ transplant recipients) (Chavarot et al., 2021), recommendation of an additional dose is encouraged since a third vaccine dose increases the seropositivity prevalence from 40% to 68% (Husain and Argyropoulos, 2021). The seropositivity is protective against symptomatic infection, while it is unlikely to carry a significant risk of adverse events (Husain and Argyropoulos, 2021).

Cancer patients benefit from third-dose vaccinations since they run a high risk of failed induction of immune memory (Peeters et al., 2021). The CANVAX Cohort Study shows that immune responses to SARS-CoV-2 vaccines are moderately impaired in patients with cancer, while antibody testing may be effective to identify immune-inert patients to receive booster vaccinations (Naranbhai et al., 2021). A third-dose BNT162b2 (Pfizer/BioNTech) vaccination demonstrated a median 3-fold increase of neutralizing antibody response with mild adverse events (Shroff et al., 2021). This should be similarly considered in cancer patients with active anti-neoplastic treatment (Peeters et al., 2021). In view of the breakthrough infections with multiple lineages of variants, immunocompromised patients are at risk of severe diseases (Deng et al., 2021; D'Amelio et al., 2021). In terms of safety, immunocompromised patients are not at an increased risk of vaccine-related adverse events (Mackintosh et al., 2021).

RECOMMENDATION FOR BREAKTHROUGH INFECTION

Breakthrough infections have been reported worldwide. Breakthrough infections are mild in healthy adults (Abbasi, 2021). Thus, management of breakthrough infections should include a thorough clinical history review to determine the presence of risk factors and appropriate actions (**Figure 1**).

Common reasons related to this include: 1) infection of variant strains (Bosch et al., 2021); 2) low circulating antibody levels as time elapsed from the previous second dose (Chemaitelly et al., 2021; Levin et al., 2021); 3) insufficient induction of antibody due to multiple comorbidities or immunocompromised state (Albach et al., 2021; Arya et al., 2021); 4) history of substance abuse (Wang et al., 2022); and 5) old age (Butt et al., 2021; Glatman-Freedman et al., 2021).

Within 6 months after the last dose of mRNA-based vaccination, the antibody is still protective against COVID-19 disease (El Sahly et al., 2021; Thomas et al., 2021). Consideration of vaccine-resistant variant strain infection should be the top priority. Low serum antibody level leading to insufficient protective efficacy is the common reason for breakthrough infections. Booster vaccination after 6 months, regardless of antibody level, should be considered (Ebinger et al., 2021; Gobbi et al., 2021; Liao et al., 2021; Turner et al., 2021), though there is evidence suggesting breakthrough infections do not necessarily correlate with lack of vaccine-induced immunity (Duarte et al., 2021).

Patients with multiple comorbidities leading to the immunocompromised state should consider booster vaccination, with either homologous or heterologous vaccines (Baker et al., 2021; Schmidt et al., 2021). The multiple comorbidities include solid organ transplant patients on immunosuppressants (Werbel et al., 2021), autoimmune diseases with failed induction of antibody despite full-dose vaccination schedule (Yahav et al., 2021), chronic kidney disease with renal replacement therapy (Boyarsky et al., 2021a; Boyarsky et al., 2021b; Bensouna et al., 2021; Kamar et al., 2021; Peled et al., 2021; Werbel et al., 2021), poorly controlled hypertension, diabetes mellitus (Brosh-Nissimov et al., 2021), and cancers (Peeters et al., 2021).

CONCLUSION

This paper provides an updated evaluation of booster vaccination: its necessity, concerns, and benefits on variant strains. It gives a detailed description of the efficacy and safety of vaccination on variant strains. However, there are limited data on the effect of booster vaccination on Omicron strains at the time of writing, and Omicron-strain vaccination has been under development by vaccine manufacturers; thus, clinical trials of newly modified vaccines have not started. Booster vaccination brings benefits to the waning immunity and protective efficacy of COVID-19 vaccines. Booster vaccination induces immunological memory by elevating circulating anti-SARS-CoV-2 antibody level. Its associated risk is manageable, while risk–benefit analysis should be evaluated by medical health staff to manage comorbidities and rule out contraindications for vaccinations. Special populations should have an alternative vaccination schedule to boost their protective antibodies against multiple lineages of SARS-CoV-2 variants. These patients are not at a higher risk of vaccine-associated adverse events. More research is required on the schedule of booster vaccination and the type of booster vaccine for special populations.

CHECKLIST DECLARATION

1) Not in contravention of the European Respiratory Society (ERS) policy on tobacco
2) No funding on this project, and therefore no issue of copyright transfer
3) Completed International Committee of Medical Journal Editors (ICMJE) conflict of interest disclosure and submitted as an attachment
4) Consented submission with email address specified

AUTHOR CONTRIBUTIONS

Literature search, study designs, figures, data collections, data analysis, data interpretation, and manuscript writing were done by MZY, MY, and C-LL. MZY and MY contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fddsv.2022.858006/full#supplementary-material

REFERENCES

Abbasi, J. (2021). COVID-19 mRNA Vaccines Blunt Breakthrough Infection Severity. *JAMA* 326 (6), 473. doi:10.1001/jama.2021.12619

Abu-Farha, R., Mukattash, T., Itani, R., Karout, S., Khojah, H. M. J., Abed Al-Mahmood, A., et al. (2021). Willingness of Middle Eastern Public to Receive COVID-19 Vaccines. *Saudi Pharm. J.* 29 (7), 734–739. doi:10.1016/j.jsps.2021.05.005

Abu-Raddad, L. J., Chemaitelly, H., and Butt, A. A.National Study Group for C-V (2021). Effectiveness of the BNT162b2 Covid-19 Vaccine against the B.1.1.7 and B.1.351 Variants. *N. Engl. J. Med.* 385 (2), 187–189. doi:10.1056/nejmc2104974

Al Kaabi, N., Zhang, Y., Xia, S., Yang, Y., Al Qahtani, M. M., Abdulrazzaq, N., et al. (2021). Effect of 2 Inactivated SARS-CoV-2 Vaccines on Symptomatic COVID-19 Infection in Adults: A Randomized Clinical Trial. *JAMA* 326 (1), 35–45. doi:10.1001/jama.2021.8565

Albach, F. N., Burmester, G. R., and Biesen, R. (2021). Successful BNT162b2 Booster Vaccinations in a Patient with Rheumatoid Arthritis and Initially Negative Antibody Response. *Ann. Rheum. Dis.* 80 (10), 1361–1362. doi:10.1136/annrheumdis-2021-220834

Alter, G., Yu, J., Liu, J., Chandrashekar, A., Borducchi, E. N., Tostanoski, L. H., et al. (2021). Immunogenicity of Ad26.COV2.S Vaccine against SARS-CoV-2 Variants in Humans. *Nature* 596 (7871), 268–272. doi:10.1038/s41586-021-03681-2

Arbel, R., Hammerman, A., Sergienko, R., Friger, M., Peretz, A., Netzer, D., et al. (2021). BNT162b2 Vaccine Booster and Mortality Due to Covid-19. *N. Engl. J. Med.* 385 (26), 2413–2420. doi:10.1056/nejmoa2115624

Arya, A., Li, M., Aburjania, N., Singh, P., Royer, T., Moss, S., et al. (2021). COVID-19 in Solid Organ Transplantation: Disease Severity and Clinical Update. *Transplant. Proc.* 53 (4), 1227–1236. doi:10.1016/j.transproceed.2021.02.014

Asghar Abdoli, R. A., Aminianfar, H., Kianmehr, Z., Azimi, E., Emamipour, N., Jamshidi, H., et al. (2021). Safety and Potency of COVIran Barekat Inactivated Vaccine Candidate for SARS-CoV-2: A Preclinical Study. *bioRxiv*.

Baker, M. C., Mallajosyula, V., Davis, M. M., Boyd, S. D., Nadeau, K. C., and Robinson, W. H. (2021). Effective Viral Vector SARS-CoV-2 Booster Vaccination in a Patient with Rheumatoid Arthritis after Initial Ineffective mRNA Vaccine Response. *Arthritis Rheumatol.* 74 (3), 541–542. doi:10.1002/art.41978

Bar-On, Y. M., Goldberg, Y., Mandel, M., Bodenheimer, O., Freedman, L., Kalkstein, N., et al. (2021). Protection of BNT162b2 Vaccine Booster against Covid-19 in Israel. *N. Engl. J. Med.* 385 (15), 1393–1400. doi:10.1056/nejmoa2114255

Barchuk, A., Cherkashin, M., Bulina, A., Berezina, N., Rakova, T., Kuplevatskaya, D., et al. (2021). Vaccine Effectiveness against Referral to Hospital and Severe Lung Injury Associated with COVID-19: A Population-Based Case-Control Study in St. Petersburg, Russia. *medRxiv*.

Barda, N., Dagan, N., Ben-Shlomo, Y., Kepten, E., Waxman, J., Ohana, R., et al. (2021). Safety of the BNT162b2 mRNA Covid-19 Vaccine in a Nationwide Setting. *N. Engl. J. Med.* 385 (12), 1078–1090. doi:10.1056/nejmoa2110475

Barouch, D. H., Stephenson, K. E., Sadoff, J., Yu, J., Chang, A., Gebre, M., et al. (2021). Durable Humoral and Cellular Immune Responses 8 Months after Ad26.COV2.S Vaccination. *N. Engl. J. Med.* 385 (10), 951–953. doi:10.1056/nejmc2108829

Barrett, J. R., Belij-Rammerstorfer, S., Dold, C., Ewer, K. J., Folegatti, P. M., Gilbride, C., et al. (2021). Phase 1/2 Trial of SARS-CoV-2 Vaccine ChAdOx1 nCoV-19 with a Booster Dose Induces Multifunctional Antibody Responses. *Nat. Med.* 27 (2), 279–288. doi:10.1038/s41591-020-01179-4

Benotmane, I., Gautier, G., Perrin, P., Olagne, J., Cognard, N., Fafi-Kremer, S., et al. (2021). Antibody Response after a Third Dose of the mRNA-1273 SARS-CoV-2 Vaccine in Kidney Transplant Recipients with Minimal Serologic Response to 2 Doses. *JAMA* 326 (11), 1063–1065. doi:10.1001/jama.2021.12339

Bensouna, I., Caudwell, V., Kubab, S., Acquaviva, S., Pardon, A., Vittoz, N., et al. (2021). SARS-CoV-2 Antibody Response after a Third Dose of the BNT162b2 Vaccine in Patients Receiving Maintenance Hemodialysis or Peritoneal Dialysis. *Am. J. Kidney Dis.* 79 (2), 185–192. doi:10.1053/j.ajkd.2021.08.005

Bergwerk, M., Gonen, T., Lustig, Y., Amit, S., Lipsitch, M., Cohen, C., et al. (2021). Covid-19 Breakthrough Infections in Vaccinated Health Care Workers. *N. Engl. J. Med.* 385 (16), 1474–1484. doi:10.1056/nejmoa2109072

BIOTECH, B. (2021). Fact Sheet for Vaccination Recipients and Caregivers: Covaxin SARS-CoV-2 Vaccine. Available from: https://www.bharatbiotech.com/images/covaxin/covaxin-factsheet.pdf.

Borobia, A. M., Carcas, A. J., Pérez-Olmeda, M., Castaño, L., Bertran, M. J., García-Pérez, J., et al. (2021). Immunogenicity and Reactogenicity of BNT162b2 Booster in ChAdOx1-S-Primed Participants (CombiVacS): a Multicentre, Open-Label, Randomised, Controlled, Phase 2 Trial. *Lancet* 398 (10295), 121–130. doi:10.1016/S0140-6736(21)01420-3

Bosch, W., Cowart, J. B., Bhakta, S., Carter, R. E., Wadei, H. M., Shah, S. Z., et al. (2021). COVID-19 Vaccine-Breakthrough Infections Requiring Hospitalization in Mayo Clinic Florida through August 2021. *Clin. Infect. Dis.* 2, ciab932. doi:10.1093/cid/ciab932

Boyarsky, B. J., Werbel, W. A., Avery, R. K., Tobian, A. A. R., Massie, A. B., Segev, D. L., et al. (2021). Antibody Response to 2-Dose SARS-CoV-2 mRNA Vaccine Series in Solid Organ Transplant Recipients. *JAMA* 325 (21), 2204–2206. doi:10.1001/jama.2021.7489

Boyarsky, B. J., Werbel, W. A., Avery, R. K., Tobian, A. A. R., Massie, A. B., Segev, D. L., et al. (2021). Immunogenicity of a Single Dose of SARS-CoV-2 Messenger RNA Vaccine in Solid Organ Transplant Recipients. *JAMA* 325 (17), 1784–1786. doi:10.1001/jama.2021.4385

Brosh-Nissimov, T., Orenbuch-Harroch, E., Chowers, M., Elbaz, M., Nesher, L., Stein, M., et al. (2021). BNT162b2 Vaccine Breakthrough: Clinical Characteristics of 152 Fully Vaccinated Hospitalized COVID-19 Patients in Israel. *Clin. Microbiol. Infect.* 27 (11), 1652–1657. doi:10.1016/j.cmi.2021.06.036

Bruxvoort, K. J., Sy, L. S., Qian, L., Ackerson, B. K., Luo, Y., Lee, G. S., et al. (2021). Effectiveness of mRNA-1273 against delta, Mu, and Other Emerging Variants of SARS-CoV-2: Test Negative Case-Control Study. *BMJ* 375, e068848. doi:10.1136/bmj-2021-068848

Burki, T. (2021). Booster Shots for COVID-19-The Debate Continues. *Lancet Infect. Dis.* 21 (10), 1359–1360. doi:10.1016/s1473-3099(21)00574-0

Butt, A. A., Nafady-Hego, H., Chemaitelly, H., Abou-Samra, A.-B., Khal, A. A., Coyle, P. V., et al. (2021). Outcomes Among Patients with Breakthrough SARS-CoV-2 Infection after Vaccination. *Int. J. Infect. Dis.* 110, 353–358. doi:10.1016/j.ijid.2021.08.008

Campbell, F., Archer, B., Laurenson-Schafer, H., Jinnai, Y., Konings, F., Batra, N., et al. (2021). Increased Transmissibility and Global Spread of SARS-CoV-2 Variants of Concern as at June 2021. *Euro Surveill.* 26 (24), 1. doi:10.2807/1560-7917.ES.2021.26.24.2100509

Cavalcanti, E., Isgrò, M. A., Rea, D., Di Capua, L., Trillò, G., Russo, L., et al. (2021). Vaccination Strategy and Anti - SARS-CoV-2 S Titers in Healthcare Workers of the INT - IRCCS "Fondazione Pascale" Cancer Center (Naples, Italy). *Infect. Agents Cancer* 16 (1), 32. doi:10.1186/s13027-021-00375-2

CDC, U. (2021). CDC Statement on ACIP Booster Recommendations. Available from:https://www.cdc.gov/media/releases/2021/p0924-booster-recommendations-.html.

CDC, U. (2021). Comparative Effectiveness of Moderna, Pfizer-BioNTech, and Janssen (Johnson & Johnson) Vaccines in Preventing COVID-19 Hospitalizations Among Adults without Immunocompromising Conditions — United States, March–August 2021. Morbidity and Mortality Weekly Report. *MMWR* 70 (38), 1337–1343.

Cerqueira-Silva, T., de Araújo Oliveira, V., Pescarini, J., Júnior, J. B., Machado, T. M., Ortiz, R. F., et al. (2021). The Effectiveness of Vaxzevria and CoronaVac Vaccines: A Nationwide Longitudinal Retrospective Study of 61 Million Brazilians (VigiVac-COVID19). *medRxiv*.

Charmet, T., Schaeffer, L., Grant, R., Galmiche, S., Chény, O., Von Platen, C., et al. (2021). Impact of Original, B.1.1.7, and B.1.351/P.1 SARS-CoV-2 Lineages on Vaccine Effectiveness of Two Doses of COVID-19 mRNA Vaccines: Results from a Nationwide Case-Control Study in France. *The Lancet Reg. Health - Europe* 8, 100171. doi:10.1016/j.lanepe.2021.100171

Chavarot, N., Morel, A., Leruez-Ville, M., Vilain, E., Divard, G., Burger, C., et al. (2021). Weak Antibody Response to Three Doses of mRNA Vaccine in Kidney Transplant Recipients Treated with Belatacept. *Am. J. Transpl.* 21 (12), 4043–4051. doi:10.1111/ajt.1681410.1111/ajt.16814

Chemaitelly, H., Tang, P., Hasan, M. R., AlMukdad, S., Yassine, H. M., Benslimane, F. M., et al. (2021). Waning of BNT162b2 Vaccine Protection against SARS-CoV-2 Infection in Qatar. *N. Engl. J. Med.* 385 (24), e83. doi:10.1056/nejmoa2114114

Choudhary, O. P., Choudhary, P., and Singh, I. (2021). India's COVID-19 Vaccination Drive: Key Challenges and Resolutions. *Lancet Infect. Dis.* 21 (11), 1483–1484. doi:10.1016/s1473-3099(21)00567-3

Chung, H., He, S., Nasreen, S., Sundaram, M. E., Buchan, S. A., Wilson, S. E., et al. (2021). Effectiveness of BNT162b2 and mRNA-1273 Covid-19 Vaccines against Symptomatic SARS-CoV-2 Infection and Severe Covid-19 Outcomes in Ontario, Canada: Test Negative Design Study. *BMJ* 374, n1943. doi:10.1136/bmj.n1943

Ciabattini, A., Pastore, G., Fiorino, F., Polvere, J., Lucchesi, S., Pettini, E., et al. (2021). Evidence of SARS-CoV-2-specific Memory B Cells Six Months after Vaccination with the BNT162b2 mRNA Vaccine. *Front. Immunol.* 12, 740708. doi:10.3389/fimmu.2021.740708

ClinicalTrials.Gov (2021). A Phase 1/2 Safety and Immunogenicity Trial of COVID-19 Vaccine COVIVAC - NCT04830800. Available from: https://clinicaltrials.gov/ct2/show/NCT04830800.

ClinicalTrials.Gov (2021). A Safety and Immunogenicity Study of Inactivated SARS-CoV-2 Vaccine (Vero Cells) in Healthy Population Aged 18 Years and above (COVID-19) - NCT04758273. Available from: https://clinicaltrials.gov/ct2/show/NCT04758273.

ClinicalTrials.Gov (2021). A Study to Evaluate MVC-Cov1901 Vaccine against COVID-19 in Adult (COVID-19) - NCT04695652. Available from: https://clinicaltrials.gov/ct2/show/NCT04695652.

ClinicalTrials.Gov (2021). A Study to Evaluate Safety and Immunogenicity of Inactivated SARS-CoV-2 Vaccine (Vero Cells) in Healthy Population Aged 18 Years and above (COVID-19) - NCT04756323. Available from: https://clinicaltrials.gov/ct2/show/NCT04756323.

ClinicalTrials.Gov (2021). A Study to Evaluate the Efficacy, Safety and Immunogenicity of SARS-CoV-2 Vaccine (Vero Cells), Inactivated in Healthy Adults Aged 18 Years and Older (COVID-19) - NCT04852705. Available from: https://clinicaltrials.gov/ct2/show/NCT04852705.

ClinicalTrials.Gov (2021). A Study to Evaluate the Safety and Immunogenicity of MVC-Cov1901 against COVID-19. Available from: https://clinicaltrials.gov/ct2/show/NCT04487210.

ClinicalTrials.Gov (2021). An Open Study on the Safety, Tolerability, and Immunogenicity of "Sputnik Light" Vaccine - NCT04713488. Available from: https://www.clinicaltrials.gov/ct2/show/NCT04713488.

ClinicalTrials.Gov (2021). Immunogenicity, Efficacy and Safety of QazCovid-In® COVID-19 Vaccine -NCT04691908. Available from: https://www.clinicaltrials.gov/ct2/show/NCT04691908.

ClinicalTrials.Gov (2021). Reactogenicity, Safety and Immunogenicity of QazCovid-In® COVID-19 Vaccine - NCT04530357. Available from: https://clinicaltrials.gov/ct2/show/study/NCT04530357?draw=2.

ClinicalTrials.Gov (2021). Safety and Immunogenicity Study of an Inactivated SARS-CoV-2 Vaccine for Preventing against COVID-19 - NCT04412538. Available from: https://www.clinicaltrials.gov/ct2/show/NCT04412538.

ClinicalTrials.Gov (2021). Study to Evaluate Efficacy, Immunogenicity and Safety of the Sputnik-Light (SPUTNIK-LIGHT) - NCT04741061. Available from: https://www.clinicaltrials.gov/ct2/show/NCT04741061.

ClinicalTrials.Gov (2021). The Efficacy, Safety and Immunogenicity Study of Inactivated SARS-CoV-2 Vaccine for Preventing against COVID-19 - NCT04659239. Available from: https://www.clinicaltrials.gov/ct2/show/NCT04659239.

Collie, S., Champion, J., Moultrie, H., Bekker, L.-G., and Gray, G. (2022). Effectiveness of BNT162b2 Vaccine against Omicron Variant in South Africa. *N. Engl. J. Med.* 386 (5), 494–496. doi:10.1056/nejmc2119270

Connolly, C. M., Teles, M., Frey, S., Boyarsky, B. J., Alejo, J. L., Werbel, W. A., et al. (2021). Booster-dose SARS-CoV-2 Vaccination in Patients with Autoimmune Disease: a Case Series. *Ann. Rheum. Dis.* 81 (2), 291–293. doi:10.1136/annrheumdis-2021-221206

Cromer, D., Juno, J. A., Khoury, D., Reynaldi, A., Wheatley, A. K., Kent, S. J., et al. (2021). Prospects for Durable Immune Control of SARS-CoV-2 and Prevention of Reinfection. *Nat. Rev. Immunol.* 21 (6), 395–404. doi:10.1038/s41577-021-00550-x

D'Amelio, R., Asero, R., Cassatella, M. A., Lagana, B., Lunardi, C., Migliorini, P., et al. (2021). Anti-COVID-19 Vaccination in Patients with Autoimmune-Autoinflammatory Disorders and Primary/Secondary Immunodeficiencies: The Position of the Task Force on Behalf of the Italian Immunological Societies. *Biomedicines* 9 (9), 1. doi:10.3390/biomedicines9091163

Dan, J. M., Mateus, J., Kato, Y., Hastie, K. M., Yu, E. D., Faliti, C. E., et al. (2021). Immunological Memory to SARS-CoV-2 Assessed for up to 8 Months after Infection. *Science* 371 (6529), 1. doi:10.1126/science.abf4063

den Hartog, G., Vos, E. R. A., van den Hoogen, L. L., van Boven, M., Schepp, R. M., Smits, G., et al. (2021). Persistence of Antibodies to SARS-CoV-2 in Relation to Symptoms in a Nationwide Prospective Study. *Clin. Infect. Dis.* 73 (12), 2155–2162. doi:10.1093/cid/ciab172

Deng, X., Evdokimova, M., O'Brien, A., Rowe, C. L., Clark, N. M., Harrington, A., et al. (2021). Breakthrough Infections with Multiple Lineages of SARS-CoV-2 Variants Reveals Continued Risk of Severe Disease in Immunosuppressed Patients. *Viruses* 13 (9), 1. doi:10.3390/v13091743

Dey, A., Chozhavel Rajanathan, T. M., Chandra, H., Pericherla, H. P. R., Kumar, S., Choonia, H. S., et al. (2021). Immunogenic Potential of DNA Vaccine Candidate, ZyCoV-D against SARS-CoV-2 in Animal Models. *bioRxiv*.

Duarte, L. F., Gálvez, N. M. S., Iturriaga, C., Melo-González, F., Soto, J. A., Schultz, B. M., et al. (2021). Immune Profile and Clinical Outcome of Breakthrough Cases after Vaccination with an Inactivated SARS-CoV-2 Vaccine. *Front. Immunol.* 12, 742914. doi:10.3389/fimmu.2021.742914

Dyer, O. (2021). Covid-19: Rich Countries' Booster Plans Will Impede Global Vaccination, Experts Say. *BMJ* 374, n2353. doi:10.1136/bmj.n2353

Ebinger, J. E., Fert-Bober, J., Printsev, I., Wu, M., Sun, N., Prostko, J. C., et al. (2021). Antibody Responses to the BNT162b2 mRNA Vaccine in Individuals Previously Infected with SARS-CoV-2. *Nat. Med.* 27 (6), 981–984. doi:10.1038/s41591-021-01325-6

El Sahly, H. M., Baden, L. R., Essink, B., Doblecki-Lewis, S., Martin, J. M., Anderson, E. J., et al. (2021). Efficacy of the mRNA-1273 SARS-CoV-2 Vaccine at Completion of Blinded Phase. *N. Engl. J. Med.* 385 (19), 1774–1785. doi:10.1056/nejmoa2113017

EMA (2021). Signal Assessment Report on Embolic and Thrombotic Events (SMQ) with COVID-19 Vaccine (ChAdOx1-S [recombinant]) – COVID-19 Vaccine AstraZeneca (Other Viral Vaccines). Available from: https://www.ema.europa.eu/en/documents/prac-recommendation/signal-assessment-report-embolic-thrombotic-events-smq-covid-19-vaccine-chadox1-s-recombinant-covid_en.pdf.

EMA (2021). Summary of Product Characteristics - Astrazeneca Covid-Vaccine. Available from: https://www.ema.europa.eu/en/documents/product-information/vaxzevria-previously-covid-19-vaccine-astrazeneca-epar-product-information_en.pdf.

England, P. H. (2021). SARS-CoV-2 Variants of Concern and Variants under Investigation in England Technical Briefing 17. Available from: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/1001354/Variants_of_Concern_VOC_Technical_Briefing_17.pdf.

Erice, A., Varillas-Delgado, D., and Caballero, C. (2022). Decline of Antibody Titres 3 Months after Two Doses of BNT162b2 in Non-immunocompromised Adults. *Clin. Microbiol. Infect.* 28 (1), 139.e1–139.e4. doi:10.1016/j.cmi.2021.08.023

Flaxman, A., Marchevsky, N. G., Jenkin, D., Aboagye, J., Aley, P. K., Angus, B., et al. (2021). Reactogenicity and Immunogenicity after a Late Second Dose or a Third Dose of ChAdOx1 nCoV-19 in the UK: a Substudy of Two Randomised Controlled Trials (COV001 and COV002). *Lancet* 398 (10304), 981–990. doi:10.1016/S0140-6736(21)01699-8

Furer, V., Eviatar, T., Zisman, D., Peleg, H., Paran, D., Levartovsky, D., et al. (2021). Immunogenicity and Safety of the BNT162b2 mRNA COVID-19 Vaccine in Adult Patients with Autoimmune Inflammatory Rheumatic Diseases and in the General Population: a Multicentre Study. *Ann. Rheum. Dis.* 80 (10), 1330–1338. doi:10.1136/annrheumdis-2021-220647

Gagne, M., Corbett, K. S., Flynn, B. J., Foulds, K. E., Wagner, D. A., Andrew, S. F., et al. (2021). Protection from SARS-CoV-2 Delta One Year after mRNA-1273 Vaccination in Nonhuman Primates Is Coincident with an Anamnestic Antibody Response in the Lower Airway. *bioRxiv*.

Galmiche, S. N. L., Tartour, E., de Lamballerie, X., Wittkop, L., Loubet, P., and Launay, O. (2021). Immunological and Clinical Efficacy of COVID-19 Vaccines in Immunocompromised Populations: a Systematic Review. *Clin. Microbiol. Infect.* 28 (2), 163–177. doi:10.1016/j.cmi.2021.09.036

Glatman-Freedman, A., Bromberg, M., Dichtiar, R., Hershkovitz, Y., and Keinan-Boker, L. (2021). The BNT162b2 Vaccine Effectiveness against New COVID-19 Cases and Complications of Breakthrough Cases: A Nation-wide Retrospective Longitudinal Multiple Cohort Analysis Using Individualised Data. *EBioMedicine* 72, 103574. doi:10.1016/j.ebiom.2021.103574

Gluck, V., Grobecker, S., Kostler, J., Tydykov, L., Bertok, M., Weidlich, T., et al. (2021). Immunity after COVID-19 and Vaccination: Follow-Up Study over 1 Year Among Medical Personnel. *Infection*, 1–8. doi:10.1007/s15010-021-01703-9

Gobbi, F., Buonfrate, D., Moro, L., Rodari, P., Piubelli, C., Caldrer, S., et al. (2021). Antibody Response to the BNT162b2 mRNA COVID-19 Vaccine in Subjects with Prior SARS-CoV-2 Infection. *Viruses* 13 (3). doi:10.3390/v13030422

Gray, G. E., Collie, S., Garrett, N., Goga, A., Champion, J., Zylstra, M., et al. (2021). Vaccine Effectiveness against Hospital Admission in South African Health Care Workers Who Received a Homologous Booster of Ad26.COV2 during an Omicron COVID19 Wave: Preliminary Results of the Sisonke 2 Study. *medRxiv*.

Hall, V. J., Foulkes, S., Saei, A., Andrews, N., Oguti, B., Charlett, A., et al. (2021). COVID-19 Vaccine Coverage in Health-Care Workers in England and

Effectiveness of BNT162b2 mRNA Vaccine against Infection (SIREN): a Prospective, Multicentre, Cohort Study. *Lancet* 397 (10286), 1725–1735. doi:10.1016/S0140-6736(21)00790-X

Havervall, S., Marking, U., Greilert-Norin, N., Ng, H., Gordon, M., Salomonsson, A.-C., et al. (2021). Antibody Responses after a Single Dose of ChAdOx1 nCoV-19 Vaccine in Healthcare Workers Previously Infected with SARS-CoV-2. *EBioMedicine* 70, 103523. doi:10.1016/j.ebiom.2021.103523

Hillus, D., Schwarz, T., Tober-Lau, P., Hastor, H., Thibeault, C., Kasper, S., et al. (2021). Safety, Reactogenicity, and Immunogenicity of Homologous and Heterologous Prime-Boost Immunisation with ChAdOx1-nCoV19 and BNT162b2: A Prospective Cohort Study. *medRxiv*.

Hitchings, M. D. T., Ranzani, O. T., Dorion, M., D'Agostini, T. L., de Paula, R. C., de Paula, O. F. P., et al. (2021). Effectiveness of the ChAdOx1 Vaccine in the Elderly during SARS-CoV-2 Gamma Variant Transmission in Brazil. *medRxiv*.

Hsieh, S.-M., Liu, W.-D., Huang, Y.-S., Lin, Y.-J., Hsieh, E.-F., Lian, W.-C., et al. (2021). Safety and Immunogenicity of a Recombinant Stabilized Prefusion SARS-CoV-2 Spike Protein Vaccine (MVC COV1901) Adjuvanted with CpG 1018 and Aluminum Hydroxide in Healthy Adults: A Phase 1, Dose-Escalation Study. *EClinicalMedicine* 38, 100989. doi:10.1016/j.eclinm.2021.100989

Husain, S. A., and Argyropoulos, C. P. (2021). Boosters and Optimizing SARS-CoV-2 Vaccine for Transplantation: No Time to Wait. *Am. J. Transpl.* 22 (1), 328–329. doi:10.1111/ajt.16788

IRCT (2021). Comparison of the Safety, Efficacy and Immunogenicity of Fakhravac and Sinopharm SARS-CoV-2 Vaccines, in Adults Aged 18 and over; a Phase III Randomised, Non-inferiority Clinical Trial. Available from: https://en.irct.ir/trial/57980.

IRCT (2021). Phase 2 Trial of Safety and Immunogenicity of 10 Micro Gram Inactivated SARS-CoV-2 Vaccine (FAKHRAVAC), Two Doses Two Weeks Apart in Adults Aged 18-70 Years: a Randomized, Double-Blind, Placebo-Controlled, Clinical Trial. Available from: https://en.irct.ir/trial/56027.

Jara, A., Undurraga, E. A., Gonzalez, C., Paredes, F., Fontecilla, T., Jara, G., et al. (2021). Effectiveness of an Inactivated SARS-CoV-2 Vaccine in Chile. *N. Engl. J. Med.* 385 (10), 875–884. doi:10.1056/nejmoa2107715

Jeewandara, C., Kamaladasa, A., Pushpakumara, P. D., Jayathilaka, D., Aberathna, I. S., Danasekara, D. R. S. R., et al. (2021). Immune Responses to a Single Dose of the AZD1222/Covishield Vaccine in Health Care Workers. *Nat. Commun.* 12 (1), 4617. doi:10.1038/s41467-021-24579-7

Kamar, N., Abravanel, F., Marion, O., Couat, C., Izopet, J., and Del Bello, A. (2021). Three Doses of an mRNA Covid-19 Vaccine in Solid-Organ Transplant Recipients. *N. Engl. J. Med.* 385 (7), 661–662. doi:10.1056/nejmc2108861

Kang, Y., Li, Y., Sun, L., Deng, A., Hu, T., Zhang, J., et al. (2021). Effectiveness of Inactivated COVID-19 Vaccines against COVID-19 Pneumonia and Severe Illness Caused by the B.1.617.2 (Delta) Variant: Evidence from an Outbreak in Guangdong, China. *SSRN* [Epub ahead of print]. doi:10.2139/ssrn.3895639

Kant, S., and Geetha, D. (2021). Impact of Rituximab on Humoral Response to COVID-19 Booster Vaccine and Antibody Kinetics in Patients with Anti-neutrophil Cytoplasmic Antibody Vasculitis. *Kidney Int.* 100 (5), 1124–1127. doi:10.1016/j.kint.2021.08.020

Kant, S., Kronbichler, A., Salas, A., Bruchfeld, A., and Geetha, D. (2021). Timing of COVID-19 Vaccine in the Setting of Anti-CD20 Therapy: A Primer for Nephrologists. *Kidney Int. Rep.* 6 (5), 1197–1199. doi:10.1016/j.ekir.2021.03.876

Kent, S. J., and Juno, J. A. (2021). Vaccination after Prior COVID-19 Infection: Implications for Dose Sparing and Booster Shots. *EBioMedicine* 72, 103586. doi:10.1016/j.ebiom.2021.103586

Kherabi, Y., Fiolet, T., Rozencwajg, S., Salaün, J.-P., and Peiffer-Smadja, N. (2021). COVID-19 Vaccine Boosters: What Do We Know So Far? *Anaesth. Crit. Care Pain Med.* 40, 100959. doi:10.1016/j.accpm.2021.100959

Khoury, D. S., Cromer, D., Reynaldi, A., Schlub, T. E., Wheatley, A. K., Juno, J. A., et al. (2021). Neutralizing Antibody Levels Are Highly Predictive of Immune protection from Symptomatic SARS-CoV-2 Infection. *Nat. Med.* 27 (7), 1205–1211. doi:10.1038/s41591-021-01377-8

Kozlovskaya, L. I., Piniaeva, A. N., Ignatyev, G. M., Gordeychuk, I. V., Volok, V. P., Rogova, Y. V., et al. (2021). Long-term Humoral Immunogenicity, Safety and Protective Efficacy of Inactivated Vaccine against COVID-19 (CoviVac) in Preclinical Studies. *Emerging Microbes & Infections* 10 (1), 1790–1806. doi:10.1080/22221751.2021.1971569

Krammer, F., Srivastava, K., Team, P., and Simon, V. (2021). Robust Spike Antibody Responses and Increased Reactogenicity in Seropositive Individuals after a Single Dose of SARS-CoV-2 mRNA Vaccine. *medRxiv*, 1.

Kumar, N. P., Padmapriyadarsini, C., Uma Devi, K. R., Banurekha, V. V., Nancy, A., Girish Kumar, C. P., et al. (2021). Antibody Responses to the BBV152 Vaccine in Individuals Previously Infected with SARS-CoV-2: A Pilot Study. *Indian J. Med. Res.* 153 (5&6), 671–676. doi:10.4103/ijmr.IJMR_2066_21

Lee, H. K., Knabl, L., Knabl, L., Kapferer, S., Pateter, B., Walter, M., et al. (2021). Robust Immune Response to the BNT162b mRNA Vaccine in an Elderly Population Vaccinated 15 Months after Recovery from COVID-19. *medRxiv*.

Lefevre, B., Tondeur, L., Madec, Y., Grant, R., Lina, B., van der Werf, S., et al. (2021). Beta SARS-CoV-2 Variant and BNT162b2 Vaccine Effectiveness in Long-Term Care Facilities in France. *Lancet Healthy Longev* 2 (11), e685–e687. doi:10.1016/s2666-7568(21)00230-0

Levin, E. G., Lustig, Y., Cohen, C., Fluss, R., Indenbaum, V., Amit, S., et al. (2021). Waning Immune Humoral Response to BNT162b2 Covid-19 Vaccine over 6 Months. *N. Engl. J. Med.* 385 (24), e84. doi:10.1056/nejmoa2114583

Li, X.-N., Huang, Y., Wang, W., Jing, Q.-L., Zhang, C.-H., Qin, P.-Z., et al. (2021). Effectiveness of Inactivated SARS-CoV-2 Vaccines against the Delta Variant Infection in Guangzhou: a Test-Negative Case-Control Real-World Study. *Emerging Microbes & Infections* 10 (1), 1751–1759. doi:10.1080/22221751.2021.1969291

Liao, Y., Zhang, Y., Zhao, H., Pu, J., Zhao, Z., Li, D., et al. (2021). Intensified Antibody Response Elicited by Boost Suggests Immune Memory in Individuals Administered Two Doses of SARS-CoV-2 Inactivated Vaccine. *Emerging Microbes & Infections* 10 (1), 1112–1115. doi:10.1080/22221751.2021.1937328

Liu, C., Lee, J., Ta, C., Soroush, A., Rogers, J. R., Kim, J. H., et al. (2021). A Retrospective Analysis of COVID-19 mRNA Vaccine Breakthrough Infections – Risk Factors and Vaccine Effectiveness. *medRxiv*.

Liu, H., Wei, P., Zhang, Q., Aviszus, K., Linderberger, J., Yang, J., et al. (2021). The Lambda Variant of SARS-CoV-2 Has a Better Chance than the Delta Variant to Escape Vaccines. *bioRxiv*.

Liu, X., Shaw, R. H., Stuart, A. S. V., Greenland, M., Aley, P. K., Andrews, N. J., et al. (2021). Safety and Immunogenicity of Heterologous versus Homologous Prime-Boost Schedules with an Adenoviral Vectored and mRNA COVID-19 Vaccine (Com-COV): a Single-Blind, Randomised, Non-inferiority Trial. *The Lancet* 398 (10303), 856–869. doi:10.1016/s0140-6736(21)01694-9

Logunov, D. Y., Dolzhikova, I. V., Shcheblyakov, D. V., Tukhvatulin, A. I., Zubkova, O. V., Dzharullaeva, A. S., et al. (2021). Safety and Efficacy of an rAd26 and rAd5 Vector-Based Heterologous Prime-Boost COVID-19 Vaccine: an Interim Analysis of a Randomised Controlled Phase 3 Trial in Russia. *The Lancet* 397 (10275), 671–681. doi:10.1016/s0140-6736(21)00234-8

Lopez Bernal, J., Andrews, N., Gower, C., Gallagher, E., Simmons, R., Thelwall, S., et al. (2021). Effectiveness of Covid-19 Vaccines against the B.1.617.2 (Delta) Variant. *N. Engl. J. Med.* 385 (7), 585–594. doi:10.1056/nejmoa2108891

Lozano-Ojalvo, D., Camara, C., Lopez-Granados, E., Nozal, P., Del Pino-Molina, L., Bravo-Gallego, L. Y., et al. (2021). Differential Effects of the Second SARS-CoV-2 mRNA Vaccine Dose on T Cell Immunity in Naive and COVID-19 Recovered Individuals. *Cell Rep.* 36 (8), 109570. doi:10.1016/j.celrep.2021.109570

Ltd SLSC (2021). COVID-19 Vaccine (Vero Cell), Inactivated (Brief Version) Conditional Market Authorization. Available from: https://www.covidvaccine.gov.hk/pdf/CoronaVac_ENG_PI_brief.pdf.

Lumley, S. F., Wei, J., O'Donnell, D., Stoesser, N. E., Matthews, P. C., Howarth, A., et al. (2021). The Duration, Dynamics, and Determinants of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antibody Responses in Individual Healthcare Workers. *Clin. Infect. Dis.* 73 (3), e699–e709. doi:10.1093/cid/ciab004

Mackintosh, J. A., Lipman, M., Lowe, D. M., and Renzoni, E. A. (2021). Protecting the Vulnerable: SARS-CoV-2 Vaccination in Immunosuppressed Patients with Interstitial Lung Disease. *Lancet Respir. Med.* 9 (9), 947–949. doi:10.1016/s2213-2600(21)00326-x

Madhi, S. A., Baillie, V., Cutland, C. L., Voysey, M., Koen, A. L., Fairlie, L., et al. (2021). Efficacy of the ChAdOx1 nCoV-19 Covid-19 Vaccine against the B.1.351 Variant. *N. Engl. J. Med.* 384 (20), 1885–1898. doi:10.1056/NEJMoa2102214

Malik, J. A., Mulla, A. H., Farooqi, T., Pottoo, F. H., Anwar, S., and Rengasamy, K. R. R. (2021). Targets and Strategies for Vaccine Development against SARS-CoV-2. *Biomed. Pharmacother.* 137, 111254. doi:10.1016/j.biopha.2021.111254

Mallapaty, S. (2021). Iran Hopes to Defeat COVID with home-grown Crop of Vaccines. *Nature* 596 (7873), 475. doi:10.1038/d41586-021-02216-z

Mega, E. R. (2021). Can Cuba Beat COVID with its Homegrown Vaccines?. *Nature* [Epub ahead of print]. doi:10.1038/d41586-021-01126-4

Menni, C., Klaser, K., May, A., Polidori, L., Capdevila, J., Louca, P., et al. (2021). Vaccine Side-Effects and SARS-CoV-2 Infection after Vaccination in Users of the COVID Symptom Study App in the UK: a Prospective Observational Study. *Lancet Infect. Dis.* 21 (7), 939–949. doi:10.1016/s1473-3099(21)00224-3

Mevorach, D., Anis, E., Cedar, N., Bromberg, M., Haas, E. J., Nadir, E., et al. (2021). Myocarditis after BNT162b2 mRNA Vaccine against Covid-19 in Israel. *N. Engl. J. Med.* 385 (23), 2140–2149. doi:10.1056/nejmoa2109730

Moghaddar, M., Radman, R., and Macreadie, I. (2021). Severity, Pathogenicity and Transmissibility of Delta and Lambda Variants of SARS-CoV-2, Toxicity of Spike Protein and Possibilities for Future Prevention of COVID-19. *Microorganisms* 9 (10), 1. doi:10.3390/microorganisms9102167

Moghnieh, R., Mekdashi, R., El-Hassan, S., Abdallah, D., Jisr, T., Bader, M., et al. (2021). Immunogenicity and Reactogenicity of BNT162b2 Booster in BBIBP-CorV-Vaccinated Individuals Compared with Homologous BNT162b2 Vaccination: Results of a Pilot Prospective Cohort Study from Lebanon. *Vaccine* 39 (46), 6713–6719. doi:10.1016/j.vaccine.2021.10.007

Momin, T., Kansagra, K., Patel, H., Sharma, S., Sharma, B., Patel, J., et al. (2021). Safety and Immunogenicity of a DNA SARS-CoV-2 Vaccine (ZyCoV-D): Results of an Open-Label, Non-randomized Phase I Part of Phase I/II Clinical Study by Intradermal Route in Healthy Subjects in India. *EClinicalMedicine* 38, 101020. doi:10.1016/j.eclinm.2021.101020

Munro, A. P. S., Janani, L., Cornelius, V., Aley, P. K., Babbage, G., Baxter, D., et al. (2021). Safety and Immunogenicity of Seven COVID-19 Vaccines as a Third Dose (Booster) Following Two Doses of ChAdOx1 nCov-19 or BNT162b2 in the UK (COV-BOOST): A Blinded, Multicentre, Randomised, Controlled, Phase 2 Trial. *Lancet* 398 (10318), 2258–2276. doi:10.1016/S0140-6736(21)02717-3

Naaber, P., Tserel, L., Kangro, K., Sepp, E., Jürjenson, V., Adamson, A., et al. (2021). Dynamics of Antibody Response to BNT162b2 Vaccine after Six Months: a Longitudinal Prospective Study. *Lancet Reg. Health - Europe* 10, 100208. doi:10.1016/j.lanepe.2021.100208

NACI (2021). Recommendations on the Use of COVID-19 Vaccines. Available from: https://www.canada.ca/en/public-health/services/immunization/national-advisory-committee-on-immunization-naci/recommendations-use-covid-19-vaccines.html?hq_e=el&hq_m=2188311&hq_l=1&hq_v=91d220e044.

Naranbhai, V., Pernat, C. A., Gavralidis, A., St Denis, K. J., Lam, E. C., Spring, L. M., et al. (2021). Immunogenicity and Reactogenicity of SARS-CoV-2 Vaccines in Patients with Cancer: The CANVAX Cohort Study. *J. Clin. Oncol.* 40 (1), 12–23. doi:10.1200/JCO.21.01891

Nasreen, S., Chung, H., He, S., Brown, K. A., Gubbay, J. B., Buchan, S. A., et al. (2022). Effectiveness of COVID-19 Vaccines against Symptomatic SARS-CoV-2 Infection and Severe Outcomes with Variants of Concern in Ontario. *Nat. Microbiol.* 7 (3), 379–385. doi:10.1038/s41564-021-01053-0

Nasreen, S., Chung, H., He, S., Brown, K. A., Gubbay, J. B., Buchan, S. A., et al. (2021). Effectiveness of mRNA and ChAdOx1 COVID-19 Vaccines against Symptomatic SARS-CoV-2 Infection and Severe Outcomes with Variants of Concern in Ontario. *medRxiv*, 2021.

Neidleman, J., Luo, X., McGregor, M., Xie, G., Murray, V., Greene, W. C., et al. (2021). mRNA Vaccine-Induced T Cells Respond Identically to SARS-CoV-2 Variants of Concern but Differ in Longevity and Homing Properties Depending on Prior Infection Status. *Elife* 10, 10. doi:10.7554/eLife.72619

Palacios, R., Patiño, E. G., de Oliveira Piorelli, R., Conde, M. T. R. P., Batista, A. P., Zeng, G., et al. (2020). Double-Blind, Randomized, Placebo-Controlled Phase III Clinical Trial to Evaluate the Efficacy and Safety of Treating Healthcare Professionals with the Adsorbed COVID-19 (Inactivated) Vaccine Manufactured by Sinovac - PROFISCOV: A Structured Summary of a Study Protocol for a Randomised Controlled Trial. *Trials* 21 (1), 853. doi:10.1186/s13063-020-04775-4

Peeters, M., Verbruggen, L., Teuwen, L., Vanhoutte, G., Vande Kerckhove, S., Peeters, B., et al. (2021). Reduced Humoral Immune Response after BNT162b2 Coronavirus Disease 2019 Messenger RNA Vaccination in Cancer Patients under Antineoplastic Treatment. *ESMO Open* 6 (5), 100274. doi:10.1016/j.esmoop.2021.100274

Pegu, A., O'Connell, S. E., Schmidt, S. D., O'Dell, S., Talana, C. A., Lai, L., et al. (2021). Durability of mRNA-1273 Vaccine-Induced Antibodies against SARS-CoV-2 Variants. *Science* 373 (6561), 1372–1377. doi:10.1126/science.abj4176

Peled, Y., Ram, E., Lavee, J., Segev, A., Matezki, S., Wieder-Finesod, A., et al. (2021). Third Dose of the BNT162b2 Vaccine in Heart Transplant Recipients: Immunogenicity and Clinical Experience. *J. Heart Lung Transpl.* 41 (2), 148–157. doi:10.1016/j.healun.2021.08.010

Perkmann, T., Perkmann-Nagele, N., Mucher, P., Radakovics, A., Repl, M., Koller, T., et al. (2021). Initial SARS-CoV-2 Vaccination Response Can Predict Booster Response for BNT162b2 but Not for AZD1222. *Int. J. Infect. Dis.* 110, 309–313. doi:10.1016/j.ijid.2021.07.063

Pouwels, K. B., Pritchard, E., Matthews, P. C., Stoesser, N., Eyre, D. W., Vihta, K. D., et al. (2021). Effect of Delta Variant on Viral burden and Vaccine Effectiveness against New SARS-CoV-2 Infections in the UK. *Nat. Med.* 27 (12), 2127–2135. doi:10.1038/s41591-021-01548-7

Powers, A. C., Aronoff, D. M., and Eckel, R. H. (2021). COVID-19 Vaccine Prioritisation for Type 1 and Type 2 Diabetes. *Lancet Diabetes Endocrinol.* 9 (3), 140–141. doi:10.1016/s2213-8587(21)00017-6

PriyankaChoudhary, O. P., Singh, I., and Patra, G. (2020). Aerosol Transmission of SARS-CoV-2: The Unresolved Paradox. *Trav. Med. Infect. Dis.* 37, 101869. doi:10.1016/j.tmaid.2020.101869

Quiroga, B., Soler, M. J., Ortiz, A., Vaquera, S. M., Mantecon, C. J. J., Useche, G., et al. (2021). Safety and Immediate Humoral Response of COVID-19 Vaccines in Chronic Kidney Disease Patients: the SENCOVAC Study. *Nephrol. Dial. Transpl.* 12, gfab313. doi:10.1093/ndt/gfab313

Ramasamy, M. N., Minassian, A. M., Ewer, K. J., Flaxman, A. L., Folegatti, P. M., Owens, D. R., et al. (2021). Safety and Immunogenicity of ChAdOx1 nCoV-19 Vaccine Administered in a Prime-Boost Regimen in Young and Old Adults (COV002): a Single-Blind, Randomised, Controlled, Phase 2/3 Trial. *Lancet* 396 (10267), 1979–1993. doi:10.1016/S0140-6736(20)32466-1

Ranzani, O. T., Hitchings, M. D. T., Dorion, M., D'Agostini, T. L., de Paula, R. C., de Paula, O. F. P., et al. (2021). Effectiveness of the CoronaVac Vaccine in Older Adults during a Gamma Variant Associated Epidemic of Covid-19 in Brazil: Test Negative Case-Control Study. *BMJ* 374, n2015. doi:10.1136/bmj.n2015

Reis, B. Y., Barda, N., Leshchinsky, M., Kepten, E., Hernan, M. A., Lipsitch, M., et al. (2021). Effectiveness of BNT162b2 Vaccine against Delta Variant in Adolescents. *N. Engl. J. Med.* 385 (22), 2101–2103. doi:10.1056/nejmc2114290

Reuters (2021). Cuba Says Abdala Vaccine 92.28% Effective against Coronavirus. Available from: https://www.reuters.com/business/healthcare-pharmaceuticals/cuba-says-abdala-vaccine-9228-effective-against-coronavirus-2021-06-21/.

RPCEC (2021). ABDALA Clinical Study - Phase III. Available from: https://rpcec.sld.cu/en/trials/RPCEC00000359-En.

Ryzhikov, A. B., Ryzhikov, E. A., Bogryantseva, M. P., Danilenko, E. D., Imatdinov, I. R., Nechaeva, E. A., et al. (2021). Immunogenicity and Protectivity of the Peptide Candidate Vaccine against SARS-CoV-2. *Ann. RAMS* 76 (1), 5–19. doi:10.15690/vramn1528

Saadat, S., Rikhtegaran Tehrani, Z., Logue, J., Newman, M., Frieman, M. B., Harris, A. D., et al. (2021). Binding and Neutralization Antibody Titers after a Single Vaccine Dose in Health Care Workers Previously Infected with SARS-CoV-2. *JAMA* 325 (14), 1467–1469. doi:10.1001/jama.2021.3341

Sadoff, J., Gray, G., Vandebosch, A., Cardenas, V., Shukarev, G., Grinsztejn, B., et al. (2022). Final Analysis of Efficacy and Safety of Single-Dose Ad26.COV2.S. *N. Engl. J. Med.* 9, NEJMoa2117608. doi:10.1056/nejmoa2117608

Sadoff, J., Gray, G., Vandebosch, A., Cárdenas, V., Shukarev, G., Grinsztejn, B., et al. (2021). Safety and Efficacy of Single-Dose Ad26.COV2.S Vaccine against Covid-19. *N. Engl. J. Med.* 384 (23), 2187–2201. doi:10.1056/nejmoa2101544

Sapkal, G. N., Yadav, P. D., Ella, R., Deshpande, G. R., Sahay, R. R., Gupta, N., et al. (2021). Inactivated COVID-19 Vaccine BBV152/COVAXIN Effectively Neutralizes Recently Emerged B.1.1.7 Variant of SARS-CoV-2. *J. Trav. Med* 28 (4). doi:10.1093/jtm/taab051

Schaefer, G. O., Leland, R. J., and Emanuel, E. J. (2021). Making Vaccines Available to Other Countries before Offering Domestic Booster Vaccinations. *JAMA* 326 (10), 903–904. doi:10.1001/jama.2021.13226

Schmidt, T., Klemis, V., Schub, D., Schneitler, S., Reichert, M. C., Wilkens, H., et al. (2021). Cellular Immunity Predominates over Humoral Immunity after Homologous and Heterologous mRNA and Vector-Based COVID-19 Vaccine Regimens in Solid Organ Transplant Recipients. *Am. J. Transpl.* 21 (12), 3990–4002. doi:10.1111/ajt.16818

Shapiro Ben David, S., Shamir-Stein, N. a., Baruch Gez, S., Lerner, U., Rahamim-Cohen, D., and Ekka Zohar, A. (2021). Reactogenicity of a Third BNT162b2 mRNA COVID-19 Vaccine Among Immunocompromised Individuals and Seniors - A Nationwide Survey. *Clin. Immunol.* 232, 108860. doi:10.1016/j.clim.2021.108860

Sheikh, A., McMenamin, J., Taylor, B., Robertson, C., Public Health, S., and the, E. I. I. C. (2021). SARS-CoV-2 Delta VOC in Scotland: Demographics, Risk of Hospital Admission, and Vaccine Effectiveness. *Lancet* 397 (10293), 2461–2462. doi:10.1016/S0140-6736(21)01358-1

Shroff, R. T., Chalasani, P., Wei, R., Pennington, D., Quirk, G., Schoenle, M. V., et al. (2021). Immune Responses to Two and Three Doses of the BNT162b2 mRNA Vaccine in Adults with Solid Tumors. *Nat. Med.* 27 (11), 2002–2011. doi:10.1038/s41591-021-01542-z

Shrotri, M., Navaratnam, A. M. D., Nguyen, V., Byrne, T., Geismar, C., Fragaszy, E., et al. (2021). Spike-antibody Waning after Second Dose of BNT162b2 or ChAdOx1. *The Lancet* 398 (10298), 385–387. doi:10.1016/s0140-6736(21)01642-1

Soiza, R. L., Scicluna, C., and Thomson, E. C. (2021). Efficacy and Safety of COVID-19 Vaccines in Older People. *Age Ageing* 50 (2), 279–283. doi:10.1093/ageing/afaa274

Stowe, J. A. N., Gower, C., Gallagher, E., Utsi, L., Simmons, R., et al. (2021). *Effectiveness of COVID-19 Vaccines against Hospital Admission with the Delta (B.1.617.2) Variant*. Public Health England. Available from: https://khub.net/documents/135939561/479607266/Effectiveness+of+COVID-19+vaccines+against+hospital+admission+with+the+Delta+%28B.1.617.2%29+variant.pdf/1c213463-3997-ed16-2a6f-14e5deb0b997?t=1623689315431.

Tang, P., Hasan, M. R., Chemaitelly, H., Yassine, H. M., Benslimane, F. M., Khatib, H. A. A., et al. (2021). BNT162b2 and mRNA-1273 COVID-19 Vaccine Effectiveness against the Delta (B.1.617.2) Variant in Qatar. *medRxiv*, 2021.

Tang, P., Hasan, M. R., Chemaitelly, H., Yassine, H. M., Benslimane, F. M., Al Khatib, H. A., et al. (2021). BNT162b2 and mRNA-1273 COVID-19 Vaccine Effectiveness against the SARS-CoV-2 Delta Variant in Qatar. *Nat. Med.* 27 (12), 2136–2143. doi:10.1038/s41591-021-01583-4

Tanriover, M. D., Doğanay, H. L., Akova, M., Güner, H. R., Azap, A., Akhan, S., et al. (2021). Efficacy and Safety of an Inactivated Whole-Virion SARS-CoV-2 Vaccine (CoronaVac): Interim Results of a Double-Blind, Randomised, Placebo-Controlled, Phase 3 Trial in Turkey. *The Lancet* 398 (10296), 213–222. doi:10.1016/s0140-6736(21)01429-x

The Lancet Infectious Diseases, D. (2021). COVID-19 Vaccine Equity and Booster Doses. *Lancet Infect. Dis.* 21 (9), 1193. doi:10.1016/s1473-3099(21)00486-2

Thomas, S. J., Moreira, E. D., Jr., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., et al. (2021). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine through 6 Months. *N. Engl. J. Med.* 385 (19), 1761–1773. doi:10.1056/nejmoa2110345

Thompson, M. G., Stenehjem, E., Grannis, S., Ball, S. W., Naleway, A. L., Ong, T. C., et al. (2021). Effectiveness of Covid-19 Vaccines in Ambulatory and Inpatient Care Settings. *N. Engl. J. Med.* 385 (15), 1355–1371. doi:10.1056/nejmoa2110362

Torjesen, I. (2021). Covid-19: Omicron May Be More Transmissible Than Other Variants and Partly Resistant to Existing Vaccines, Scientists Fear. *BMJ* 375, n2943. doi:10.1136/bmj.n2943

Tseng, H. F., Ackerson, B. K., Luo, Y., Sy, L. S., Talarico, C. A., Tian, Y., et al. (2022). Effectiveness of mRNA-1273 against SARS-CoV-2 Omicron and delta Variants. *medRxiv*.

Turner, J. S., Kim, W., Kalaidina, E., Goss, C. W., Rauseo, A. M., Schmitz, A. J., et al. (2021). SARS-CoV-2 Infection Induces Long-Lived Bone Marrow Plasma Cells in Humans. *Nature* 595 (7867), 421–425. doi:10.1038/s41586-021-03647-4

Uriu, K., Kimura, I., Shirakawa, K., Takaori-Kondo, A., Nakada, T. A., Kaneda, A., et al. (2021). Neutralization of the SARS-CoV-2 Mu Variant by Convalescent and Vaccine Serum. *N. Engl. J. Med.* 385 (25), 2397–2399. doi:10.1056/nejmc2114706

USCDC (2021). ACIP COVID-19 Vaccines. Available from: https://www.cdc.gov/vaccines/acip/meetings/downloads/slides-2021-04-23/06-COVID-Oliver-508.pdf.

USCDC (2021). COVID-19 Vaccines while Pregnant or Breastfeeding. Available from: https://www.cdc.gov/coronavirus/2019-ncov/vaccines/recommendations/pregnancy.html.

USCDC (2021). Interim Clinical Considerations for Use of COVID-19 Vaccines Currently Approved or Authorized in the United States. Available from: https://www.cdc.gov/vaccines/covid-19/clinical-considerations/covid-19-vaccines-us.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fvaccines%2Fcovid-19%2Finfo-by-product%2Fclinical-considerations.html#Contraindications.

USFDA (2021). Fact Sheet for Healthcare Providers Administering Vaccine (Vaccination Providers) Emergency Use Authorization (Eua) of the Janssen Covid-19 Vaccine to Prevent Coronavirus Disease 2019 (Covid-19). Available from: https://www.fda.gov/media/146304/download.

USFDA (2021). Fact Sheet for Healthcare Providers Administering Vaccine (Vaccination Providers) Emergency Use Authorization (Eua) of the Moderna Covid-19 Vaccine to Prevent Coronavirus Disease 2019 (Covid-19). Available from: https://www.fda.gov/media/144637/download.

USFDA (2021). Fact Sheet for Healthcare Providers Administering Vaccine (Vaccination Providers) Emergency Use Authorization (Eua) of the Pfizer-Biontech Covid-19 Vaccine to Prevent Coronavirus Disease 2019 (Covid-19). Available from: https://www.fda.gov/media/144413/download.

Valdes-Balbin, Y., Santana-Mederos, D., Quintero, L., Fernández, S., Rodriguez, L., Sanchez Ramirez, B., et al. (2021). SARS-CoV-2 RBD-Tetanus Toxoid Conjugate Vaccine Induces a Strong Neutralizing Immunity in Preclinical Studies. *ACS Chem. Biol.* 16 (7), 1223–1233. doi:10.1021/acschembio.1c00272

Van De Pas, R., Widdowson, M.-A., Ravinetto, R., N Srinivas, P., N Srinivas, T. J., Fofana, T. O., et al. (2022). COVID-19 Vaccine Equity: a Health Systems and Policy Perspective. *Expert Rev. Vaccin.* 21 (1), 25–36. doi:10.1080/14760584.2022.2004125

Vicenti, I., Gatti, F., Scaggiante, R., Boccuto, A., Zago, D., Basso, M., et al. (2021). Single-dose BNT162b2 mRNA COVID-19 Vaccine Significantly Boosts Neutralizing Antibody Response in Health Care Workers Recovering from Asymptomatic or Mild Natural SARS-CoV-2 Infection. *Int. J. Infect. Dis.* 108, 176–178. doi:10.1016/j.ijid.2021.05.033

Vie Pce (2021). Aprueba El Cecmed El Autorizo De Uso De Emergencia Del Candidato Vacunal Cubano Abdala. Available from: https://www.cecmed.cu/noticias/aprueba-cecmed-autorizo-uso-emergencia-candidato-vacunal-cubano-abdala.

Voysey, M., Costa Clemens, S. A., Madhi, S. A., Weckx, L. Y., Folegatti, P. M., Aley, P. K., et al. (2021). Single-dose Administration and the Influence of the Timing of the Booster Dose on Immunogenicity and Efficacy of ChAdOx1 nCoV-19 (AZD1222) Vaccine: a Pooled Analysis of Four Randomised Trials. *Lancet* 397 (10277), 881–891. doi:10.1016/S0140-6736(21)00432-3

Wang, H., Zhang, Y., Huang, B., Deng, W., Quan, Y., Wang, W., et al. (2020). Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent Protection against SARS-CoV-2. *Cell* 182 (3), 713–721. doi:10.1016/j.cell.2020.06.008

Wang, L., Wang, Q., Davis, P. B., Volkow, N. D., and Xu, R. (2022). Increased Risk for COVID-19 Breakthrough Infection in Fully Vaccinated Patients with Substance Use Disorders in the United States between December 2020 and August 2021. *World Psych.* 21, 124–132. doi:10.1002/wps.20921

Wang, M., Zhang, L., Li, Q., Wang, B., Liang, Z., Sun, Y., et al. (2021). Reduced Sensitivity of the SARS-CoV-2 Lambda Variant to Monoclonal Antibodies and Neutralizing Antibodies Induced by Infection and Vaccination. *Emerg. Microbes Infect.* 11, 1–30. doi:10.1080/22221751.2021.2008775

Wang, Q., Zhuang, P., Huang, H., Li, L., Liu, L., Webber, H. C., et al. (2020). Mouse γ-Synuclein Promoter-Mediated Gene Expression and Editing in Mammalian Retinal Ganglion Cells. *J. Neurosci.* 40 (20), 3896–3914. doi:10.1523/jneurosci.0102-20.2020

Wei, J., Stoesser, N., Matthews, P. C., Ayoubkhani, D., Studley, R., Bell, I., et al. (2021). Antibody Responses to SARS-CoV-2 Vaccines in 45,965 Adults from the General Population of the United Kingdom. *Nat. Microbiol.* 6 (9), 1140–1149. doi:10.1038/s41564-021-00947-3

Werbel, W. A., Boyarsky, B. J., Ou, M. T., Massie, A. B., Tobian, A. A. R., Garonzik-Wang, J. M., et al. (2021). Safety and Immunogenicity of a Third Dose of SARS-CoV-2 Vaccine in Solid Organ Transplant Recipients: A Case Series. *Ann. Intern. Med.* 174 (9), 1330–1332. doi:10.7326/l21-0282

WHO (2021). COVID-19 Subcommittee of the WHO Global Advisory Committee on Vaccine Safety (GACVS): Updated Guidance Regarding Myocarditis and Pericarditis Reported with COVID-19 mRNA Vaccines. Available from: https://www.who.int/news/item/09-07-2021-gacvs-guidance-myocarditis-pericarditis-covid-19-mrna-vaccines.

WHO (2021). Statement of the WHO Global Advisory Committee on Vaccine Safety (GACVS) COVID-19 Subcommittee on Reports of Guillain-Barré Syndrome (GBS) Following Adenovirus Vector COVID-19 Vaccines. Available from: https://www.who.int/news/item/26-07-2021-statement-of-the-who-gacvs-covid-19-subcommittee-on-gbs.

WHO (2021). The Sinovac-CoronaVac COVID-19 Vaccine: What You Need to Know. Available from: https://www.who.int/news-room/feature-stories/detail/the-sinovac-covid-19-vaccine-what-you-need-to-know.

WHO (2021). Tracking SARS-CoV-2 Variants. Available from: https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/.

Wu, S., Huang, J., Zhang, Z., Wu, J., Zhang, J., Hu, H., et al. (2021). Safety, Tolerability, and Immunogenicity of an Aerosolised Adenovirus Type-5 Vector-Based COVID-19 Vaccine (Ad5-nCoV) in Adults: Preliminary Report of an Open-Label and Randomised Phase 1 Clinical Trial. *Lancet Infect. Dis.* 21 (12), 1654–1664. doi:10.1016/s1473-3099(21)00396-0

Xia, S., Duan, K., Zhang, Y., Zhao, D., Zhang, H., Xie, Z., et al. (2020). Effect of an Inactivated Vaccine against SARS-CoV-2 on Safety and Immunogenicity Outcomes. *JAMA* 324 (10), 951–960. doi:10.1001/jama.2020.15543

Xia, S., Zhang, Y., Wang, Y., Wang, H., Yang, Y., Gao, G. F., et al. (2021). Safety and Immunogenicity of an Inactivated SARS-CoV-2 Vaccine, BBIBP-CorV: a Randomised, Double-Blind, Placebo-Controlled, Phase 1/2 Trial. *Lancet Infect. Dis.* 21 (1), 39–51. doi:10.1016/s1473-3099(20)30831-8

Xie, X., Han, J. B., Ma, G., Feng, X. L., Li, X., Zou, Q. C., et al. (2021). Emerging SARS-CoV-2 B.1.621/Mu Variant Is Prominently Resistant to Inactivated Vaccine-Elicited Antibodies. *Zool Res.* 42 (6), 789–791. doi:10.24272/j.issn.2095-8137.2021.343

Yahav, D., Rozen-Zvi, B., Mashraki, T., Atamna, A., Ben-Zvi, H., Bar-Haim, E., et al. (2021). Immunosuppression Reduction when Administering a Booster Dose of the BNT162b2 mRNA SARS-CoV-2 Vaccine in Kidney Transplant Recipients without Adequate Humoral Response Following Two Vaccine Doses: Protocol for a Randomised Controlled Trial (BECAME Study). *BMJ Open* 11 (10), e055611. doi:10.1136/bmjopen-2021-055611

Yan, Z. P., Yang, M., and Lai, C. L. (2021). COVID-19 Vaccines: A Review of the Safety and Efficacy of Current Clinical Trials. *Pharmaceuticals (Basel)* 14 (5). doi:10.3390/ph14050406

Yan, Z., Yang, M., and Lai, C.-L. (2021). COVID-19 Vaccinations: A Comprehensive Review of Their Safety and Efficacy in Special Populations. *Vaccines* 9 (10), 1097. doi:10.3390/vaccines9101097

Yan, Z., Yang, M., and Lai, C.-L. (2021). Long COVID-19 Syndrome: A Comprehensive Review of its Effect on Various Organ Systems and Recommendation on Rehabilitation Plans. *Biomedicines* 9 (8), 966. doi:10.3390/biomedicines9080966

Yang, S., Li, Y., Dai, L., Wang, J., He, P., Li, C., et al. (2021). Safety and Immunogenicity of a Recombinant Tandem-Repeat Dimeric RBD-Based Protein Subunit Vaccine (ZF2001) against COVID-19 in Adults: Two Randomised, Double-Blind, Placebo-Controlled, Phase 1 and 2 Trials. *Lancet Infect. Dis.* 21 (8), 1107–1119. doi:10.1016/s1473-3099(21)00127-4

Yigit, M., Ozkaya-Parlakay, A., Cosgun, Y., Ince, Y. E., Bulut, Y. E., and Senel, E. (2021). Should a Third Booster Dose Be Scheduled after Two Doses of CoronaVac? A Single-center Experience. *J. Med. Virol.* 94 (1), 287–290. doi:10.1002/jmv.27318

Yigit, M., Ozkaya-Parlakay, A., and Senel, E. (2021). Evaluation of COVID-19 Vaccine Refusal in Parents. *Pediatr. Infect. Dis. J.* 40 (4), e134–e136. doi:10.1097/inf.0000000000003042

Zhao, X., Zheng, A., Li, D., Zhang, R., Sun, H., Wang, Q., et al. (2021). Neutralisation of ZF2001-Elicited Antisera to SARS-CoV-2 Variants. *Lancet Microbe* 2 (10), e494. doi:10.1016/s2666-5247(21)00217-2

Zhou, Y., Zhang, J., Wu, W., Liang, M., and Wu, Q.-S. (2021). Willingness to Receive Future COVID-19 Vaccines Following the COVID-19 Epidemic in Shanghai, China. *BMC Public Health* 21 (1), 1103. doi:10.1186/s12889-021-11174-0

Zhukova, A., Blassel, L., Lemoine, F., Morel, M., Voznica, J., and Gascuel, O. (2020). Origin, Evolution and Global Spread of SARS-CoV-2. *C R Biol.* [Epub ahead of print]. doi:10.5802/crbiol.29

Zuckerman, N., Nemet, I., Kliker, L., Atari, N., Lustig, Y., Bucris, E., et al. (2021). The SARS-CoV-2 Lambda Variant and its Neutralisation Efficiency Following Vaccination with Comirnaty, Israel, April to June 2021. *Euro Surveill.* 26 (45), 1. doi:10.2807/1560-7917.ES.2021.26.45.2100974

Рыжиков ЕАР, А. Б., БоҐрянцева, М. Ш., Усова, С. В., Даниленко, Е. Д., Нечаева, Е. А., Шьянков, О. В., et al. (2021). *A Single Blind, Placebo-Controlled Randomized Study of the Safety, Reactogenicity and Immunogenicity of the "EpiVacCorona" Vaccine for the Prevention of COVID-19, in Volunteers Aged 18–60 Years (Phase I–II)*. Moscow: Russian Journal of Infection and Immunity.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Drug Discovery

REVIEW
published: 30 March 2022
doi: 10.3389/fddsv.2022.837587

The Need for Speed and Efficiency: A Brief Review of Small Molecule Antivirals for COVID-19

*Ana C. Puhl, Thomas R. Lane, Fabio Urbina and Sean Ekins**

Collaborations Pharmaceuticals, Inc., Raleigh, NC, United States

While we currently have multiple highly effective vaccines approved for use against SARS-CoV-2 in the USA and other countries, there are far fewer small molecule antivirals approved to date. The emergence of the latest SARS-CoV-2 variant, Omicron which is heavily mutated in the spike protein, is also raising concerns about the effectiveness of these current vaccines and increasing the call for more therapeutic options. At the time of writing only remdesivir is approved by the FDA while molnupiravir (already approved in the United Kingdom) and Paxlovid (PF-07321332) have emergency use authorizations from the FDA. Repurposed molecules, such as dexamethasone and baricitinib, have been authorized for emergency use in some countries and are used in combination with remdesivir. After 2 years we are only now starting to see the progression of further molecules through animal models to assess their efficacy before clinical trials. As datasets accumulate from both *in vitro* and *in vivo* animal efficacy models, this may allow us to understand the physicochemical properties necessary for antiviral activity and enable the search for additional antivirals. We now summarize 25 small molecule drugs that are either approved, in the process of approval or in the pipeline for COVID which have both *in vitro* and *in vivo* data. We demonstrate that these drugs are structurally diverse and cover a wide chemistry space. This information may aid our understanding of what it takes to be a promising treatment for COVID-19 and propose how to discover antivirals faster and more efficiently for the next pandemic.

Keywords: SARS-CoV-2, antiviral, cytokine storm, COVID-19, drug discovery pipeline, repurposing

OPEN ACCESS

Edited by:
Bruno Villoutreix, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
José L Medina-Franco, National Autonomous University of Mexico, Mexico
Olivier Terrier, UMR5308 Centre International de Recherche en Infectiologie (CIRI), France
Giuseppe Felice Mangiatordi, Italian National Research Council, Italy

***Correspondence:**
Sean Ekins
sean@collaborationspharma.com

Specialty section:
This article was submitted to Anti-Infective Agents, a section of the journal Frontiers in Drug Discovery

Received: *16 December 2021*
Accepted: *27 January 2022*
Published: *30 March 2022*

Citation:
Puhl AC, Lane TR, Urbina F and Ekins S (2022) The Need for Speed and Efficiency: A Brief Review of Small Molecule Antivirals for COVID-19. Front. Drug. Discov. 2:837587. doi: 10.3389/fddsv.2022.837587

INTRODUCTION

At the time of writing, we are still in the midst of a major a global health crisis caused by the virus Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) that was originally reported in Wuhan, China in late 2019 (Coronaviridae, 2020; Wu et al., 2020). This virus causes the disease COVID-19 [3] and shares aspects of pathology and pathogenesis with the earlier Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) (Liu et al., 2020a). SARS-CoV-2, SARS-CoV and MERS-CoV belong to the same family (Coronaviridae) and genus (*Betacoronavirus*). SARS-CoV-2 results in cough, loss of smell and taste, respiratory distress and pneumonia as well as a host of other symptoms (Pan et al., 2020) including extrapulmonary events characterized by a sepsis-like disease collectively called 2019 coronavirus disease (COVID-19) (WHO, 2020). SARS-CoV-2 directly interacts with angiotensin converting enzyme 2 (ACE2) receptor in many cell types (Brann et al., 2020; Bunyavanich et al., 2020; Sungnak et al., 2020; Whitcroft and Hummel, 2020). SARS-CoV-2 rapidly spread worldwide prompting the World Health

GRAPHICAL ABSTRACT | Table of contents graphic.

Organization to declare the outbreak a pandemic, with more than 1.5 million cases confirmed in less than 100 days.[4] The high infection rate has caused considerable stress on global healthcare systems leading to over 6 M deaths from COVID-19 at the time of writing (January 2022, World Health Organization COVID-19 dashboard).

In the USA, there are 3 vaccines available to protect against SARS-CoV-2 (Huang et al., 2021; Kyriakidis et al., 2021; Rehman et al., 2021), and globally there are over 20 vaccines currently approved (Craven, 2021). COVID-19 continues to represent an ongoing public health crisis for which vaccines represent our first line of defense. The recent identification in South Africa (and subsequently in other countries) of a new strain B.1.1.529 named Omicron as a variant of concern due to its heavily mutated nature with over 30 changes to the spike protein alone suggests it may reduce vaccine efficacy (Callaway, 2021) although those who received boosters may be better protected. This rapidly developing scenario would suggest the urgent need for other therapeutic approaches to address this and future variants.

There are limited options when it comes to small molecule antivirals, with only remdesivir being FDA approved currently in the US. Several other already approved drugs were quickly touted by the popular press, such as hydroxychloroquine and ivermectin, based on either limited *in vitro* or clinical data and subsequent clinical trials have demonstrated their resounding lack of efficacy (Galan et al., 2021; Vallejos et al., 2021). There have been extensive repurposing efforts since the pandemic began and numerous computational approaches have proposed drugs to be tested. Much of this early work has been reviewed by us and others previously (Ekins et al., 2020; Muratov et al., 2021). As the general public have observed that vaccines for COVID-19 were developed rapidly in months, there is the unrealistic expectation that antiviral small molecule drugs can also be developed as rapidly. Unfortunately, those in the industry accept that it normally takes a decade or more for a small molecule to progress through the various stages from drug discovery to the clinic at a cost in excess of $1 billion (Paul et al., 2010). Repurposing already FDA approved drugs may be expected to progress much more rapidly.

We were keen to evaluate small molecules which have both *in vitro* activity and have been tested *in vivo* against one of the various SARS-CoV-2 animal models (mouse, hamster or non-human primate etc.) (Muñoz-Fontela et al., 2020). While this evaluation is likely not comprehensive and because of the fast-moving nature of COVID-19 research it will almost certainly be rapidly outdated. Our goal is therefore to understand the classes of molecules that have shown promise to date. Obviously, there are major pharmaceutical companies, with seemingly unlimited resources and capabilities, involved in identifying molecules (e.g., Pfizer) or licensing them from others (e.g., Merck). Those outside of these larger pharmaceutical companies in either smaller companies or increasingly in academia need to find a way to collaborate with those who have the capabilities to test molecules under BSL3 conditions *in vitro* and *in vivo*. This will require different skill sets such as coordinating complex, multidimensional projects and may include multiple international partners which may add other issues related to funding and intellectual property.

While thousands of papers (nearly 200,000 articles in PubMed at the time of writing) have been written relating to COVID-19, it would be impossible to compress this knowledge into a single review. For example, there are also likely thousands of clinical trials globally, which is outside the scope of this effort. Instead, we will describe *in vitro* screening efforts and the molecules derived from these screens that progressed to *in vivo* models as this is more manageable and valuable for future drug discovery efforts.

In Vitro Screening

Early in the SARS-CoV-2 pandemic many of the repurposing efforts used FDA approved drugs that had previously been shown to have antiviral activity against other related viruses. Several of these drugs had low μM activity and a selectivity index (SI) greater than 10 in Vero cells, including nitazoxanide (EC_{50} 2.12 μM), remdesivir (EC_{50} 0.77 μM, and chloroquine (EC_{50} 1.13 μM). This work alone represented one of the earliest articles describing the use of remdesivir and chloroquine (Wang et al., 2020a). While chloroquine was identified early on, the derivative hydroxychloroquine was in multiple clinical trials in China by February 2020. It is also worth noting that the *in vitro* activity of hydroxychloroquine (EC_{50} 4.51 μM) was not as potent as chloroquine when assessed at four different multiplicities of infection (Liu et al., 2020b). However, it is likely this work generated significant interest in this compound that led to the subsequent numerous clinical trials. Other groups confirmed this activity in Vero cells and also demonstrated activity in Caco-2 but not Calu-3 cells (Clementi et al., 2020). Several groups showed similar activity for remdesivir in Vero cells (EC_{50} 1.65 μM), with increased activity in human epithelial cultures (EC_{50} 0.01 μM) and Calu-3 (EC_{50} 0.28 μM) (Pruijssers et al., 2020). Additional molecules were identified including eight artemisinins and lumefantrine (EC_{50} 23.50 μM) which were tested in Vero cells and time of addition studies suggested this was working post entry (Cao et al., 2020). While not a focus of the current analysis, natural products were also tested *in vitro,* such as lycorine (EC_{50} 0.31 μM) and oxysophoridine (EC_{50} 0.18 μM), and many of these had activity in Vero cells with increased potency over drugs like gemcitabine (EC_{50} 1.24 μM) and chloroquine (EC_{50} 1.38 μM) (Zhang et al., 2020a). Larger collections of molecules were also tested in Vero cells which resulted in identification of niclosamide (IC_{50} 0.28 μM) and ciclesonide (IC_{50} 4.33 μM) as hits (Jeon et al.,

2020). Additionally, the antiviral tilorone (IC_{50} 4 μM) was identified as an early hit (Jeon et al., 2020) and has previously been shown to have similar activity against MERS (Ekins and Madrid, 2020) and Ebola (Ekins et al., 2015a) [as has remdesivir (de Wit et al., 2020)]. An earlier preprint (Jeon et al., 2020) also included the antimalarial pyronaridine (IC_{50} 31 μM). The FDA approved antiparasitic, ivermectin (IC_{50} 2.8 μM) was also shown to have *in vitro* activity in Vero cells, which likely also sparked early interest in this molecule (Caly et al., 2020). 12,000 clinical stage or FDA approved compounds in the ReFRAME library were screened against Vero cells. 21 hits were identified with promising dose response relationships in Vero cells including apilimod (EC_{50} 0.023 μM) and clofazimine (EC_{50} 0.310 μM) (Riva et al., 2020). Further, the PIKfyve kinase inhibitor apilimod was tested in 293T cells (EC_{50} 0.012 μM) and Huh-7 cells (0.088 μM) (Riva et al., 2020). A second group demonstrated how SARS-CoV-2 modified phosphorylation in infected cells and proposed kinase inhibitors as important (Bouhaddou et al., 2020) including apilimod which showed activity in Vero (IC_{50} < 0.08 μM) and in A549 cells (IC_{50} 0.007 μM) (Bouhaddou et al., 2020). A protein interaction map identified FDA and clinical stage compounds binding to sigma-1 and 2 receptors which act as host factors, with the most potent compound identified in Vero cells being PB28 (IC_{90} 280 nM) (Gordon et al., 2020a). Much of this early *in vitro* screening work was in Vero cells, and when promising compounds are tested in human cells, they may have very different activity likely due to the lack of the host protein TMPRSS2 (Shulla et al., 2011; Hoffmann et al., 2020; Shang et al., 2020).

Other cell types have also been used for larger screens, such as a quantitative HTS in Huh7 cells, which tested 1425 compounds and identified 11 novel compounds with activity IC_{50} < 1 μM including lactoferrin which showed potent activity (IC_{50} 308 nM) (Mirabelli et al., 2020). An enzyme-linked immunosorbent assay (ELISA) and cell viability assay screen of 1528 compounds led to 19 hits, out of which 4 were the most active in Vero cells and included cetilistat (EC_{50} 1.13 μM), diiodhydroyquinoline (EC_{50} 1.38 μM), abiraterone acetate (EC_{50} 1.94 μM) and bexarotene (EC_{50} 2.01 μM) (Yuan et al., 2020). A screen of the Prestwick library in hPSC lung organoids identified imatinib (EC_{50} 4.86 μM), mycophenolic acid (EC_{50} 0.15 μM) and quinacrine (EC_{50} 2.83 μM) (Han et al., 2020). This was followed by testing in mice treated with these 3 drugs infected with SARS-CoV-2 pseudovirus and showed significant decreases in infected cells (Han et al., 2020). This is of interest for several reasons, one being that others had not observed *in vitro* SARS-CoV-2 activity for quinacrine in Vero cells (Jeon et al., 2020). Quinacrine and tilorone have also previously been demonstrated to possess activity against Ebola infected HeLa cells (Ekins et al., 2015a) and not Vero cells (Lane et al., 2019a) and we more recently have tested these compounds in several cell types infected with SARS-CoV-2 (Puhl et al., 2021a). Target-based screens have also been performed, with a FRET-based screen of M^{pro} which assessed 10,000 compounds, finding 7 primary hits and one of these being ebselen (IC_{50} 0.67 μM) which also had activity in Vero cells (EC_{50} 4.67 μM) (Jin et al., 2020a). It should be noted that from all this predominantly *in vitro* testing, very few compounds have progressed to *in vivo* animal models of SARS-CoV-2 infection.

Antivirals of Most Interest

Remdesivir

There are currently few small-molecule drugs approved for COVID-19 (Hall et al., 2021), including remdesivir (Eastman et al., 2020), which as described above originally demonstrated activity in Vero cells (Wang et al., 2020a; Pruijssers et al., 2020), human epithelial cells and in Calu-3 cells (Pruijssers et al., 2020) infected with SARS-CoV-2 prior to clinical testing. Remdesivir represents a "repurposed prodrug" which was originally developed for Hepatitis C virus, then repurposed for treating Ebola virus (EBOV) and has since reached clinical trials for EBOV (Mulangu et al., 2019). Remdesivir's target specificity and potency towards RNA polymerase was noted early on (Gordon et al., 2020b), as it causes irreversible chain termination. A primary human lung epithelium infection model and a lung organoid model were used to show remdesivir could suppress viral infection (Mulay et al., 2020). Remdesivir was therefore repurposed very quickly (Wang et al., 2020a; Pruijssers et al., 2020) obtaining an emergency use authorization and then FDA approval in less than a year (Eastman et al., 2020). Subsequently, there have been many clinical studies for remdesivir, but the effectiveness of this drug is far from comprehensive (Wang et al., 2020b; Goldman et al., 2020; Spinner et al., 2020; Barratt-Due et al., 2021) and yet still it is the only small molecule approved by the FDA (while molnupiravir and paxlovid have emergency use authorizations) for use alone against COVID-19. This antiviral is severely limited by its requirement to be administered I.V. and its use is therefore restricted to a hospital setting. We are aware of remdesivir oral formulations being tested so these may overcome the limitations in future.

Molnupiravir

Molnupiravir (EIDD-2801, MK4482) is a prodrug that was identified as an inhibitor of influenza A and respiratory syncytial virus acting as an RNA mutagen and like remdesivir was initially developed as a hepatitis C inhibitor in the early 2000s. This was shown early on in the pandemic to be active *in vitro* against SARS-CoV-2 and progressed to *in vivo* testing in mice and hamster (Sheahan et al., 2020; Rosenke et al., 2021; Wahl et al., 2021). We are not aware of any clinical trial (NCT04392219) data that has been peer reviewed yet for this molecule although Merck have obtained emergency use authorization from the FDA. Molnupiravir was initially reported to have reduced the risk of hospitalization or death by approximately 50% compared to placebo for patients with mild or moderate COVID-19 (NCT04575597) (Anon, 2021), although this was recently adjusted to 30% (Anon, 2021b) and may impact its ultimate approval. Molnupiravir is approved in Britain for use in people with mild to moderate COVID-19 and at least one risk factor for developing severe illness, such as obesity, older age diabetes, and heart disease.

Paxlovid

The rapid development of the potent M^{pro} inhibitor PF-07321332 and clinical testing demonstrates the capabilities of a major pharma. However, it is worth pointing out that its

development also began nearly 20 years earlier from a potent M^{pro} inhibitor for SARS-CoV-1. This compound was also a known P-glycoprotein substrate requiring it to be tested *in vitro* in Vero E6 cells with a P-gp inhibitor as these cells express high levels of this efflux transporter (Owen et al., 2021). The molecule is also a substrate for CYP3A4. Clinically this compound is used in combination with the protease inhibitor ritonavir to inhibit its metabolism and has been branded as Paxlovid (Owen et al., 2021). Like for molnupiravir, we are not aware of any clinical trial data that has been peer reviewed and published yet for this molecule at the time of writing, although Pfizer have also obtained an FDA emergency use authorization. This drug has been reported to reduce the risk of hospitalization or death by 89% compared to placebo in non-hospitalized high-risk adults with COVID-19 in interim analysis of phase 2/3 EPIC-HR study in which no deaths were reported in patients who received Paxlovid compared to 10 deaths in patients who received placebo. One of the major limitations of this drug is its complex synthesis and limited supply of the clinical material. Therefore, efforts to develop inhibitors that are more readily synthesized may be ideal and there is considerable activity around developing additional M^{pro} inhibitors such as GC376 (Dampalla et al., 2021).

PF-00835231

PF-00835231 is potent inhibitor of M^{pro}, which binds covalently to the protease and is administered i. v. PF-00835231 is an analog of rupintrivir, a human rhinovirus (HRV) Mpro inhibitor. PF-07304814 is a phosphate prodrug that is rapidly converted *in vivo* to the active moiety, PF-00835231, which exhibits high selectivity over human proteases, acts as a broad-spectrum protease inhibitor and demonstrates potent antiviral activity *in vivo* (Boras et al., 2021). PF-07304814 exhibits an encouraging preclinical profile that has the ADME, safety, and once converted to PF-00835231, SARS-CoV-2 antiviral activity to support progression to the clinic as a COVID-19 single-agent antiviral treatment. The favorable profile of PF-07304814 enabled the rapid progression to clinical trials (NCT04627532 and NCT04535167) (Boras et al., 2021).

Favipiravir

Favipiravir is an approved antiviral in Japan for pandemic influenza and has also demonstrated some activity against Ebola and other viruses *in vivo* animal models, suggesting a broad-spectrum activity. Like molnupiravir, favipiravir leads to mutations in the viral RNA (Driouich et al., 2021). Favipiravir is not potent and often requires high doses leading to some toxicity in animal models. To date most of these SARS-CoV-2 *in vivo* studies have been in hamster (Kaptein et al., 2020; Driouich et al., 2021; Touret et al., 2021).

Dexamethasone

As inflammation is one of the hallmarks in COVID-19 and particularly severe in hospitalized patients, the role of anti-inflammatory agents such as steroids was studied early on. By July 2020 a clinical trial had showed the effectiveness of dexamethasone by decreasing mortality in patients requiring supplemental oxygen or mechanical ventilation (Group et al., 2021). More recent trials in patients with moderate to severe infection against SARS-CoV-19 showed survival benefits of using dexamethasone when given in addition to the standard of care (Tomazini et al., 2020). We are not aware of any *in vitro* or *in vivo* studies for this compound that enabled its progression to clinical trials.

Fluvoxamine

Like dexamethasone, other molecules have apparently progressed to clinical trials without apparent *in vitro* or *in vivo* testing against SARS-CoV-2. One such molecule is the selective serotonin reuptake inhibitor fluvoxamine, which is used to treat obsessive compulsive disorder and depression, with promising recent clinical trial results against SARS-CoV-2. This molecule has been shown to bind the sigma receptor, reduces inflammation and protects against septic shock in mice (Rosen et al., 2019). The first small trial was a double blind randomized fully remote contactless clinical trial with 80 patients treated with fluvoxamine 100 mg and 72 patients with a placebo, dosed 3 times a day. Patients on fluvoxamine had lower odds of clinical deterioration (Lenze et al., 2020). The most recent study described a clinical trial performed in Brazil with 741 patients given fluvoxamine 100 mg twice daily for 10 days. Amongst high-risk patients with early diagnosed COVID-19 hospitalization was reduced (Reis et al., 2021). There are several likely mechanisms for fluvoxamine against SARS-CoV-2 (Sukhatme et al., 2021). One of them is that sigma 1 receptor agonists like fluvoxamine and fluoxetine are lysosomotropic (Hallifax and Houston, 2007; Kazmi et al., 2013). Given the lysosomal egress of β-coronaviruses from infected cells, lysosomotropic drugs like fluvoxamine could have antiviral effects in the virus laden lysosomes (Homolak and Kodvanj, 2020).

Pyronaridine

A machine learning model was used to identify pyronaridine tetraphosphate (Ekins et al., 2015b) for testing against EBOV and subsequently this molecule inhibited EBOV and Marburg *in vitro* as well as demonstrating significant efficacy in the mouse-adapted EBOV (ma-EBOV) model (Ekins et al., 2018; Lane et al., 2019a; Lane et al., 2019b). Pyronaridine was identified as a possible virus entry inhibitor (Lane and Ekins, 2020). Pyronaridine tetraphosphate is used as an antimalarial in several countries as part of a combination therapy with artesunate (Pyramax). Pyronaridine alone also demonstrated significant activity in the guinea pig-adapted model of EBOV infection (Lane et al., 2020a). It has been recently shown that this compound possesses *in vitro* activity against SARS-CoV-2 (Bae et al., 2020; Jeon et al., 2020; Puhl et al., 2021a) and pyronaridine is in a clinical trial administered in combination with artesunate. Using A549-ACE2 cells, which support SARS-CoV2 growth to about 10^7 PFU/ml, pyronaridine showed SARS-CoV-2 inhibition demonstrating IC_{50} 0.23 μM and a good selectivity index and binding to SARS-CoV-2 spike RBD (K_d 0.62 μM) (Puhl et al., 2021a) while more recently it has been shown to inhibit Pl^{pro} (IC_{50} 1.8 μM) (Puhl et al., 2021b). Pyronaridine is of particular interest as the C_{max} data for pyronaridine in our previous mouse

pharmacokinetics studies (i.p. dosing) suggested that plasma levels that are above the average IC_{50} observed for SARS-CoV-2 inhibition *in vitro* (Lane et al., 2019b) can be reached with dosing well below the maximum tolerated dose. Pyronaridine also has excellent *in vitro* ADME properties with a long half-life that makes a single dose treatment possible (Lane et al., 2019b; Puhl et al., 2021a). We have recently assessed the *in vivo* efficacy of pyronaridine in a K18-hACE2 mouse model of COVID-19 (Puhl et al., 2021b) where it resulted in a decreased viral load and improved lung histopathology. Cytokine and chemokine analysis showed increased INF-1β levels and decreased IL-6, CXCL1, CCL4 (Puhl et al., 2021b) (**Table 1**).

Kinase Inhibitors

Many FDA-approved kinase inhibitors have previously been proposed as broad-spectrum antiviral therapies (Baranov et al., 2020) as they have multiple host protein targets required for the viral life cycle, replication, and infection of multiple virus types. Kinase inhibitors also possess anti-inflammatory and cytokine inhibitory activity properties which may address the lung damage from respiratory virus infections (Baranov et al., 2020). One of the earliest molecules to be computationally repurposed using a knowledge graph approach was the AAK1 and JAK1/2 kinase inhibitor baricitinib (Richardson et al., 2020). The mechanism was eventually validated *in vitro* and in human patients (Stebbing et al., 2020; Lenz et al., 2021) as well as in combination with hydroxychloroquine (Titanji et al., 2021). This molecule was granted an FDA emergency use authorization in combination with remdesivir. Subsequently, protein kinase inhibitors have been proposed for treating SARS-CoV-2 and have demonstrated *in vitro* (Riva et al., 2020; Weisberg et al., 2020; Baranov et al., 2020; Hoang et al., 2021; Zhang et al., 2020b; Zhao et al., 2020) and *in vivo* activity while several are also in clinical trials (Raghuvanshi and Bharate, 2021; Drayman et al., 2021). We also recently screened a panel of 45 kinase inhibitors in a model of SARS-CoV-2 in the β-coronavirus murine hepatitis virus (MHV), a model of SARS-CoV-2 infection, and identified 10 compounds with activity (Puhl et al., 2021c). 2 compounds demonstrated activity against SARS-CoV-2, HCov-229E and MHV (entrectinib and vandetanib) for which the main mechanism remains to be elucidated (Puhl et al., 2021c). Imatinib, masitinib and vandetanib are all kinase inhibitors that have similar low μM *in vitro* activity against SARS-CoV-2. Out of these, only masitinib (Drayman et al., 2021) showed a decrease in viral load in mice, while vandetanib reduced lung inflammation in mice and altered cytokine levels indicative that this might be useful to address the cytokine storm in COVID-19 (Puhl et al., 2021c). Imatinib did not decrease viral load in hamsters infected with SARS-CoV-2 (Touret et al., 2021) (**Table 1**) and clinically it did not decrease the time to discontinuation of ventilation and supplemental oxygen from more than 48 consecutive hours in patients with COVID-19 that required supplemental oxygen (Aman et al., 2021).

Quaternary Ammonium Compounds

A text mining approach undertaken by our team and collaborators recently described hundreds of molecules that have been identified with antiviral effects against coronaviruses in the literature (Baker et al., 2020). One of these was cetylpyridinium chloride, the quaternary ammonium compound used in mouthwashes and nasal sprays which destroys the viral capsid upon direct contact (Baker et al., 2020). Several pilot clinical trials have also suggested the utility of this and other mouthwashes to destroy the virus (Eduardo et al., 2021; Seneviratne et al., 2021). Others have shown a more than 98% reduction of SARS-CoV-2 S pseudovirion entry in 293/hACE2 cells when the cells were treated with lysosomotropic agents increasing endosomal pH, such as ammonium chloride and bafilomycin A (Ou et al., 2021).

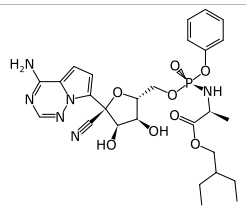
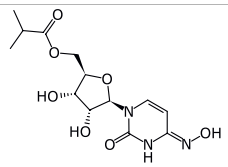
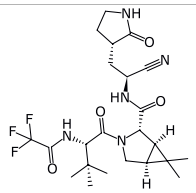
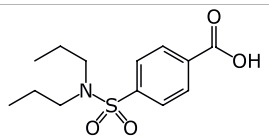
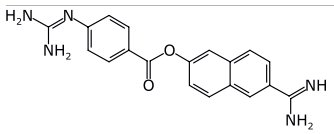
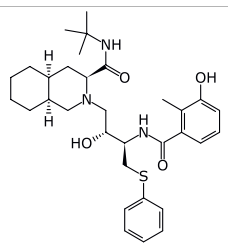
Phospholidosis

A recent article (Tummino et al., 2021) focused on compounds originally identified from a repurposing screen that showed no relationship between sigma 1 receptor potency and SARS-CoV-2 antiviral activity. They demonstrated that for cationic amphiphilic drugs (CADs), phospholipidosis was observed in cells and correlated strongly with their *in vitro* antiviral activity. We recently discussed (Lane and Ekins, 2021) this study and pointed out that compounds with a basic pKa (>6.5) and cLogP of >2 tend to be lysosomotropic and accumulate in the lysosomes (Nadanaciva et al., 2011), which is a key for many phospholipidosis-inducing compounds. 4 phospholipidosis-inducing drugs (amiodarone, sertraline, PB28 and tamoxifen–**Table 1**) that showed *in vitro* activity were tested in a 3-days mouse efficacy model for SARS-CoV-2 infection and these did not show efficacy as measured by viral load (and neither did elacridar a compound that is not a CAD) (Tummino et al., 2021). We pointed out that phospholipidosis may not even be relevant in the mouse model for SARS-CoV-2 as the authors showed amiodarone offers neither antiviral protection nor hallmarks of phospholipidosis. Others have described how CADs accumulate in different subcellular compartments (e.g., mitochondria and lysosomes) (Vater et al., 2017a; Vater et al., 2017b) and basic amines may also lead to accumulation in these compartments and change the pH, inhibiting entry for some viruses. This approach has been proposed as a strategy to decrease SARS-CoV-2 viral infection (Gorshkov et al., 2021). In summary, many CADs have antiviral activity and induce phospholipidosis during chronic treatment and yet this toxicity is reversible and therefore manageable (Salata et al., 2017).

Drug Transporters

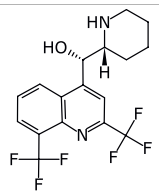
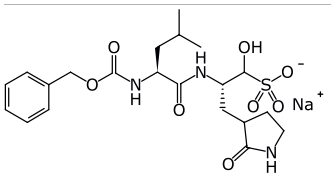
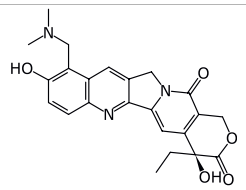
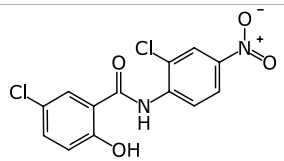
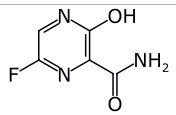
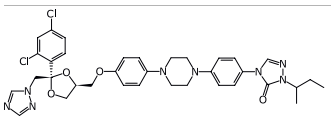
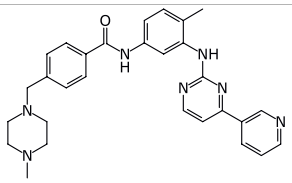
CADs are also of interest because of potential for interfering with human drug transporters. Cationic compounds with SARS-CoV-2 antiviral activity (chloroquine, hydroxychloroquine and quinacrine) inhibited OCT and MATE transporters *in vitro* (Martinez-Guerrero et al., 2021). An independent study has also evaluated 25 drugs used in COVID-19 clinical trials to assess the potential for drug-drug interactions (Yee et al., 2021). Transporters can also be targeted to reach viral sanctuary sites such as the brain and testes. As an example, the human equilibrative nucleoside transporters 1 and 2 (ENT) are of interest because their substrates may gain entry to the testes and other sites. We are at the early stages for understanding these structure-inhibitor and structure-substrate relationships for these

TABLE 1 | Inhibitors of SARS-CoV-2 *in vitro* and *in vivo* in mouse or hamster.

Structure	Name	*In vitro* activity	*In vivo* activity	Target/ Mechanism	Class
	Remdesivir	Vero cells EC_{50} 0.77 μM (Wang et al., 2020a) EC_{50} 1.65 μM (Pruijssers et al., 2020) Vero E6 EC_{50} 1.2 μM (Touret et al., 2021) EC_{90} 1.5 μM (Touret et al., 2021) CC_{50} > 20 μM (Touret et al., 2021) human epithelial cell culture EC_{50} 0.01 μM (Pruijssers et al., 2020) Calu-3 EC_{50} 0.28 μM (Pruijssers et al., 2020)	Mouse—prophylactic and therapeutic dosing infected with SARS-CoV-2 MA^{10} lung viral load significantly decreased (Martinez et al., 2021)	RNA polymerase irreversible chain termination	Active
	Molnupiravir (EIDD-2801, MK-4482)	Vero cells IC_{50} 0.3 μM (Sheahan et al., 2020) Calu-3 IC_{50} 0.08 μM (Sheahan et al., 2020)	C57BL/6 Mice showed decreased virus lung titers and improved histopathology scores (Sheahan et al., 2020) Human lung only mice showed decreased virus titers (>4 log in some caes) 24h, 48 after exposure or 12 h before exposure and improved histopathology (Wahl et al., 2021) Hamster model dosed orally 250 mg/kg/day—1 log reduction in viral RNA and virus lung titers (2 logs) seen in pre-infection or post-infection models (Rosenke et al., 2021)	RNA mutagen	Active
	PF-07321332—(Paxlovid when dosed with ritonavir)	M^{pro} 3.11 nM (Owen et al., 2021) Vero E6 enACE2 74.5 nM (Owen et al., 2021) A459 -ACE2 77.9 nM (Owen et al., 2021)	Statistically significant, reduction of weight loss, > 1 log reduction in virus titer and improved lung histolopathology score at 300 mg/kg dose using SARS-CoV-2 MA^{10} infection in mice (Owen et al., 2021)	M^{pro} inhibitor	Active
	Probenecid	NHBE cells IC50 = 0.0013 μM Vero E6 cells IC50 0.75 μM (Murray et al., 2021)	Hamsters demonstrated a 4–5 log reduction in virus versus control (Murray et al., 2021)	Host (OAT3 inhibitor)	Active
	Nafamostat	Calu-3 2B4 cells IC_{50} 2.2 nM (Li et al., 2021)	2 log reduction in lung tissue viral titer, inhibited weight loss. Protection and increased survival was also seen in K18-hACE2 mice (Li et al., 2021)	Host (TMPRSS2 inhibitor)	Active
	Nelfinavir	Vero E6 cells IC_{50} 3.3 μM CC50 12.3 uM (Jan et al., 2021)	In hamster 30 mg/kg/day significantly reduced lung viral titer after 3 days (Jan et al., 2021)	M^{pro} inhibitor	Active

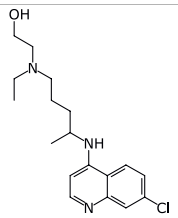
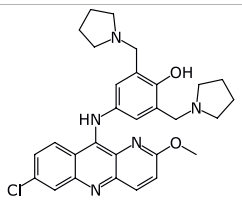
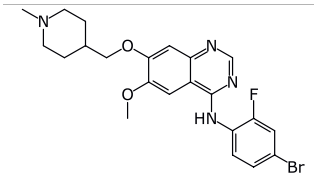
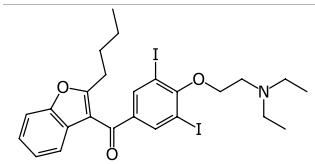
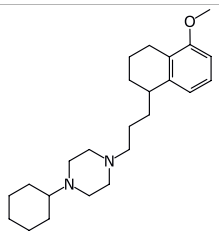
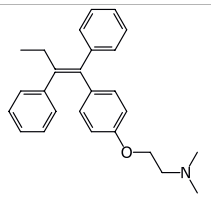
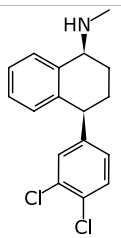
(Continued on following page)

TABLE 1 | (*Continued*) Inhibitors of SARS-CoV-2 *in vitro* and *in vivo* in mouse or hamster.

Structure	Name	*In vitro* activity	*In vivo* activity	Target/ Mechanism	Class
	Mefloquine	Vero E6 cells IC_{50} 3.2 μM CC_{50} > 10 μM (Jan et al., 2021) Vero E6 IC_{50} 7.11 μM and CC_{50} 18.53 μM (Weston et al., 2020)	In hamster 30 mg/kg/day significantly reduced lung viral titer after 3 days (Jan et al., 2021)	Host	Active
	GC-376	Vero cells EC_{50} 0.91 μM (Hung et al., 2020) M^{pro} (K_i 12 nM) (Hung et al., 2020)	K18 hACE2 transgenic mouse showed modest activity, reduced viral load, 5 log reduction of virus in brain (Caceres et al., 2021)	M^{pro}	Active
	Topotecan	In A4549-ACE2 cells it does not inhibit viral replication but it dampens expression of cytokines (IL-6, CXCL2, CXCL3, CXCL8 EGR1, TNFAIP3) (Ho et al., 2021)	K18-hACE2 mice treatment significantly increased survival, decreased inflammatory gene expression in the lung (Ho et al., 2021)	Host	Active
	Niclosamide	Vero E6 cells EC_{50} 0.030 μg/ml (Brunaugh et al., 2021)	Niclosamide-lysozyme tested in hACE2 transgenic mice at day 10 a statistical significant reduction in viral load was seen (Brunaugh et al., 2021)	Host	Active
	Favipiravir	Vero E6 EC_{50} 204 μM (Driouich et al., 2021) Caco-2 no activity (Driouich et al., 2021)	Hamster model 25 mg/day significantly reduced lung infectious titers and viral RNA (Touret et al., 2021) Hamster model at doses from 300 mg/kg oral or 600 mg/kg or 1000 mg/kg ip decreased viral load by day 4 (Kaptein et al., 2020) Hamster model dosed 18.75, 37.5 or 75 mg/kg 3 times/day demonstrated reduced viral load and lung histopathology at the higher concentrations (Driouich et al., 2021)	RNA polymerase and RNA mutagen	Active
	Itraconazole	Vero E6 cells demonstrated significant inhibition at 1 μM (Liesenborghs et al., 2021a; Liesenborghs et al., 2021b)	In the hamster model at either 30 mg/kg/day or 70 mg/kg/day oral dosed did not reduce viral load or decrease inflammation (Liesenborghs et al., 2021a; Liesenborghs et al., 2021b)	Host	Inactive
	Imatinib	Vero E6 cells EC_{50} 2.5 μM, EC_{90} 5.1 μM, CC_{50} > 40 μM (Touret et al., 2021) Human airway epithelial cells—no activity (Touret et al., 2021) Vero E6 IC_{50} 3.24 μM and CC_{50} > 30.86 μM (Weston et al., 2020)	In hamster at doses upto 32 mg/kg BID no significant differences between treated and untreated animals (Touret et al., 2021)	Host	Inactive

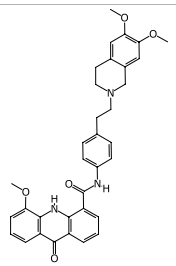
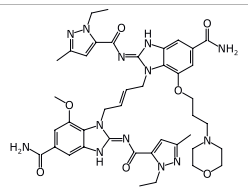
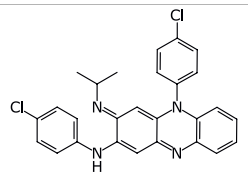
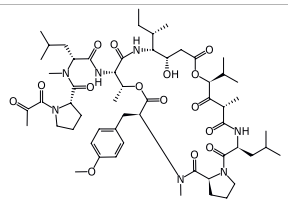
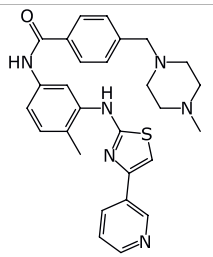
(Continued on following page)

TABLE 1 | (*Continued*) Inhibitors of SARS-CoV-2 *in vitro* and *in vivo* in mouse or hamster.

Structure	Name	*In vitro* activity	*In vivo* activity	Target/ Mechanism	Class
	Hydroxychloroquine	Vero E6 IC_{50} 9.21 μM and CC_{50} > 50 μM (Weston et al., 2020)	Hamster and macaque showed no effect at doses up to 50 mg/kg (Rosenke et al., 2020) Hamster at 50 mg/kg showed no significant effect on viral load (Kaptein et al., 2020) increased INF-1β levels and decreased IL-6, CXCL1, CCL4 (Puhl et al., 2021b)	Host	Inactive
	Pyronaridine	Vero 76 or Vero E6 cells - inactive (Bae et al., 2020) Caco-2 cells EC_{90} 5.49 μM CC_{50} 51.65 μM (Bae et al., 2020) A549-ACE2 (pretreatment) IC_{50} 0.232 μM CC_{50} 11.53 μM (Puhl et al., 2021a) PI^{pro} IC_{50} 1.8 μM (Puhl et al., 2021b)	K18-hACE2 mouse model showed significant decreased viral load and improved lung histopathology after a single 75 mg/kg i.p. dose (Puhl et al., 2021b) Pyronaridine	PI^{pro} and host	Active
	Vandetanib	A549-ACE2 IC_{50} 0.79 μM no sign of cytotoxicity Caco-2 EC_{90} 2 μM CC_{50} 4.1 μM (Puhl et al., 2021c)	K18-hACE2 mouse model no effect on viral load but statistically significantly reduced inflammation in lungs after 25 mg/kg Increased INF-1β in lung, decreased IL-6, IL-10, TNF-α, CCL3 (Puhl et al., 2021c)	Host	Active
	Amiodarone	A549-ACE2 IC_{50} 166 nM Vero E6 IC_{50} 602 nM (Tummino et al., 2021)	Not active in mouse (Tummino et al., 2021)	Host	Inactive
	PB28	A549-ACE2 IC_{50} 407 nM Vero E6 IC_{50} 676 nM (Tummino et al., 2021) Vero cells IC_{90} 280 nM (Gordon et al., 2020c)	Not active in mouse (Tummino et al., 2021)	Host (Sigma receptor- 1 and -2)	Inactive
	Tamoxifen	Vero E6 IC_{50} 34.12 μM and CC_{50} 37.96 μM (Weston et al., 2020) A549-ACE2 IC_{50} 275 nM Vero E6 IC_{50} 2.570 μM (Tummino et al., 2021)	Not active in mouse (Tummino et al., 2021)	Host	Inactive
	Sertraline	A549-ACE2 IC50 134 nM Vero E6 IC_{50} 2.291 μM (Tummino et al., 2021)	Not active in mouse (Tummino et al., 2021)	Host	Inactive

(Continued on following page)

TABLE 1 | (*Continued*) Inhibitors of SARS-CoV-2 *in vitro* and *in vivo* in mouse or hamster.

Structure	Name	*In vitro* activity	*In vivo* activity	Target/ Mechanism	Class
	Elacridar	A549-ACE2 IC_{50} 3.890 μM Vero E6 IC_{50} 575 nM (Tummino et al., 2021)	Not active in mouse (Tummino et al., 2021)	Host	Inactive
	diABZI-4	A549-ACE2 cells 0.1 μM leads to >2 log PFU decrease (Humphries et al., 2021)	0.5 mg/kg increased survival of mice dosed 3 h pre-treatment or 12 post treatment intranasally (Humphries et al., 2021)	Host (STING activator)	Active
	Clofazimine	Vero E6 cells EC_{50} 0.31 μM (Yuan et al., 2021)	25 mg/kg oral dose lowered viral load >1 log PFU in hamster lung when dosed prophylactically or therapeutically (Yuan et al., 2021)	Host and viral helicase	Active
	Plitidepsin	VeroE6 IC_{90} 1.76 nM in hACE2-HEK293T cells IC_{90} 0.88 nM (White et al., 2021)	Lowered mouse lung viral load 2 log PFU after 0.3 mg/kg daily dosing for 3 days (White et al., 2021)	Host (eEF1A)	Active
	Masitinib	A549-ACE2 cells EC_{50} 3.2 μM (Drayman et al., 2021)	25 mg/kg twice a day lowered mouse lung viral load >2 log PFU and improved survival (Drayman et al., 2021)	M^{pro} inhibitor	Active

transporters (Miller et al., 2021a; Miller et al., 2021b; Miller et al., 2021c) and most recently evaluated remdesivir and molnupiravir (Miller et al., 2021a). This illustrated how these transporters may have a role in the efficacy of these compounds and how it may differ for each based on the affinity for these transporters.

Targeting the Cytokine Storm

It has been demonstrated that SARS-CoV-2 causes an imbalance in the human immune system which may lead to a cytokine and chemokine storm (Costela-Ruiz et al., 2020) impacting PDGF, VEGF (Huang et al., 2020), IL-6 (Herold et al., 2020; Wang et al., 2021), IL-8, IL-10 (Wang et al., 2021), TNF-α (Wang et al., 2021) and IFN-α and -β (Molony et al., 2017; Hu et al., 2018; Zhang et al., 2020c; Xia et al., 2020; Yuen et al., 2020). Furthermore, an impaired type I interferon response has already been observed in COVID-19 (Hadjadj et al., 2020), which is followed by increased circulating levels of IL-6 and TNF-α. This may also result in acute respiratory distress syndrome (ARDS), coagulation disorders, and eventually multiple organ failure (Costela-Ruiz et al., 2020; Chen et al., 2021). Disease severity is linked to a highly

TABLE 2 | A. Calculated and predicted physicochemical properties for SAR-CoV-2 inhibitors. pKa and LogP calculated with ChemAxon software (Budapest, Hungary). Predictions using a machine learning model (Lane et al., 2020b) for lysosomotropic activity are highlighted in the last 3 columns, CAD paper = (Tummino et al., 2021) known lysosomotropic = (Nadanaciva et al., 2011; Kazmi et al., 2013).

ID	apKa1	apKa2	bpKa1	bpKa2	logP	Molecular weight	in vivo activity	Based on phospholipidosis criteria	Based on CAD paper	Known to be lysosomotropic	Predicted Active	Prediction Score	Applicability Score
amiodarone			9.08	-3.01	7.64	645.32	inactive	lyso	lyso	yes	YES	1.226	1.000
elacridar	13.88	15.92	8.36	-4.1	6.81	563.654	inactive	lyso	lyso	unknown	NO	0.461	0.527
hydroxychloroquine	15.59		9.76	7.28	2.89	335.88	inactive	not lyso	lyso	yes	YES	1.453	1.000
imatinib	12.69	16.33	7.84	4.27	4.38	493.615	inactive	lyso	lyso	yes	NO	0.410	1.000
itraconazole			3.91	1.94	7.31	705.64	inactive	not lyso	not lyso	unknown	NO	0.428	0.419
PB28			9.06	2.97	5.36	370.581	inactive	lyso	lyso	unknown	YES	0.747	0.522
sertraline			9.56		5.15	306.23	inactive	lyso	lyso	yes	YES	1.027	1.000
tamoxifen			8.76	-7.17	6.35	371.524	inactive	lyso	lyso	yes	YES	1.153	1.000
clofazimine	16.15		6.63	2.3	7.3	473.4	active	not lyso	lyso	unknown	YES	0.556	0.438
diABZI-4	13.91	14.52	6.96	2.96	0.41	849.954	active	not lyso	not lyso	unknown	YES	0.703	0.514
favipiravir	9.39	13.58	-3.68	-5.22	0.25	157.104	active	not lyso	not lyso	unknown	NO	0.228	0.516
GC376	-0.88	12.06	-1.5	-3.21	-0.55	507.53	active	not lyso	not lyso	unknown	NO	0.381	0.500
masitinib	15.12	16.41	7.84	4.22	4.97	498.65	active	lyso	lyso	unknown	YES	0.539	0.809
mefloquine	13.79		9.46	-0.85	4.11	378.318	active	lyso	lyso	unknown	NO	0.472	0.468
molnupiravir	8.21	9.8	-3.66	-3.79	-0.36	329.309	active	not lyso	not lyso	unknown	NO	0.382	0.397
nafamostat			11.32	8.24	2.15	347.378	active	not lyso	lyso	unknown	NO	0.427	0.327
nelfinavir	9.32	14.13	8.18	-0.91	4.72	567.79	active	lyso	lyso	unknown	NO	0.302	0.378
niclosamide	6.89	14.61	-4.43	-7.05	3.91	327.12	active	not lyso	not lyso	unknown	NO	0.436	0.509
PF-07321332	7.1	11.51	-1.57	-3.01	0.44	499.535	active	not lyso	not lyso	unknown	NO	0.438	0.367
plitidepsin	11.01	11.98	-3.1	-3.18	3.98	1110.357	active	not lyso	not lyso	unknown	NO	0.378	0.358
probenecid	3.53				2.44	285.36	active	not lyso	not lyso	unknown	NO	0.273	0.514
pyronaridine	7.96	19.39	10.08	9.15	4.22	518.06	active	lyso	lyso	unknown	YES	0.663	0.506
remdesivir	6.18	12.13	20.98	0.65	0.2	604.601	active	not lyso	not lyso	unknown	NO	0.392	0.357
topotecan	8	11.72	9.75	1.74	-0.33	421.453	active	not lyso	not lyso	unknown	YES	0.505	0.372
Vandetanib	14.35		9.13	4.45	4.54	475.362	active	lyso	lyso	unknown	YES	0.608	0.605

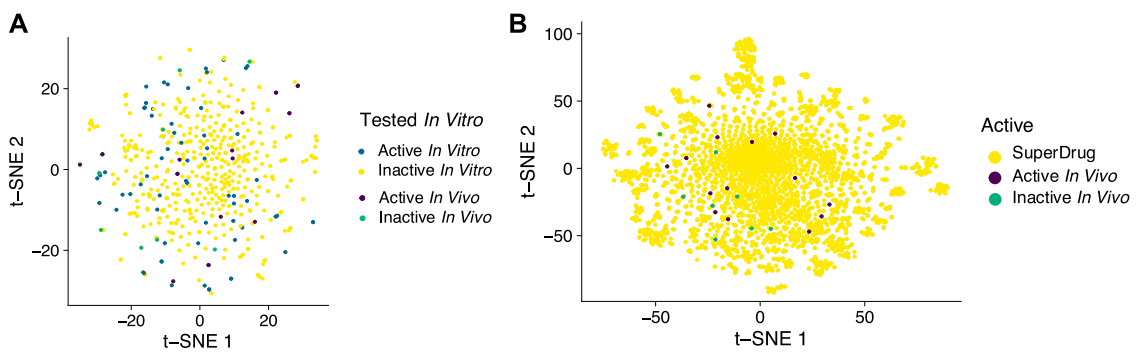


FIGURE 1 | t-SNE plots using 1024-bit ECFP6 fingerprints as input features to visualize compounds tested *in vivo* versus **(A)**. compounds tested *in vitro* and used for a machine learning model, **(B)**. versus approved drugs in SuperDrug to illustrate the coverage of molecular property space.

dysregulated innate immune response, which is broadly characterized by a delayed interferon I (IFN-I) and IFN-III response relative to symptom onset and possibly peak virus replication, and the production of an exuberant inflammatory response (Lowery et al., 2021), exacerbated proinflammatory cytokine production and in extensive cellular infiltrates in the respiratory tract, resulting in lung pathology (Lowery et al., 2021). Gene expression in human lung only mice demonstrated interferon-stimulated genes and inflammatory cytokines including IL6, CXCL8, CXCL10, TNF and CCL5 were induced from infected lung tissue (Wahl et al., 2021). Hence, in this mouse model, SARS-CoV-2 causes an upregulation of the innate immune response.

Most recently, a randomized, placebo-controlled trial of Janus-kinase inhibition using tofacitinib, has been reported to improve COVID-19 survival, in the presence of background glucocorticoid treatment (received by 89% of patients) (Guimaraes et al., 2021). Antivirals that can dampen the cytokine storm in a selective manner would provide a useful therapeutic approach for treating patients, for example vandetanib (Puhl et al., 2021c) (**Table 1**).

Physiochemical Properties of Antivirals

What should the perfect COVID drug look like? In an ideal world, a molecule that directly addresses one or more viral target as well as having host effects to modulate the cytokine storm would be considered promising. We have focused on a small set of compounds which are predominantly already approved drugs or drug candidates, that we have separated into those that demonstrated some degree of *in vivo* efficacy in various animal models for SARS-CoV-2 and those that do not. Interestingly, 17 out of 25 molecules displayed activity against

host targets or mechanisms (**Table 1**). Our criteria are broad so that we can capture molecules that may have a direct antiviral effect or a host effect. Therefore, viral load reduction alone was not a solo-criteria and in some cases, molecules showed improvements in histopathology alone, and these were considered active. Clearly there has been some concern around CADs and whether they represent a waste of resources based on a small *in vitro*: *in vivo* analysis (Tummino et al., 2021). Our analysis of physiochemical properties for these 25 drugs that have been tested *in vitro* and *in vivo* (**Table 2**) clearly shows a wide range of logP and pKa values, such that there are molecules that comply with requirements for phospholipidosis [basic pKa (>6.5) and cLogP of >2) and CADs (cLogP (≥3) and pKa (≥7.4)] in both the *in vivo* active and inactive groups (**Table 2**). This perhaps provides a larger and more convincing dataset than this earlier study (Tummino et al., 2021) to demonstrate why we should pursue CADs as antivirals alongside other classes of molecules.

Computational Approaches to Guide Drug Discovery: Machine Learning Models

To date we have collated hundreds of drugs that have *in vitro* data against this virus primarily in Vero cells (Wang et al., 2020a; Liu et al., 2020b; Jeon et al., 2020; Jin et al., 2020b). This has enabled machine learning models and even with these relatively modest datasets we have shown that such models can be used to select additional compounds for testing (Gawriljuk et al., 2021). In addition, we can use these molecules that have been tested to date to visualize the *in vivo* in active and inactive molecules (**Figure 1A**) and these appear to show the coverage of this property space is relatively even and not clustered in any particular area. Similarly, the *in vivo* active and inactive molecules are well dispersed in the thousands of molecules in the SuperDrug database (**Figure 1B**). Selecting compounds close to the active molecules in these property spaces may be one way to help select additional compounds for future testing. Other sources of *in vitro* data are available as groups have screened libraries such as the NIH NCATS OpenData portal (Anon, 2022) and this resource could be used for modeling. In addition, as datasets are built up specifically for antiviral targets like M^{pro}, PL^{pro} or others, these could be used for target specific machine leaning models that could be combined with the whole cell models.

Impact Beyond COVID-19

Several *in vivo* studies of small molecule drugs have described the direct measurement of cytokine and chemokine levels or gene expression patterns as an attempt to understand these drugs and their effects on inflammation. The utility of this is that we may be able to identify molecules with a specific pattern of increased or decreased cytokine levels that may be the mirror image for biomarkers for other lung or other diseases. This is analogous to the connectivity map (CMap), which brings together data on genes and thousands of drugs and disease states used in several repurposing projects to identify new uses for old drugs (Lamb et al., 2006; Lamb, 2007; Zimmer et al., 2010; Subramanian et al., 2017). There is therefore the potential to find new drugs that could be potentially useful for these other (lung) diseases which may not currently have viable treatments or with limited treatment options (e.g. lung fibrosis).

Addressing COVID-19 Symptoms

Several of the symptoms of COVID-19 include impacts on the peripheral nervous system. Olfactory dysfunction was described early on as well as the diagnosis and management of these symptoms (Whitcroft and Hummel, 2020). Loss of the sense of smell (hyposmia/anosmia) and/or taste (hypogeusia/ageusia) have been widely reported (Lechien et al., 2020; Mao et al., 2020), can predict SARS-CoV-2 infection and have been added to the list of major symptoms (Menni et al., 2020) as well as the Center for disease Control and Prevention's website of symptoms (CDC, 2020). 64–67% of those testing positive (>7,000) in a study in the US and United Kingdom described a loss of smell and taste (Menni et al., 2020). Surveys have shown that taste and smell dysfunction may be an early symptom of COVID-19 in over 50% of those questioned (Mercante et al., 2020) and another study reported resolution of these symptoms in 48.7% of patients within 4 weeks of onset (Boscolo-Rizzo et al., 2020). While at first glance these may not seem as severe as other symptoms of the virus such as fever and cough, they can be long-lived based on what we know of other viral infections (Suzuki et al., 2007). There are considerable ongoing efforts in drug and vaccine discovery for COVID-19 (with hundreds of drugs in various stages of research and clinical trials ongoing), yet there is relatively little discussion of how SARS-CoV-2 might be causing these specific symptoms (Bilinska and Butowt, 2020) or even whether these could be targets for pharmaceutical intervention. To date, there have not been proposals to mitigate the taste and smell symptoms as a treatment strategy. Besides SARS-CoV-2 directly interacting with angiotensin converting enzyme 2 (ACE2) receptor in the nasal epithelium (Whitcroft and Hummel, 2020), nasal goblet cells (Sungnak et al., 2020) and olfactory mucosa (Brann et al., 2020) there have been few alternative suggestions of how the virus might be causing these symptoms or how to treat them. For example, decreased IL-6 improved smell and taste in COVID-19 patients (Cazzolla et al., 2020). SARS-CoV-2 infection of non-neuronal cell types has been proposed to lead to olfactory dysfunction in COVID-19 patients (Brann et al., 2020). One group has recently proposed that ACE2-independent pathways may be involved such that alternative viral receptors may yet be identified (Bilinska and Butowt, 2020). Significant differences in the level of gene expression of ACE2 in different age groups (Bunyavanich et al., 2020) may explain differences in susceptibility. There is considerable previous discussion for the side effects of drugs including the impact of ageing (Schiffman, 1997; Schiffman et al., 2002) and anosmia and agusia (Schiffman and Doty, 2015; Schiffman, 2018) diseases, which include drug treatment for other diseases (including other viruses such as influenza) (Schiffman, 1983a; Schiffman, 1983b). The exact mechanisms by which SARS-CoV-2 and other neurotropic or neuro-invasive viruses impair these chemical senses are not yet fully understood (Bilinska and Butowt, 2020; Xydakis et al., 2020). Interestingly, while many papers have discussed the role of

GPCR's in the role of taste and smell (Meunier et al., 2020), there has been no discussion on whether these receptors themselves could be directly or indirectly affected by SARS-CoV-2. Further research into the likely many mechanisms responsible for chemosensory losses may provide insights into the virus and provide new knowledge for the development of treatments. This would also point to the need for more investment in this research area. Clearly, prior to COVID-19 few patients were seen with sensorineural viral anosmia which limited clinical research. We now have an abundance of research subjects (Xydakis et al., 2020) and it would be important to take advantage of this situation as it could inform how we address future coronaviruses. Certainly, there are many other important symptoms associated with COVID-19 gathering some public attention such as hair loss (trichodynia and telogen effluvium) that need to be addressed (Rossi et al., 2021; Sharquie and Jabbar, 2021; Starace et al., 2021). Small molecules addressing these many COVID-19 symptoms may also provide future generations of antivirals that can further differentiate themselves from remdesivir, molnupiravir and paxlovid.

The Need for Global Collaborations

One of the challenges we identified from the very outset of the pandemic was identifying laboratories that could perform the *in vitro* and *in vivo* testing of efficacy for small molecules. Many of the collaborators we had previously worked with for different viruses were setting up testing against SAR-CoV-2, with much of the work done in Vero cells initially. We ran into the challenge of using Vero cells with 3 compounds identified previously for Ebola virus, which had more cytotoxicity in Vero versus human cell lines such as HeLa (Bae et al., 2020). There was a long lag time before human cell line models became accessible for testing with SARS-CoV-2. Subsequently, we have been keen to assess a molecules' activity against as many cell lines as possible (Puhl et al., 2021a). The potential for P-glycoprotein to have a role in effluxing compounds out of Vero cells as noted by Pfizer (Owen et al., 2021) is one issue, another might be the lack of an interferon response in Vero cells. As an example, pyronaridine does not appear to be a P-gp substrate based on testing in Caco-2 cells (Lane et al., 2019b) so this may be having a lesser impact. From our experience we found the lack of a clear coordinated US (and for that matter global) response has led to silo-ing of capabilities such that there are initiatives that could probably assist companies in developing small molecule antivirals, but these are relatively difficult to gain entry to. There were many US government agencies as well as philanthropic organizations and websites that promoted calls for molecules and research for COVID (e.g. NIAID, BARDA etc). After numerous submissions in response to government agencies, foundations, and requests for funding there was little in the way of responses. This could be because of the strict filter implemented in order to wade through the massive numbers of applications or it could be the criteria set for antivirals has been set too high e.g., direct acting molecules were likely preferred over host targeting molecules, or molecules with known mechanisms of action were preferred over those with no known mechanism. We have been fortunate to be able to coordinate testing and collaborations with many other global academic laboratories, driven by the shared desire to find molecules that could be rapidly brought to patients, rather than by a financial return. We are also starting to see others share their experiences of COVID drug discovery such as the COVID Moonshot and we can likely learn from these for future efforts (von Delft et al., 2021).

Future Prospects: Accelerating the Drug Discovery Pipeline

After spending a significant amount of our time over the past 2 years on COVID-19 drug discovery what have we learnt? First, few laboratories still have a comprehensive drug-discovery pipeline for the disease. While many academic laboratories are experts at the biology or the animal model development, they lack compounds and are reliant on big pharmaceutical companies to supply them. This requires academics to access pharmaceutical companies who may already have molecules with antiviral activity. This relationship is symbiotic: the academics likely get funded for the experiments and the industry obtains the data they need from key opinion leaders. Other approaches are available in the USA such as the NIAID antiviral testing capabilities which contracts out the testing to academic laboratories. When COVID-19 was identified, *in vitro* testing in Vero cells was available after several months followed by Caco-2 and Calu-3 cells. We have been able to additionally obtain testing against A549 cells at several academic laboratories in the USA and Brazil. Being able to submit molecules for testing against a panel of human and animal cells from the outset would be ideal in future and potentially speed up identification of molecules and perhaps filter out compounds that may be less useful. We initially, could not move pyronaridine forward until we could also demonstrate the molecule was active against SARS-CoV-2 in human cells, just as it showed activity differences between Vero and Hela cells for EBOV. There also needs to be a clear pathway for anyone to route their molecules through an *in vitro-in vivo* profiling service provided globally. Even now this does not really exist with each country likely focused on their own researchers. In the USA, there is a patchwork of government agencies such as BARDA, NIH etc. each advertising resources wanting to identify small molecules and using websites to solicit information. This is a one-way street and submitting information does not guarantee a response. What is needed is a truly global initiative with a transparent pathway from the very outset to counter the next pandemic virus.

One wonders how much has been financially invested by researchers and small companies without any guarantee of funding or reward, in the hope that they can hit the lottery and find a molecule that they can then convince a large pharma company to license. For every small company that licenses a drug to a major pharma there are likely many hundreds that are currently striving to get to that point. Increasingly, the bar will continue to be set higher. Remdesivir is a relatively low bar to overcome (e.g., find a drug that can demonstrate efficacy that is not administered i. v.). Molnupiravir will be harder (find a drug that likely will not have the mutation concerns or require less frequent dosing), and paxlovid harder still (find a drug as potent,

orally delivered that does not interfere with CYP3A4). After these there will likely be combinations of these and other drugs as we have seen for HIV and hepatitis C. Already there have been efforts to predict synergistic combinations (Jin et al., 2021)We will then probably move into finding drugs that specifically address some of the symptoms like loss of smell and taste, decrease inflammation as well as impact long COVID. This will unfortunately require a long wait for those patients suffering from these symptoms now.

We will likely see these newer drugs brought to market more rapidly than any other drugs to date because of the unmet need. This in turn will set expectations for faster review for drugs for other diseases like cancer which have generally been expedited, but COVID-19 has clearly identified the 'need for speed' in drug discovery. There is also a need for efficiency and there was certainly redundancy of testing as many groups tried the same or similar compounds or experiments. This may point to a need for some degree of coordination of efforts being needed. As the Omicron variant suggests, we will need to move very quickly to keep up with this moving target of COVID-19. The next pandemic could be worse so we will need to be ready with broad spectrum antivirals. We cannot afford to rest on our laurels as often happens after outbreaks make the news (e.g. Ebola, Zika, flu). Our global medicine cabinet needs more antivirals targeting different mechanisms and we therefore should not be too selective about avoiding compounds that may appear undesirable such as CADs or impact cytokines. These characteristics of molecules may turn out to be desirable features. Recent publications while this was in review, include additional compounds like cannabidiol which has an EC_{50} ~1 μM and can significantly inhibit viral replication in mouse lungs when treated for a week before infection (Nguyen et al., 2022). The mechanism appears to be *via* induction of the interferon pathway. We would expect to see many other molecules identified that may possess promising direct antiviral or host effects, but these may have to overcome considerable hurdles to compete with the drugs that are either approved or emergency authorized currently. The situation with COVID-19 is dynamic and we have also seen the recent removal of authorizations for two monoclonal antibodies by the FDA as they are infective against Omicron. This also points to the need for continual drug discovery and development of antivirals for COVID-19 and why it may be important to understand the physicochemical properties and potential mechanisms of molecules that have been identified to date, so we can learn from them.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

We kindly acknowledge NIH funding R44GM122196 from NIH NIGMS as well as our many collaborators on the studies described. Susan S. Schiffman and Stephen H Wright are thanked for stimulating discussions. Mindy Davis and others are gratefully acknowledged for assistance with the NIAID virus screening capabilities. Collaborations Pharmaceuticals, Inc. has utilized the non-clinical and pre-clinical services program offered by the National Institute of Allergy and Infectious Diseases.

REFERENCES

Puhl, A. C., Gomes, G. F., Damasceno, S., Fritch, E. J., Levi, J. A., Johnson, N. J., et al. Vandetanib Reduces Inflammatory Cytokines and Ameliorates COVID-19 in Infected Mice. BIORXIV/2021/472155 (2021)

Aman, J., Duijvelaar, E., Botros, L., Kianzad, A., Schippers, J. R., Smeele, P. J., et al. (2021). Imatinib in Patients with Severe COVID-19: a Randomised, Double-Blind, Placebo-Controlled, Clinical Trial. *Lancet Respir. Med.* 9 (9), 957–968. doi:10.1016/S2213-2600(21)00237-X

Anon (2021b). "Merck and Ridgeback Biotherapeutics Provide Update on Results from MOVe-OUT Study of Molnupiravir, an Investigational Oral Antiviral Medicine," in *At Risk Adults with Mild-To-Moderate COVID-19*. Available at https://www.merck.com/news/merck-and-ridgeback-biotherapeutics-provide-update-on-results-from-move-out-study-of-molnupiravir-an-investigational-oral-antiviral-medicine-in-at-risk-adults-with-mild-to-moderate-covid-19/.

Anon (2021a). Merck and Ridgeback's Investigational Oral Antiviral Molnupiravir Reduced the Risk of Hospitalization or Death by Approximately 50 Percent Compared to Placebo for Patients with Mild or Moderate COVID-19 in Positive Interim Analysis of Phase 3 Study. Available at https://www.merck.com/news/merck-and-ridgebacks-investigational-oral-antiviral-molnupiravir-reduced-the-risk-of-hospitalization-or-death-by-approximately-50-percent-compared-to-placebo-for-patients-with-mild-or-moderat/.

Anon (2022). NCATS OpenData portal in. Available at https://opendata.ncats.nih.gov/covid19/index.html.

Bae, J.-Y., Lee, G. E., Park, H., Cho, J., Kim, Y.-E., Lee, J.-Y., et al. (2020). Pyronaridine and Artesunate Are Potential Antiviral Drugs against COVID-19 and Influenza. *bioRxiv* 0728, 225102. doi:10.1101/2020.07.28.225102

Baker, N., Williams, A. J., Tropsha, A., and Ekins, S. (2020). Repurposing Quaternary Ammonium Compounds as Potential Treatments for COVID-19. *Pharm. Res.* 37, 104. doi:10.1007/s11095-020-02842-8

Baranov, M. V., Bianchi, F., and van den Bogaart, G. (2020). The PIKfyve Inhibitor Apilimod: A Double-Edged Sword against COVID-19. *Cells* 10 (1), 30. doi:10.3390/cells10010030

Barratt-Due, A., Olsen, I. C., Nezvalova-Henriksen, K., Kåsine, T., Lund-Johansen, F., Hoel, H., et al. (2021). Evaluation of the Effects of Remdesivir and Hydroxychloroquine on Viral Clearance in COVID-19. *Ann. Intern. Med.* 174 (9), 1261–1269. doi:10.7326/M21-0653

Bilinska, K., and Butowt, R. (2020). Anosmia in COVID-19: A Bumpy Road to Establishing a Cellular Mechanism. *ACS Chem. Neurosci.* doi:10.1021/acschemneuro.0c00406

Boras, B., Jones, R. M., Anson, B. J., Arenson, D., Aschenbrenner, L., Bakowski, M. A., et al. (2021). Preclinical Characterization of an Intravenous Coronavirus 3CL Protease Inhibitor for the Potential Treatment of COVID19. *Nat. Commun.* 12 (1), 6055. doi:10.1038/s41467-021-26239-2

Boscolo-Rizzo, P., Borsetto, D., Fabbris, C., Spinato, G., Frezza, D., Menegaldo, A., et al. (2020). Evolution of Altered Sense of Smell or Taste in Patients with Mildly Symptomatic COVID-19. *JAMA Otolaryngol. Head Neck Surg.* doi:10.1001/jamaoto.2020.1379

Bouhaddou, M., Memon, D., Meyer, B., White, K. M., Rezelj, V. V., Correa Marrero, M., et al. (2020). The Global Phosphorylation Landscape of SARS-CoV-2 Infection. *Cell* 182 (3), 685–712. doi:10.1016/j.cell.2020.06.034

Brann, D. H., Tsukahara, T., Weinreb, C., Lipovsek, M., Van den Berge, K., Gong, B., et al. (2020). Non-neuronal Expression of SARS-CoV-2 Entry Genes in the Olfactory System Suggests Mechanisms Underlying COVID-19-Associated Anosmia. *Sci. Adv.* 6, eabc5801. doi:10.1126/sciadv.abc5801

Brunaugh, A. D., Seo, H., Warnken, Z., Ding, L., Seo, S. H., and Smyth, H. D. C. (2021). Development and Evaluation of Inhalable Composite Niclosamide-Lysozyme Particles: A Broad-Spectrum, Patient-Adaptable Treatment for Coronavirus Infections and Sequalae. *PLoS One* 16 (2), e0246803. doi:10.1371/journal.pone.0246803

Bunyavanich, S., Do, A., and Vicencio, A. (2020). Nasal Gene Expression of Angiotensin-Converting Enzyme 2 in Children and Adults. *JAMA* 323, 2427. doi:10.1001/jama.2020.8707

Caceres, C. J., Cardenas-Garcia, S., Carnaccini, S., Seibert, B., Rajao, D. S., Wang, J., et al. (2021). Efficacy of GC-376 against SARS-CoV-2 Virus Infection in the K18 hACE2 Transgenic Mouse Model. *Sci. Rep.* 11 (1), 9609. doi:10.1038/s41598-021-89013-w

Callaway, E. (2021). Heavily Mutated Omicron Variant Puts Scientists on Alert. *Nature* 600, 21. doi:10.1038/d41586-021-03552-w

Caly, L., Druce, J. D., Catton, M. G., Jans, D. A., and Wagstaff, K. M. (2020). The FDA-Approved Drug Ivermectin Inhibits the Replication of SARS-CoV-2 *In Vitro*. *Antiviral Res.* 178, 104787. doi:10.1016/j.antiviral.2020.104787

Cao, R., Hu, H., Li, Y., Wang, X., Xu, M., Liu, J., et al. (2020). Anti-SARS-CoV-2 Potential of Artemisinins *In Vitro*. *ACS Infect. Dis.* 6 (9), 2524–2531. doi:10.1021/acsinfecdis.0c00522

Cazzolla, A. P., Lovero, R., Lo Muzio, L., Testa, N. F., Schirinzi, A., Palmieri, G., et al. (2020). Taste and Smell Disorders in COVID-19 Patients: Role of Interleukin-6. *ACS Chem. Neurosci.* 11 (17), 2774–2781. doi:10.1021/acschemneuro.0c00447

Cdc: Coronavirus Disease 2019 (COVID-19). In, (2020)

Chen, R., Lan, Z., Ye, J., Pang, L., Liu, Y., Wu, W., et al. (2021). Cytokine Storm: The Primary Determinant for the Pathophysiological Evolution of COVID-19 Deterioration. *Front. Immunol.* 12, 589095. doi:10.3389/fimmu.2021.589095

Clementi, N., Criscuolo, E., Diotti, R. A., Ferrarese, R., Castelli, M., Dagna, L., et al. (2020). Combined Prophylactic and Therapeutic Use Maximizes Hydroxychloroquine Anti-SARS-CoV-2 Effects *In Vitro*. *Front. Microbiol.* 11, 1704. doi:10.3389/fmicb.2020.01704

Coronaviridae, V. (2020). The Species Severe Acute Respiratory Syndrome-Related Coronavirus: Classifying 2019-nCoV and Naming it SARS-CoV-2. *Nat. Microbiol.* 5 (4), 536–544. doi:10.1038/s41564-020-0695-z

Costela-Ruiz, V. J., Illescas-Montes, R., Puerta-Puerta, J. M., Ruiz, C., and Melguizo-Rodríguez, L. (2020). SARS-CoV-2 Infection: The Role of Cytokines in COVID-19 Disease. *Cytokine Growth Factor. Rev.* 54, 62–75. doi:10.1016/j.cytogfr.2020.06.001

Dampalla, C. S., Zheng, J., Perera, K. D., Wong, L.-Y. R., Meyerholz, D. K., Nguyen, H. N., et al. (2021). Postinfection Treatment with a Protease Inhibitor Increases Survival of Mice with a Fatal SARS-CoV-2 Infection. *Proc. Natl. Acad. Sci. USA* 118 (29), e2101555118. doi:10.1073/pnas.2101555118

de Wit, E., Feldmann, F., Cronin, J., Jordan, R., Okumura, A., Thomas, T., et al. (2020). Prophylactic and Therapeutic Remdesivir (GS-5734) Treatment in the Rhesus Macaque Model of MERS-CoV Infection. *Proc. Natl. Acad. Sci. USA* 117, 6771–6776. doi:10.1073/pnas.1922083117

Drayman, N., DeMarco, J. K., Jones, K. A., Azizi, S.-A., Froggatt, H. M., Tan, K., et al. (2021). Masitinib Is a Broad Coronavirus 3CL Inhibitor that Blocks Replication of SARS-CoV-2. *Science* 373, 931–936. doi:10.1126/science.abg5827

Driouich, J.-S., Cochin, M., Lingas, G., Moureau, G., Touret, F., Petit, P.-R., et al. (2021). Favipiravir Antiviral Efficacy against SARS-CoV-2 in a Hamster Model. *Nat. Commun.* 12 (1), 1735. doi:10.1038/s41467-021-21992-w

Eastman, R. T., Roth, J. S., Brimacombe, K. R., Simeonov, A., Shen, M., Patnaik, S., et al. (2020). Remdesivir: A Review of its Discovery and Development Leading to Emergency Use Authorization for Treatment of COVID-19. *ACS Cent. Sci.* 6 (5), 672–683. doi:10.1021/acscentsci.0c00489

Eduardo, F. d. P., Corrêa, L., Heller, D., Daep, C. A., Benitez, C., Malheiros, Z., et al. (2021). Salivary SARS-CoV-2 Load Reduction with Mouthwash Use: A Randomized Pilot Clinical Trial. *Heliyon* 7 (6), e07346. doi:10.1016/j.heliyon.2021.e07346

Ekins, S., Freundlich, J. S., Clark, A. M., Anantpadma, M., Davey, R. A., and Madrid, P. (2015). Machine Learning Models Identify Molecules Active against the Ebola Virus *In Vitro*. *F1000Res* 4, F1000Res1091. doi:10.12688/f1000research.7217.2

Ekins, S., Freundlich, J. S., Clark, A. M., Anantpadma, M., Davey, R. A., and Madrid, P. (2015). Machine Learning Models Identify Molecules Active against the Ebola Virus *In Vitro*. *F1000Res* 4, F1000Res1091. doi:10.12688/f1000research.7217.3

Ekins, S., Lingerfelt, M. A., Comer, J. E., Freiberg, A. N., Mirsalis, J. C., O'Loughlin, K., et al. (2018). Efficacy of Tilorone Dihydrochloride against Ebola Virus Infection. *Antimicrob. Agents Chemother.* 62 (2). doi:10.1128/AAC.01711-17

Ekins, S., and Madrid, P. B. (2020). Tilorone, a Broad-Spectrum Antiviral for Emerging Viruses. *Antimicrob. Agents Chemother.* 64 (5), e00440–20. doi:10.1128/AAC.00440-20

Ekins, S., Mottin, M., Ramos, P. R. P. S., Sousa, B. K. P., Neves, B. J., Foil, D. H., et al. (2020). Déjà Vu: Stimulating Open Drug Discovery for SARS-CoV-2. *Drug Discov. Today* 25 (5), 928–941. doi:10.1016/j.drudis.2020.03.019

Galan, L. E. B., Santos, N. M. D., Asato, M. S., Araújo, J. V., de Lima Moreira, A., Araújo, A. M. M., et al. (2021). Phase 2 Randomized Study on Chloroquine, Hydroxychloroquine or Ivermectin in Hospitalized Patients with Severe Manifestations of SARS-CoV-2 Infection. *Pathog. Glob. Health* 115 (4), 235–242. doi:10.1080/20477724.2021.1890887

Gawriljuk, V. O., Zin, P. P. K., Puhl, A. C., Zorn, K. M., Foil, D. H., Lane, T. R., et al. (2021). Machine Learning Models Identify Inhibitors of SARS-CoV-2. *J. Chem. Inf. Model.* 61 (9), 4224–4235. doi:10.1021/acs.jcim.1c00683

Goldman, J. D., Lye, D. C. B., Hui, D. S., Marks, K. M., Bruno, R., Montejano, R., et al. (2020). Remdesivir for 5 or 10 Days in Patients with Severe Covid-19. *N. Engl. J. Med.* 383 (19), 1827–1837. doi:10.1056/NEJMoa2015301

Gordon, C. J., Tchesnokov, E. P., Woolner, E., Perry, J. K., Feng, J. Y., Porter, D. P., et al. (2020). Remdesivir Is a Direct-Acting Antiviral that Inhibits RNA-dependent RNA Polymerase from Severe Acute Respiratory Syndrome Coronavirus 2 with High Potency. *J. Biol. Chem.* 295 (20), 6785–6797. doi:10.1074/jbc.RA120.013679

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., O'Meara, M. J., et al. (2020). A SARS-CoV-2-Human Protein-Protein Interaction Map Reveals Drug Targets and Potential Drug-Repurposing. *bioRxiv*. doi:10.1101/2020.03.22.002386

Gordon, D. E., Jang, G. M., Bouhaddou, M., Xu, J., Obernier, K., White, K. M., et al. (2020). A SARS-CoV-2 Protein Interaction Map Reveals Targets for Drug Repurposing. *Nature* 583 (7816), 459–468. doi:10.1038/s41586-020-2286-9

Gorshkov, K., Chen, C. Z., Bostwick, R., Rasmussen, L., Tran, B. N., Cheng, Y.-S., et al. (2021). The SARS-CoV-2 Cytopathic Effect Is Blocked by Lysosome Alkalizing Small Molecules. *ACS Infect. Dis.* 7 (6), 1389–1408. doi:10.1021/acsinfecdis.0c00349

Group, R. C., Horby, P., Lim, W. S., Emberson, J. R., Mafham, M., Bell, J. L., et al. (2021). Dexamethasone in Hospitalized Patients with Covid-19. *N. Engl. J. Med.* 384 (8), 693–704. doi:10.1056/NEJMoa2021436

Guimaraes, P. O., Quirk, D., Furtado, R. H., Maia, L. N., Saraiva, J. F., Antunes, M. O., et al. (2021). Tofacitinib in Patients Hospitalized with Covid-19 Pneumonia. *N. Engl. J. Med.* 385 (5), 406–415. doi:10.1056/NEJMoa2101643

Hadjadj, J., Yatim, N., Barnabei, L., Corneau, A., Boussier, J., Smith, N., et al. (2020). Impaired Type I Interferon Activity and Inflammatory Responses in Severe COVID-19 Patients. *Science* 369 (6504), 718–724. doi:10.1126/science.abc6027

Hall, M. D., Anderson, J. M., Anderson, A., Baker, D., Bradner, J., Brimacombe, K. R., et al. (2021). Report of the National Institutes of Health SARS-CoV-2 Antiviral Therapeutics Summit. *J. Infect. Dis.* 224 (Suppl. ment_1), S1–S21. doi:10.1093/infdis/jiab305

Hallifax, D., and Houston, J. B. (2007). Saturable Uptake of Lipophilic Amine Drugs into Isolated Hepatocytes: Mechanisms and Consequences for Quantitative Clearance Prediction. *Drug Metab. Dispos* 35 (8), 1325–1332. doi:10.1124/dmd.107.015131

Han, Y., Yang, L., Duan, X., Duan, F., Nilsson-Payant, B. E., Yaron, T. M., et al. (2020). Identification of Candidate COVID-19 Therapeutics Using hPSC-Derived Lung Organoidsids. *bioRxiv*. doi:10.1101/2020.05.05.079095

Herold, T., Jurinovic, V., Arnreich, C., Lipworth, B. J., Hellmuth, J. C., von Bergwelt-Baildon, M., et al. (2020). Elevated Levels of IL-6 and CRP Predict the Need for Mechanical Ventilation in COVID-19. *J. Allergy Clin. Immunol.* 146 (1), 128e4–136. doi:10.1016/j.jaci.2020.05.008

Ho, J. S. Y., Mok, B. W., Campisi, L., Jordan, T., Yildiz, S., Parameswaran, S., et al. (2021). TOP1 Inhibition Therapy Protects against SARS-CoV-2-Induced Lethal Inflammation. *Cell* 184 (10), 2618–2632. doi:10.1016/j.cell.2021.03.051

Hoang, T. N., Pino, M., Boddapati, A. K., Viox, E. G., Starke, C. E., Upadhyay, A. A., et al. (2021). Baricitinib Treatment Resolves Lower-Airway Macrophage Inflammation and Neutrophil Recruitment in SARS-CoV-2-Infected Rhesus Macaques. *Cell* 184 (2), 460–475. doi:10.1016/j.cell.2020.11.007

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181 (2), 271e8–280. doi:10.1016/j.cell.2020.02.052

Homolak, J., and Kodvanj, I. (2020). Widely Available Lysosome Targeting Agents Should Be Considered as Potential Therapy for COVID-19. *Int. J. Antimicrob. Agents* 56 (2), 106044. doi:10.1016/j.ijantimicag.2020.106044

Hu, R., Xia, C. Q., Butfiloski, E., and Clare-Salzler, M. (2018). Effect of High Glucose on Cytokine Production by Human Peripheral Blood Immune Cells and Type I Interferon Signaling in Monocytes: Implications for the Role of Hyperglycemia in the Diabetes Inflammatory Process and Host Defense against Infection. *Clin. Immunol.* 195, 139–148. doi:10.1016/j.clim.2018.06.003

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical Features of Patients Infected with 2019 Novel Coronavirus in Wuhan, China. *The Lancet* 395 (10223), 497–506. doi:10.1016/S0140-6736(20)30183-5

Huang, H.-Y., Wang, S.-H., Tang, Y., Sheng, W., Zuo, C.-J., Wu, D.-W., et al. (2021). Landscape and Progress of Global COVID-19 Vaccine Development. *Hum. Vaccin. Immunother.* 17, 3276–3280. doi:10.1080/21645515.2021.1945901

Humphries, F., Shmuel-Galia, L., Jiang, Z., Wilson, R., Landis, P., Ng, S.-L., et al. (2021). A Diamidobenzimidazole STING Agonist Protects against SARS-CoV-2 Infection. *Sci. Immunol.* 6 (59), eabi9002. doi:10.1126/sciimmunol.abi9002

Hung, H. C., Ke, Y. Y., Huang, S. Y., Huang, P. N., Kung, Y. A., Chang, T. Y., et al. (2020). Discovery of M Protease Inhibitors Encoded by SARS-CoV-2. *Antimicrob. Agents Chemother.* 64 (9). doi:10.1128/AAC.00872-20

Jan, J. T., Cheng, T. R., Juang, Y. P., Ma, H. H., Wu, Y. T., Yang, W. B., et al. (2021). Identification of Existing Pharmaceuticals and Herbal Medicines as Inhibitors of SARS-CoV-2 Infection. *Proc. Natl. Acad. Sci. U S A.* 118 (5). doi:10.1073/pnas.2021579118

Craven, J.: COVID-19 Vaccine Tracker. In, (2021)

Jeon, S., Ko, M., Lee, J., Choi, I., Byun, S. Y., Park, S., et al. (2020). Identification of Antiviral Drug Candidates against SARS-CoV-2 from FDA-Approved Drugs. *Antimicrob. Agents Chemother.* 64, e00819–20. doi:10.1128/AAC.00819-20

Jin, W., Stokes, J. M., Eastman, R. T., Itkin, Z., Zakharov, A. V., Collins, J. J., et al. (2021). Deep Learning Identifies Synergistic Drug Combinations for Treating COVID-19. *Proc. Natl. Acad. Sci. U S A.* 118 (39). doi:10.1073/pnas.2105070118

Jin, Z., Du, X., Xu, Y., Deng, Y., Liu, M., Zhao, Y., et al. (2020). Structure of Mpro from SARS-CoV-2 and Discovery of its Inhibitors. *Nature* 582 (7811), 289–293. doi:10.1038/s41586-020-2223-y

Jin, Z., Du, X., Xu, Y., Deng, Y., Liu, M., Zhao, Y., et al. (2020). Structure of M(pro) from SARS-CoV-2 and Discovery of its Inhibitors. *Nature* 582, 289–293. doi:10.1038/s41586-020-2223-y

Kaptein, S. J. F., Jacobs, S., Langendries, L., Seldeslachts, L., Ter Horst, S., Liesenborghs, L., et al. (2020). Favipiravir at High Doses Has Potent Antiviral Activity in SARS-CoV-2–infected Hamsters, whereas Hydroxychloroquine Lacks Activity. *Proc. Natl. Acad. Sci. USA* 117 (43), 26955–26965. doi:10.1073/pnas.2014441117

Kazmi, F., Hensley, T., Pope, C., Funk, R. S., Loewen, G. J., Buckley, D. B., et al. (2013). Lysosomal Sequestration (Trapping) of Lipophilic Amine (Cationic Amphiphilic) Drugs in Immortalized Human Hepatocytes (Fa2N-4 Cells). *Drug Metab. Dispos* 41 (4), 897–905. doi:10.1124/dmd.112.050054

Kyriakidis, N. C., López-Cortés, A., González, E. V., Grimaldos, A. B., and Prado, E. O. (2021). SARS-CoV-2 Vaccines Strategies: a Comprehensive Review of Phase 3 Candidates. *NPJ Vaccin.* 6 (1), 28. doi:10.1038/s41541-021-00292-w

Lamb, J., Crawford, E. D., Peck, D., Modell, J. W., Blat, I. C., Wrobel, M. J., et al. (2006). The Connectivity Map: Using Gene-Expression Signatures to Connect Small Molecules, Genes, and Disease. *Science* 313 (5795), 1929–1935. doi:10.1126/science.1132939

Lamb, J. (2007). The Connectivity Map: a New Tool for Biomedical Research. *Nat. Rev. Cancer* 7 (1), 54–60. doi:10.1038/nrc2044

Lane, T. R., Comer, J. E., Freiberg, A. N., Madrid, P. B., and Ekins, S. (2019). Repurposing Quinacrine against Ebola Virus InfectionIn Vivo. *Antimicrob. Agents Chemother.* 63, e01142–19. doi:10.1128/AAC.01142-19

Lane, T. R., Dyall, J., Mercer, L., Goodin, C., Foil, D. H., Zhou, H., et al. (2020). Repurposing Pyramax® for the Treatment of Ebola Virus Disease: Additivity of the Lysosomotropic Pyronaridine and Non-lysosomotropic Artesunate. *Antiviral Res.* 182, 104908. doi:10.1016/j.antiviral.2020.104908

Lane, T. R., and Ekins, S. (2021). Defending Antiviral Cationic Amphiphilic Drugs that May Cause Drug-Induced Phospholipidosis. *J. Chem. Inf. Model.* 61, 4125–4130. doi:10.1021/acs.jcim.1c00903

Lane, T. R., and Ekins, S. (2020). Toward the Target: Tilorone, Quinacrine, and Pyronaridine Bind to Ebola Virus Glycoprotein. *ACS Med. Chem. Lett.* 11, 1653–1658. doi:10.1101/2020.05.26.11818210.1021/acsmedchemlett.0c00298

Lane, T. R., Massey, C., Comer, J. E., Anantpadma, M., Freundlich, J. S., Davey, R. A., et al. (2019). Repurposing the Antimalarial Pyronaridine Tetraphosphate to Protect against Ebola Virus Infection. *Plos Negl. Trop. Dis.* 13 (11), e0007890. doi:10.1371/journal.pntd.0007890

Lane, T. R., Massey, C., Comer, J. E., Freiberg, A. N., Zhou, H., Dyall, J., et al. (2020). Pyronaridine Tetraphosphate Efficacy against Ebola Virus Infection in guinea Pig. *Antiviral Res.* 181, 104863. doi:10.1016/j.antiviral.2020.104863

Lechien, J. R., Chiesa-Estomba, C. M., De Siati, D. R., Horoi, M., Le Bon, S. D., Rodriguez, A., et al. (2020). Olfactory and Gustatory Dysfunctions as a Clinical Presentation of Mild-To-Moderate Forms of the Coronavirus Disease (COVID-19): a Multicenter European Study. *Eur. Arch. Otorhinolaryngol.* doi:10.1007/s00405-020-05965-1

Lenz, H.-J., Richardson, P., and Stebbing, J. (2021). The Emergence of Baricitinib: A Story of Tortoises versus Hares. *Clin. Infect. Dis.* 72 (7), 1251–1252. doi:10.1093/cid/ciaa940

Lenze, E. J., Mattar, C., Zorumski, C. F., Stevens, A., Schweiger, J., Nicol, G. E., et al. (2020). Fluvoxamine vs Placebo and Clinical Deterioration in Outpatients with Symptomatic COVID-19. *JAMA* 324 (22), 2292–2300. doi:10.1001/jama.2020.22760

Li, K., Meyerholz, D. K., Bartlett, J. A., and McCray, P. B., Jr. (2021). The TMPRSS2 Inhibitor Nafamostat Reduces SARS-CoV-2 Pulmonary Infection in Mouse Models of COVID-19. *mBio* 12 (4), e0097021. doi:10.1128/mBio.00970-21

Liesenborghs, L., Spriet, I., Jochmans, D., Belmans, A., Gyselinck, I., Teuwen, L. A., et al. (2021). Corrigendum to "itraconazole for COVID-19: Preclinical Studies and a Proof-Of-Concept Randomized Clinical Trial Laurens. *EBioMedicine* 69, 103454. doi:10.1016/j.ebiom.2021.103454

Liesenborghs, L., Spriet, I., Jochmans, D., Belmans, A., Gyselinck, I., Teuwen, L. A., et al. (2021). Itraconazole for COVID-19: Preclinical Studies and a Proof-Of-Concept Randomized Clinical Trial. *EBioMedicine* 66, 103288. doi:10.1016/j.ebiom.2021.103288

Liu, J., Cao, R., Xu, M., Wang, X., Zhang, H., Hu, H., et al. (2020). Hydroxychloroquine, a Less Toxic Derivative of Chloroquine, Is Effective in Inhibiting SARS-CoV-2 Infection *In Vitro*. *Cell Discov* 6, 16. doi:10.1038/s41421-020-0156-0

Liu, J., Zheng, X., Tong, Q., Li, W., Wang, B., Sutter, K., et al. (2020). Overlapping and Discrete Aspects of the Pathology and Pathogenesis of the Emerging Human Pathogenic Coronaviruses SARS-CoV, MERS-CoV, and 2019-nCoV. *J. Med. Virol.* 92, 491–494. doi:10.1002/jmv.25709

Lowery, S. A., Sariol, A., and Perlman, S. (2021). Innate Immune and Inflammatory Responses to SARS-CoV-2: Implications for COVID-19. *Cell Host Microbe* 29 (7), 1052–1062. doi:10.1016/j.chom.2021.05.004

Mao, L., Jin, H., Wang, M., Hu, Y., Chen, S., He, Q., et al. (2020). Neurologic Manifestations of Hospitalized Patients with Coronavirus Disease 2019 in Wuhan, China. *JAMA Neurol.* doi:10.1001/jamaneurol.2020.1127

Martinez, D. R., Schafer, A., Leist, S. R., Li, D., Gully, K., Yount, B., et al. (2021). Prevention and Therapy of SARS-CoV-2 and the B.1.351 Variant in Mice. *Cell Rep* 36 (4), 109450. doi:10.1016/j.celrep.2021.109450

Martinez-Guerrero, L., Zhang, X., Zorn, K. M., Ekins, S., and Wright, S. H. (2021). Cationic Compounds with SARS-CoV-2 Antiviral Activity and Their Interaction with Organic Cation Transporter/Multidrug and Toxin Extruder Secretory Transporters. *J. Pharmacol. Exp. Ther.* 379, 96–107. In press. doi:10.1124/jpet.121.000619

Menni, C., Valdes, A. M., Freidin, M. B., Sudre, C. H., Nguyen, L. H., Drew, D. A., et al. (2020). Real-time Tracking of Self-Reported Symptoms to Predict Potential COVID-19. *Nat. Med.* doi:10.1038/s41591-020-0916-2

Mercante, G., Ferreli, F., De Virgilio, A., Gaino, F., Di Bari, M., Colombo, G., et al. (2020). Prevalence of Taste and Smell Dysfunction in Coronavirus Disease 2019. *JAMA Otolaryngol. Head Neck Surg.* doi:10.1001/jamaoto.2020.1155

Meunier, N., Briand, L., Jacquin-Piques, A., Brondel, L., and Penicaud, L. (2020). COVID 19-Induced Smell and Taste Impairments: Putative Impact on Physiology. *Front. Physiol.* 11, 625110. doi:10.3389/fphys.2020.625110

Miller, S. R., Lane, T. R., Zorn, K. M., Ekins, S., Wright, S. H., and Cherrington, N. J. (2021). Multiple Computational Approaches for Predicting Drug Interactions with Human Equilibrative Nucleoside Transporter 1. *Drug Metab. Dispos* 49 (7), 479–489. doi:10.1124/dmd.121.000423

Miller, S. R., McGrath, M. E., Zorn, K. M., Ekins, S., Wright, S. H., and Cherrington, N. J. (2021). Remdesivir and EIDD-1931 Interact with Human Equilibrative Nucleoside Transporters 1 and 2: Implications for Reaching SARS-CoV-2 Viral Sanctuary Sites. *Mol. Pharmacol.* 100, 548–557. In press. doi:10.1124/molpharm.121.000333

Miller, S. R., Zhang, X., Hau, R. K., Jilek, J. L., Jennings, E. Q., Galligan, J. J., et al. (2021). Predicting Drug Interactions with Human Equilibrative Nucleoside Transporters 1 and 2 Using Functional Knockout Cell Lines and Bayesian Modeling. *Mol. Pharmacol.* 99 (2), 147–162. doi:10.1124/molpharm.120.000169

Mirabelli, C., Wotring, J. W., Zhang, C. J., McCarty, S. M., Fursmidt, R., Frum, T., et al. (2020). Morphological Cell Profiling of SARS-CoV-2 Infection Identifies Drug Repurposing Candidates for COVID-19. *bioRxiv*. doi:10.1101/2020.05.27.117184

Molony, R. D., Nguyen, J. T., Kong, Y., Montgomery, R. R., Shaw, A. C., and Iwasaki, A. (2017). Aging Impairs Both Primary and Secondary RIG-I Signaling for Interferon Induction in Human Monocytes. *Sci. Signal.* 10 (509). doi:10.1126/scisignal.aan2392

Mulangu, S., Dodd, L. E., Davey, R. T., Jr., Tshiani Mbaya, O., Proschan, M., Mukadi, D., et al. (2019). A Randomized, Controlled Trial of Ebola Virus Disease Therapeutics. *N. Engl. J. Med.* 381 (24), 2293–2303. doi:10.1056/NEJMoa1910993

Mulay, A., Konda, B., Garcia, G., Yao, C., Beil, S., Sen, C., et al. (2020). SARS-CoV-2 Infection of Primary Human Lung Epithelium for COVID-19 Modeling and Drug Discovery. *bioRxiv*. doi:10.1101/2020.06.29.174623

Muñoz-Fontela, C., Dowling, W. E., Funnell, S. G. P., Gsell, P.-S., Riveros-Balta, A. X., Albrecht, R. A., et al. (2020). Animal Models for COVID-19. *Nature* 586 (7830), 509–515. doi:10.1038/s41586-020-2787-6

Muratov, E. N., Amaro, R., Andrade, C. H., Brown, N., Ekins, S., Fourches, D., et al. (2021). A Critical Overview of Computational Approaches Employed for COVID-19 Drug Discovery. *Chem. Soc. Rev.* 50, 9121–9151. doi:10.1039/d0cs01065k

Murray, J., Hogan, R. J., Martin, D. E., Blahunka, K., Sancilio, F. D., Balyan, R., et al. (2021). Probenecid Inhibits SARS-CoV-2 Replication *In Vivo* and *In Vitro*. *Sci. Rep.* 11 (1), 18085. doi:10.1038/s41598-021-97658-w

Nadanaciva, S., Lu, S., Gebhard, D. F., Jessen, B. A., Pennie, W. D., and Will, Y. (2011). A High Content Screening Assay for Identifying Lysosomotropic Compounds. *Toxicol. Vitro* 25 (3), 715–723. doi:10.1016/j.tiv.2010.12.010

Nguyen, L. C., Yang, D., Nicolaescu, V., Best, T. J., Gula, H., Saxena, D., et al. (2022). Cannabidiol Inhibits SARS-CoV-2 Replication through Induction of the Host ER Stress and Innate Immune Responses. *Sci. Adv.* eabi6110.

Ou, X., Liu, Y., Lei, X., Li, P., Mi, D., Ren, L., et al. (2021). Author Correction: Characterization of Spike Glycoprotein of SARS-CoV-2 on Virus Entry and its Immune Cross-Reactivity with SARS-CoV. *Nat. Commun.* 12 (1), 2144. doi:10.1038/s41467-021-22614-1

Owen, D. R., Allerton, C. M. N., Anderson, A. S., Aschenbrenner, L., Avery, M., Berritt, S., et al. (2021). An Oral SARS-CoV-2 M Pro Inhibitor Clinical Candidate for the Treatment of COVID-19. *Science* 374, 1586–1593. doi:10.1126/science.abl4784

Pan, Y., Guan, H., Zhou, S., Wang, Y., Li, Q., Zhu, T., et al. (2020). Initial CT Findings and Temporal Changes in Patients with the Novel Coronavirus Pneumonia (2019-nCoV): a Study of 63 Patients in Wuhan, China. *Eur. Radiol.* 30, 3306–3309. doi:10.1007/s00330-020-06731-x

Paul, S. M., Mytelka, D. S., Dunwiddie, C. T., Persinger, C. C., Munos, B. H., Lindborg, S. R., et al. (2010). How to Improve R&D Productivity: the Pharmaceutical Industry's Grand challenge. *Nat. Rev. Drug Discov.* 9 (3), 203–214. doi:10.1038/nrd3078

Pruijssers, A. J., George, A. S., Schäfer, A., Leist, S. R., Gralinksi, L. E., Dinnon, K. H., 3rd, et al. (2020). Remdesivir Inhibits SARS-CoV-2 in Human Lung Cells and Chimeric SARS-CoV Expressing the SARS-CoV-2 RNA Polymerase in Mice. *Cel Rep.* 32 (3), 107940. doi:10.1016/j.celrep.2020.107940

Puhl, A. C., Fritch, E. J., Lane, T. R., Tse, L. V., Yount, B. L., Sacramento, C. Q., et al. (2021). Repurposing the Ebola and Marburg Virus Inhibitors Tilorone, Quinacrine, and Pyronaridine: *In Vitro* Activity against SARS-CoV-2 and Potential Mechanisms. *ACS Omega* 6 (11), 7454–7468. doi:10.1021/acsomega.0c05996

Puhl, A. C., Gomes, G. F., Damasceno, S., Godoy, A. S., Noske, G. D., Nakamura, A. M., et al. (2021). Pyronaridine Protects against SARS-CoV-2 in Mouse. *bioRxiv* 0930, 462449. doi:10.1101/2021.09.30.462449

Raghuvanshi, R., and Bharate, S. B. (2021). Recent Developments in the Use of Kinase Inhibitors for Management of Viral Infections. *J. Med. Chem.* 65, 893–921. doi:10.1021/acs.jmedchem.0c01467

Rehman, M. F. U., Fariha, C., Anwar, A., Shahzad, N., Ahmad, M., Mukhtar, S., et al. (2021). Novel Coronavirus Disease (COVID-19) Pandemic: A Recent Mini Review. *Comput. Struct. Biotechnol. J.* 19, 612–623. doi:10.1016/j.csbj.2020.12.033

Reis, G., Dos Santos Moreira-Silva, E. A., Silva, D. C. M., Thabane, L., Milagres, A. C., Ferreira, T. S., et al. (2021). Effect of Early Treatment with Fluvoxamine on Risk of Emergency Care and Hospitalisation Among Patients with COVID-19: the TOGETHER Randomised, Platform Clinical Trial. *Lancet Glob. Health*. doi:10.1016/S2214-109X(21)00448-4

Richardson, P., Griffin, I., Tucker, C., Smith, D., Oechsle, O., Phelan, A., et al. (2020). Baricitinib as Potential Treatment for 2019-nCoV Acute Respiratory Disease. *The Lancet* 395 (10223), e30–e31. doi:10.1016/S0140-6736(20)30304-4

Riva, L., Yuan, S., Yin, X., Martin-Sancho, L., Matsunaga, N., Pache, L., et al. (2020). Discovery of SARS-CoV-2 Antiviral Drugs through Large-Scale Compound Repurposing. *Nature* 586, 113–119. doi:10.1038/s41586-020-2577-1

Rosen, D. A., Seki, S. M., Fernández-Castañeda, A., Beiter, R. M., Eccles, J. D., Woodfolk, J. A., et al. (2019). Modulation of the Sigma-1 Receptor-IRE1 Pathway Is Beneficial in Preclinical Models of Inflammation and Sepsis. *Sci. Transl. Med.* 11 (478). doi:10.1126/scitranslmed.aau5266

Rosenke, K., Hansen, F., Schwarz, B., Feldmann, F., Haddock, E., Rosenke, R., et al. (2021). Orally Delivered MK-4482 Inhibits SARS-CoV-2 Replication in the Syrian Hamster Model. *Nat. Commun.* 12 (1), 2295. doi:10.1038/s41467-021-22580-8

Rosenke, K., Jarvis, M. A., Feldmann, F., Schwarz, B., Okumura, A., Lovaglio, J., et al. (2020). Hydroxychloroquine Prophylaxis and Treatment Is Ineffective in Macaque and Hamster SARS-CoV-2 Disease Models. *JCI Insight* 5 (23). doi:10.1172/jci.insight.143174

Rossi, A., Magri, F., Sernicola, A., Michelini, S., Caro, G., Muscianese, M., et al. (2021). Telogen Effluvium after SARS-CoV-2 Infection: A Series of Cases and Possible Pathogenetic Mechanisms. *Skin Appendage Disord.* 21, 1–5. doi:10.1159/000517223

Salata, C., Calistri, A., Parolin, C., Baritussio, A., and Palù, G. (2017). Antiviral Activity of Cationic Amphiphilic Drugs. *Expert Rev. Anti-infective Ther.* 15 (5), 483–492. doi:10.1080/14787210.2017.1305888

Schiffman, S. S. (2015). "Influence of Drugs on Taste Function," in *Handbook of Olfaction and Gustation*. Editor R. L. Doty (John Wiley & Sons). doi:10.1002/9781118971758.ch40

Schiffman, S. S. (2018). Influence of Medications on Taste and Smell. *World J. Otorhinolaryngol. Head Neck Surg.* 4 (1), 84–91. doi:10.1016/j.wjorl.2018.02.005

Schiffman, S. S. (1983). Taste and Smell in Disease (First of Two Parts). *N. Engl. J. Med.* 308 (21), 1275–1279. doi:10.1056/NEJM198305263082107

Schiffman, S. S. (1983). Taste and Smell in Disease (Second of Two Parts). *N. Engl. J. Med.* 308 (22), 1337–1343. doi:10.1056/NEJM198306023082207

Schiffman, S. S. (1997). Taste and Smell Losses in normal Aging and Disease. *JAMA* 278 (16), 1357–1362. doi:10.1001/jama.1997.03550160077042

Schiffman, S. S., Zervakis, J., Graham, B. G., and Westhall, H. L. (2002). "Age-related Chemosensory Losses: Effect of Medications," in *Chemistry of Taste*. Editor P. G. D. Paredes (Washington DC: American Chemical Society). doi:10.1021/bk-2002-0825.ch008

Seneviratne, C. J., Balan, P., Ko, K. K. K., Udawatte, N. S., Lai, D., Ng, D. H. L., et al. (2021). Efficacy of Commercial Mouth-Rinses on SARS-CoV-2 Viral Load in

Saliva: Randomized Control Trial in Singapore. *Infection* 49 (2), 305–311. doi:10.1007/s15010-020-01563-9

Shang, J., Wan, Y., Luo, C., Ye, G., Geng, Q., Auerbach, A., et al. (2020). Cell Entry Mechanisms of SARS-CoV-2. *Proc. Natl. Acad. Sci. USA* 117 (21), 11727–11734. doi:10.1073/pnas.2003138117

Sharquie, K. E., and Jabbar, R. I. (2021). COVID-19 Infection Is a Major Cause of Acute Telogen Effluvium. *Ir J. Med. Sci.* doi:10.1007/s11845-021-02754-5

Sheahan, T. P., Sims, A. C., Zhou, S., Graham, R. L., Pruijssers, A. J., Agostini, M. L., et al. (2020). An Orally Bioavailable Broad-Spectrum Antiviral Inhibits SARS-CoV-2 in Human Airway Epithelial Cell Cultures and Multiple Coronaviruses in Mice. *Sci. Transl. Med.* 12 (541), eabb5883. doi:10.1126/scitranslmed.abb5883

Shulla, A., Heald-Sargent, T., Subramanya, G., Zhao, J., Perlman, S., and Gallagher, T. (2011). A Transmembrane Serine Protease Is Linked to the Severe Acute Respiratory Syndrome Coronavirus Receptor and Activates Virus Entry. *J. Virol.* 85 (2), 873–882. doi:10.1128/JVI.02062-10

Spinner, C. D., Gottlieb, R. L., Criner, G. J., Arribas López, J. R., Cattelan, A. M., Soriano Viladomiu, A., et al. (2020). Effect of Remdesivir vs Standard Care on Clinical Status at 11 Days in Patients with Moderate COVID-19. *JAMA* 324 (11), 1048–1057. doi:10.1001/jama.2020.16349

Starace, M., Iorizzo, M., Sechi, A., Alessandrini, A. M., Carpanese, M., Bruni, F., et al. (2021). Trichodynia and Telogen Effluvium in COVID-19 Patients: Results of an International Expert Opinion Survey on Diagnosis and Management. *JAAD Int.* 5, 11–18. doi:10.1016/j.jdin.2021.07.006

Stebbing, J., Krishnan, V., Bono, S., Ottaviani, S., Casalini, G., Richardson, P. J., et al. (2020). Mechanism of Baricitinib Supports Artificial Intelligence-predicted Testing in COVID -19 Patients. *EMBO Mol. Med.* 12 (8), e12697. doi:10.15252/emmm.202012697

Subramanian, A., Narayan, R., Corsello, S. M., Peck, D. D., Natoli, T. E., Lu, X., et al. (2017). A Next Generation Connectivity Map: L1000 Platform and the First 1,000,000 Profiles. *Cell* 171 (6), 1437–1452. doi:10.1016/j.cell.2017.10.049

Sukhatme, V. P., Reiersen, A. M., Vayttaden, S. J., and Sukhatme, V. V. (2021). Fluvoxamine: A Review of its Mechanism of Action and its Role in COVID-19. *Front. Pharmacol.* 12 (763). doi:10.3389/fphar.2021.652688

Sungnak, W., Huang, N., Huang, N., Bécavin, C., Berg, M., Queen, R., et al. (2020). SARS-CoV-2 Entry Factors Are Highly Expressed in Nasal Epithelial Cells Together with Innate Immune Genes. *Nat. Med.* 26 (5), 681–687. doi:10.1038/s41591-020-0868-6

Suzuki, M., Saito, K., Min, W. P., Vladau, C., Toida, K., Itoh, H., et al. (2007). Identification of Viruses in Patients with Postviral Olfactory Dysfunction. *Laryngoscope* 117 (2), 272–277. doi:10.1097/01.mlg.0000249922.37381.1e

Titanji, B. K., Farley, M. M., Mehta, A., Connor-Schuler, R., Moanna, A., Cribbs, S. K., et al. (2021). Use of Baricitinib in Patients with Moderate to Severe Coronavirus Disease 2019. *Clin. Infect. Dis.* 72 (7), 1247–1250. doi:10.1093/cid/ciaa879

Tomazini, B. M., Maia, I. S., Cavalcanti, A. B., Berwanger, O., Rosa, R. G., Veiga, V. C., et al. (2020). Effect of Dexamethasone on Days Alive and Ventilator-free in Patients with Moderate or Severe Acute Respiratory Distress Syndrome and COVID-19. *JAMA* 324 (13), 1307–1316. doi:10.1001/jama.2020.17021

Touret, F., Driouich, J.-S., Cochin, M., Petit, P. R., Gilles, M., Barthélémy, K., et al. (2021). Preclinical Evaluation of Imatinib Does Not Support its Use as an Antiviral Drug against SARS-CoV-2. *Antiviral Res.* 193, 105137. doi:10.1016/j.antiviral.2021.105137

Tummino, T. A., Rezelj, V. V., Fischer, B., Fischer, A., O'Meara, M. J., Monel, B., et al. (2021). Drug-induced Phospholipidosis Confounds Drug Repurposing for SARS-CoV-2. *Science* 373 (6554), 541–547. doi:10.1126/science.abi4708

Vallejos, J., Zoni, R., Bangher, M., Villamandos, S., Bobadilla, A., Plano, F., et al. (2021). Ivermectin to Prevent Hospitalizations in Patients with COVID-19 (IVERCOR-COVID19) a Randomized, Double-Blind, Placebo-Controlled Trial. *BMC Infect. Dis.* 21 (1), 635. doi:10.1186/s12879-021-06348-5

Vater, M., Möckl, L., Gormanns, V., Fademrecht, C. S., Mallmann, A. M., Ziegart-Sadowska, K., et al. (2017). Erratum: Corrigendum: New Insights into the Intracellular Distribution Pattern of Cationic Amphiphilic Drugs. *Sci. Rep.* 7, 46011. doi:10.1038/srep46011

Vater, M., Möckl, L., Gormanns, V., Schultz Fademrecht, C., Mallmann, A. M., Ziegart-Sadowska, K., et al. (2017). New Insights into the Intracellular Distribution Pattern of Cationic Amphiphilic Drugs. *Sci. Rep.* 7, 44277. doi:10.1038/srep44277

von Delft, F., Calmiano, M., Chodera, J., Griffen, E., Lee, A., London, N., et al. (2021). A white-knuckle Ride of Open COVID Drug Discovery. *Nature* 594 (7863), 330–332. doi:10.1038/d41586-021-01571-1

Wahl, A., Gralinski, L. E., Johnson, C. E., Yao, W., Kovarova, M., Dinnon, K. H., 3rd, et al. (2021). SARS-CoV-2 Infection Is Effectively Treated and Prevented by EIDD-2801. *Nature* 591 (7850), 451–457. doi:10.1038/s41586-021-03312-w

Wang, J., Yang, X., Li, Y., Huang, J.-a., Jiang, J., and Su, N. (2021). Specific Cytokines in the Inflammatory Cytokine Storm of Patients with COVID-19-Associated Acute Respiratory Distress Syndrome and Extrapulmonary Multiple-Organ Dysfunction. *Virol. J.* 18 (1), 117. doi:10.1186/s12985-021-01588-y

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., et al. (2020). Remdesivir and Chloroquine Effectively Inhibit the Recently Emerged Novel Coronavirus (2019-nCoV) *In Vitro*. *Cell Res* 30 (3), 269–271. doi:10.1038/s41422-020-0282-0

Wang, Y., Zhang, D., Du, G., Du, R., Zhao, J., Jin, Y., et al. (2020). Remdesivir in Adults with Severe COVID-19: a Randomised, Double-Blind, Placebo-Controlled, Multicentre Trial. *The Lancet* 395 (10236), 1569–1578. doi:10.1016/S0140-6736(20)31022-9

Weisberg, E., Parent, A., Yang, P. L., Sattler, M., Liu, Q., Liu, Q., et al. (2020). Repurposing of Kinase Inhibitors for Treatment of COVID-19. *Pharm. Res.* 37 (9), 167. doi:10.1007/s11095-020-02851-7

Weston, S., Coleman, C. M., Haupt, R., Logue, J., Matthews, K., Li, Y., et al. (2020). Broad Anti-coronavirus Activity of Food and Drug Administration-Approved Drugs against SARS-CoV-2 *In Vitro* and SARS-CoV *In Vivo*. *J. Virol.* 94 (21). doi:10.1128/JVI.01218-20

Whitcroft, K. L., and Hummel, T. (2020). Olfactory Dysfunction in COVID-19: Diagnosis and Management. *JAMA*. doi:10.1001/jama.2020.83910.1001/jama.2020.8391

White, K. M., Rosales, R., Yildiz, S., Kehrer, T., Miorin, L., Moreno, E., et al. (2021). Plitidepsin Has Potent Preclinical Efficacy against SARS-CoV-2 by Targeting the Host Protein eEF1A. *Science* 371 (6532), 926–931. doi:10.1126/science.abf4058

Who: Naming the Coronavirus Disease (COVID-2019) and the Virus that Causes it. In, (2020)

Wu, F., Zhao, S., Yu, B., Chen, Y.-M., Wang, W., Song, Z.-G., et al. (2020). Author Correction: A New Coronavirus Associated with Human Respiratory Disease in China. *Nature* 580 (7803), E7. doi:10.1038/s41586-020-2202-3

Xia, H., Cao, Z., Xie, X., Zhang, X., Chen, J. Y., Wang, H., et al. (2020). Evasion of Type I Interferon by SARS-CoV-2. *Cel Rep* 33 (1), 108234. doi:10.1016/j.celrep.2020.108234

Xydakis, M. S., Dehgani-Mobaraki, P., Holbrook, E. H., Geisthoff, U. W., Bauer, C., Hautefort, C., et al. (2020). Smell and Taste Dysfunction in Patients with COVID-19. *Lancet Infect. Dis*. doi:10.1016/S1473-3099(20)30293-0

Yee, S. W., Vora, B., Oskotsky, T., Zou, L., Jakobsen, S., Enogieru, O. J., et al. (2021). Drugs in COVID-19 Clinical Trials: Predicting Transporter-Mediated Drug-Drug Interactions Using *In Vitro* Assays and Real-World Data. *Clin. Pharmacol. Ther.* 110 (1), 108–122. doi:10.1002/cpt.2236

Yuan, S., Chan, J. F. W., Chik, K. K. H., Chan, C. C. Y., Tsang, J. O. L., Liang, R., et al. (2020). Discovery of the FDA-Approved Drugs Bexarotene, Cetilistat, Diiodohydroxyquinoline, and Abiraterone as Potential COVID-19 Treatments with a Robust Two-Tier Screening System. *Pharmacol. Res.* 159, 104960. doi:10.1016/j.phrs.2020.104960

Yuan, S., Yin, X., Meng, X., Chan, J. F., Ye, Z. W., Riva, L., et al. (2021). Clofazimine Broadly Inhibits Coronaviruses Including SARS-CoV-2. *Nature* 593 (7859), 418–423. doi:10.1038/s41586-021-03431-4

Yuen, C. K., Lam, J. Y., Wong, W. M., Mak, L. F., Wang, X., Chu, H., et al. (2020). SARS-CoV-2 Nsp13, Nsp14, Nsp15 and Orf6 Function as Potent Interferon Antagonists. *Emerg. Microbes Infect.* 9 (1), 1418–1428. doi:10.1080/22221751.2020.1780953

Zhang, Q., Bastard, P., Liu, Z., Le Pen, J., Moncada-Velez, M., Chen, J., et al.U. T. C. I. Group (2020). Inborn Errors of Type I IFN Immunity in Patients with Life-Threatening COVID-19. *Science* 370 (6515). doi:10.1126/science.abd4570

Zhang, Q., Chen, C. Z., Swaroop, M., Xu, M., Wang, L., Lee, J., et al. (2020). Heparan Sulfate Assists SARS-CoV-2 in Cell Entry and Can Be Targeted by Approved Drugs *In Vitro*. *Cel Discov* 6 (1), 80. doi:10.1038/s41421-020-00222-5

Zhang, Y.-N., Zhang, Q.-Y., Li, X.-D., Xiong, J., Xiao, S.-Q., Wang, Z., et al. (2020). Gemcitabine, Lycorine and Oxysophoridine Inhibit Novel Coronavirus (SARS-CoV-2) in Cell Culture. *Emerging Microbes & Infections* 9 (1), 1170–1173. doi:10.1080/22221751.2020.1772676

Zhao, H., Mendenhall, M., and Deininger, M. W. (2020). Imatinib Is Not a Potent Anti-SARS-CoV-2 Drug. *Leukemia* 34 (11), 3085–3087. doi:10.1038/s41375-020-01045-9

Zimmer, M., Lamb, J., Ebert, B. L., Lynch, M., Neil, C., Schmidt, E., et al. (2010). The Connectivity Map Links Iron Regulatory Protein-1-Mediated Inhibition of Hypoxia-Inducible Factor-2a Translation to the Anti-inflammatory 15-deoxy-delta12,14-prostaglandin J2. *Cancer Res.* 70 (8), 3071–3079. doi:10.1158/0008-5472.CAN-09-2877

Conflict of Interest: SE is CEO, AP, TRL, FU are employees of Collaborations Pharmaceuticals, Inc. Collaborations Pharmaceuticals, Inc. has obtained FDA orphan drug designations for pyronaridine, tilorone and quinacrine for use against Ebola. CPI have also filed a provisional patent for use of these molecules against Marburg and other viruses.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

MINI REVIEW
published: 13 April 2022
doi: 10.3389/fddsv.2022.892057

COVID-19 Therapies: Protease Inhibitions and Novel Degrader Strategies

Michèle Reboud-Ravaux and Chahrazade El Amri*

Sorbonne Université, Institut de Biologie Paris Seine (IBPS), CNRS UMR 8256, Inserm ERL U1164, Paris, France

The global spread of severe acute respiratory syndrome corona virus-2 (SARS-CoV-2) variants is alarming. In addition to vaccines, effective antiviral agents are urgently needed to combat corona virus disease 2019 (COVID-19). In this review, we will give insights on several canonical approaches using current medicinal chemistry. They target host (TMPRSS2, cathepsins B/L, furin) and viral ($3CL^{pro}$ and PL^{Pro}) proteases involved in virus cell entry and virus production, respectively. Innovative mechanisms of drug action are now explored whereby the drug triggers a cellular event that reduces the level of disease-implicated protein or RNA. The potential therapeutic power of induced degradations of viral proteins by PROTACs and of RNA by RIBOTACs for the treatment of COVID-19 will be discussed. Degraders of host cell RNA-binding proteins (RNA-PROTACs) may also constitute a therapeutical opportunity. First applicated to oncology, these novel technologies may be of a particular interest to obtain therapeutics susceptible to act on mutated viruses.

Keywords: SARS-CoV-2, host proteases, virus proteases, inhibitors, degraders, PROTACs, RIBOTACs

OPEN ACCESS

Edited by:
Bruno Villoutreix, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Caroline Demeret, Institut Pasteur, France
Abdel-Majid Khatib, Institut National de la Santé et de la Recherche Médicale (INSERM), France

****Correspondence:***
Michèle Reboud-Ravaux
michele.reboud@sorbonne-universite.fr

Specialty section:
This article was submitted to Anti-Infective Agents, a section of the journal Frontiers in Drug Discovery

Received: *08 March 2022*
Accepted: *24 March 2022*
Published: *13 April 2022*

Citation:
Reboud-Ravaux M and El Amri C (2022) COVID-19 Therapies: Protease Inhibitions and Novel Degrader Strategies. Front. Drug. Discov. 2:892057. doi: 10.3389/fddsv.2022.892057

INTRODUCTION

Due to the global outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), pharmaceutical companies and academic institutions are actively pursuing efforts to develop new vaccines, repurpose existing drugs and discover of small inhibitors. Host and viral proteases have been especially targeted by inhibitors to block their functions in virus entry and cell cycle. High local concentrations of these inhibitors are needed to obtain a large clinical benefit but they could facilitate off-target binding and side-effects (Adjei, 2006). Such occupancy-based strategy is based on inhibitor binding into an active site. Other putative protein targets to combat COVID-19 are devoid of binding site. Their control can be obtained by decreasing their cellular level in so-called event-driven pharmacology (Cromm and Crews, 2017). These novel therapeutic approaches comprise nucleotide-based techniques [small interfering RNA (siRNA), antisens oligonucleotides, genome editing CRISPR-Cas9 strategy], and targeted protein degradation (TPD) techniques. TPDs examplified by PROteolysis TArgeting Chimeras (PROTACs), molecular glues, LYsosome-TArgeting Chimeras (LYTACs) and Antibody-based PROTACs (AbTACs) (Alabi and Crews, 2021; Bond and Crews, 2021) may constitute a new area of drug discovery to combat SARS-CoV-2 and the uncontrolled spread of virus variants. Other new chemical modalities use RNA-PROTACs that target specific RNAs to degrade RNA-binding proteins for their degradation (Ghidini et al., 2021). The degradation of the viral RNA itself can be induced with ribonuclease targeting chimeras (RIBOTACs) (Di Giorgio and Duca, 2020).

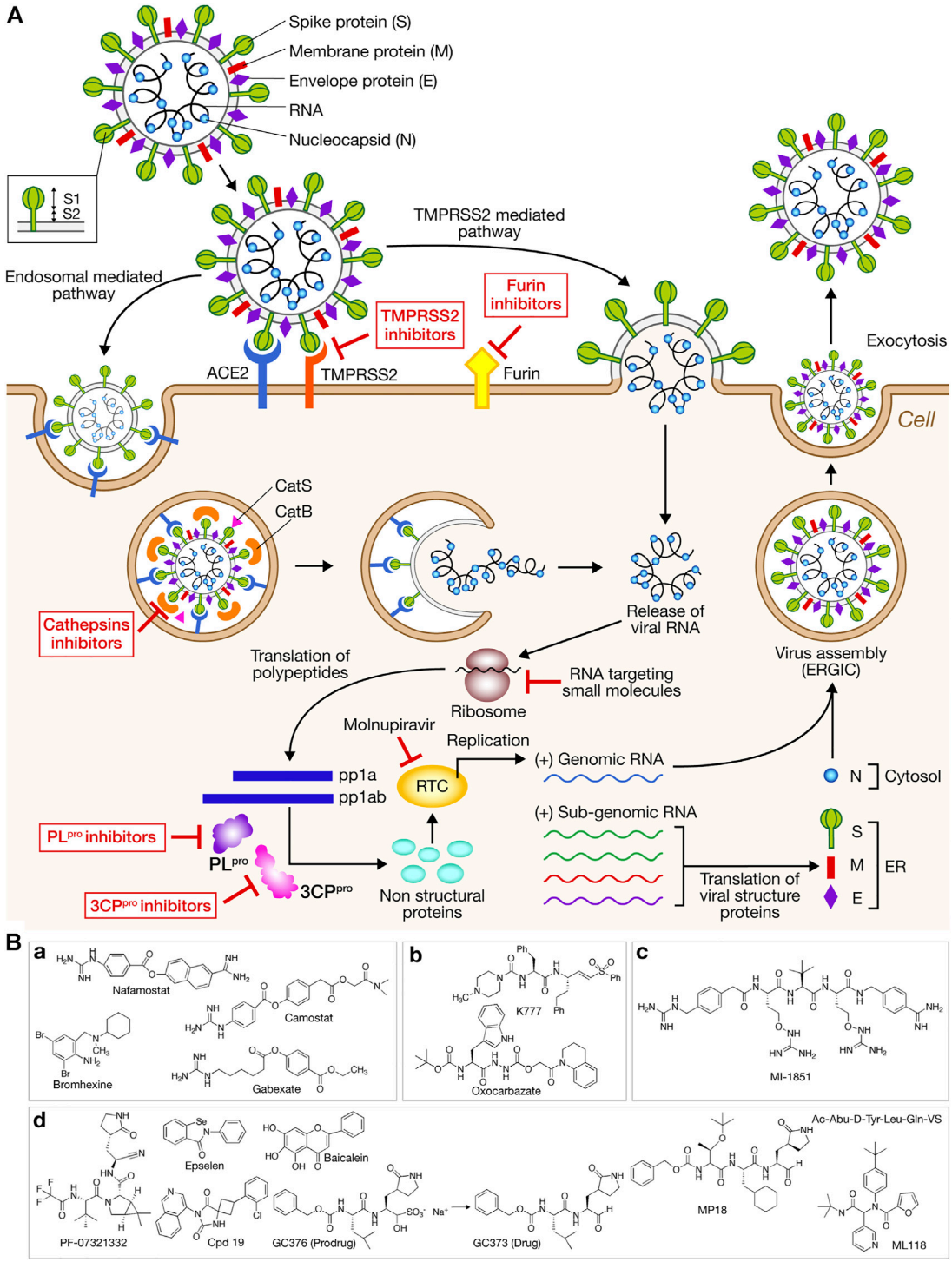


FIGURE 1 | (A) Simplified diagram of life cycle of SARS-CoV-2 and host and viral targets for antiviral development. The host's machinery is used to translate the released single-stranded positive RNA into a large polyprotein. After autocatalytic cleavage of $3CL^{pro}$, the polyprotein is cleaved at 14 different sites with 11 of these by $3CL^{pro}$. The intracellular virus replication is followed by the release of the newly packaged SARS-CoV-2. Cellular and viral proteases are highlighted as potential targets for antiviral development. PROTACs or molecular glues drugs may potentially target proteases such as $3CP^{pro}$, or proteins such as the envelope protein E. RNA targeting molecules such as RIBOTAC and RNA PROTAC may also constitute antivirals. The sites of action of molnupiravir and RNA targeting small molecules are also indicated. ACE2: angiotensin-converting enzyme 2; CatL: cathepsin L; CatS: cathepsin S; pp1a, pp1ab: polyproteins; RTC: replication transcription complex; RER: rough endoplasmic reticulum; ERGIC: endoplasmic-Golgi intermediate compartment. **(B)** Protease inhibitors. (a) TMPRSS2 inhibitors. (b) Cathepsin L inhibitors. (c) Furin inhibitor MI-1851. (d) $3CL^{pro}$ inhibitors. PF-0732132 is found in the drug paxlovid. MPI8 inhibits both $3CL^{pro}$ and CatL.

VIRUS CELL CYCLE AND THERAPEUTICAL APPROACHES

The enveloped SARS-CoV-2 is single-stranded positive-sense RNA virus. Its genome encodes Non-structural proteins Nsps (Nsp1-Nsp16) (replicase complex), nine accessory proteins (ORFs) and four major structural proteins: S (Spike), E (Envelope) M (Membrane), and N (Nucleocapsid) (Arya et al., 2021) (**Figure 1A**). Nsps are produced by processing by viral proteases, papain-like protease PL^{pro} (Nsp5) and main protease $3CL^{pro}$ (Nsp3), of the polyproteins pp1a and pp1ab. Virus entry into the host cells occurs *via* two pathways: endocytosis or membrane fusion after protein S binding to the cellular angiotensin-converting enzyme 2 receptor (ACE2) (**Figure 1A**). The homo-trimeric spike glycoprotein that protrudes from the viral surface has two major subunits, the S1 subunit implicated in receptor recognition and the membrane-anchored S2 subunit mediating fusion between the viral and the host cell membranes. The host protease cleavage site called S1/S2 is located at the border between S1 and S2 subunits. The concerted action of ACE2 binding and S protein processing by the transmembrane serine protease 2 (TMPSSR2) induces irreversible conformational changes that promote virus-cell fusion (Senapati et al., 2021). Several other host proteases have been suggested to promote cell entry, cathepsins B/L and furin (**Figure 1A**). The uncoating by nucleocapsid degradation allows the release of the viral RNA into the cytoplasm to be translated. Two overlapping open reading frames (ORFs) encodes for polyproteins pp1a and pp1ab that are processed by the proteases $3CL^{pro}$ and PL^{pro} leading to Nsp 1–16 which form the replicase/transcriptase complex (RTC). The subgenomic RNAs are translated in the four structural proteins and some accessory proteins. New viral particles are assembled at intracellular membranes. Host and viral proteases as well as RNAs constitute targets to obtain anti-SARS-CoV-2 agents; protein and RNA degraders may also lead to next-generation of drugs (**Figure 1A**).

HOST PROTEASES

Virus cell entry occurs *via* two independent pathways, endosomal mediated and TMPRSS2 mediated (**Figure 1A**). Among type II transmembrane proteases of the human respiratory tract known to cleave surface proteins of respiratory virus, the activity of TMPRSS2 was found the most crucial for SARS-CoV-2 entry and pathogenesis (Hoffmann et al., 2020b). TMPRSS2 aided by TMPRSS4 also facilitates virus entry into human small intestinal enterocytes participating to clinical complications (Zang et al., 2020). By priming the spike protein, TMPRSS2 facilitates the fusion of viral and host membranes whereas endosomal cathepsin B/L facilitates the fusion of viral and endosomal membranes (**Figure 1A**). These proteases can work independently. Recently, a suboptimal S1/S2 spike cleavage and inability to utilize TMPRSS2 was observed for the Omicron BA.1 variant that bears multiple spike mutations compared to the Delta one, thus favoring the endocytic pathway (Meng et al., 2022).

Camostat mesylate is a clinical TMPSSR2 inhibitor (phase I) that can partially block SARS-CoV-2 entry into cell lung line Calu-3, without cytotoxicity. This TMPSRRS2 inhibitor has the potential to treat COVID-19 in humans (Hoffmann et al., 2020b). Several other TMPRSS2 inhibitors are in clinical or pre-clinical phases: nafanostat approved in Japan (phase II), bromhexine (phase IV) and gabexate (preclinical phase) (**Figure 1Ba**). The TMPRSS2 main exosite is a novel target for inhibitors (Singh et al., 2020). Whereas TMPRSS2 acts locally at host cell membrane, the cysteine protease cathepsin L (CatL) with its acidic optimum pH is the major protease that cleaves the virus S1 subunit within endosomes (Ou et al., 2020). CatS is the major endosomal protease that mediates antigen presentation and antibody production (Beers et al., 2005). About 10 FDA approved drugs have an inhibitory activity against CatL but no available drug can specifically inhibit CatL (Dana and Pathak, 2020). Among them, are found oxocarbazate and the vinylsulfone K777 (pre-clinical phase) (**Figure 1Bb**). A combination of TMPRSS2 and cathepsins B/L inhibitors could lead to a complete blockade of viral entry due to a strong synergy (Hoffmann et al., 2020b; Liu T. et al., 2020; Padmanabhan et al., 2020; Hashimoto et al., 2021). Cathepsin L and $3CL^{pro}$ share common structural and electrochemical similarities. The dual non-covalent inhibitor MPI8 that inhibits the viral $3CL^{pro}$ and the host cathepsin L selectively versus cathepsins B or K is a potent antiviral *in vitro* (Cao et al., 2022; Ma et al., 2022) (**Figures 1Bb,d**).

Emerging evidence suggests that furin plays a critical role in viral entry and propagation. SARS-CoV-2 bears a polybasic sequence PRRAR at the S1/S2 cleavage site that can be cleaved by furin (Hoffmann et al., 2020a; Peacock et al., 2021). Several peptide-based and small-molecule inhibitors of furin have been developed (Dahms et al., 2021; Osman et al., 2022). A combination of the furin inhibitor MI-1851 (**Figure 1Bc**) with various TMPRSS2 inhibitors enhances the antiviral potency (Bestle et al., 2020). A novel antibody against furin cleavage site constitutes a suitable approach to decrease viral infectivity (Spelios et al., 2022).

VIRUS PROTEASES

The viral 3-chymotrypsin-like protease ($3CL^{pro}$ or M^{pro}) and papain-like protease (PL^{pro}), and the RNA-dependent-RNA polymerase (RdRP) appeared as traditional targets to combat virus. Two oral antiviral treatments have been approved with molnupiravir targeting RdRP developed by Merck, and nirmatrelvir (PF-07321332) in paxlovid targeting $3CL^{pro}$ developed by Pfizer (**Figures 1A,B**). The third drug, remdesivir developed by Gilead targeting RNA polymerase is less accessible (expensive intravenous infusions).

$3CL^{pro}$ and PL^{pro} activities represent rate-limiting steps in viral replication (Cannalire et al., 2022). Active $3CL^{pro}$ is a homodimer containing a noncanonical Cys145-His41 dyad whereas $3CL^{pro}$ contains the classic Cys112-His273-Asp287 triad for papain-like proteases. $3CL^{pro}$ cleaves the C-terminal region of the precursor protein at 11 sites whereas PL cleaves the N-terminal region of the

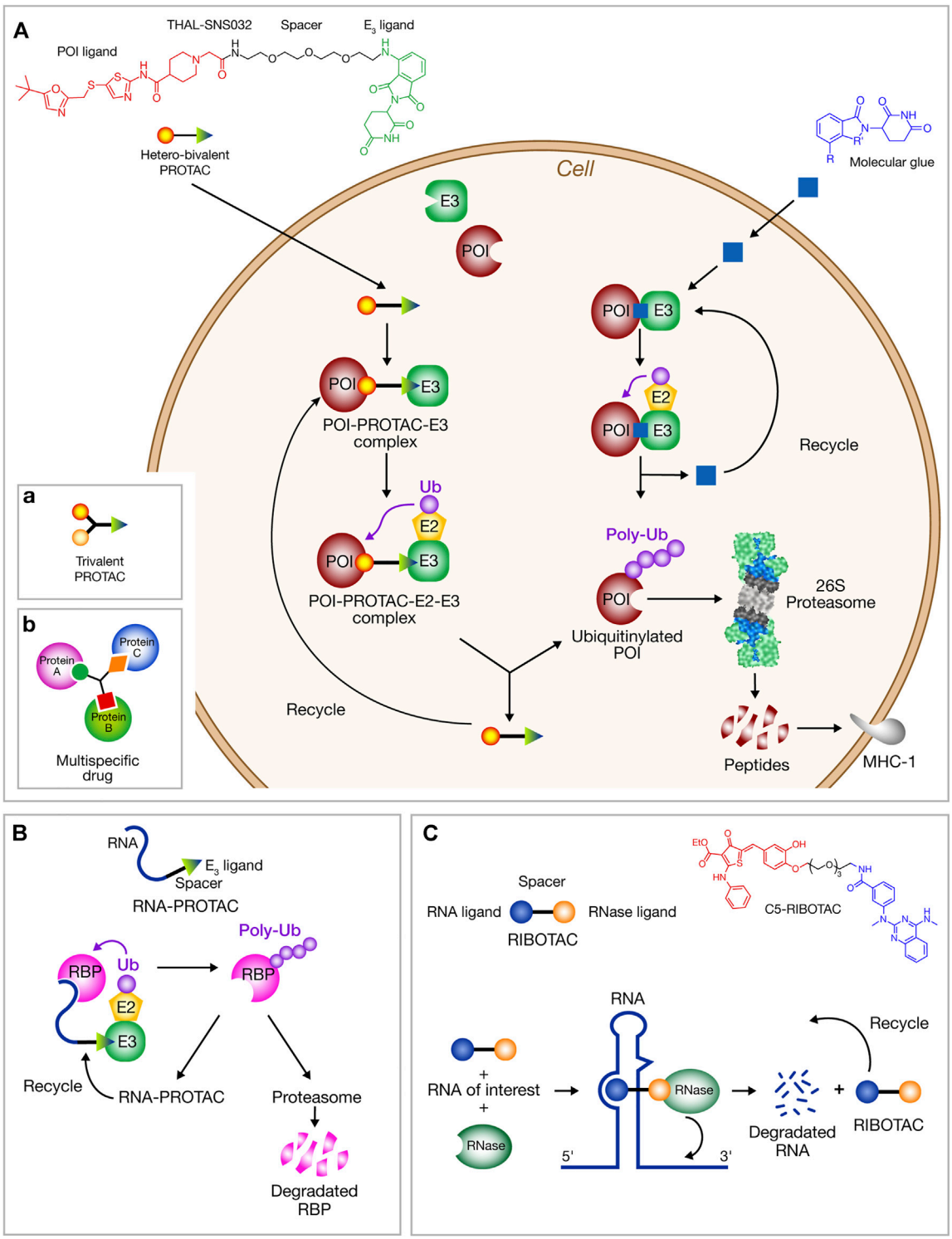


FIGURE 2 | Schematic representation of degrader strategies. **(A)** Targeted protein degradation by chimeric PROTACs and molecular glues. Hetero-bifunctional PROTACs and molecular glues induce the proximity of a protein target (POI) and biological effector (E3) allowing POI ubiquitination and its targeting to proteasome for degradation. PROTAC and molecular glue are released allowing their subsequent use in a new cycle of POI induced degradation. The capability of PROTACs to induce MHC-I peptides is outlined. The chemical structure of THAL-SNS032 (PROTAC) and immunomodulators IMiDs (molecular glues) are shown. Schematic structure of: (a) trivalent PROTACs, (b) multitarget drugs. **(B)** RNA-PROTACs direct RBPs to proteasomal degradation. A short oligonucleotide binds to the RNA domain of the RBP. Linked by a spacer to a motif susceptible to binds an E3 ligase, it mediates RBP ubiquitination and degradation. **(C)** RIBOTACs induce the degradation of RNA itself.

viral precursor protein at three sites. $3CL^{pro}$ shows glutamine-specific cleavage activity not observed in human proteases making it an interesting target (Ullrich and Nitsche 2020). Nearly 200 3D-structures of $3CL^{pro}$ have been released favoring identification of new inhibitors by structure-based rational design or virtual screening of large collections of molecules (Liu Y. et al., 2020; Zhang et al., 2020; Singh and Villoutreix, 2021; Luttens et al., 2022). A lot of structurally diverse compounds (synthetic or natural) displaying $3CL^{pro}$ inhibitory activity are summarized in earlier reviews (Akaji and Konno, 2020; Chen et al., 2021) (**Figure 1Bd**). Many are covalent peptidomimetics such as Ac-Abu-D-Tyr-Leu-Gln-VS (Rut et al., 2021) but non-covalent ones are now reported (Han et al., 2022) (**Figure 1Bd**). Efforts have been made to avoid peptidyl secondary amides (Yamamoto et al., 2022). Ultra-large virtual screening identifies an inhibitor showing comparable efficacy as PF-07321332 against SARS-CoV-2, and antiviral efficacy against SARS-CoV-1 and MERS (non-covalent Cpd 19) (Luttens et al., 2022). No peptidic compound is reported as orally available.

Fewer inhibitors of PL^{pro} are known and potent and specific inhibitors are still needed (Ma and Wang, 2022). Additionally, PL^{pro} contributes to immune escape by cleaving post-translational modified host proteins involved in innate immune response, (ubiquitin and ubiquitin-like protein ISG15 from interferon (IFN) responsive factor) (Freitas et al., 2020).

TARGETED PROTEIN DEGRADATIONS

Protease inhibitors rely on the accessible binding sites. The emerging TPD technologies can target proteins devoid of binding site, categorized as "undruggable" before. They draw inspiration from natural proteasomal protein degradation to specifically eliminate disease-relevant proteins. They are based on the design of small molecules called "degraders" able to induce the proteasomal degradation of the targeted protein. Two major types are known: PROteolysis TArgeting Chimeras (PROTACs) firstly developed in 2001 by Crews and Deshaies (Sakamoto et al., 2001; Sakamoto et al., 2003), and non-chimeric molecules known as molecular glues (Lu et al., 2014). In both cases, the association between the protein of interest (POI) and an E3 ubiquitin ligase is induced allowing the ubiquitin transfer to the POI and its subsequent degradation by proteasome (**Figure 2A**). The feasibility of PROTAC technology had led to clinical trials using the first oral PROTACs [ARV-110 (phase I) and ARV-471 (phase II)] for prostate and breast cancer treatment (Arvinas, Inc., 2019a; Arvinas, Inc., 2019b), and more recently, KT-474 (Kymera Therapeutics) and NX-2127 (Nurix Therapeutics) for autoimmune disorders and B-cell malignancies treatment, respectively (Qi et al., 2021). Several molecular glues such as the immunomodulatory "ImID" small molecules that bind to E3 ligase cereblon (thalidomide, lenalidomide and pomalidomide) have been approved for liquid cancers and four other ones are in clinical trials (Chamberlain and Hamann, 2019).

PROTACs are hetero-bifunctional small molecules comprising two ligands connected by an organic linker, a ligand targets the POI and the other an E3 ligase (Bondeson et al., 2018; Burslem and Crews, 2020; Reboud-Ravaux, 2021). Compared to nucleic-acid strategies, PROTACs have the advantage to lead to an acute and reversible reduction of the targeted protein cell level and a reversible chemical extinction of all properties of POI (Verma et al., 2020). Molecular glues are monovalent small molecules (MW < 200 Da) that do not require a binding pocket on the POI and are good therapeutic candidates for undruggable proteins. They reshape the surface of an E3 ligase inducing the binding of a protein leading to assembly of a possibly cooperative ternary complex. Novel mechanisms for their action have been described (Alabi, 2021) such as polymerization enhancing interaction with a E3 ligase or, for bulky and aggregated proteins, an induced degradation *via* autophagy (Alabi, 2021). New degrader technologies such as LYTACs and AbTACs are developed to broaden the spectrum of protein targets to extracellular and membrane proteins (Alabi, 2021; Lin et al., 2021).

The TPD technologies can now be considered as newly emerging antiviral strategies that may counteract pathogen viruses by inducing the degradation of either viral or host protein targets (Alabi and Crews, 2021; Verma, 2021; Grohmann et al., 2021; Desantis and Giracci, 2022). For example, a NS3/4A protease degrader of the hepatitis C virus (HCV) has been reported (de Wispelaere et al., 2019). Anti-influenza activity was reported for oseltamivir-based PROTAC derivatives (Zhou et al., 2021). Protein degraders for SARS-CoV-2 $3CL^{pro}$ were hypothesized (Liu Y. et al., 2020). Design of PROTAC structures were obtained by computer modeling of the interaction between $3CL^{pro}$ and cereblon E3 ligase (Shaheer et al., 2021). The countereffect of potential deubiquitinase action of PL^{pro} is not experimentally evidenced. In parallel, a novel and potential capability of PROTAC compounds as anti-SARS-CoV-2 has been reported with an antiviral PROTAC targeting the envelope protein E that acts as viroporin. (Martinez-Ortiz and Zhou, 2020). This non-glycosylated envelope protein is a feasible target since inhibition of SARS coronavirus envelope protein ion channel affects several virus functions such as virulence, membrane permeabilizing activity and the viral assembly (Pervushin et al., 2009). Moreover, viral epitopes derived from the proteasomal degradation of protein E can be presented to MHC-I and promote the generation of antibodies against the viral protein (Jensen et al., 2018) (**Figure 2A**). This may result in the development of host T-cell activity against the viral protein. In view to combat resistance to viral mutations, host-directed antivirals are also promising. Four first-in-class indomethacin (INM)-based-PROTACs inhibit SARS-CoV-2 replication and exhibit broad-spectrum anti-viral activity in the Coronaviridae family (Desantis et al., 2021). THAL-SNS032 is a commercial cyclin-dependent-kinase 9 (CDK9)-directed PROTAC that has anti-human cytomegalovirus (HCV) activity. It inhibits SARS-CoV-2 replication (Hahn et al., 2021). Targeting androgen regulation of TMPRSS2 and ACE2 is a possible strategy to combat COVID-19 (Qiao et al., 2020; Deng et al., 2021). Androgen receptor-

inhibitory therapies might reduce susceptibility to COVID-19 symptoms and mortality (Stopsack et al., 2020). Besides inhibitors (e.g., darolutamide, enzalutamide, flutamide and apalutamide), PROTACs targeting the androgen receptor could be protective against COVID since increased mortality and morbidity is observed in men (Wadman, 2020).

RNA-PROTACS AND RIBOTACS

Targeting conserved viral RNA structures and sequences is a novel approach to inhibit viral infection and progression. Interactions between RNA and small molecules are poorly understood rendering RNA difficult to target (Costales et al., 2020; Hegde et al., 2021). Nevertheless, RNA-PROTACs have been designed and synthesized producing degraders of RNA-binding proteins (RBPs) whose defects are observed in many diseases (**Figure 2B**) (Ghidini et al., 2021). The RNA binding site of RBPs can be used to produce RNA-PROTACs. These chimeric structures are composed of a small-RNA mimics that docks the RNA binding site of the RBP and of a peptide able to recruit the E3 ligase. This delivery of peptide to its target site by the oligonucleotide successfully provokes RBP degradation in cancer cells (Ghidini et al., 2021). Several RBPs in the host cells are predicted to bind to the SARS-CoV-2 RNA genome (Sun et al., 2021).

Ribonuclease targeting chimeras (RIBOTACs) are chimeric molecules inducing the degradation of RNA itself (**Figure 2C**), able to destroy cancer associated RNA (Kargbo, 2020) or SARS-CoV-2 RNA structures sequences (Di Giorgio and Duca, 2020; Haniff et al., 2020; Hegde et al., 2021). The PROTAC concept to the RNA field, firstly developed by the Disney group has now been extended (Costales et al., 2020; Liu Y. et al., 2020). The small molecule RIBOTAC has been shown to reduce SARS-CoV-2 RNA levels in a cellular model (Haniff et al., 2020). Using a 15-nucleotide complementary antisense oligonucleotide (ASO) linked to an RNase L recruiter the viral titer was reduced in virus-infected Vero E6 cells (Su et al., 2021).

DISCUSSION

Intensive efforts of the scientific community are needed to face potential future pandemics in order to discover broad-spectrum antiviral agents with new scaffolds and better resistance profiles. Viral $3CL^{Pro}$ and RdRP show the highest degree of conservation across different CoVs and are among the most characterized SARS-CoV-2 targets. Two small molecules nirmatrelvir (PF-07321332) in paxlovid targeting $3CL^{pro}$ and molnupiravir targeting RdRP were recently introduced in clinics. Having no known homolog in host cell, $3CL^{pro}$ remains an ideal target to identify new efficient inhibitors with broad-spectrum activity against coronavirus. Being catalytically inactive in the monomeric form, the development of dimerization inhibitors may also potentially lead to a new class of compounds as previously observed with the homodimeric HIV-1 protease (Bannwarth et al., 2009) and recently for SARS-CoV main protease (Goyal and Goyal, 2020). The underexplored PL^{pro} is less conserved across CoVs family and could appear as less attractive in view of future CoV outbreaks. The antiviral efficacy of protease inhibitors could benefit of the use of drug combination therapy (host TMPRRS2 and cathepsins B/L) (Padmanabhan et al., 2020) or of multitarget drugs binding simultaneously to viral $3CL^{pro}$ and host cathepsin L (Ma et al., 2022).

The increasing understanding of proteasomal protein degradation and RNA biology provides powerful and practical opportunities for the development of novel anti-SARS-CoV-2 agents. TPD using PROTACs and molecular glues may target not only viral or host enzymes but also a large variety of "undruggable" proteins such as structural proteins as previously suggested (Martinez-Ortiz and Zhou, 2020) or potentially many other ones. PROTACs have been applied to degrade a large variety of proteins, cytoplasmic, nuclear, membrane-bound and multipass transmembranes ones as well as "hard to drug" proteins (e.g., KRAS and Myc families) (Bond and Crews, 2021). In the case of $3CL^{pro}$, developing PROTACs using existing enzyme inhibitors could combine occupancy-driven and event-driven technologies avoiding enzyme accumulation in infected cells and lowering side-effects since PROTACs are recycled. They have many advantages over traditional protein inhibitors (Burslem and Crews, 2020). Acting in a catalytic manner allowing for substoichiometric usage, their effect persists until the POI reaccumulates. Off-target side effects and toxicity may be reduced. Nevertheless, the PROTAC large molecular weight ("beyond rule of 5") may result in low bioavalaibility. Modifications of the linkers can efficiently enhance cell permeability and activity (Klein et al., 2021). Click-chemistry can also be used for intracellular PROTAC synthesis (Lebraud et al., 2016; Schiedel et al., 2018). Recent developments of PROTAC technology (kinetic and pharmacokinetic studies, covalence, resistance, new ligands) favor the increase of PROTAC repertoire (Hu and Crews, 2022). Pro-drug PROTACs could be beneficial by improving clinical delivery and metabolic stability (Wei et al., 2021). Recently trivalent PROTACs with their branched trifunctional scaffold (**Figure 2Aa**) were designed and proved to enhance protein degradation *via* combined avidity and cooperativity (Imaide et al., 2021; Zheng et al., 2021). By augmenting the binding valency, various multispecific agents are now prospectively developed opening up to various applications beyond PROTACs (**Figure 2Ab**) (Deshaies, 2020). Such newly created drugs acting on different proteins may possibly perturb the viral cycle for example at the protein assembly level. They could be directed towards distinct domains of the same protein, or even two distinct proteins belonging to a multiprotein complex. The low-molecular-weight molecular glues may have also utility by targeting undruggable proteins and complexes implicated in virus cell cycle. They can be utilized according different mechanisms to induce degradation of neosubstrates (Alabi, 2021). A better understanding of protein-protein interfaces will greatly facilitate rational design of molecular glues (Kozicka and Thomä, 2021).

Structure-function studies of viral non-structural, structural and accessory proteins as well as those implicated in interaction with cell proteins are essential to select potential targets for drug development against COVID-19 (Gorgulla et al., 2021; Singh and Villoutreix, 2021). The discovery of small ligands is needed, even very poor binding ones susceptible to be introduced in PROTACs, molecular glues or other multispecific agents (Mayor-Ruiz et al., 2020). The avidity and cooperativity in ternary complexes (POI-PROTAC-E3) compensate for low binary binding affinities or poor cellular permability allowing for the use of weak, non-functional ligands (Imaide et al., 2021). In silico screening platforms comprising ultra-large-scale ones, artificial intelligence and machine learning techniques are essential to discover novel protein ligands. (Gupta and Mohanty, 2021; Villoutreix, 2021; Luttens et al., 2022). As well, the innovative RNA-targeting strategies could lead to promising developments that will benefit of RNA-targeting drug discovery platforms (Warner et al., 2018).

AUTHOR CONTRIBUTIONS

MR-R conceived, planned and wrote the manuscript. CE edited and contributed to the manuscript organization. The two authors critically revised the paper and approved the submitted version.

FUNDING

This work was supported by the Institut National pour Recherche Médicale (INSERM), Sorbonne-Université and the Centre National de la Recherche Scientifique (CNRS).

ACKNOWLEDGMENTS

We wish to thank Sophie Gournet (Plate-forme Illustration & Graphisme; IBPS, Sorbonne Université) for performing schematics and drawings for this mini-review.

REFERENCES

Adjei, A. A. (2006). What Is the Right Dose? the Elusive Optimal Biologic Dose in Phase I Clinical Trials. *J. Clin. Oncol.* 24, 4054–4055. doi:10.1200/JCO.2006.07.4658

Akaji, K., and Konno, H. (2020). Design and Evaluation of Anti-SARS-coronavirus Agents Based on Molecular Interactions with the Viral Protease. *Molecules* 25, 3920. doi:10.3390/molecules25173920

Alabi, S. B., and Crews, C. M. (2021). Major Advances in Targeted Protein Degradation: PROTACs, LYTACs, and MADTACs. *J. Biol. Chem.* 296, 100647. doi:10.1016/j.jbc.2021.100647

Alabi, S. (2021). Novel Mechanisms of Molecular Glue-Induced Protein Degradation. *Biochemistry* 60, 2371–2373. doi:10.1021/acs.biochem.1c00353

Arvinas, Inc. (2019a) Clinical Trial of ARV-471 in Patients wither/HER2-Locally Advanced or Metastatic Breast Cancer (mBC). In Patients with Metastatic Castration Resistant Prostate Cancer (mCRPC). Available at: https//clinical trials.gov/ct2/show/NCT94072952. (October 23).

Arvinas, Inc. (2019b) Trial of ARV-110 in Patients with Metastatic Castration Resistant Prostate Cancer (mCRPC). Available at: https//clinical trials.gov/ct2/show/NCT03888612. (March, 2019).

Arya, R., Kumari, S., Pandey, B., Mistry, H., Bihani, S. C., Das, A., et al. (2021). Structural Insights into SARS-CoV-2 Proteins. *J. Mol. Biol.* 433, 166725. doi:10.1016/j.jmb.2020.11.024

Bannwarth, L., Rose, T., Dufau, L., Vanderesse, R., Dumond, J., Jamart-Grégoire, B., et al. (2009). Dimer Disruption and Monomer Sequestration by Alkyl Tripeptides Are Successful Strategies for Inhibiting Wild-type and Multidrug-Resistant Mutated HIV-1 Proteases. *Biochemistry* 48, 379–387. doi:10.1021/bi801422u

Beers, C., Burich, A., Kleijmeer, M. J., Griffith, J. M., Wong, P., and Rudensky, A. Y. (2005). Cathepsin S Controls MHC Class II-Mediated Antigen Presentation by Epithelial Cells *In Vivo*. *J. Immunol.* 174 (3), 1205–1212. doi:10.4049/jimmunol.174.3.1205

Bestle, D., Heindl, M. R., Limburg, H., Van Lam van, T., Pilgram, O., Moulton, H., et al. (2020). TMPRSS2 and Furin Are Both Essential for Proteolytic Activation of SARS-CoV-2 in Human Airway Cells. *Life Sci. Alliance* 3, e202000786. doi:10.26508/lsa.202000786

Bond, M. J., and Crews, C. M. (2021). Proteolysis Targeting Chimeras (PROTACs) Come of Age: Entering the Third Decade of Targeted Protein Degradation. *RSC Chem. Biol.* 2, 725–742. doi:10.1039/d1cb00011j

Bondeson, D. P., Smith, B. E., Burslem, G. M., Buhimschi, A. D., Hines, J., Jaime-Figueroa, S., et al. (2018). Lessons in PROTAC Design from Selective Degradation with a Promiscuous Warhead. *Cel Chem. Biol.* 25, 78–87. doi:10.1016/j.chembiol.2017.09.010

Burslem, G. M., and Crews, C. M. (2020). Proteolysis-targeting Chimeras as Therapeutics and Tools for Biological Discovery. *Cell* 181, 102–114. doi:10.1016/j.cell.2019.11.031

Cannalire, R., Cerchia, C., Beccari, A. R., Di Leva, F. S., and Summa, V. (2022). Targeting SARS-CoV-2 Proteases and Polymerase for COVID-19 Treatment: State of the Art and Future Opportunities. *J. Med. Chem.* 65, 2716–2746. doi:10.1021/acs.jmedchem.0c01140

Cao, W., Cho, C.-C. D., Geng, Z. Z., Shaabani, N., Ma, X. R., Vatansever, E. C., et al. (2022). Evaluation of SARS-CoV-2 Main Protease Inhibitors Using a Novel Cell-Based Assay. *ACS Cent. Sci.* 8, 192–204. doi:10.1021/acscentsci.1c00910

Chamberlain, P. P., and Hamann, L. G. (2019). Development of Targeted Protein Degradation Therapeutics. *Nat. Chem. Biol.* 15, 937–944. doi:10.1038/s41589-019-0362-y

Chen, W., Wang, Z., Wang, Y., and Li, Y. (2021). Natural Bioactive Molecules as Potential Agents against SARS-CoV-2. *Front. Pharmacol.* 12, 702472. doi:10.3389/fphar.2021.702472

Costales, M. G., Aikawa, H., Li, Y., Childs-Disney, J. L., Abegg, D., Hoch, D. G., et al. (2020). Small-molecule Targeted Recruitment of a Nuclease to Cleave an Oncogenic RNA in a Mouse Model of Metastatic Cancer. *Proc. Natl. Acad. Sci. U.S.A.* 117, 2406–2411. doi:10.1073/pnas.1914286117

Cromm, P. M., and Crews, C. M. (2017). Targeted Protein Degradation: from Chemical Biology to Drug Discovery. *Cel Chem. Biol.* 24, 1181–1190. doi:10.1016/j.chembiol.2017.05.024

Dahms, S. O., Haider, T., Klebe, G., Steinmetzer, T., and Brandstetter, H. (2021). OFF-State-Specific Inhibition of the Proprotein Convertase Furin. *ACS Chem. Biol.* 16, 1692–1700. doi:10.1021/acschembio.1c00411

Dana, D., and Pathak, S. K. (2020). A Review of Small Molecule Inhibitors and Functional Probes of Human Cathepsin L. *Molecules* 25, 698. doi:10.3390/molecules25030698

de Wispelaere, M., Du, G., Donovan, K. A., Zhang, T., Eleuteri, N. A., Yuan, J. C., et al. (2019). Small Molecule Degraders of the Hepatitis C Virus Protease Reduce Susceptibility to Resistance Mutations. *Nat. Commun.* 10, 3468. doi:10.1038/s41467-019-11429-w

Deng, Q., Rasool, R. U., Russell, R. M., Natesan, R., and Asangani, I. A. (2021). Targeting Androgen Regulation of TMPRSS2 and ACE2 as a Therapeutic Strategy to Combat COVID-19. *iScience* 24, 102254. doi:10.1016/j.isci.2021.102254

Desantis, J., and Goracci, L. (2022). Proteolysis Targeting Chimeras in Antiviral Research. *Future Med. Chem.* 14 (7), 459–462. doi:10.4155/fmc-2022-0005

Desantis, J., Mercorelli, B., Celegato, M., Croci, F., Bazzacco, A., Baroni, M., et al. (2021). Indomethacin-based PROTACs as Pan-Coronavirus Antiviral Agents. *Eur. J. Med. Chem.* 226, 113814. doi:10.1016/j.ejmech.2021.113814

Deshaies, R. J. (2020). Multispecific Drugs Herald a New Era of Biopharmaceutical Innovation. *Nature* 580, 329–338. doi:10.1038/s41586-020-2168-1

Di Giorgio, A., and Duca, M. (2020). New Chemical Modalities Enabling Specific RNA Targeting and Degradation: Application to SARS-CoV-2 RNA. *ACS Cent. Sci.* 6, 1647–1650. doi:10.1021/acscentsci.0c01187

Freitas, B. T., Durie, I. A., Murray, J., Longo, J. E., Miller, H. C., Crich, D., et al. (2020). Characterization and Noncovalent Inhibition of the Deubiquitinase and DeISGylase Activity of SARS-CoV-2 Papain-like Protease. *ACS Infect. Dis.* 6, 2099–2109. doi:10.1021/acsinfecdis.0c00168

Ghidini, A., Cléry, A., Halloy, F., Allain, F. H. T., and Hall, J. (2021). RNA-PROTACs: Degraders of RNA-Binding Proteins. *Angew. Chem. Int. Ed.* 60, 3163–3169. doi:10.1002/anie.202012330

Gorgulla, C., Padmanabha Das, K. M., Leigh, K. E., Cespugli, M., Fischer, P. D., Wang, Z.-F., et al. (2021). A Multi-Pronged Approach Targeting SARS-CoV-2 Proteins Using Ultra-large Virtual Screening. *iScience* 24 (2), 102021. doi:10.1016/j.isci.2020.102021

Goyal, B., and Goyal, D. (2020). Targeting the Dimerization of the Main Protease of Coronaviruses: A Potential Broad-Spectrum Therapeutic Strategy. *ACS Comb. Sci.* 22 (6), 297–305. doi:10.1021/acscombsci.0c00058

Grohmann, C., Marapana, D. S., and Ebert, G. (2021). Targeted Protein Degradation at the Host-Pathogen Interface. *Mol. Microbiol.* 117, 670–681. doi:10.1111/mmi.14849

Gupta, P., and Mohanty, D. (2021). SMMPPI: a Machine Learning-Based Approach for Prediction of Modulators of Protein-Protein Interactions and its Application for Identification of Novel Inhibitors for RBD:hACE2 Interactions in SARS-CoV-2. *Brief Bioinform* 22, bbab111. doi:10.1093/bib/bbab111

Hahn, F., Hamilton, S. T., Wangen, C., Wild, M., Kicuntod, J., Brückner, N., et al. (2021). Development of a PROTAC-Based Targeting Strategy Provides a Mechanistically Unique Mode of Anti-cytomegalovirus Activity. *Int. J. Mol. Sci.* 22, 12858. doi:10.3390/ijms222312858

Han, S. H., Goins, C. M., Arya, T., Shin, W.-J., Maw, J., Hooper, A., et al. (2022). Structure-Based Optimization of ML300-Derived, Noncovalent Inhibitors Targeting the Severe Acute Respiratory Syndrome Coronavirus 3CL Protease (SARS-CoV-2 3CLpro). *J. Med. Chem.* 65, 2880–2904. doi:10.1021/acs.jmedchem.1c00598

Haniff, H. S., Tong, Y., Liu, X., Chen, J. L., Suresh, B. M., Andrews, R. J., et al. (2020). Targeting the SARS-CoV-2 RNA Genome with Small Molecule Binders and Ribonuclease Targeting Chimera (RIBOTAC) Degraders. *ACS Cent. Sci.* 6, 1713–1721. doi:10.1021/acscentsci.0c00984

Hashimoto, R., Sakamoto, A., Deguchi, S., Yi, R., Sano, E., Hotta, A., et al. (2021). Dual Inhibition of TMPRSS2 and Cathepsin B Prevents SARS-CoV-2 Infection in iPS Cells. *Mol. Ther. - Nucleic Acids* 26, 1107–1114. doi:10.1016/j.omtn.2021.10.016

Hegde, S., Tang, Z., Zhao, J., and Wang, J. (2021). Inhibition of SARS-CoV-2 by Targeting Conserved Viral RNA Structures and Sequences. *Front. Chem.* 9, 802766. doi:10.3389/fchem.2021.802766

Hoffmann, M., Kleine-Weber, H., and Pöhlmann, S. (2020a). A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol. Cel* 78, 779–784. e5. doi:10.1016/j.molcel.2020.04.022

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020b). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181, 271–280. e8. doi:10.1016/j.cell.2020.02.052

Hu, Z., and Crews, C. M. (2022). Recent Developments in PROTAC-Mediated Protein Degradation: From Bench to Clinic. *Chembiochem* 23, e202100270. doi:10.1002/cbic.202100270

Imaide, S., Riching, K. M., Makukhin, N., Vetma, V., Whitworth, C., Hughes, S. J., et al. (2021). Trivalent PROTACs Enhance Protein Degradation *via* Combined Avidity and Cooperativity. *Nat. Chem. Biol.* 17, 1157–1167. doi:10.1038/s41589-021-00878-4

Jensen, S. M., Potts, G. K., Ready, D. B., and Patterson, M. J. (2018). Specific MHC-I Peptides Are Induced Using PROTACs. *Front. Immunol.* 9, 2697. doi:10.3389/fimmu.2018.02697

Kargbo, R. B. (2020). SMARCA2/4 PROTAC for Targeted Protein Degradation and Cancer Therapy. *ACS Med. Chem. Lett.* 11, 1797–1798. doi:10.1021/acsmedchemlett.0c00347

Klein, V. G., Bond, A. G., Craigon, C., Lokey, R. S., and Ciulli, A. (2021). Amide-to-Ester Substitution as a Strategy for Optimizing PROTAC Permeability and Cellular Activity. *J. Med. Chem.* 64, 18082–18101. doi:10.1021/acs.jmedchem.1c01496

Kozicka, Z., and Thomä, N. H. (2021). Haven't Got a Glue: Protein Surface Variation for the Design of Molecular Glue Degraders. *Cel Chem. Biol.* 28, 1032–1047. doi:10.1016/j.chembiol.2021.04.009

Lebraud, H., Wright, D. J., Johnson, C. N., and Heightman, T. D. (2016). Protein Degradation by In-Cell Self-Assembly of Proteolysis Targeting Chimeras. *ACS Cent. Sci.* 2 (12), 927–934. doi:10.1021/acscentsci.6b00280

Lin, J., Jin, J., Shen, Y., Zhang, L., Gong, G., Bian, H., et al. (2021). Emerging Protein Degradation Strategies: Expanding the Scope to Extracellular and Membrane Proteins. *Theranostics* 11, 8337–8349. doi:10.7150/thno.62686

Liu, T., Luo, S., Libby, P., and Shi, G.-P. (2020). Cathepsin L-Selective Inhibitors: A Potentially Promising Treatment for COVID-19 Patients. *Pharmacol. Ther.* 213, 107587. doi:10.1016/j.pharmthera.2020.107587

Liu, Y., Liang, C., Xin, L., Ren, X., Tian, L., Ju, X., et al. (2020). The Development of Coronavirus 3C-like Protease (3CLpro) Inhibitors from 2010 to 2020. *Eur. J. Med. Chem.* 206, 112711. doi:10.1016/j.ejmech.2020.112711

Lu, G., Middleton, R. E., Sun, H., Naniong, M., Ott, C. J., Mitsiades, C. S., et al. (2014). The Myeloma Drug Lenalidomide Promotes the Cereblon-dependent Destruction of Ikaros Proteins. *Science* 343, 305–309. doi:10.1126/science10.1126/science.1244917

Luttens, A., Gullberg, H., Abdurakhmanov, E., Vo, D. D., Akaberi, D., Talibov, V. O., et al. (2022). Ultralarge Virtual Screening Identifies SARS-CoV-2 Main Protease Inhibitors with Broad-Spectrum Activity against Coronaviruses. *J. Am. Chem. Soc.* 144, 2905–2920. doi:10.1021/jacs.1c08402

Ma, C., and Wang, J. (2022). Validation and Invalidation of SARS-CoV-2 Papain-like Protease Inhibitors. *ACS Pharmacol. Transl. Sci.* 5, 102–109. doi:10.1021/acsptsci.1c00240

Ma, X. R., Alugubelli, Y. R., Ma, Y., Vatansever, E. C., Scott, D. A., Qiao, Y., et al. (2022). MPI8 Is Potent against SARS-CoV-2 by Inhibiting Dually and Selectively the SARS-CoV-2 Main Protease and the Host Cathepsin L**. *ChemMedChem* 17, e202100456. doi:10.1002/cmdc.202100456

Martinez-Ortiz, W., and Zhou, M.-M. (2020). Could PROTACs Protect Us from COVID-19? *Drug Discov. Today* 25, 1894–1896. doi:10.1016/j.drudis.2020.08.007

Mayor-Ruiz, C., Bauer, S., Brand, M., Kozicka, Z., Siklos, M., Imrichova, H., et al. (2020). Rational Discovery of Molecular Glue Degraders via Scalable Chemical Profiling. *Nat. Chem. Biol.* 16, 1199–1207. doi:10.1038/s41589-020-0594-x

Meng, B., Abdullahi, A., Ferreira, I. A. T. M., Goonawardane, N., Saito, A., Kimura, I., et al. (2022). Altered TMPRSS2 Usage by SARS-CoV-2 Omicron Impacts Tropism and Fusogenicity. *Nature* 603 (7902), 706–714. doi:10.1038/s41586-022-04474-x

Osman, E. E. A., Rehemtulla, A., and Neamati, N. (2022). Why All the Fury over Furin? *J. Med. Chem.* 65, 2747–2784. doi:10.1021/acs.jmedchem.1c00518

Ou, X., Liu, Y., Lei, X., Li, P., Mi, D., Ren, L., et al. (2020). Characterization of Spike Glycoprotein of SARS-CoV-2 on Virus Entry and its Immune Cross-Reactivity with SARS-CoV. *Nat. Commun.* 11, 1620. doi:10.1038/s41467-020-15562-9

Padmanabhan, P., Desikan, R., and Dixit, N. M. (2020). Targeting TMPRSS2 and Cathepsin B/L Together May Be Synergistic against SARS-CoV-2 Infection. *Plos Comput. Biol.* 16, e1008461. doi:10.1371/journal.pcbi.1008461

Peacock, T. P., Goldhill, D. H., Zhou, J., Baillon, L., Frise, R., Swann, O. C., et al. (2021). The Furin Cleavage Site in the SARS-CoV-2 Spike Protein Is Required for Transmission in Ferrets. *Nat. Microbiol.* 6, 899–909. doi:10.1038/s41564-021-00908-w

Pervushin, K., Tan, E., Parthasarathy, K., Lin, X., Jiang, F. L., Yu, D., et al. (2009). Structure and Inhibition of the SARS Coronavirus Envelope Protein Ion Channel. *Plos Pathog.* 5, e1000511. doi:10.1371/journal.ppat.1000511

Qi, S.-M., Dong, J., Xu, Z.-Y., Cheng, X.-D., Zhang, W.-D., Qin, J.-J., et al. (2021). PROTAC: An Effective Targeted Protein Degradation Strategy for Cancer Therapy. *Front. Pharmacol.* 12, 692574. doi:10.3389/fphar.2021.692574

Qiao, Y., Wang, X.-M., Mannan, R., Pitchiaya, S., Zhang, Y., Wotring, J. W., et al. (2020). Targeting Transcriptional Regulation of SARS-CoV-2 Entry Factors ACE2 and TMPRSS2. *Proc. Natl. Acad. Sci. U.S.A.* 118, e2021450118. doi:10.1073/pnas.2021450118

Reboud-Ravaux, M. (2021). Dégradation induite des protéines par des molécules PROTAC et stratégies apparentées : développements à visée thérapeutique. *Biologie Aujourd'hui* 215, 25–43. doi:10.1051/jbio/2021007

Rut, W., Groborz, K., Zhang, L., Sun, X., Zmudzinski, M., Pawlik, B., et al. (2021). SARS-CoV-2 Mpro Inhibitors and Activity-Based Probes for Patient-Sample Imaging. *Nat. Chem. Biol.* 17 (2), 222–228. doi:10.1038/s41589-020-00689-z

Sakamoto, K. M., Kim, K. B., Kumagai, A., Mercurio, F., Crews, C. M., and Deshaies, R. J. (2001). Protacs: Chimeric Molecules that Target Proteins to the Skp1-Cullin-F Box Complex for Ubiquitination and Degradation. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8554–8559. doi:10.1073/pnas.141230798

Sakamoto, K. M., Kim, K. B., Verma, R., Ransick, A., Stein, B., Crews, C. M., et al. (2003). Development of Protacs to Target Cancer-Promoting Proteins for Ubiquitination and Degradation. *Mol. Cell Proteomics* 2, 1350–1358. doi:10.1074/mcp.t300009-mcp200

Schiedel, M., Herp, D., Hammelmann, S., Swyter, S., Lehotzky, A., Robaa, D., et al. (2018). Chemically Induced Degradation of Sirtuin 2 (Sirt2) by a Proteolysis Targeting Chimera (PROTAC) Based on Sirtuin Rearranging Ligands (SirReals). *J. Med. Chem.* 61, 482–491. doi:10.1021/acs.jmedchem.6b01872

Senapati, S., Banerjee, P., Bhagavatula, S., Kushwaha, P. P., and Kumar, S. (2021). Contributions of Human ACE2 and TMPRSS2 in Determining Host-Pathogen Interaction of COVID-19. *J. Genet.* 100, 12. doi:10.1007/s12041-021-01262-w

Shaheer, M., Singh, R., and Sobhia, M. E. (2021). Protein Degradation: a Novel Computational Approach to Design Protein Degrader Probes for Main Protease of SARS-CoV-2. *J. Biomol. Struct. Dyn.* 30, 1–13. doi:10.1080/07391102.2021.1953601

Singh, N., Decroly, E., Khatib, A.-M., and Villoutreix, B. O. (2020). Structure-based Drug Repositioning over the Human TMPRSS2 Protease Domain: Search for Chemical Probes Able to Repress SARS-CoV-2 Spike Protein Cleavages. *Eur. J. Pharm. Sci.* 153, 105495. doi:10.1016/j.ejps.2020.105495

Singh, N., and Villoutreix, B. O. (2021). Resources and Computational Strategies to advance Small Molecule SARS-CoV-2 Discovery: Lessons from the Pandemic and Preparing for Future Health Crises. *Comput. Struct. Biotechnol. J.* 19, 2537–2548. doi:10.1016/j.csbj.2021.04.059

Spelios, M. G., Capanelli, J. M., and Li, A. W. (2022). A Novel Antibody against the Furin Cleavage Site of SARS-CoV-2 Spike Protein: Effects on Proteolytic Cleavage and ACE2 Binding. *Immunol. Lett.* 242, 1–7. doi:10.1016/j.imlet.2022.01.002

Stopsack, K. H., Mucci, L. A., Antonarakis, E. S., Nelson, P. S., and Kantoff, P. W. (2020). *TMPRSS2* and COVID-19: Serendipity or Opportunity for Intervention? *Cancer Discov.* 10, 779–782. doi:10.1158/2159-8290.CD-20-0451

Su, X., Ma, W., Feng, D., Cheng, B., Wang, Q., Guo, Z., et al. (2021). Efficient Inhibition of SARS-CoV-2 Using Chimeric Antisense Oligonucleotides through RNase L Activation**. *Angew. Chem. Int. Ed.* 60, 21662–21667. doi:10.1002/anie.202105942

Sun, L., Li, P., Ju, X., Rao, J., Huang, W., Ren, L., et al. (2021). *In Vivo* Structural Characterization of the SARS-CoV-2 RNA Genome Identifies Host Proteins Vulnerable to Repurposed Drugs. *Cell* 184, 1865–1883. e20. doi:10.1016/j.cell.2021.02.008

Ullrich, S., and Nitsche, C. (2020). The SARS-CoV-2 Main Protease as Drug Target. *Bioorg. Med. Chem. Lett.* 30, 127377. doi:10.1016/j.bmcl.2020.127377

Verma, R. (2021). Exploiting Ubiquitin Ligases for Induced Target Degradation as an Antiviral Strategy. *Adv. Exp. Med. Biol.* 1322, 339–357. doi:10.1007/978-981-16-0267-2_13

Verma, R., Mohl, D., and Deshaies, R. J. (2020). Harnessing the Power of Proteolysis for Targeted Protein Inactivation. *Mol. Cel* 77, 446–460. doi:10.1016/j.molcel.2020.01.010

Villoutreix, B. O. (2021). Post-pandemic Drug Discovery and Development: Facing Present and Futures Challenges. *Front. Drug Discov.*, 1. article 728469. doi:10.3389/fdds.2021.728469

Wadman, M. (2020). Sex Hormones Signal Why Virus Hits Men Harder. *Science* 368, 1038–1039. doi:10.1126/science.368.6489.35610.1126/science.368.6495.1038

Warner, K. D., Hajdin, C. E., and Weeks, K. M. (2018). Principles for Targeting RNA with Drug-like Small Molecules. *Nat. Rev. Drug Discov.* 17, 547–558. doi:10.1038/nrd.2018.93

Wei, M., Zhao, R., Cao, Y., Wei, Y., Li, M., Dong, Z., et al. (2021). First Orally Bioavailable Prodrug of Proteolysis Targeting Chimera (PROTAC) Degrades Cyclin-dependent Kinases 2/4/6 *In Vivo*. *Eur. J. Med. Chem.* 209, 112903. doi:10.1016/j.ejmech.2020.112903

Yamamoto, K. Z., Yasuo, N., and Sekijima, M. (2022). Screening for Inhibitors of Main Protease in SARS-CoV-2: In Silico and *In Vitro* Approach Avoiding Peptidyl Secondary Amides. *J. Chem. Inf. Model.* 62, 350–358. doi:10.1021/acs.jcim.1c01087

Zang, R., Castro, M. F. G., McCune, B. T., Zeng, Q., Rothlauf, P. W., Sonnek, N. M., et al. (2020). TMPRSS2 and TMPRSS4 Promote SARS-CoV-2 Infection of Human Small Intestinal Enterocytes. *Sci. Immunol.* 5, eabc3582. doi:10.1126/sciimmunol.abc3582

Zhang, L., Lin, D., Sun, X., Curth, U., Drosten, C., Sauerhering, L., et al. (2020). Crystal Structure of SARS-CoV-2 Main Protease Provides a Basis for Design of Improved α-ketoamide Inhibitors. *Science* 368, 409–412. doi:10.1126/science.abb3405

Zheng, M., Huo, J., Gu, X., Wang, Y., Wu, C., Zhang, Q., et al. (2021). Rational Design and Synthesis of Novel Dual PROTACs for Simultaneous Degradation of EGFR and PARP. *J. Med. Chem.* 64, 7839–7852. doi:10.1021/acs.jmedchem.1c00649

Zhou, H., Wu, S., and Xu, Z. (2021) CN112592331.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

REVIEW
published: 25 April 2022
doi: 10.3389/fddsv.2022.898035

Developing Small-Molecule Inhibitors of Protein-Protein Interactions Involved in Viral Entry as Potential Antivirals for COVID-19

Peter Buchwald[1,2]*

[1]Diabetes Research Institute, Miller School of Medicine, University of Miami, Miami, FL, United States, [2]Department of Molecular and Cellular Pharmacology, Miller School of Medicine, University of Miami, Miami, FL, United States

Blocking protein-protein interactions (PPIs) involved in the initiation of the cell attachment and entry of viruses is an important antiviral mechanism of action including for neutralizing antibodies. Doing it with small-molecule inhibitors (SMIs) is challenging, as it is for all other PPIs, and might require the exploration of chemical space beyond that of typical drug-like structures. However, it could lead to new antiviral agents suitable for oral administration and acting on alternative targets, considerations that are essential for the development of widely acceptable and broad-spectrum preventive or curative therapeutics. Fostemsavir, an antiretroviral that acts via blocking of the gp120–CD4 PPI, supports the feasibility of the concept. Here, a brief review of relevant drug design considerations is presented together with a summary of the progress made toward the identification of SMIs targeting the PPI between the SARS-CoV-2 spike protein and ACE2 that initiates the viral attachment and cellular entry of this coronavirus causing the COVID-19 pandemic. SMIs identified in various screening assays that were also confirmed to have antiviral activity in a live virus or pseudovirus assay with an IC_{50} < 30 μM so far include several organic dyes (methylene blue, Evans blue, Congo red, direct violet 1), verteporfin, DRI-C23041, and cannabigerolic and cannabidiolic acids. While specificity and activity profiles still need improvement, results so far already provide proof-of-principle evidence for the feasibility of SMIs targeting the SARS-CoV-2-S–hACE2 PPI. Methylene blue, which is approved for clinical use, is orally bioactive, and could act by multiple mechanisms of action, might have potential for repurposing for COVID-19 prevention and treatment.

Keywords: antiviral, coronavirus, fostemsavir, methylene blue, protein-protein interaction, SARS-cov-2, spike protein, variants of concern

OPEN ACCESS

Edited by:
Bruno Villoutreix, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Daniela Trisciuzzi, University of Bari Aldo Moro, Italy
Phillippe Roche, Cancer Research Center of Marseille/ CNRS, France

***Correspondence:**
Peter Buchwald
pbuchwald@med.miami.edu

Specialty section:
This article was submitted to Anti-Infective Agents, a section of the journal Frontiers in Drug Discovery

Received: *16 March 2022*
Accepted: *31 March 2022*
Published: *25 April 2022*

Citation:
Buchwald P (2022) Developing Small-Molecule Inhibitors of Protein-Protein Interactions Involved in Viral Entry as Potential Antivirals for COVID-19. Front. Drug. Discov. 2:898035. doi: 10.3389/fddsv.2022.898035

INTRODUCTION

New drugs introduced during the past century, such as antibacterials (penicillin, 1943) anti-inflammatories (cortisol, 1952), antipsychotics (chlorpromazine, 1953), contraceptives (norethindrone, 1960), anxiolytics (diazepam, 1963), immunosuppressant (cyclosporin A, 1983), antidepressants (fluoxetine, 1987), TNFα-inhibitors (infliximab, 1998), and PD-1–PD-L1 inhibitors (pembrolizumab, nivolumab, 2014)—all shown with their first year of US market approval, are responsible for most of the unprecedented medical progress that happened since then and have completely altered the way life is conducted in industrialized nations. However, truly effective

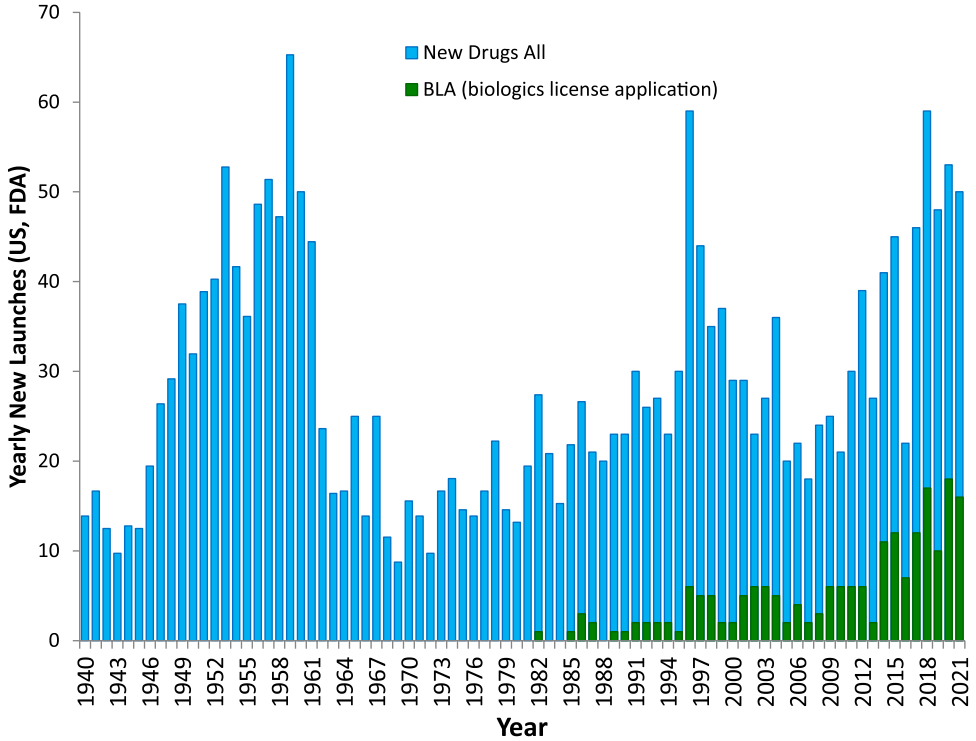


FIGURE 1 | The number of all new drugs launched annually in the United States with FDA approval. The number of all new drugs are shown as blue columns with that of new biologics (approved biologic license applications, BLAs) as superimposed green columns. Except for a few peaks in the 1950s, 1990s, and the last decade, it has been quite steady in the 20–30 per year range. Graphic prepared based on data from (Reuben, 1996; Mullard, 2016a; 2020).

antivirals are still lacking, as the recent coronavirus-inflicted COVID-19 pandemic made painfully clear. The search for antivirals has its own particular challenges, as viruses hijack the reproduction machinery of their host organisms, but progress in drug discovery and development as a whole has been frustratingly slow due to a variety of problems (Proudfoot, 2002; Munos, 2009; Paul et al., 2010; Scannell et al., 2012).

Despite enormous increases in research and development (R&D) investments, the number of newly introduced drugs in the United States remained stubbornly stagnant since the 1960s staying around 20–30 per year (**Figure 1**) and ~85% of them represented no or only modest improvements (Wolff, 1995) demonstrating a pervasive need for innovation. This is probably best illustrated by the fact that the number of new drugs approved by the United States Food and Drug Administration (FDA) that were developed per $1 billion of R&D spending in the drug industry (inflation-adjusted) has been decreasing exponentially since 1950, being steadily halved about every 9 years (Scannell et al., 2012). This is mainly due to the highly increased regulatory burden, the unrealistic public expectation of no side effects, the need to outperform existing old drugs, and the depletion of effective new targets for traditional drug design approaches (Walters et al., 2011; Bodor and Buchwald, 2012; Scannell et al., 2012). Regarding the last, it is commonly estimated that there are only about 500 to 1,500 human protein targets that are both "druggable" and "disease modifying", i.e., only about 2–7% of the ~20,000 canonical (nonmodified) human proteins encoded by individual genes (Hopkins and Groom, 2002; Russ and Lampel, 2005). In general agreement with this, a survey of small-molecule drug targets counted ~550 human proteins (plus another ~180 non-human ones) (Santos et al., 2017). Thus, we are probably beginning to run out of traditional protein targets, at least human ones, and quite likely most low-hanging fruits among such targets that can provide therapeutic benefits have already been picked.

SMALL-MOLECULE INHIBITORS OF PROTEIN-PROTEIN INTERACTIONS

Protein-protein interactions (PPIs), the focus of the present review, represent possible additional, alternate targets as evidenced by the increasing number of clinically approved biologics targeting them (**Figure 1**). For example, one of the latest such successes was the development of cancer immunotherapies targeting immune checkpoint PPIs such as CD80–CTLA4 and PD-1–PD-L1, which has been named *Science* Breakthrough of the Year in 2013 (Couzin-Frankel, 2013). Unfortunately, PPIs are difficult to modulate with small molecules as the corresponding protein interfaces tend to lack well-defined ligand-binding sites where sufficiently strong interactions can take place to ensure the energy of interaction

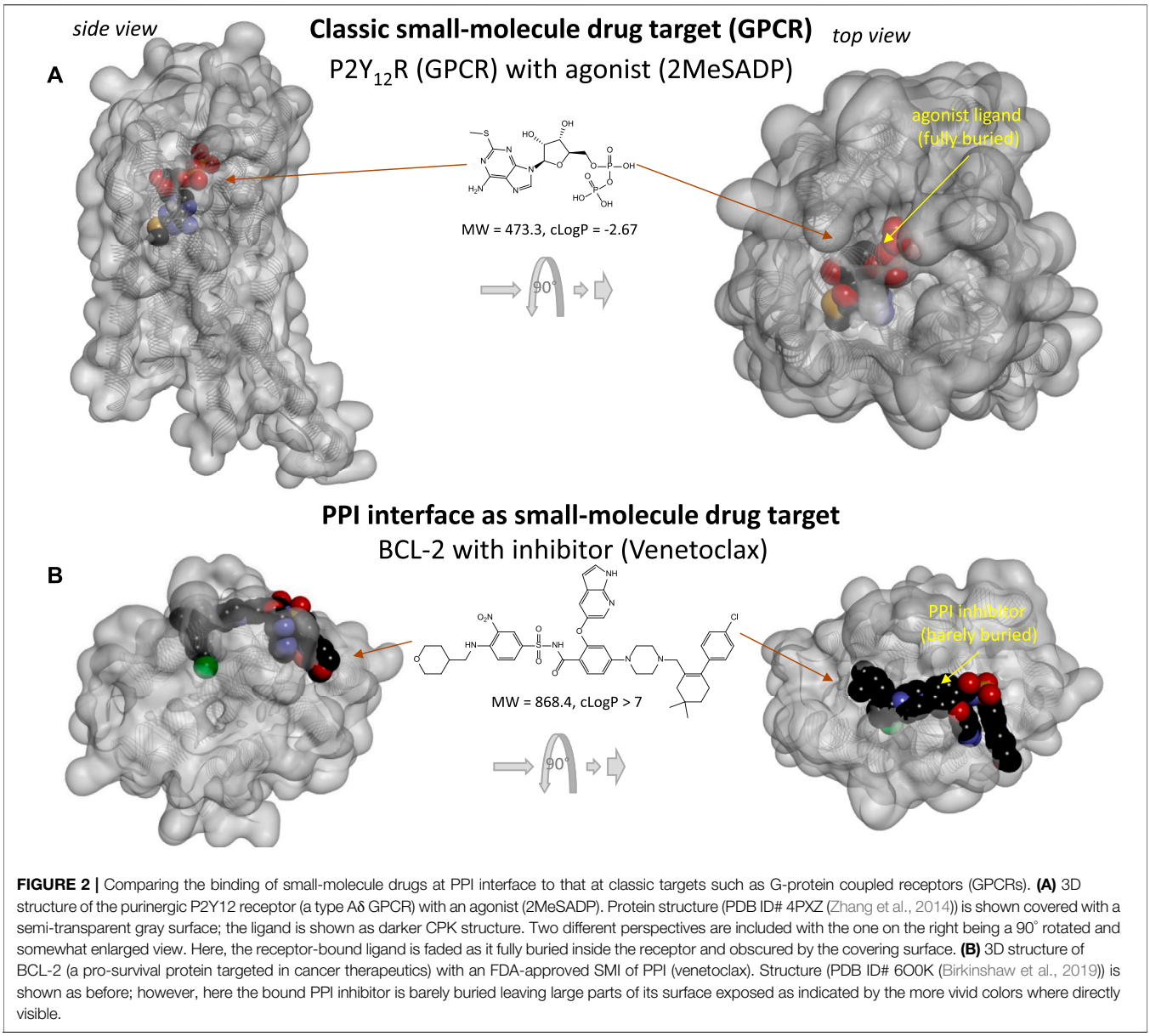


FIGURE 2 | Comparing the binding of small-molecule drugs at PPI interface to that at classic targets such as G-protein coupled receptors (GPCRs). **(A)** 3D structure of the purinergic P2Y12 receptor (a type Aδ GPCR) with an agonist (2MeSADP). Protein structure (PDB ID# 4PXZ (Zhang et al., 2014)) is shown covered with a semi-transparent gray surface; the ligand is shown as darker CPK structure. Two different perspectives are included with the one on the right being a 90° rotated and somewhat enlarged view. Here, the receptor-bound ligand is faded as it fully buried inside the receptor and obscured by the covering surface. **(B)** 3D structure of BCL-2 (a pro-survival protein targeted in cancer therapeutics) with an FDA-approved SMI of PPI (venetoclax). Structure (PDB ID# 6O0K (Birkinshaw et al., 2019)) is shown as before; however, here the bound PPI inhibitor is barely buried leaving large parts of its surface exposed as indicated by the more vivid colors where directly visible.

needed for high affinity binding. Nevertheless, the sheer number of such PPIs, estimated to be in the 300,000 (Zhang et al., 2012; Cheng et al., 2018) to 650,000 (Stumpf et al., 2008) range for humans, implies that a considerable number should still be druggable. Drugs need to be quite potent to be able to compete with naturally present ligands, to be sufficiently specific for their intended target, and to not need unacceptably high doses. Typically, they need to have affinities in the mid-nanomolar range. For example, the median value for all approved drugs has been estimated to be around 20 nM (Overington et al., 2006), which corresponds to a free energy of binding of $\Delta G^0 = -RT{\cdot}\ln K_D = -5.94{\cdot}\log_{10} K_D$ [kJ/mol] = 45.7 kJ/mol. To achieve such high energy, small-molecule endogenous agonists and drugs of classic targets such as G-protein coupled receptors (GPCRs) typically bind at binding sites that are fully buried and allow interactions along the entire ligand surface (**Figure 2A**) (Buchwald, 2019). Since PPI interfaces tend to be relatively large and flat surfaces that lack such well-defined deep pockets, strong binding is difficult to achieve here with small molecules, as interactions are limited to only parts of the total ligand surface. This is illustrated in **Figure 2**, which compares the 3D structure of a typical fully buried small-molecule agonist at a classic GPCR target (purinergic P2Y12 receptor) with that of a surface-bound small-molecule inhibitor (SMI) of a PPI (venetoclax bound to BCL-2).

Not surprisingly, binding pockets on protein-protein interfaces that are suitable to accommodate small molecules are indeed considerably smaller than those of traditional protein-ligand interactions (Fuller et al., 2009). Typically, existing drugs target a single binding pocket with an average

volume of ~300 Å^3, whereas SMIs of PPIs target multiple (3–5) smaller pockets (~100 Å^3) (Fuller et al., 2009). As the achievable maximum energy is limited by the pocket size (Buchwald, 2008), adequate binding affinity at protein interfaces can only be achieved by molecules large enough to reach a sufficient number of such smaller pockets (as illustrated in **Figure 2B**). The need for larger size can also be seen from the perspective of ligand efficiency, LE, defined as the binding energy per unit size–typically the binding free energy per non-hydrogen atom (N_a), LE = $\Delta G^0/N_a$ (Hopkins et al., 2004). Typical ligand-receptor protein interactions have LE of ~1.5 kJ/atom (Hopkins et al., 2004; Hajduk, 2006; Reynolds et al., 2007; Buchwald, 2008), which corresponds to an about two-fold increase in affinity (decrease in K_D or IC_{50}) with the addition of each (non-hydrogen) atom. Such high LE is almost impossible to achieve at PPI interfaces where the bound SMI ligand can interact only along part of its surface (**Figure 2B**). Thus, to achieve the free energy needed for 20 nM binding (45.7 kJ/mol) with an LE of 1 kJ/atom, structures with more than 45 non-hydrogen atoms are needed, which is already larger than desired for typical "druggability". SMIs of PPIs were indeed found to be larger than classic drugs including receptor ligands, ion channel modulators, and enzyme inhibitors (Neugebauer et al., 2007).

On the other hand, biologics, such as antibodies and fusion proteins, can interact with proteins along a broader surface and a variety of epitopes without having to rely solely on druggable pockets to achieve adequate affinity and specificity. An increasing number of biologics are being used clinically as they can be highly specific (**Figure 1**); however, they cannot cross cell membranes, thus cannot reach intracellular targets (Verdine and Walensky, 2007; Hughes et al., 2011; Neklesa et al., 2017), and their protein nature also causes solubility, stability, route of administration (i.e., no oral bioavailability), and biodistribution limitations. Further, since they are foreign proteins, they can act as antigens and elicit strong immune responses in some recipients (Suntharalingam et al., 2006; Wadman, 2006; Leader et al., 2008). All these problems are further exacerbated by their typically long elimination half-lives, which makes it difficult to rapidly eliminate unwanted effects when they occur (Huck et al., 2018). Not surprisingly, FDA-approved biologics encountered more post-market safety issues than did small-molecule drugs (Downing et al., 2017). SMIs of PPIs may represent viable alternatives lacking these problems, if the difficulties related to affinity/specificity can be overcome. While such SMIs were not pursued until relatively recently because they were considered unlikely to be successful due to the aforementioned challenges, during the last 2 decades, it has become clear that SMIs can be effective against at least some PPIs. Most small-molecule PPI modulators are SMIs (i.e., antagonists)—our sole focus here, as so far there are only a limited number of identified small-molecule PPI 'agonists' (i.e., enhancers or stabilizers) (Thiel et al., 2012; Milroy et al., 2014; Andrei et al., 2017). SMIs of PPIs, as antagonists in general, can be orthosteric, directly interfering with the interface and competing with the protein ligand, or allosteric, binding away from the interface but causing sufficient conformational change to block binding of the protein ligand.

Tens of PPI-targeting SMIs have reached preclinical development (Arkin and Wells, 2004; Wells and McClendon, 2007; Wilson, 2009; Buchwald, 2010; Arkin et al., 2014; Milroy et al., 2014; Song and Buchwald, 2015; Scott et al., 2016), and three are approved by the FDA for clinical use: lifitegrast (Gadek et al., 2002), venetoclax (Souers et al., 2013), and fostemsavir (Meanwell et al., 2018) (**Figure 3**). **Lifitegrast** (SAR 1118) is a LFA-1–ICAM-1 inhibitor developed first at Sunesis (Zhong et al., 2012) from a series originating at Genentech (Gadek et al., 2002) and then clinically by SARcode/Shire; it was approved by the FDA for the treatment of dry eye in 2016 (Xiidra) (Scott et al., 2016). **Venetoclax** (ABT-199) is part of a small-molecule series developed by Abbott and later AbbVie and designed to target PPIs in the B cell lymphoma 2 (BCL-2) family (Souers et al., 2013). It received FDA approval in 2015 for treatment of chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), and later acute myeloid leukemia (AML) (Venclexta, Venclyxto) (Mullard, 2016b). **Fostemsavir** (BMS-663068) is a water soluble prodrug of temsavir developed by Bristol-Myers Squibb that acts by blocking gp120 binding to CD4 to limit HIV attachment and entry; it was approved by the FDA for clinical use in the US in 2020 as an antiretroviral for adults living with HIV/AIDS (Rukobia) (Meanwell et al., 2018). Finally, **maraviroc** (Selzentry) is an antiretroviral that can be considered an allosteric SMI of the gp120–CCR5 PPI as it targets CCR5 and stabilizes a conformation no longer recognized by the HIV envelope (Melby and Westby, 2009; Tan et al., 2013). These successes, and particularly that of fostemsavir reemphasize the feasibility of SMIs of PPIs as drug discovery strategy for antivirals. Such SMIs could yield novel therapies that are not only more patient friendly than antibodies (i.e., suitable for oral or inhaled administration), but also less immunogenic, more controllable (shorter half-life/better biodistribution), and possibly even less strain- and mutation-sensitive.

TARGETING SARS-COV-2 SPIKE PPIS AS ANTIVIRAL STRATEGY

SARS-CoV-2—Background

While human coronaviruses (CoVs), enveloped positive-stranded RNA viruses mostly responsible for upper respiratory and digestive tract infections, have been circulating for long, SARS-CoV-2 (severe acute respiratory syndrome-coronavirus 2), the most recent one to emerge, became particularly infamous by being the most infectious agent in a century (Tiwari et al., 2020) and the one responsible for the COVID-19 pandemic that caused hundreds of millions of infections and millions of deaths worldwide (Matheson and Lehner, 2020; Shang et al., 2021; V'Kovski et al., 2021). SARS-CoV-2 is one of the seven CoVs known to infect humans, four of which (HCoV 229E, OC43, NL63, and HKU1) are responsible for about a third of the common cold cases and three that are highly pathogenic and caused recent epidemics associated with considerable mortality: SARS-CoV(-1) (2002–2003, ~10% mortality), MERS-CoV (2012, ~35% mortality), and now SARS-CoV-2 (2019-), which is less lethal but more transmissible (Guy et al., 2020; Rajgor et al.,

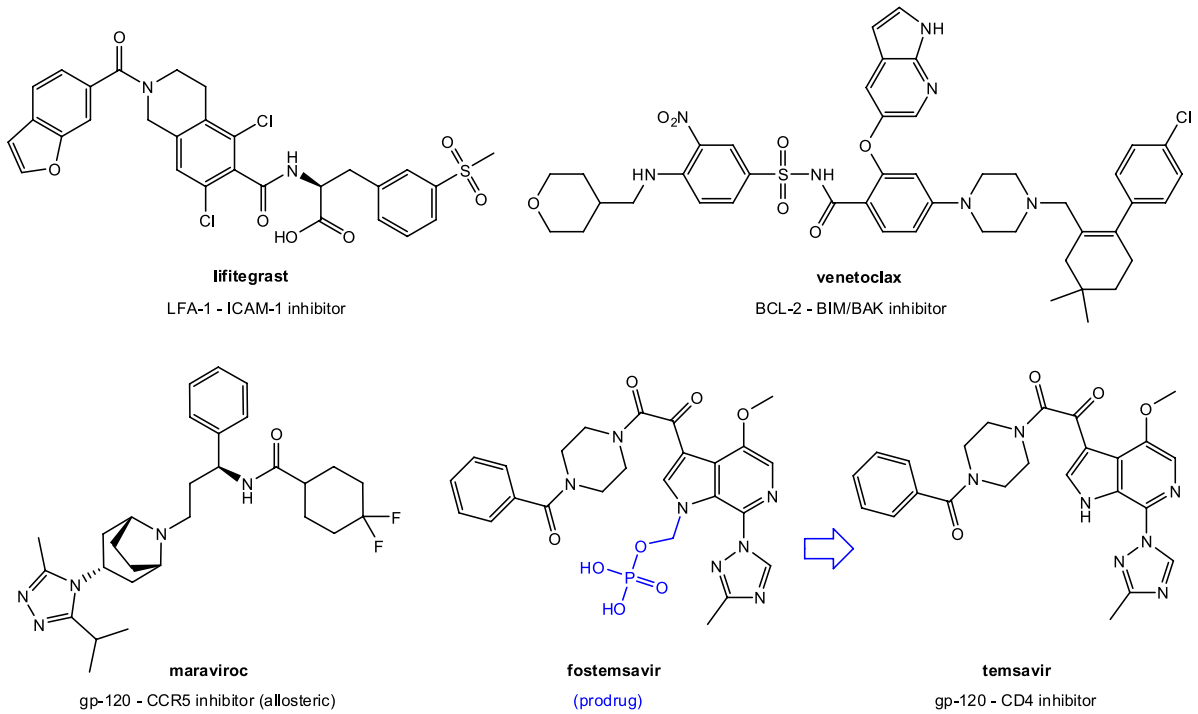


FIGURE 3 | SMIs of PPIs approved for clinical use by the FDA. In addition to lifitegrast (an LFA-1–ICAM-1 inhibitor) and venetoclax (a BCL-2–BIM/BAK inhibitor), they include two anti (retro)virals: maraviroc, an allosteric CCR5 inhibitor, and fostemsavir, a prodrug of temsavir, a gp-120–CD4 PPI inhibitor.

2020). While estimates vary, about 3% of the individuals infected with the original SARS-CoV-2 strain needed hospitalization, and the average infection fatality ratio (IFR, percentage of those infected that do not survive) was around 0.5% but in a strongly age-dependent manner increasing exponentially from 0.001 to 0.002% in <20 years old to 10–20% in those >80–90 years old (Salje et al., 2020; O'Driscoll et al., 2021; COVID-19 Forecasting Team, 2022). While difficult to estimate due to changes in vaccination status and treatment options (Bhattacharyya and Hanage, 2022), it has been considerably, several-fold reduced with the more later emerged *omicron* (B.1.1.529) variant, but likely remained higher than that of influenza (IFR << 0.1%) (Liu et al., 2022; Matsuyama, 2022).

CoVs, which are classified into four genera (α-, *ß*-, γ-, and δ-CoV), initiate infection with the binding of their spike (S) protein to cell surface receptors followed by membrane fusion and virus entry. For SARS-CoV(-1) and SARS-CoV-2 (as well as HCoV-NL63), the receptor is angiotensin converting enzyme 2 (ACE2) (Lan et al., 2020; Shang et al., 2020; Sivaraman et al., 2021; Zhang et al., 2021). For MERS-CoV, it is dipeptidyl peptidase 4 (DPP4), and for HCoV-229E, human aminopeptidase N (APN; CD13) (V'Kovski et al., 2021). Some *ß*-coronaviruses (e.g., HCoV-OC43) bind to sialic acid receptors (Tortorici et al., 2019). Thus, blockade of the SARS-CoV-2-S–hACE2 PPI can disrupt infection efficiency, and abrogation of this interaction is a main goal in the development of vaccines and neutralizing antibodies (nAbs) for the COVID-19 pandemic (Jiang et al., 2020; Lv et al., 2020; Tai et al., 2020). In fact, the spike protein is the principal target of nAbs generated following infection by SARS-CoV-2, with the majority of those identified so far recognizing epitopes within the receptor-binding domain (RBD) that binds ACE2 (Sui et al., 2014; Lv et al., 2020; Wang et al., 2020; Wu et al., 2020; Yuan et al., 2020). The spike protein also is the SARS-CoV-2 component of mRNA and adenovirus-based vaccines approved for use (Harvey et al., 2021).

The SARS-CoV-2 spike protein is a homotrimer with monomer units of ~180 kDa; it is highly glycosylated and is post-translationally cleaved into an S1 and S2 subunit. S1 consists of the amino-terminal domain and the RBD and is responsible for binding to ACE2; S2 includes the trimeric core and is responsible for membrane fusion (Ou et al., 2020; Wang et al., 2020). The RBD located within the S1 domain is known to switch between a standing-up and a lying-down position for receptor binding and immune evasion, respectively (Gil et al., 2020; Shang et al., 2020). Notably, there is a multi-basic furin cleavage site at the S1-S2 boundary, which is unique within b-lineage betacoronaviruses and sarbecoviruses, and is important for the increased infectivity and virulence facilitating the conformational change required for receptor binding (Coutard et al., 2020; Hoffmann et al., 2020; Harvey et al., 2021). It is also an important part of the discussions surrounding the controversies regarding the possible origins of this CoV (Cohen, 2021; Ambati et al., 2022).

There are several possible targets for therapeutic interventions in the CoV lifecycle: viral attachment and entry, uncoating, gRNA replication, translation in ER and Golgi, assembly, and virion release (Guy et al., 2020; V'Kovski et al., 2021; Zhao et al., 2022).

Viral attachment and entry are particularly promising among them because they are the first steps in the replication cycle and take place at relatively accessible extracellular sites (Melby and Westby, 2009). They are also well suited for a PPI inhibition focused approach, the subject of the present review. However, targeting viral entry also has its own challenges, as the envelope and fusion glycoproteins are usually the most variable of all virus-encoded proteins. Indeed, the amino acid sequences can vary both within and between individuals, making the spectrum of antiviral activity for any entry inhibitor an important consideration (Melby and Westby, 2009). RNA viruses are known to accumulate mutations over time yielding antibody resistance and requiring the use of antibody cocktails to avoid mutational escape (Baum et al., 2020). Not surprisingly, several SARS-CoV-2 mutants have already emerged some being variants of concern (VOC) with increased transmissibility, higher disease severity, and resistance to neutralizing antibodies, including those elicited by current vaccines (Cai et al., 2021; Gobeil et al., 2021; Harvey et al., 2021; Kupferschmidt, 2021; Wibmer et al., 2021). Currently, as labeled by the WHO, these include *alpha* (B.1.1.7; first identified in UK, Sep 2020), *beta* (B.1.351; South Africa, May 2020), *gamma* (P.1; Brazil, November 2020), *delta* (B.1.617.2; India, October 2020), and *omicron* (B.1.1.529; multi/S. Africa, November 2021). Emergence of escape variants is likely to continue as the accumulation of RBD mutations is facilitated by the structural plasticity at the RBD-ACE2 interface, further eroding the activities of therapeutic antibodies and serums of vaccine recipients (Nabel et al., 2022).

Therapeutic Need for Small-Molecule Antivirals

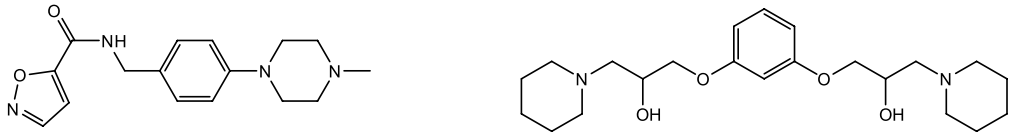
Based on the above, it would be particularly important to have broadly cross-reactive agents that can neutralize a wide range of antigenically disparate viruses (Sui et al., 2014). SARS-CoV(-1) and SARS-CoV-2 share close to 80% amino acid identity in their S proteins, raising the possibility of conserved immunogenic surfaces on these antigens, as supported by the identification of some antibodies of possibly broader activity (Lv et al., 2020; Wec et al., 2020; Starr et al., 2021; Martinez et al., 2022; Park et al., 2022) such as the more recently identified RBD-specific antibody DH1047 (Martinez et al., 2022) or the ACE2-mimicking S2K146 (Park et al., 2022). Nevertheless, most SARS-CoV antibodies are not cross-reactive; for example, none of the 206 RBD-specific monoclonal antibodies derived from single B cells of eight SARS-CoV-2 infected individuals in one study cross-reacted with SARS-CoV(-1) or MERS-CoV RBDs (Ju et al., 2020). As already discussed, targeting such PPIs with SMIs is undoubtedly more challenging, but if successful, it could lead to alternative antiviral treatment options with possible benefits including less strain-specific activity.

Despite the undeniable success of the COVID-19 vaccination program, there remains a considerable need to develop new antivirals and especially oral ones, as a significant portion of the population is either unwilling to be vaccinated or unable to do so due to pre-existing medical conditions. Effective oral treatments could have significant impact on this pandemic as they can be taken easily following the first symptoms. Remdesivir, the first small-molecule COVID-19 drug approved by the FDA, must be given intravenously. Considerable effort and financial resources have been invested in the repurposing of approved drugs as possible small-molecule antiviral agents for SARS-CoV-2, but with only minimal success so far. For example, the large WHO Solidarity trial found that repurposed antiviral drugs including hydroxychloroquine, remdesivir, lopinavir, and interferon-β1 had little or no effect on hospitalized COVID-19 patients, as indicated by overall mortality, initiation of ventilation, and duration of hospital stay (WHO Solidarity Trial Consortium et al., 2020). Further, a paper suggested that most drugs identified in many of the screening assays as possibility for being repurposed against SARS-CoV-2 are not working because they inhibit in the *in vitro* assay by being cationic amphiphilic drugs that cause phospholipidosis, which, however, does not translate into *in vivo* activity (Tummino et al., 2021). This observation has been questioned and should be treated with caution as many of these molecules have both *in vitro* and *in vivo* efficacy with no reported phospholipidosis (Lane and Ekins, 2021).

Regardless, there is an ongoing need to not just repurpose existing drugs but develop novel ones that can combat such infections (Zhao et al., 2022). Recently, two new drugs with classic antiviral mechanisms (i.e., inhibition of protease activity or viral reproduction) have shown promise and granted emergency use authorization by the United States Food and Drug Administration (FDA) for the treatment of COVID-19: molnupiravir (Jayk Bernal et al., 2021) and nirmatrelvir (part of the nirmatrelvir/ritonavir combination Paxlovid) (Owen et al., 2021). Molnupiravir is a prodrug of the synthetic nucleoside derivative N4-hydroxycytidine that exerts antiviral action through introduction of copying errors during viral RNA replication. It was developed originally for the treatment of influenza at Emory University and acquired by Ridgeback Biotherapeutics, who later partnered with Merck (Jayk Bernal et al., 2021). Nirmatrelvir (PF-07321332) is an inhibitor of the SARS-CoV-2 main protease (M^{pro}) developed at Pfizer starting from PF-00835231, an inhibitor of recombinant SARS-CoV(-1) M^{pro} identified during the response to the 2002 SARS outbreak (Owen et al., 2021). It showed very promising clinical results as Paxlovid (nirmatrelvir/ritonavir). In addition, AT-527, a double prodrug of a guanosine nucleotide analog, derived from Atea Pharmaceuticals' nucleotide prodrug platform and shown to be efficacious and well tolerated in hepatitis C virus (HCV) infected subjects (Good et al., 2021), was also pursued, but it was not successful in its first clinical trial. Lessons learned from RNA viruses so far proved that the size and quality of existing antiviral libraries needs to be increased and diversified and polymerase and protease drugs need to be complemented with others targeting different viral proteins (Edwards et al., 2022).

Small-Molecule PPI Targeting Approaches

Following the outbreak of COVID-19, due to the immense therapeutic need generated by the pandemic it created, tremendous screening and drug discovery efforts were invested into the identification of effective preventive or therapeutic



SSAA092E2

N-(4-(4-methylpiperazin-1-yl)benzyl)isoxazole-5-carboxamide

VE607

3,3'-(1,3-phenylenebis(oxy))bis(1-(piperidin-1-yl)propan-2-ol)

FIGURE 4 | Compounds identified before the COVID-19 pandemic as possible SMIs of the SARS-CoV(-1)-S–hACE2 PPI. SSAA09E2 and VE607 have been identified as viral entry inhibitors for SARS-CoV(-1) with low micromolar activity, see text for details.

antiviral interventions in both academic and industrial settings (Ghosh et al., 2020; Shyr et al., 2020; Xiu et al., 2020; Su et al., 2021; Zhao et al., 2022). Here, those directed at identifying SMIs of the SARS-CoV-2-S–hACE2 PPI will be highlighted briefly; some of the earliest ones have been summarized in (Chang et al., 2021). Various screening campaigns have been conducted aiming to identify promising hits mainly from repositionable (repurposable) drug and existing chemical libraries. Assays used (often after virtual screening, i.e., *in silico* preselection typically via molecular docking in AutoDock or Glide) included ELISA (enzyme-linked immunosorbent assay) types (Carino et al., 2020; Bojadzic et al., 2021b; Fu et al., 2021), AlphaLISA (Hanson et al., 2020), Luminex bead-based (Tsegay et al., 2021), surface plasmon resonance (SPR) (Day et al., 2021; Yu et al., 2021; Zhu et al., 2021), affinity selection-mass spectrometry (van Breemen et al., 2022), NanoBiT (Xiong et al., 2021; Yu et al., 2021), CEBIT (condensate-aided enrichment of biomolecular interactions in test tubes) (Pei et al., 2022), and others. Some possible natural product inhibitor have been highlighted in (Ma et al., 2021); however, most are just molecular docking based hypotheses. Considering that several publications relied solely on *in silico* derived hypotheses or just one *in vitro* (often cell-free) inhibitory assay, here, only those compounds will be highlighted first that inhibited this PPI *in vitro* and have concentration-dependent antiviral activity confirmed in a live virus or pseudovirus assay with a sufficiently promising IC_{50}.

In fact, following the emergence of the SARS-CoV(-1) epidemic in the early 2000s, a few groups already performed high-throughput screening (HTS) assays to identify possible antiviral candidates targeting various early steps in its cell invasion. As part of this, some putative SMI candidates of viral entry have been identified, including, for example, **SSAA09E2** (from a screening using a SARS/HIV-luc pseudotyped virus infection assay; pseudovirus IC_{50} 9.7 μM) (Adedeji et al., 2013) and **VE607** (from a screening using protection from SARS-CoV-induced cytopathic effects, CPE, in Vero cells as a phenotypic indicator; live virus IC_{50} 1.6 μM) (Kao et al., 2004) (see structures in **Figure 4**). Other inhibitory small-molecule candidates acting by different mechanism have also been identified; they include, for example, SSAA09E1, SSAA09E3 (Adedeji et al., 2013); MP576, HE602 (Kao et al., 2004); ARB 05–018137, ARB 05–090614 (Severson et al., 2007); K22 (Lundin et al., 2014); and others–see reviews in (Du et al., 2009; Gil et al., 2020; Xiu et al., 2020). Most of these had low micromolar activity (Xiu et al., 2020); however, none of them led to approved preventive or curative therapies for human CoV diseases mainly because in addition to their relatively low (i.e., not nanomolar) potency, they were also not particularly suitable for clinical translatability. They could not pass the preclinical development stage and enter clinical trials due to their poor bioavailability, safety, and pharmacokinetics (Xiu et al., 2020).

SMIs of the SARS-CoV-2-S–hACE2 PPI with confirmed antiviral activity in a live virus or pseudovirus assay having IC_{50} < 30 μM are from the studies listed below in approximate chronological order of their corresponding publications (structures shown in **Figure 5**). Whenever possible, therapeutic (selectivity) index (TI, SI) estimates are also included as an indicator of the relative safety, as it quantifies the separation between toxic and effective concentrations, TI = TC_{50}/IC_{50}.

- A computational screening interrogating 57,641 compounds followed by SPR screening of a library of 3,141 compounds by Day and co-workers at Griffith University, Australia identified three candidates showing concentration-dependent antiviral activity *in vitro*: Evans blue, lifitegrast (**Figure 3**), and lumacaftor (Day et al., 2021) (March 2021). Of these, **Evans blue** was the most promising candidate and the only one with IC_{50} < 30 μM; it had a K_D of 2 μM for SARS-CoV-2-S and inhibited SARS-CoV-2 infection in Vero E6 cells with an IC_{50} of 28 μM. According to the authors, it was also non-toxic for up to 1 mM (TC_{50} > 1,000 μM), suggesting a sufficiently large therapeutic index (TI > 30).
- Our work at the University of Miami, Florida, United States identified several organic dyes (Congo red, direct violet 1, Evans blue) and novel druglike compounds (DRI-C23041, DRI-C91005) that inhibited the interaction of ACE2 with the spike proteins of SARS-CoV-2 as well as SARS-CoV(-1) with low micromolar activity in cell-free ELISA-type assays (IC_{50}'s of 0.2–3.0 μM) (Bojadzic et al., 2021b) (May 2021). Of these, **DRI-C23041**, **Congo red**, and **direct violet 1** (**Figure 5**) were also confirmed to inhibit the entry of two different spike-bearing pseudoviruses into HEK293/Vero E6 cells with IC_{50}'s of 5.6/7.4, 20.3/27.4, and 35.8/16.4 μM, respectively. They were also relatively noncytotoxic in the same assay having TC_{50} > 400 μM for DRI-C23041 (i.e., TI > 70) and >100 μM for Congo red

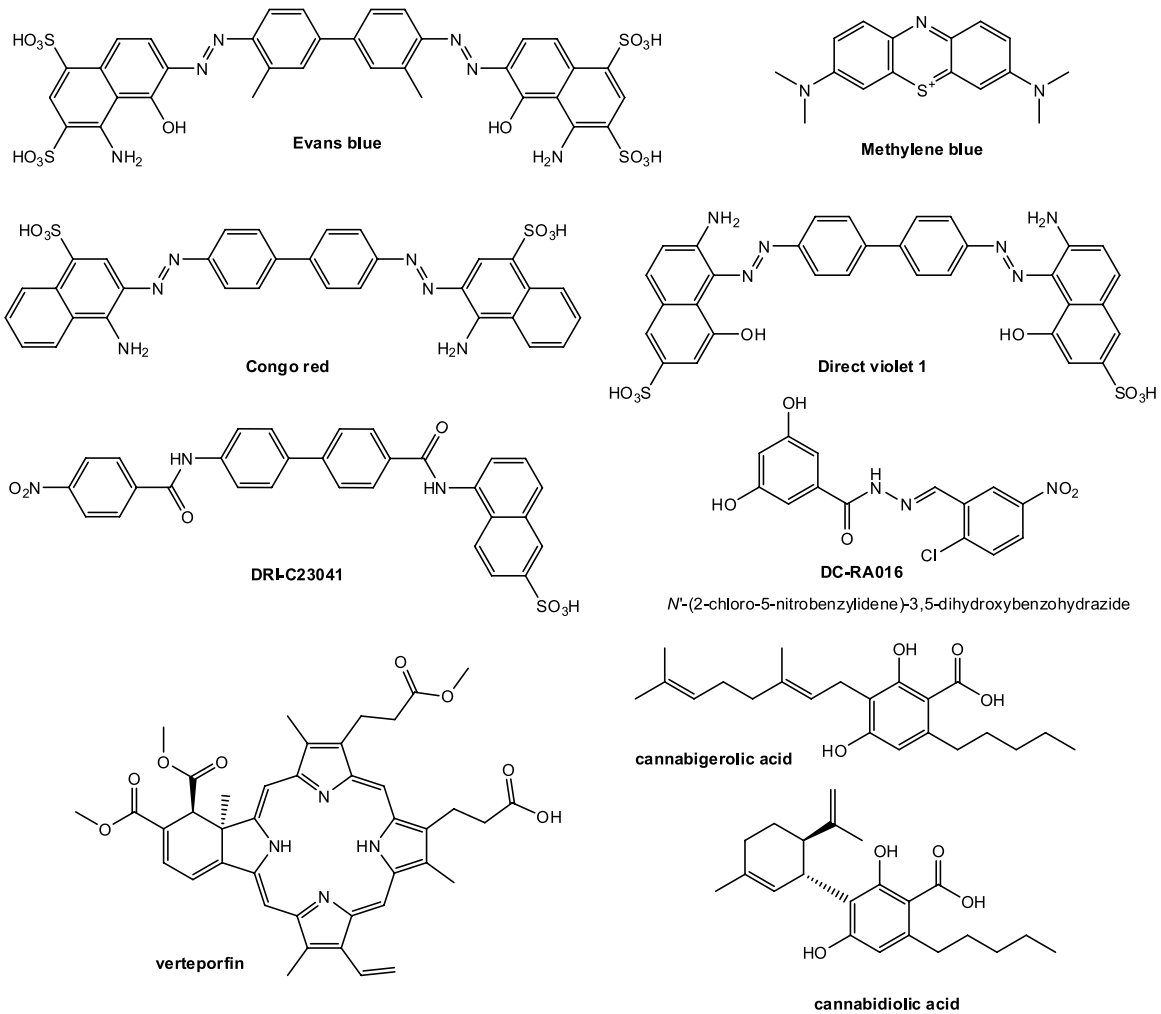


FIGURE 5 | Compounds identified so far since the outbreak of COVID-19 as possible SMIs of the SARS-CoV-2-S–hACE2 PPI. Only compounds that have been confirmed to have antiviral activity in a live virus or pseudovirus assay with promising enough activity (IC_{50} < 30 μM) are shown.

and direct violet 1 (TI > 5). **Evans blue**, which was the best hit in the work from Day, was identified as an inhibitor, but was not tested here in viral assays as other compounds were more active.

- During this screening, we also identified **methylene blue** as a SMI and confirmed that it had a quite promising IC_{50} of 3.5 μM in this viral assay (Bojadzic et al., 2021a) (January 2021). This is of possible interest as a methylene blue is an inexpensive and widely available drug approved by the FDA for the treatment of methemoglobinemia and used for other medical applications. It was also identified by several other groups as having anti-SARS-CoV-2 activity and confirmed to have low micromolar activity in concentration-response studies including with live viruses, possibly due to additional multiple mechanisms of action (Gendrot et al., 2020; Cagno et al., 2021; Gendrot et al., 2021; Murer et al., 2022). Methylene blue seems to be a promiscuous PPI inhibitor with low micromolar activity and a relatively narrow TI, but with multiple evidence suggesting that it clearly inhibits SARS-CoV-2 including VOCs such as *delta* (B.1.617.2) (Chuang et al., 2022).
- Fu and co-workers at the New York University School of Medicine, United States screened a library of 958 FDA-approved drugs using ELISA-based HTS, and identified five drugs, N-acetylcysteine (NAC), tiopronin (TPR), verteporfin (VP), calcitriol, and racecadotril, to inhibit RBD–ACE2 interaction at both low and high concentrations (Fu et al., 2021) (July 2021). Of these, **verteporfin** (Visudyne) significantly inhibited pseudovirus entry into hACE2 overexpressing HEK293T cells (IC_{50} < 0.1 μM) while having a half cytotoxic concentration TC_{50} ≈ 10 μM (implying TI > 100). Before this work, verteporfin was confirmed by another group to potently inhibit the cytopathic effect produced by SARS-CoV-2 infection with an IC_{50} < 0.31 μM with indications that the porphyrin ring structure binds the ACE2 receptor (Gu et al., 2021) (December 2020).

- Xiong and co-workers at the Chinese Academy of Sciences, Shanghai and Beijing, China and collaborators virtually screened and filtered compounds from the SPECS database and then purchased 109 selected candidates for follow-up biological testing including NanoBiT and SPR assays to check their ability to block the SARS-CoV-2-S-RBD–ACE2 PPI (Xiong et al., 2021) (Sep 2021). From these, they highlighted two inhibitors as sufficiently promising in pseudovirus assays with some separation between efficacy and cytotoxicity: **DC-RA016** (ZINC125276) (IC_{50} = 22.4 μM) and DC-RA052 (IC_{50} = 68 μM). The IC_{50} for DC-RA016 was, however, somewhat overstated due to the way the concentration-response curve was fitted (with a non-zero bottom) as this compound barely caused 50% inhibition at 100 μM (Figure 4B in (Xiong et al., 2021)). Nevertheless, its structure was included here for illustration (**Figure 5**).
- Finally, van Breeman and co-workers at Oregon State University, Corvallis, OR, United States used affinity selection-mass spectrometry for the discovery of botanical ligands to the SARS-CoV-2 spike protein and found cannabinoid acids from hemp (*Cannabis sativa*) to be allosteric as well as orthosteric ligands with micromolar affinity for the spike protein (van Breemen et al., 2022) (January 2022). In follow-up virus neutralization assays, **cannabigerolic acid** and **cannabidiolic acid** prevented infection of human epithelial cells by a pseudovirus expressing the SARS-CoV-2 spike protein and prevented entry of live SARS-CoV-2 into cells including for variants B.1.1.7 and B.1.351 with IC_{50}'s of 21 and 23 μM (7.7 and 8.4 μg/ml) in the pseudovirus and 67 and 103 μM (24 and 37 μg/ml) in the live virus assay for cannabidiolic and cannabigerolic acid, respectively, where their cytotoxicities were not yet significant (van Breemen et al., 2022). Cannabidiolic acid seems to have a TC_{50} around 80 μg/ml (Fig. S4 in (van Breemen et al., 2022)) giving TI ≈ 10.

Some of the other works that identified SMI hits but did not include confirmation in viral assay or the inhibitory activity in these assays was not sufficiently potent include (again, in chronological order of their corresponding publications):

- Carino and co-workers at the University of Perugia, Italy used *in silico* prescreening followed by *in vitro* confirmation using a commercial SARS-CoV-2 spike inhibitor screening assay kit and found that naturally occurring and clinically available triterpenoids, such as glycyrrhetinic and oleanolic acids, as well as primary and secondary bile acids and their amidated derivatives, such as glyco-ursodeoxycholic acid and semi-synthetic derivatives such as obeticholic acid, reduced the RBD–ACE2 binding (Carino et al., 2020) (October 2020). However, these compounds showed only weak activity and concentration dependence. None of them caused 50% reduction at the highest concentration tested (10 μM). Activities were not confirmed in viral or pseudoviral assays.
- The group of Hanson an co-workers at the National Center for Advancing Translational Sciences (NCATS), National Institutes of Health (NIH), Bethesda, MD, United States used an AlphaLISA assay based HTS of 3,384 small-molecule drugs and preclinical compounds suitable for repurposing and identified 25 possible hits (Hanson et al., 2020) (November 2020). However, of these only corilagin was validated in cherry-picking as showing activity against ACE2–RBD with an IC_{50} of 5.5 μM, and there was no confirmation in viral assays.
- Zhu and co-workers at Peking Union Medical College, Beijing, China used SPR to screen a library of 960 compounds and identified demethylzeylasteral as having promisingly high affinities for S-RBD and ACE2 (K_D of 1.0 and 1.7 μM for S-RBD and ACE2, respectively) (Zhu et al., 2021) (Dec. 2020). In a pseudovirus assay, it inhibited entry of SARS-CoV-2 pseudovirus into HEK293T cells to "a certain extent" at nontoxic concentration (7% inhibition at 0.37 μM).
- Yu and co-workers at the Shanghai University of Traditional Chinese Medicine, Shanghai, China used SPR and NanoBit assays to verify the spike protein-binding activity of compounds selected via virtual screening from traditional Chinese medicines and then their inhibitory activities on SARS-CoV-2-S-RBD–ACE2 PPI (Yu et al., 2021) (May 2021). They found glycyrrhizic acid to be the most efficient and nontoxic broad-spectrum anti-CoV SMI with a K_D of 0.87 μM toward SARS-CoV-2-S1 as suggested by SPR, but an IC_{50} of only 22 μM for disrupting the corresponding PPI in the NanoBiT assessment. There was no confirmation in viral assay.
- Tsegay and co-workers at Seattle Children's Research Institute, Seattle, WA, United States screened 2,701 compounds from an "FDA-approved drug screening library" for their ability to inhibit the binding of recombinant SARS-CoV-2 spike to hACE2 in a Luminex bead-based assay and identified 56 that inhibited in a concentration-dependent manner (June 2021) (Tsegay et al., 2021). Best SMIs were thiostrepton, oxytocin, nilotinib, and hydroxycamptothecin with IC_{50}'s in the 4–9 μM range, but there were no cell-based activity or toxicity assessments.
- Pei and co-workers from Tsinghua University, Beijing, China used CEBIT to screen 2572 FDA approved drugs for their ability to inhibit this PPI and identified six candidate compounds that were confirmed by SPR to bind with K_D of 17–780 μM: varenicline, sennoside A, quercetin, quinacrine, methylene blue, and sunitinib (Pei et al., 2022) (March 2022).

In addition to SMIs, peptide-based inhibitors of PPIs are also a possibility–see (Lee et al., 2019; Wang et al., 2021; Trisciuzzi et al., 2022) for recent reviews. Some peptide disruptors have also been reported for SARS-CoV-2–hACE2, but so far none have been very effective (Gil et al., 2020; Xiu et al., 2020; Zhang et al., 2020). A stapled peptide approach carried out at the University of Southern Denmark, Odense, Denmark showed some promise

with an IC_{50} of 3.6 μM for inhibition of the PPI, but no cell-based confirmations were performed (Maas et al., 2021). Relatively high affinity peptide binders of the SARS-CoV-2 spike RBD (K_D: 80–970 nM) have been identified by affinity selection-mass spectrometry from a screening of 800 million synthetic peptides at the Massachusetts Institute of Technology (MIT), Cambridge, MA, United States ; however, they turned out to not compete for ACE2 binding (Pomplun et al., 2021). Because of bioavailability, metabolic instability (short half-life), lack of membrane permeability, and other issues, developing peptides into clinically approved drugs is difficult and rarely pursued (Otvos and Wade, 2014; Henninot et al., 2018)—a main reason why we focused here on small-molecule compounds that represent an approach much more likely to ultimately transition into clinical development.

SUMMARY AND OUTLOOK

Blocking of PPIs involved in the initiation of cell attachment and entry of CoVs can provide efficient antiviral therapeutics and is the main mechanism of action of biologics such as neutralizing antibodies. SMIs face more challenges to achieve this, as they do for all other PPIs; however, they could lead to new alternative antiviral agents that are suitable for oral administration and act by a different mechanism of action than existing small-molecule antivirals such as protease or viral reproduction inhibitors. Oral bioavailability is highly desirable to achieve widespread usage and compliance (Neklesa et al., 2017), and oral therapeutics are much more suitable for long-term use and/or broadly acceptable preventive use (including for transmission control of viral diseases) than any other routes of administration (Cochrane et al., 1999; Moia et al., 2013). Broadly specific activity is also of considerable interest as it could make possible mutation resistant, multi-strain, or even pan-CoV inhibition. While it is usually difficult if not impossible to achieve with antibodies that tend to be target-specific, it could be more achievable with SMIs. For example, we have shown that while the corresponding antibodies did not cross-react for the human vs. mouse CD40–CD40L PPI, our SMIs did and even maintained similar potencies (Margolles-Clark et al., 2009; Bojadzic and Buchwald, 2019). The impact of SARS-CoV-2 variants on spike and RBD structure and on nAb activity, which could also affect SMIs, has been summarized recently (Nabel et al., 2022). Computational simulations of SARS-CoV-2 spike flexibility and its interactions with other proteins are being carried out and should provide helpful tools for future screening efforts (Pedebos and Khalid, 2022).

SMIs of the SARS-CoV-2-S–hACE2 PPI identified so far and summarized above provide proof-of-principle evidence for the feasibility of such a small-molecule approach, but it remains to be seen if they can ultimately lead to clinically usable therapies as specificity and activity profiles still need improvement. While specific goals vary somewhat depending on the specifics of the project, small-molecule drug candidates are generally expected to have, among others: • potency in at least the hundred-nanomolar range (i.e., IC_{50} < 100 nM meaning pK_i > 7) (the median of existing drugs being ~20 nM); • adequate selectivity/specificity (>20× versus other targets is a reasonable minimum and >100× is desirable); • good safety profile (TI > 30 and optimally >100 in early studies plus passing of all toxicity studies); • adequate solubility and partition properties (needed to achieve acceptable formulation and desired delivery to the intended target); and • acceptable oral bioavailability and duration of action (somewhat flexible, but oral bioavailability $F\%$ > 30% and elimination half-life $t_{1/2}$ > 4 h are reasonable goals) (Williams, 2005; Smith and O'Donnell, 2007; Bodor and Buchwald, 2012). Some of these are undoubtedly more difficult to achieve with small molecules targeting PPIs than with those targeting classic drug targets such as GPCRs, ion channels, and enzymes that have pre-formed domains (pockets) to bind their natural ligands with good affinity and specificity. Problems related to lack of good binding pockets and thus a relatively low ligand efficiency (LE) have been reviewed briefly earlier (*Small-Molecule Inhibitors of Protein-Protein Interactions*; see also illustration in **Figure 2**). Because of this, SMIs of PPIs tend to be larger structures than classic drugs (Neugebauer et al., 2007), and it is now well-recognized that the chemical space of existing drugs and corresponding screening libraries does not correspond well with that of promising SMIs of PPIs (Pagliaro et al., 2004; Neugebauer et al., 2007; Reynès et al., 2010; Sperandio et al., 2010; Morelli et al., 2011). Fortunately, computational prescreening including exploration of relevant physicochemical properties can provide valuable information (Villoutreix et al., 2012; Trisciuzzi et al., 2019) and there are now databases, such as TIMBAL (Higueruelo et al., 2009), 2P2I (Bourgeas et al., 2010), or iPPI-DB (Labbe et al., 2016; Torchet et al., 2021), that contain an increasing number of 3D structures for protein-protein and protein-inhibitor complexes. These can make computationally enriched library selection much more successful, which has been shown to accelerate hit discovery (Milhas et al., 2016). A chemical library of >10,000 compounds dedicated to PPI inhibition has been developed (Fr-PPIChem) and is freely available upon request for experimental screening against PPIs (Bosc et al., 2020).

Larger structures, often with multiple aromatic rings, are usually better suited for effective PPI inhibition (Che et al., 2006; Fletcher and Hamilton, 2006; Hershberger et al., 2007); however, these tend to violate the widely used "rule-of-five" (Ro5) criteria, which includes MW < 500 (Lipinski et al., 1997; Lipinski, 2004) and has been widely used to guide candidate selection and ensure adequate oral bioavailability and ADME (absorption, distribution, metabolism, and excretion) profile. Nevertheless, an increasing number of new drugs have been launched lately (including venetoclax and fostemsavir discussed earlier) that significantly violate these empirical rules proving that oral bioavailability can be achieved even in the "beyond the rule-of-five" chemical space (DeGoey et al., 2017; Doak and Kihlberg, 2017). Along these lines, it is instructive to highlight that the first promising lead during the development of venetoclax (ABT-199) was ABT-737, which was so far from being suitable for formulation as a drug that one of its developers jokingly described it as having "the biophysical properties of brick dust" (Mullard, 2016b).

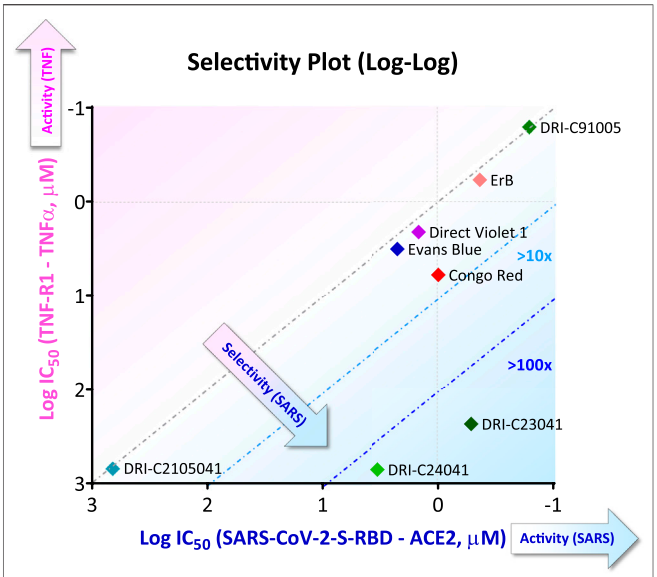


FIGURE 6 | Illustrative selectivity plot comparing inhibitory activity of SMIs against two PPIs. Selectivity plot showing the inhibitory activity of some of the compounds found to inhibit the SARS-CoV-2-S-RBD–hACE2 as the targeted PPI (quantified as log IC_{50}) versus that against another non-targeted PPI (here, TNF-R1–TNF-α PPI). More active and selective compounds are clustered toward the lower right corner. Figure prepared with data from (Bojadzic et al., 2021b).

Similarly, the incredibly tedious process of medicinal chemistry optimization that was required to make the original lead of the series that ultimately led to fostemsavir (BMS-663068) as a clinical product is nicely described in detail in (Meanwell et al., 2018).

Hits obtained so far for this PPI (**Figure 5**) reemphasize that our approach relying on the chemical space of organic dyes as a starting point when screening for SMIs of PPIs makes sense. For example, Evans blue which was the best hit identified from a HTS of >3,000 compounds selected after *in silico* prescreening of ~60,000 structures (Day et al., 2021), came up as a hit from our screening of a much smaller library of <100 dyes (Bojadzic et al., 2021b). For obvious reasons, organic dyes have good affinity for proteins (Hunger, 2003), and they contain *privileged structures* for protein binding (Che et al., 2006; Fletcher and Hamilton, 2006; Hershberger et al., 2007). Thus, contrary to commonly available drug-like libraries, they are a good starting point to identify SMIs of PPIs. We have even found organic dyes that are promiscuous PPI inhibitors (Ganesan et al., 2011; Ganesan and Buchwald, 2013). Of course, organic dyes are not particularly suitable for therapeutic development because of their strong color (plus, for azo dyes, their quick metabolic degradation (Levine, 1991; Feng et al., 2012)). Nevertheless, we have shown in at least one case (the CD40–CD40L PPI, a member of the TNF superfamily) that new drug-like SMIs can be developed by first using this chemical space to identify the molecular scaffold required for activity and then removing the color-causing chromophore(s) while retaining PPI inhibitory activity (Chen et al., 2017; Bojadzic et al., 2018). Specificity can be an issue with dyes, and indeed most dyes found here as promising SMIs of the SARS-CoV-2-S–hACE PPI seem to be quite non-specific as the specificity plot shown in **Figure 6** illustrates (data from (Bojadzic et al., 2021b)). Also, many azo-containing dyes are likely PAINS (pan-assay interference compounds) that can show up as false positives in screening assays (Baell and Walters, 2014; Aldrich et al., 2017); thus, they need to be treated carefully to ensure, for example, that the PPI inhibitory activity seen is not due to aggregation/polymolecular conglomeration. Nevertheless, medicinal chemistry optimization for specificity should still be feasible, and it is worth remembering that modern medicinal chemistry emerged in the early 20th century from the synthetic dye industry of the late 19th century (mostly in Germany at that time) (Paterson, 1984). Following the discovery of the first synthetic dye in 1856, Paul Ehrlich (1854–1915) (Drews, 2004; Bosch and Rosich, 2008), who acquired his medical doctor's degree with a thesis on "the theory and practice of histological staining", laid the foundations of chemotherapy. The analogy between the azo –N=N– bond common in many of these dyes and the arsenic bond –As=As– led to his search of arsenicals and ultimately to the discovery of arsphenamine (Salvarsan, no. 606), the first modern antimicrobial, in 1909. A few years later, the testing of thousands of azo dye related compounds and the contributions of Gerhard Domagk (1895–1964) led to the discovery of Prontosil (1932), the first effective sulfonamide antibacterial.

None of the SMIs of SARS-CoV-2-S–hACE2 identified so far and discussed here (**Figure 5**) have reached clinical development (Zhao et al., 2022); in fact, none seem to have even been evaluated in existing preclinical animal models for SARS-CoV-2 (Takayama, 2020; Saravanan et al., 2022). Methylene blue, a phenothiazine dye we have identified as such an SMI (Bojadzic et al., 2021a), is, in fact, included in the WHO List of Essential Medicines and is orally bioactive; thus it might have some potential for repositioning for COVID-19 prevention and treatment especially as its low micromolar anti-CoV activity, possibly due to multiple mechanisms of action, has been confirmed by several other groups (Chuang et al., 2022). Overall, results summarized here provide proof-of-principle evidence for the feasibility of such SMI approaches toward antivirals that inhibit CoV attachment and entry, and they serve as a first guide of the chemical space needed to achieve this.

AUTHOR CONTRIBUTIONS

PB is the sole author; he conceived and wrote the manuscript.

ACKNOWLEDGMENTS

The author is grateful to people in his lab (Oscar Alcazar, Damir Bojadzic, and Sung-Ting Chuang) for their dedicated work on this and other projects despite the challenges imposed by the COVID-19 pandemic and the related restrictions and lockdowns.

REFERENCES

Adedeji, A. O., Severson, W., Jonsson, C., Singh, K., Weiss, S. R., and Sarafianos, S. G. (2013). Novel Inhibitors of Severe Acute Respiratory Syndrome Coronavirus Entry that Act by Three Distinct Mechanisms. *J. Virol.* 87 (14), 8017–8028. doi:10.1128/jvi.00998-13

Aldrich, C., Bertozzi, C., Georg, G. I., Kiessling, L., Lindsley, C., Liotta, D., et al. (2017). The Ecstasy and Agony of Assay Interference Compounds. *J. Med. Chem.* 60 (6), 2165–2168. doi:10.1021/acs.jmedchem.7b00229

Ambati, B. K., Varshney, A., Palú, G., Uhal, B. D., Uversky, V. N., et al. (2022). MSH3 Homology and Potential Recombination Link to SARS-CoV-2 Furin Cleavage Site. *Front.Virol.* 2, 834808. doi:10.3389/fviro.2022.834808

Andrei, S. A., Sijbesma, E., Hann, M., Davis, J., O'Mahony, G., Perry, M. W. D., et al. (2017). Stabilization of Protein-Protein Interactions in Drug Discovery. *Expert Opin. Drug Discov.* 12 (9), 925–940. doi:10.1080/17460441.2017.1346608

Arkin, M. R., Tang, Y., and Wells, J. A. (2014). Small-molecule Inhibitors of Protein-Protein Interactions: Progressing toward the Reality. *Chem. Biol.* 21 (9), 1102–1114. doi:10.1016/j.chembiol.2014.09.001

Arkin, M. R., and Wells, J. A. (2004). Small-molecule Inhibitors of Protein-Protein Interactions: Progressing towards the Dream. *Nat. Rev. Drug Discov.* 3 (4), 301–317. doi:10.1038/nrd1343

Baell, J., and Walters, M. A. (2014). Chemistry: Chemical con artists foil drug discovery. *Nature* 513 (7519), 481–483. doi:10.1038/513481a

Baum, A., Fulton, B. O., Wloga, E., Copin, R., Pascal, K. E., Russo, V., et al. (2020). Antibody Cocktail to SARS-CoV-2 Spike Protein Prevents Rapid Mutational Escape Seen with Individual Antibodies. *Science* 369 (6506), 1014–1018. doi:10.1126/science.abd0831

Bhattacharyya, R. P., and Hanage, W. P. (2022). Challenges in Inferring Intrinsic Severity of the SARS-CoV-2 Omicron Variant. *N. Engl. J. Med.* 386 (7), e14. doi:10.1056/nejmp2119682

Birkinshaw, R. W., Gong, J.-n., Luo, C. S., Lio, D., White, C. A., Anderson, M. A., et al. (2019). Structures of BCL-2 in Complex with Venetoclax Reveal the Molecular Basis of Resistance Mutations. *Nat. Commun.* 10 (1), 2385. doi:10.1038/s41467-019-10363-1

Bodor, N., and Buchwald, P. (2012). *Retrometabolic Drug Design and Targeting*. Hoboken, NJ: Wiley. doi:10.1002/9781118407738Retrometabolic Drug Design and Targeting

Bojadzic, D., Alcazar, O., and Buchwald, P. (2021a). Methylene Blue Inhibits the SARS-CoV-2 Spike-ACE2 Protein-Protein Interaction-A Mechanism that Can Contribute to its Antiviral Activity against COVID-19. *Front. Pharmacol.* 11 (1), 600372. doi:10.3389/fphar.2020.600372

Bojadzic, D., Alcazar, O., Chen, J., Chuang, S.-T., Condor Capcha, J. M., Shehadeh, L. A., et al. (2021b). Small-Molecule Inhibitors of the Coronavirus Spike: ACE2 Protein-Protein Interaction as Blockers of Viral Attachment and Entry for SARS-CoV-2. *ACS Infect. Dis.* 7 (6), 1519–1534. doi:10.1021/acsinfecdis.1c00070

Bojadzic, D., and Buchwald, P. (2019). CD40-targeting KGYY15 Peptides Do Not Efficiently Block the CD40-CD40L Interaction. *Diabetologia* 62 (11), 2158–2160. doi:10.1007/s00125-019-04996-6

Bojadzic, D., Chen, J., Alcazar, O., and Buchwald, P. (2018). Design, Synthesis, and Evaluation of Novel Immunomodulatory Small Molecules Targeting the CD40-CD154 Costimulatory Protein-Protein Interaction. *Molecules* 23 (5), 1153. doi:10.3390/molecules23051153

Bosc, N., Muller, C., Hoffer, L., Lagorce, D., Bourg, S., Derviaux, C., et al. (2020). Fr-PPIChem: An Academic Compound Library Dedicated to Protein-Protein Interactions. *ACS Chem. Biol.* 15 (6), 1566–1574. doi:10.1021/acschembio.0c00179

Bosch, F. e. l., and Rosich, L. (2008). The Contributions of Paul Ehrlich to Pharmacology: a Tribute on the Occasion of the Centenary of His Nobel Prize. *Pharmacology* 82 (3), 171–179. doi:10.1159/000149583

Bourgeas, R., Basse, M.-J., Morelli, X., and Roche, P. (2010). Atomic Analysis of Protein-Protein Interfaces with Known Inhibitors: the 2P2I Database. *PLoS One* 5 (3), e9598. doi:10.1371/journal.pone.0009598

Buchwald, P. (2019). A Receptor Model with Binding Affinity, Activation Efficacy, and Signal Amplification Parameters for Complex Fractional Response versus Occupancy Data. *Front. Pharmacol.* 10, 605. doi:10.3389/fphar.2019.00605

Buchwald, P. (2008). Glucocorticoid Receptor Binding: a Biphasic Dependence on Molecular Size as Revealed by the Bilinear LinBiExp Model. *Steroids* 73 (2), 193–208. doi:10.1016/j.steroids.2007.10.001

Buchwald, P. (2010). Small-molecule Protein-Protein Interaction Inhibitors: Therapeutic Potential in Light of Molecular Size, Chemical Space, and Ligand Binding Efficiency Considerations. *IUBMB Life* 62 (10), 724–731. doi:10.1002/iub.383

Cagno, V., Medaglia, C., Cerny, A., Cerny, T., Zwygart, A. C.-A., Cerny, E., et al. (2021). Methylene Blue Has a Potent Antiviral Activity against SARS-CoV-2 and H1N1 Influenza Virus in the Absence of UV-Activation *In Vitro*. *Sci. Rep.* 11 (1), 14295. doi:10.1038/s41598-021-92481-9

Cai, Y., Zhang, J., Xiao, T., Lavine, C. L., Rawson, S., Peng, H., et al. (2021). Structural Basis for Enhanced Infectivity and Immune Evasion of SARS-CoV-2 Variants. *Science* 373 (6555), 642–648. doi:10.1126/science.abi9745

Carino, A., Moraca, F., Fiorillo, B., Marchianò, S., Sepe, V., Biagioli, M., et al. (2020). Hijacking SARS-CoV-2/ace2 Receptor Interaction by Natural and Semi-synthetic Steroidal Agents Acting on Functional Pockets on the Receptor Binding Domain. *Front. Chem.* 8, 572885. doi:10.3389/fchem.2020.572885

C. G. Smith and J. T. O'Donnell (Editors) (2007). *The Process of New Drug Discovery and Development* (New York: Informa Healthcare). doi:10.1201/9781420004236

Chang, C.-k., Lin, S.-M., Satange, R., Lin, S.-C., Sun, S.-C., Wu, H.-Y., et al. (2021). Targeting Protein-Protein Interaction Interfaces in COVID-19 Drug Discovery. *Comput. Struct. Biotechnol. J.* 19, 2246–2255. doi:10.1016/j.csbj.2021.04.003

Che, Y., Brooks, B. R., and Marshall, G. R. (2006). Development of Small Molecules Designed to Modulate Protein-Protein Interactions. *J. Comput. Aided Mol. Des.* 20 (2), 109–130. doi:10.1007/s10822-006-9040-8

Chen, J., Song, Y., Bojadzic, D., Tamayo-Garcia, A., Landin, A. M., Blomberg, B. B., et al. (2017). Small-molecule Inhibitors of the CD40-Cd40l Costimulatory Protein-Protein Interaction. *J. Med. Chem.* 60 (21), 8906–8922. doi:10.1021/acs.jmedchem.7b01154

Cheng, F., Desai, R. J., Handy, D. E., Wang, R., Schneeweiss, S., Baraba´si, A.-L., et al. (2018). Network-based Approach to Prediction and Population-Based Validation of In Silico Drug Repurposing. *Nat. Commun.* 9 (1), 2691. doi:10.1038/s41467-018-05116-5

Chuang, S.-T., Papp, H., Kuczmog, A., Eells, R., Condor Capcha, J. M., Shehadeh, L. A., et al. (2022). Methylene Blue Is a Nonspecific Protein-Protein Interaction Inhibitor with Potential for Repurposing as an Antiviral for COVID-19. bioRxiv**,** 2022.2003. doi: doi:10.1101/2022.03.22.485299

Cochrane, G. M., Horne, R., and Chanez, P. (1999). Compliance in Asthma. *Respir. Med.* 93 (11), 763–769. doi:10.1016/s0954-6111(99)90260-3

Cohen, J. (2021). Call of the Wild. *Science* 373 (6559), 1072–1077. doi:10.1126/science.acx8984

Coutard, B., Valle, C., de Lamballerie, X., Canard, B., Seidah, N. G., and Decroly, E. (2020). The Spike Glycoprotein of the New Coronavirus 2019-nCoV Contains a Furin-like Cleavage Site Absent in CoV of the Same Clade. *Antiviral Res.* 176, 104742. doi:10.1016/j.antiviral.2020.104742

Couzin-Frankel, J. (2013). Cancer Immunotherapy. *Science* 342 (6165), 1432–1433. doi:10.1126/science.342.6165.1432

COVID-19 Forecasting Team (2022). Variation in the COVID-19 Infection-Fatality Ratio by Age, Time, and Geography during the Pre-vaccine Era: a Systematic Analysis. *Lancet* 399 epub. doi:10.1016/s0140-6736(21)02867-1

Day, C. J., Bailly, B., Guillon, P., Dirr, L., Jen, F. E.-C., Spillings, B. L., et al. (2021). Multidisciplinary Approaches Identify Compounds that Bind to Human ACE2 or SARS-CoV-2 Spike Protein as Candidates to Block SARS-CoV-2-ACE2 Receptor Interactions. *mBio* 12 (2), e03681–03620. doi:10.1128/mBio.03681-20

DeGoey, D. A., Chen, H.-J., Cox, P. B., and Wendt, M. D. (2017). Beyond the Rule of 5: Lessons Learned from AbbVie's Drugs and Compound Collection. *J. Med. Chem.* 61 (7), 2636–2651. doi:10.1021/acs.jmedchem.7b00717

Doak, B. C., and Kihlberg, J. (2017). Drug Discovery beyond the Rule of 5 - Opportunities and Challenges. *Expert Opin. Drug Discov.* 12 (2), 115–119. doi:10.1080/17460441.2017.1264385

Downing, N. S., Shah, N. D., Aminawung, J. A., Pease, A. M., Zeitoun, J.-D., Krumholz, H. M., et al. (2017). Postmarket Safety Events Among Novel Therapeutics Approved by the US Food and Drug Administration between 2001 and 2010. *JAMA* 317 (18), 1854–1863. doi:10.1001/jama.2017.5150

Drews, J. (2004). Paul Ehrlich: Magister Mundi. *Nat. Rev. Drug Discov.* 3 (9), 797–801. doi:10.1038/nrd1498

Du, L., He, Y., Zhou, Y., Liu, S., Zheng, B.-J., and Jiang, S. (2009). The Spike Protein of SARS-CoV - a Target for Vaccine and Therapeutic Development. *Nat. Rev. Microbiol.* 7 (3), 226–236. doi:10.1038/nrmicro2090

Edwards, A. M., Baric, R. S., Saphire, E. O., and Ulmer, J. B. (2022). Stopping Pandemics before They Start: Lessons Learned from SARS-CoV-2. *Science* 375 (6585), 1133–1139. doi:10.1126/science.abn1900

Feng, J., Cerniglia, C. E., and Chen, H. (2012). Toxicological Significance of Azo Dye Metabolism by Human Intestinal Microbiota. *Front. Biosci.* E4, 568–586. doi:10.2741/400

Fletcher, S., and Hamilton, A. D. (2006). Targeting Protein-Protein Interactions by Rational Design: Mimicry of Protein Surfaces. *J. R. Soc. Interf.* 3 (7), 215–233. doi:10.1098/rsif.2006.0115

Fu, W., Chen, Y., Wang, K., Hettinghouse, A., Hu, W., Wang, J.-Q., et al. (2021). Repurposing FDA-Approved Drugs for SARS-CoV-2 through an ELISA-Based Screening for the Inhibition of RBD/ACE2 Interaction. *Protein Cell* 12 (7), 586–591. doi:10.1007/s13238-020-00803-w

Fuller, J. C., Burgoyne, N. J., and Jackson, R. M. (2009). Predicting Druggable Binding Sites at the Protein-Protein Interface. *Drug Discov. Today* 14 (3-4), 155–161. doi:10.1016/j.drudis.2008.10.009

Gadek, T. R., Burdick, D. J., McDowell, R. S., Stanley, M. S., Marsters, J. C., Jr., Paris, K. J., et al. (2002). Generation of an LFA-1 Antagonist by the Transfer of the ICAM-1 Immunoregulatory Epitope to a Small Molecule. *Science* 295 (5557), 1086–1089. doi:10.1126/science.295.5557.1086

Ganesan, L., and Buchwald, P. (2013). The Promiscuous Protein Binding Ability of Erythrosine B Studied by Metachromasy (Metachromasia). *J. Mol. Recognit.* 26 (4), 181–189. doi:10.1002/jmr.2263

Ganesan, L., Margolles-Clark, E., Song, Y., and Buchwald, P. (2011). The Food Colorant Erythrosine Is a Promiscuous Protein-Protein Interaction Inhibitor. *Biochem. Pharmacol.* 81 (6), 810–818. doi:10.1016/j.bcp.2010.12.020

Gendrot, M., Andreani, J., Duflot, I., Boxberger, M., Le Bideau, M., Mosnier, J., et al. (2020). Methylene Blue Inhibits Replication of SARS-CoV-2 *In Vitro*. *Int. J. Antimicrob. Agents* 56, 106202. doi:10.1016/j.ijantimicag.2020.106202

Gendrot, M., Jardot, P., Delandre, O., Boxberger, M., Andreani, J., Duflot, I., et al. (2021). *In Vitro* evaluation of the Antiviral Activity of Methylene Blue Alone or in Combination against SARS-CoV-2. *J. Clin. Med.* 10, 3007. doi:10.3390/jcm10143007

Ghosh, A. K., Brindisi, M., Shahabi, D., Chapman, M. E., and Mesecar, A. D. (2020). Drug Development and Medicinal Chemistry Efforts toward SARS-Coronavirus and Covid-19 Therapeutics. *ChemMedChem* 15 (11), 907–932. doi:10.1002/cmdc.202000223

Gil, C., Ginex, T., Maestro, I., Nozal, V., Barrado-Gil, L., Cuesta-Geijo, M. Á., et al. (2020). COVID-19: Drug Targets and Potential Treatments. *J. Med. Chem.* 63 (21), 12359–12386. doi:10.1021/acs.jmedchem.0c00606

Gobeil, S. M.-C., Janowska, K., McDowell, S., Mansouri, K., Parks, R., Stalls, V., et al. (2021). Effect of Natural Mutations of SARS-CoV-2 on Spike Structure, Conformation, and Antigenicity. *Science* 373 (6555). doi:10.1126/science.abi6226

Good, S. S., Westover, J., Jung, K. H., Zhou, X.-J., Moussa, A., La Colla, P., et al. (2021). AT-527, a Double Prodrug of a Guanosine Nucleotide Analog, Is a Potent Inhibitor of SARS-CoV-2 *In Vitro* and a Promising Oral Antiviral for Treatment of COVID-19. *Antimicrob. Agents Chemother.* 65 (4), e02479–02420. doi:10.1128/aac.02479-20

Gu, C., Wu, Y., Guo, H., Zhu, Y., Xu, W., Wang, Y., et al. (2021). Protoporphyrin IX and Verteporfin Potently Inhibit SARS-CoV-2 Infection *In Vitro* and in a Mouse Model Expressing Human ACE2. *Sci. Bull.* 66 (9), 925–936. doi:10.1016/j.scib.2020.12.005

Guy, R. K., DiPaola, R. S., Romanelli, F., and Dutch, R. E. (2020). Rapid Repurposing of Drugs for COVID-19. *Science* 368 (6493), 829–830. doi:10.1126/science.abb9332

Hajduk, P. J. (2006). Fragment-based Drug Design: How Big Is Too Big? *J. Med. Chem.* 49 (24), 6972–6976. doi:10.1021/jm060511h

Hanson, Q. M., Wilson, K. M., Shen, M., Itkin, Z., Eastman, R. T., Shinn, P., et al. (2020). Targeting ACE2-RBD Interaction as a Platform for COVID-19 Therapeutics: Development and Drug-Repurposing Screen of an AlphaLISA Proximity Assay. *ACS Pharmacol. Transl. Sci.* 3, 1352–1360. doi:10.1021/acsptsci.0c00161

Harvey, W. T., Carabelli, A. M., Jackson, B., Gupta, R. K., Thomson, E. C., Harrison, E. M., et al. (2021). SARS-CoV-2 Variants, Spike Mutations and Immune Escape. *Nat. Rev. Microbiol.* 19 (7), 409–424. doi:10.1038/s41579-021-00573-0

Henninot, A., Collins, J. C., and Nuss, J. M. (2018). The Current State of Peptide Drug Discovery: Back to the Future? *J. Med. Chem.* 61 (4), 1382–1414. doi:10.1021/acs.jmedchem.7b00318

Hershberger, S., Lee, S.-G., and Chmielewski, J. (2007). Scaffolds for Blocking Protein-Protein Interactions. *Curr. Top. Med. Chem.* 7 (10), 928–942. doi:10.2174/156802607780906726

Higueruelo, A. P., Schreyer, A., Bickerton, G. R. J., Pitt, W. R., Groom, C. R., and Blundell, T. L. (2009). Atomic Interactions and Profile of Small Molecules Disrupting Protein-Protein Interfaces: the TIMBAL Database. *Chem. Biol. Drug Des.* 74 (5), 457–467. doi:10.1111/j.1747-0285.2009.00889.x

Hoffmann, M., Kleine-Weber, H., and Pöhlmann, S. (2020). A Multibasic Cleavage Site in the Spike Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol. Cel* 78 (4), 779–784. doi:10.1016/j.molcel.2020.04.022

Hopkins, A. L., Groom, C. R., and Alex, A. (2004). Ligand Efficiency: a Useful Metric for lead Selection. *Drug Discov. Today* 9 (10), 430–431. doi:10.1016/S1359-6446(04)03069-7

Hopkins, A. L., and Groom, C. R. (2002). The Druggable Genome. *Nat. Rev. Drug Discov.* 1 (9), 727–730. doi:10.1038/nrd892

Huck, B. R., Kötzner, L., and Urbahns, K. (2018). Small Molecules Drive Big Improvements in Immuno-Oncology Therapies. *Angew. Chem. Int. Ed.* 57 (16), 4412–4428. doi:10.1002/anie.201707816

Hughes, J., Rees, S., Kalindjian, S., and Philpott, K. (2011). Principles of Early Drug Discovery. *Br. J. Pharmacol.* 162 (6), 1239–1249. doi:10.1111/j.1476-5381.2010.01127.x

Jayk Bernal, A., Gomes da Silva, M. M., Musungaie, D. B., Kovalchuk, E., Gonzalez, A., Delos Reyes, V., et al. (2022). Molnupiravir for Oral Treatment of COVID-19 in Nonhospitalized Patients. *N. Engl. J. Med.* 386, 509–520. doi:10.1056/nejmoa2116044

Jiang, S., Zhang, X., Yang, Y., Hotez, P. J., and Du, L. (2020). Neutralizing Antibodies for the Treatment of COVID-19. *Nat. Biomed. Eng.* 4 (12), 1134–1139. doi:10.1038/s41551-020-00660-2

Ju, B., Zhang, Q., Ge, J., Wang, R., Sun, J., Ge, X., et al. (2020). Human Neutralizing Antibodies Elicited by SARS-CoV-2 Infection. *Nature* 584, 115–119. doi:10.1038/s41586-020-2380-z

J. Wilson, A. (2009). Inhibition of Protein-Protein Interactions Using Designed Molecules. *Chem. Soc. Rev.* 38 (12), 3289–3300. doi:10.1039/b807197g

Kao, R. Y., Tsui, W. H. W., Lee, T. S. W., Tanner, J. A., Watt, R. M., Huang, J.-D., et al. (2004). Identification of Novel Small-Molecule Inhibitors of Severe Acute Respiratory Syndrome-Associated Coronavirus by Chemical Genetics. *Chem. Biol.* 11 (9), 1293–1299. doi:10.1016/j.chembiol.2004.07.013

K. Hunger (Editor) (2003). *Industrial Dyes. Chemistry, Properties, Applications* (Weinheim: Wiley VCH). doi:10.1002/3527602011

Kupferschmidt, K. (2021). Evolving Threat. *Science* 373 (6557), 844–849. doi:10.1126/science.373.6557.844

Labbé, C. M., Kuenemann, M. A., Zarzycka, B., Vriend, G., Nicolaes, G. A. F., Lagorce, D., et al. (2016). iPPI-DB: an Online Database of Modulators of Protein-Protein Interactions. *Nucleic Acids Res.* 44 (D1), D542–D547. doi:10.1093/nar/gkv982

Lan, J., Ge, J., Yu, J., Shan, S., Zhou, H., Fan, S., et al. (2020). Structure of the SARS-CoV-2 Spike Receptor-Binding Domain Bound to the ACE2 Receptor. *Nature* 581 (7807), 215–220. doi:10.1038/s41586-020-2180-5

Lane, T. R., and Ekins, S. (2021). Defending Antiviral Cationic Amphiphilic Drugs that May Cause Drug-Induced Phospholipidosis. *J. Chem. Inf. Model.* 61 (9), 4125–4130. doi:10.1021/acs.jcim.1c00903

Leader, B., Baca, Q. J., and Golan, D. E. (2008). Protein Therapeutics: a Summary and Pharmacological Classification. *Nat. Rev. Drug Discov.* 7 (1), 21–39. doi:10.1038/nrd2399

Lee, A. C.-L., Harris, J. L., Khanna, K. K., and Hong, J.-H. (2019). A Comprehensive Review on Current Advances in Peptide Drug Development and Design. *Int. J. Mol. Sci.* 20 (10), 2383. doi:10.3390/ijms20102383

Levine, W. G. (1991). Metabolism of Azo Dyes: Implication for Detoxication and Activation. *Drug Metab. Rev.* 23 (3-4), 253–309. doi:10.3109/03602539109029761

Lipinski, C. A. (2004). Lead- and Drug-like Compounds: the Rule-Of-Five Revolution. *Drug Discov. Today Tech.* 1 (4), 337–341. doi:10.1016/j.ddtec.2004.11.007

Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J. (1997). Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv. Drug Deliv. Rev.* 23, 3–25. doi:10.1016/s0169-409x(00)00129-010.1016/s0169-409x(96)00423-1

Liu, Y., Yu, Y., Zhao, Y., and He, D. (2022). Reduction in the Infection Fatality Rate of Omicron (B.1.1.529) Variant Compared to Previous Variants in South Africa. *SSRN*. doi:10.2139/ssrn.4010080

Lundin, A., Dijkman, R., Bergström, T., Kann, N., Adamiak, B., Hannoun, C., et al. (2014). Targeting Membrane-Bound Viral RNA Synthesis Reveals Potent Inhibition of Diverse Coronaviruses Including the Middle East Respiratory Syndrome Virus. *Plos Pathog.* 10 (5), e1004166. doi:10.1371/journal.ppat.1004166

Lv, Z., Deng, Y.-Q., Ye, Q., Cao, L., Sun, C.-Y., Fan, C., et al. (2020). Structural Basis for Neutralization of SARS-CoV-2 and SARS-CoV by a Potent Therapeutic Antibody. *Science* 369 (6510), 1505–1509. doi:10.1126/science.abc5881

Ma, L.-l., Liu, H.-m., Liu, X.-m., Yuan, X.-y., Xu, C., Wang, F., et al. (2021). Screening S Protein - ACE2 Blockers from Natural Products: Strategies and Advances in the Discovery of Potential Inhibitors of COVID-19. *Eur. J. Med. Chem.* 226, 113857. doi:10.1016/j.ejmech.2021.113857

Maas, M. N., Hintzen, J. C. J., Löffler, P. M. G., and Mecinović, J. (2021). Targeting SARS-CoV-2 Spike Protein by Stapled hACE2 Peptides. *Chem. Commun.* 57 (26), 3283–3286. doi:10.1039/d0cc08387a

Margolles-Clark, E., Umland, O., Kenyon, N. S., Ricordi, C., and Buchwald, P. (2009). Small-molecule Costimulatory Blockade: Organic Dye Inhibitors of the CD40-Cd154 Interaction. *J. Mol. Med.* 87 (11), 1133–1143. doi:10.1007/s00109-009-0519-3

Martinez, D. R., Schäfer, A., Gobeil, S., Li, D., De la Cruz, G., Parks, R., et al. (2022). A Broadly Cross-Reactive Antibody Neutralizes and Protects against Sarbecovirus challenge in Mice. *Sci. Transl. Med.* 14 (629), eabj7125. doi:10.1126/scitranslmed.abj7125

Matheson, N. J., and Lehner, P. J. (2020). How Does SARS-CoV-2 Cause COVID-19? *Science* 369 (6503), 510–511. doi:10.1126/science.abc6156

Matsuyama, K. (2022). Omicron May Be Less Lethal Than Other COVID-19 Variants but It's Still 40% Deadlier Than the Flu, Scientists Say. Fortune. Available at: https://fortune.com/2022/03/03/omicron-less-lethal-covid-19-variants-40-deadlier-than-flu/March 3.

Meanwell, N. A., Krystal, M. R., Nowicka-Sans, B., Langley, D. R., Conlon, D. A., Eastgate, M. D., et al. (2018). Inhibitors of HIV-1 Attachment: the Discovery and Development of Temsavir and its Prodrug Fostemsavir. *J. Med. Chem.* 61 (1), 62–80. doi:10.1021/acs.jmedchem.7b01337

Melby, T., and Westby, M. (2009). Inhibitors of Viral Entry. *Handb. Exp. Pharmacol.* 189, 177–202. doi:10.1007/978-3-540-79086-0_7

Milhas, S., Raux, B., Betzi, S., Derviaux, C., Roche, P., Restouin, A., et al. (2016). Protein-protein Interaction Inhibition (2P2I)-Oriented Chemical Library Accelerates Hit Discovery. *ACS Chem. Biol.* 11 (8), 2140–2148. doi:10.1021/acschembio.6b00286

Milroy, L.-G., Grossmann, T. N., Hennig, S., Brunsveld, L., and Ottmann, C. (2014). Modulators of Protein-Protein Interactions. *Chem. Rev.* 114 (9), 4695–4748. doi:10.1021/cr400698c

Moia, M., Mantovani, L. G., Carpenedo, M., Scalone, L., Monzini, M. S., Cesana, G., et al. (2013). Patient Preferences and Willingness to Pay for Different Options of Anticoagulant Therapy. *Intern. Emerg. Med.* 8 (3), 237–243. doi:10.1007/s11739-012-0844-3

Morelli, X., Bourgeas, R., and Roche, P. (2011). Chemical and Structural Lessons from Recent Successes in Protein-Protein Interaction Inhibition (2P2I). *Curr. Opin. Chem. Biol.* 15 (4), 475–481. doi:10.1016/j.cbpa.2011.05.024

Mullard, A. (2016a). 2015 FDA Drug Approvals. *Nat. Rev. Drug Discov.* 15 (2), 73–76. doi:10.1038/nrd.2016.15

Mullard, A. (2020). 2019 FDA Drug Approvals. *Nat. Rev. Drug Discov.* 19 (2), 79–84. doi:10.1038/d41573-020-00001-7

Mullard, A. (2016b). Pioneering Apoptosis-Targeted Cancer Drug Poised for FDA Approval. *Nat. Rev. Drug Discov.* 15 (3), 147–149. doi:10.1038/nrd.2016.23

Munos, B. (2009). Lessons from 60 Years of Pharmaceutical Innovation. *Nat. Rev. Drug Discov.* 8 (12), 959–968. doi:10.1038/nrd2961

Murer, L., Volle, R., Andriasyan, V., Petkidis, A., Gomez-Gonzalez, A., Yang, L., et al. (2022). Identification of Broad Anti-coronavirus Chemical Agents for Repurposing against SARS-CoV-2 and Variants of Concern. *Curr. Res. Virol. Sci.* 3, 100019. doi:10.1016/j.crviro.2022.100019

Nabel, K. G., Clark, S. A., Shankar, S., Pan, J., Clark, L. E., Yang, P., et al. (2022). Structural Basis for Continued Antibody Evasion by the SARS-CoV-2 Receptor Binding Domain. *Science* 375 (6578), eabl6251. doi:10.1126/science.abl6251

Neklesa, T. K., Winkler, J. D., and Crews, C. M. (2017). Targeted Protein Degradation by PROTACs. *Pharmacol. Ther.* 174, 138–144. doi:10.1016/j.pharmthera.2017.02.027

Neugebauer, A., Hartmann, R. W., and Klein, C. D. (2007). Prediction of Protein−Protein Interaction Inhibitors by Chemoinformatics and Machine Learning Methods. *J. Med. Chem.* 50 (19), 4665–4668. doi:10.1021/jm070533j

O'Driscoll, M., Ribeiro Dos Santos, G., Wang, L., Cummings, D. A. T., Azman, A. S., Paireau, J., et al. (2021). Age-specific Mortality and Immunity Patterns of SARS-CoV-2. *Nature* 590 (7844), 140–145. doi:10.1038/s41586-020-2918-0

Otvos, L., Jr., and Wade, J. D. (2014). Current Challenges in Peptide-Based Drug Discovery. *Front. Chem.* 2, 62. doi:10.3389/fchem.2014.00062

Ou, X., Liu, Y., Lei, X., Li, P., Mi, D., Ren, L., et al. (2020). Characterization of Spike Glycoprotein of SARS-CoV-2 on Virus Entry and its Immune Cross-Reactivity with SARS-CoV. *Nat. Commun.* 11 (1), 1620. doi:10.1038/s41467-020-15562-9

Overington, J. P., Al-Lazikani, B., and Hopkins, A. L. (2006). How many Drug Targets Are There? *Nat. Rev. Drug Discov.* 5 (12), 993–996. doi:10.1038/nrd2199

O. Villoutreix, B., M. Labbe, C., Lagorce, D., Laconde, G., and Sperandio, O. (2012). A Leap into the Chemical Space of Protein-Protein Interaction Inhibitors. *Cpd* 18 (30), 4648–4667. doi:10.2174/138161212802651571

Owen, D. R., Allerton, C. M. N., Anderson, A. S., Aschenbrenner, L., Avery, M., Berritt, S., et al. (2021). An Oral SARS-CoV-2 M Pro Inhibitor Clinical Candidate for the Treatment of COVID-19. *Science* 374 (6575), 1586–1593. doi:10.1126/science.abl4784

Pagliaro, L., Felding, J., Audouze, K., Nielsen, S. J., Terry, R. B., Krog-Jensen, C., et al. (2004). Emerging Classes of Protein-Protein Interaction Inhibitors and New Tools for Their Development. *Curr. Opin. Chem. Biol.* 8 (4), 442–449. doi:10.1016/j.cbpa.2004.06.006

WHO Solidarity Trial ConsortiumPan, H., Peto, R., Henao-Restrepo, A. M., Preziosi, M. P., Sathiyamoorthy, V., et al. (2021). Repurposed Antiviral Drugs for Covid-19 - Interim WHO Solidarity Trial Results. *N. Engl. J. Med.* 384, 497–511. doi:10.1056/nejmoa2023184

M. E. Wolff (Editor) (1995). *Burger's Medicinal Chemistry and Drug Discovery* (New York: Wiley-Interscience).

Park, Y.-J., De Marco, A., Starr, T. N., Liu, Z., Pinto, D., Walls, A. C., et al. (2022). Antibody-mediated Broad Sarbecovirus Neutralization through ACE2 Molecular Mimicry. *Science* 375 (6579), 449–454. doi:10.1126/science.abm8143

Paterson, G. R. (1984). Relationships between Synthetic Dyes and Drug Entities. *Can. Bull. Med. Hist.* 1 (2), 1–23. doi:10.3138/cbmh.1.2.1

Paul, S. M., Mytelka, D. S., Dunwiddie, C. T., Persinger, C. C., Munos, B. H., Lindborg, S. R., et al. (2010). How to Improve R&D Productivity: the Pharmaceutical Industry's Grand challenge. *Nat. Rev. Drug Discov.* 9 (3), 203–214. doi:10.1038/nrd3078

Pedebos, C., and Khalid, S. (2022). Simulations of the Spike: Molecular Dynamics and SARS-CoV-2. *Nat. Rev. Microbiol.* 20 (4), 192. doi:10.1038/s41579-022-00699-9

Pei, G., Xu, W., Lan, J., Wang, X., and Li, P. (2022). CEBIT Screening for Inhibitors of the Interaction between SARS-CoV-2 Spike and ACE2. *Fundam. Res.* ePub. doi:10.1016/j.fmre.2022.01.034

Pomplun, S., Jbara, M., Quartararo, A. J., Zhang, G., Brown, J. S., Lee, Y.-C., et al. (2021). De Novo discovery of High-Affinity Peptide Binders for the SARS-CoV-2 Spike Protein. *ACS Cent. Sci.* 7 (1), 156–163. doi:10.1021/acscentsci.0c01309

Proudfoot, J. R. (2002). Drugs, Leads, and Drug-Likeness: an Analysis of Some Recently Launched Drugs. *Bioorg. Med. Chem. Lett.* 12, 1647–1650. doi:10.1016/s0960-894x(02)00244-5

Rajgor, D. D., Lee, M. H., Archuleta, S., Bagdasarian, N., and Quek, S. C. (2020). The many Estimates of the COVID-19 Case Fatality Rate. *Lancet Infect. Dis.* 20 (7), 776–777. doi:10.1016/S1473-3099(20)30244-9

Reuben, B. G. (1996). "The Consumption and Production of Pharmaceuticals," in *The Practice of Medicinal Chemistry*. Editor C. G. Wermuth (London: Academic Press), 903–938.

Reynès, C., Host, H., Camproux, A.-C., Laconde, G., Leroux, F., Mazars, A., et al. (2010). Designing Focused Chemical Libraries Enriched in Protein-Protein Interaction Inhibitors Using Machine-Learning Methods. *Plos Comput. Biol.* 6 (3), e1000695. doi:10.1371/journal.pcbi.1000695

Reynolds, C. H., Bembenek, S. D., and Tounge, B. A. (2007). The Role of Molecular Size in Ligand Efficiency. *Bioorg. Med. Chem. Lett.* 17 (15), 4258–4261. doi:10.1016/j.bmcl.2007.05.038

Russ, A. P., and Lampel, S. (2005). The Druggable Genome: an Update. *Drug Discov. Today* 10 (23-24), 1607–1610. doi:10.1016/s1359-6446(05)03666-4

Salje, H., Tran Kiem, C., Lefrancq, N., Courtejoie, N., Bosetti, P., Paireau, J., et al. (2020). Estimating the burden of SARS-CoV-2 in France. *Science* 369 (6500), 208–211. doi:10.1126/science.abc3517

Santos, R., Ursu, O., Gaulton, A., Bento, A. P., Donadi, R. S., Bologa, C. G., et al. (2017). A Comprehensive Map of Molecular Drug Targets. *Nat. Rev. Drug Discov.* 16 (1), 19–34. doi:10.1038/nrd.2016.230

Saravanan, U. B., Namachivayam, M., Jeewon, R., Huang, J.-D., and Durairajan, S. S. K. (2022). Animal Models for SARS-CoV-2 and SARS-CoV-1 Pathogenesis, Transmission and Therapeutic Evaluation. *Wjv* 11 (1), 40–56. doi:10.5501/wjv.v11.i1.40

Scannell, J. W., Blanckley, A., Boldon, H., and Warrington, B. (2012). Diagnosing the Decline in Pharmaceutical R&D Efficiency. *Nat. Rev. Drug Discov.* 11 (3), 191–200. doi:10.1038/nrd3681

Scott, D. E., Bayly, A. R., Abell, C., and Skidmore, J. (2016). Small Molecules, Big Targets: Drug Discovery Faces the Protein-Protein Interaction challenge. *Nat. Rev. Drug Discov.* 15 (8), 533–550. doi:10.1038/nrd.2016.29

Severson, W. E., Shindo, N., Sosa, M., Fletcher, T., 3rd, White, E. L., Ananthan, S., et al. (2007). Development and Validation of a High-Throughput Screen for Inhibitors of SARS CoV and its Application in Screening of a 100,000-compound Library. *J. Biomol. Screen.* 12 (1), 33–40. doi:10.1177/1087057106296688

Shang, J., Wan, Y., Luo, C., Ye, G., Geng, Q., Auerbach, A., et al. (2020). Cell Entry Mechanisms of SARS-CoV-2. *Proc. Natl. Acad. Sci. U.S.A.* 117 (21), 11727–11734. doi:10.1073/pnas.2003138117

Shang, Z., Chan, S. Y., Liu, W. J., Li, P., and Huang, W. (2021). Recent Insights into Emerging Coronavirus: SARS-CoV-2. *ACS Infect. Dis.* 7 (6), 1369–1388. doi:10.1021/acsinfecdis.0c00646

Shyr, Z. A., Gorshkov, K., Chen, C. Z., and Zheng, W. (2020). Drug Discovery Strategies for SARS-CoV-2. *J. Pharmacol. Exp. Ther.* 375 (1), 127–138. doi:10.1124/jpet.120.000123

Sivaraman, H., Er, S. Y., Choong, Y. K., Gavor, E., and Sivaraman, J. (2021). Structural Basis of SARS-CoV-2- and SARS-CoV-Receptor Binding and Small-Molecule Blockers as Potential Therapeutics. *Annu. Rev. Pharmacol. Toxicol.* 61, 465–493. doi:10.1146/annurev-pharmtox-061220-093932

Song, Y., and Buchwald, P. (2015). TNF Superfamily Protein-Protein Interactions: Feasibility of Small-Molecule Modulation. *Curr. Drug. Targets* 16, 393–408. doi:10.2174/1389450116666150223115628

Souers, A. J., Leverson, J. D., Boghaert, E. R., Ackler, S. L., Catron, N. D., Chen, J., et al. (2013). ABT-199, a Potent and Selective BCL-2 Inhibitor, Achieves Antitumor Activity while Sparing Platelets. *Nat. Med.* 19 (2), 202–208. doi:10.1038/nm.3048

Sperandio, O., Reynès, C. H., Camproux, A.-C., and Villoutreix, B. O. (2010). Rationalizing the Chemical Space of Protein-Protein Interaction Inhibitors. *Drug Discov. Today* 15 (5-6), 220–229. doi:10.1016/j.drudis.2009.11.007

Starr, T. N., Czudnochowski, N., Liu, Z., Zatta, F., Park, Y.-J., Addetia, A., et al. (2021). SARS-CoV-2 RBD Antibodies that Maximize Breadth and Resistance to Escape. *Nature* 597, 97–102. doi:10.1038/s41586-021-03807-6

Stumpf, M. P. H., Thorne, T., de Silva, E., Stewart, R., An, H. J., Lappe, M., et al. (2008). Estimating the Size of the Human Interactome. *Proc. Natl. Acad. Sci. U.S.A.* 105 (19), 6959–6964. doi:10.1073/pnas.0708078105

Su, H., Zhou, F., Huang, Z., Ma, X., Natarajan, K., Zhang, M., et al. (2021). Molecular Insights into Small-Molecule Drug Discovery for SARS-CoV-2. *Angew. Chem. Int. Ed.* 60 (18), 9789–9802. doi:10.1002/anie.202008835

Sui, J., Deming, M., Rockx, B., Liddington, R. C., Zhu, Q. K., Baric, R. S., et al. (2014). Effects of Human Anti-spike Protein Receptor Binding Domain Antibodies on Severe Acute Respiratory Syndrome Coronavirus Neutralization Escape and Fitness. *J. Virol.* 88 (23), 13769–13780. doi:10.1128/jvi.02232-14

Suntharalingam, G., Perry, M. R., Ward, S., Brett, S. J., Castello-Cortes, A., Brunner, M. D., et al. (2006). Cytokine Storm in a Phase 1 Trial of the Anti-CD28 Monoclonal Antibody TGN1412. *N. Engl. J. Med.* 355 (10), 1018–1028. doi:10.1056/nejmoa063842

Tai, W., He, L., Zhang, X., Pu, J., Voronin, D., Jiang, S., et al. (2020). Characterization of the Receptor-Binding Domain (RBD) of 2019 Novel Coronavirus: Implication for Development of RBD Protein as a Viral Attachment Inhibitor and Vaccine. *Cell. Mol. Immunol.* 17 (6), 613–620. doi:10.1038/s41423-020-0400-4

Takayama, K. (2020). *In Vitro* and Animal Models for SARS-CoV-2 Research. *Trends Pharmacol. Sci.* 41 (8), 513–517. doi:10.1016/j.tips.2020.05.005

Tan, Q., Zhu, Y., Li, J., Chen, Z., Han, G. W., Kufareva, I., et al. (2013). Structure of the CCR5 Chemokine Receptor-HIV Entry Inhibitor Maraviroc Complex. *Science* 341 (6152), 1387–1390. doi:10.1126/science.1241475

Thiel, P., Kaiser, M., and Ottmann, C. (2012). Small-molecule Stabilization of Protein-Protein Interactions: an Underestimated Concept in Drug Discovery? *Angew. Chem. Int. Ed.* 51 (9), 2012–2018. doi:10.1002/anie.201107616

Tiwari, V., Beer, J. C., Sankaranarayanan, N. V., Swanson-Mungerson, M., and Desai, U. R. (2020). Discovering Small-Molecule Therapeutics against SARS-CoV-2. *Drug Discov. Today* 25 (8), 1535–1544. doi:10.1016/j.drudis.2020.06.017

Torchet, R., Druart, K., Ruano, L. C., Moine-Franel, A., Borges, H., Doppelt-Azeroual, O., et al. (2021). The iPPI-DB Initiative: a Community-Centered Database of Protein-Protein Interaction Modulators. *Bioinformatics* 37, 89–96. doi:10.1093/bioinformatics/btaa1091

Tortorici, M. A., Walls, A. C., Lang, Y., Wang, C., Li, Z., Koerhuis, D., et al. (2019). Structural Basis for Human Coronavirus Attachment to Sialic Acid Receptors. *Nat. Struct. Mol. Biol.* 26 (6), 481–489. doi:10.1038/s41594-019-0233-y

Trisciuzzi, D., Nicolotti, O., Miteva, M. A., and Villoutreix, B. O. (2019). Analysis of Solvent-Exposed and Buried Co-crystallized Ligands: a Case Study to Support the Design of Novel Protein-Protein Interaction Inhibitors. *Drug Discov. Today* 24 (2), 551–559. doi:10.1016/j.drudis.2018.11.013

Trisciuzzi, D., Siragusa, L., Baroni, M., Autiero, I., Nicolotti, O., and Cruciani, G. (2022). Getting Insights into Structural and Energetic Properties of Reciprocal Peptide-Protein Interactions. *J. Chem. Inf. Model.* 62 (4), 1113–1125. doi:10.1021/acs.jcim.1c01343

Tsegay, K. B., Adeyemi, C. M., Gniffke, E. P., Sather, D. N., Walker, J. K., and Smith, S. E. P. (2021). A Repurposed Drug Screen Identifies Compounds that Inhibit the Binding of the COVID-19 Spike Protein to ACE2. *Front. Pharmacol.* 12, 685308. doi:10.3389/fphar.2021.685308

Tummino, T. A., Rezelj, V. V., Fischer, B., Fischer, A., O'Meara, M. J., Monel, B., et al. (2021). Drug-induced Phospholipidosis Confounds Drug Repurposing for SARS-CoV-2. *Science* 373 (6554), 541–547. doi:10.1126/science.abi4708

van Breemen, R. B., Muchiri, R. N., Bates, T. A., Weinstein, J. B., Leier, H. C., Farley, S., et al. (2022). Cannabinoids Block Cellular Entry of SARS-CoV-2 and the Emerging Variants. *J. Nat. Prod.* 85 (1), 176–184. doi:10.1021/acs.jnatprod.1c00946

Verdine, G. L., and Walensky, L. D. (2007). The challenge of Drugging Undruggable Targets in Cancer: Lessons Learned from Targeting BCL-2 Family Members. *Clin. Cancer Res.* 13 (24), 7264–7270. doi:10.1158/1078-0432.ccr-07-2184

V'kovski, P., Kratzel, A., Steiner, S., Stalder, H., and Thiel, V. (2021). Coronavirus Biology and Replication: Implications for SARS-CoV-2. *Nat. Rev. Microbiol.* 19 (3), 155–170. doi:10.1038/s41579-020-00468-6

Wadman, M. (2006). London's Disastrous Drug Trial Has Serious Side Effects for Research. *Nature* 440 (7083), 388–389. doi:10.1038/440388a

Walters, W. P., Green, J., Weiss, J. R., and Murcko, M. A. (2011). What Do Medicinal Chemists Actually Make? A 50-year Retrospective. *J. Med. Chem.* 54 (19), 6405–6416. doi:10.1021/jm200504p

Wang, C., Li, W., Drabek, D., Okba, N. M. A., van Haperen, R., Osterhaus, A. D. M. E., et al. (2020). A Human Monoclonal Antibody Blocking SARS-CoV-2 Infection. *Nat. Commun.* 11 (1), 2251. doi:10.1038/s41467-020-16256-y

Wang, X., Ni, D., Liu, Y., and Lu, S. (2021). Rational Design of Peptide-Based Inhibitors Disrupting Protein-Protein Interactions. *Front. Chem.* 9, 682675. doi:10.3389/fchem.2021.682675

Wec, A. Z., Wrapp, D., Herbert, A. S., Maurer, D. P., Haslwanter, D., Sakharkar, M., et al. (2020). Broad Neutralization of SARS-Related Viruses by Human

Monoclonal Antibodies. *Science* 369 (6504), 731–736. doi:10.1126/science.abc7424

Wells, J. A., and McClendon, C. L. (2007). Reaching for High-Hanging Fruit in Drug Discovery at Protein-Protein Interfaces. *Nature* 450 (7172), 1001–1009. doi:10.1038/nature06526

Wibmer, C. K., Ayres, F., Hermanus, T., Madzivhandila, M., Kgagudi, P., Oosthuysen, B., et al. (2021). SARS-CoV-2 501Y.V2 Escapes Neutralization by South African COVID-19 Donor Plasma. *Nat. Med.* 27, 622–625. doi:10.1038/s41591-021-01285-x

Williams, M. (2005). Systems and Integrative Biology as Alternative Guises for Pharmacology: Prime Time for an iPharm Concept? *Biochem. Pharmacol.* 70 (12), 1707–1716. doi:10.1016/j.bcp.2005.08.019

Wu, Y., Wang, F., Shen, C., Peng, W., Li, D., Zhao, C., et al. (2020). A Noncompeting Pair of Human Neutralizing Antibodies Block COVID-19 Virus Binding to its Receptor ACE2. *Science* 368 (6496), 1274–1278. doi:10.1126/science.abc2241

Xiong, J., Xiang, Y., Huang, Z., Liu, X., Wang, M., Ge, G., et al. (2021). Structure-based Virtual Screening and Identification of Potential Inhibitors of SARS-CoV-2 S-RBD and ACE2 Interaction. *Front. Chem.* 9, 740702. doi:10.3389/fchem.2021.740702

Xiu, S., Dick, A., Ju, H., Mirzaie, S., Abdi, F., Cocklin, S., et al. (2020). Inhibitors of SARS-CoV-2 Entry: Current and Future Opportunities. *J. Med. Chem.* 63 (21), 12256–12274. doi:10.1021/acs.jmedchem.0c00502

Yu, S., Zhu, Y., Xu, J., Yao, G., Zhang, P., Wang, M., et al. (2021). Glycyrrhizic Acid Exerts Inhibitory Activity against the Spike Protein of SARS-CoV-2. *Phytomedicine* 85, 153364. doi:10.1016/j.phymed.2020.153364

Yuan, M., Liu, H., Wu, N. C., Lee, C.-C. D., Zhu, X., Zhao, F., et al. (2020). Structural Basis of a Shared Antibody Response to SARS-CoV-2. *Science* 369 (6507), 1119–1123. doi:10.1126/science.abd2321

Zhang, G., Pomplun, S., Loftis, A. R., Tan, X., Loas, A., and Pentelute, B. L. (2020). Investigation of ACE2 N-Terminal Fragments Binding to SARSCoV-2 Spike RBD. bioRxiv, 999318. doi:10.1101/2020.03.19.999318

Zhang, J., Zhang, K., Gao, Z.-G., Paoletta, S., Zhang, D., Han, G. W., et al. (2014). Agonist-bound Structure of the Human P2Y12 Receptor. *Nature* 509 (7498), 119–122. doi:10.1038/nature13288

Zhang, Q. C., Petrey, D., Deng, L., Qiang, L., Shi, Y., Thu, C. A., et al. (2012). Structure-based Prediction of Protein-Protein Interactions on a Genome-wide Scale. *Nature* 490 (7421), 556–560. doi:10.1038/nature11503

Zhang, Q., Xiang, R., Huo, S., Zhou, Y., Jiang, S., Wang, Q., et al. (2021). Molecular Mechanism of Interaction between SARS-CoV-2 and Host Cells and Interventional Therapy. *Sig. Transduct. Target. Ther.* 6 (1), 233. doi:10.1038/s41392-021-00653-w

Zhao, L., Li, S., and Zhong, W. (2022). Mechanism of Action of Small-Molecule Agents in Ongoing Clinical Trials for SARS-CoV-2: a Review. *Front. Pharmacol.* 13, 840639. doi:10.3389/fphar.2022.840639

Zhong, M., Gadek, T. R., Bui, M., Shen, W., Burnier, J., Barr, K. J., et al. (2012). Discovery and Development of Potent LFA-1/ICAM-1 Antagonist SAR 1118 as an Ophthalmic Solution for Treating Dry Eye. *ACS Med. Chem. Lett.* 3 (3), 203–206. doi:10.1021/ml2002482

Zhu, Z.-L., Qiu, X.-D., Wu, S., Liu, Y.-T., Zhao, T., Sun, Z.-H., et al. (2021). Blocking Effect of Demethylzeylasteral on the Interaction between Human ACE2 Protein and SARS-CoV-2 RBD Protein Discovered Using SPR Technology. *Molecules* 26 (1), 57. doi:10.3390/molecules26010057

Conflict of Interest: The author declares the following competing financial interest(s): The University of Miami has filed a patent on some of the DRI-C compounds discussed here and their use for potential antiviral applications with PB as inventor.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

REVIEW
published: 02 May 2022
doi: 10.3389/fddsv.2022.899587

Advances in Modelling COVID-19 in Animals

Petr Nickl[1,2†], *Miles Joseph Raishbrook*[1,2†], *Linn Amanda Syding*[1†] *and Radislav Sedlacek*[1,2*]

[1]*Laboratory of Transgenic Models of Diseases, Institute of Molecular Genetics of the CAS, v.v.i, Vestec, Czechia,* [2]*Czech Centre for Phenogenomics, Institute of Molecular Genetics of the CAS, v.v.i, Vestec, Czechia*

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is a positive-sense-single stranded RNA virus and the cause of the coronavirus disease 2019 (COVID-19). The World Health Organisation has confirmed over 250 million cases with over 5.1 million deaths as a result of this pandemic since December 2019. A global outbreak of such intensity and perseverance is due to the novelty of SARS-CoV2 virus, meaning humans lack any pre-existing immunity to the virus. Humanised animal models, from rodents to primates, simulating SARS-CoV2 transmission, cell entry and immune defence in humans have already been crucial to boost understanding of its molecular mechanisms of infection, reveal at-risk populations, and study the pathophysiology *in vivo.* Focus is now turning towards using this knowledge to create effective vaccines and therapeutic agents, as well as optimise their safety for translatable use in humans. SARS-CoV2 possesses remarkable adaptability and rapid mutagenic capabilities thus exploiting innovative animal models will be pivotal to outmanoeuvre it during this pandemic. In this review, we summarise all generated SARS-CoV2-related animal models to date, evaluate their suitability for COVID-19 research, and address the current and future state of the importance of animal models in this field.

Keywords: COVID-19, mouse, model, SARS-CoV2, sensitised, humanized, mice

OPEN ACCESS

Edited by:
Bruno Villoutreix,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Venkata Ramireddy Narala,
Yogi Vemana University, India
Dhanasekaran Sakthivel,
ZIP Diagnostics Pty Ltd., Australia

****Correspondence:***
Radislav Sedlacek
radislav.sedlacek@img.cas.cz

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Anti-Infective Agents, a section of the journal Frontiers in Drug Discovery

Received: *18 March 2022*
Accepted: *14 April 2022*
Published: *02 May 2022*

Citation:
Nickl P, Raishbrook MJ, Syding LA and Sedlacek R (2022) Advances in Modelling COVID-19 in Animals. Front. Drug. Discov. 2:899587. doi: 10.3389/fddsv.2022.899587

1 COVID-19 ORIGIN AND SARS-COV2 TRANSMISSION

A pandemic is defined as a disease that is prevalent in an entire country or the world, and thus is undoubtedly the correct term for the coronavirus disease 2019 (COVID-19) outbreak. COVID-19 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) which has been pinpointed to have originated from Wuhan, China in December 2019 and has since then spread over all continents including Antarctica (Triggle et al., 2021). Before the COVID-19 outburst there were already two identified and relatively well-known human coronaviruses causing severe respiratory pneumonia namely, SARS-CoV and MERS-CoV. They both originate from bats but spilled over to intermediate hosts namely, civets and dromedary camels, respectively. The origin of SARS-CoV2 is also suggested, based on its sequence similarity to the SARS-CoV, to have originated from bats and later spilled over to an animal reservoir, however it is not yet confirmed (Forni et al., 2017). Bats are and continue to be a copious source for novel viral sequences (Jiang et al., 2022). The bat species are among one of the oldest mammals and they exhibit great diversity and are widely spread across the globe (X. M. Zhang et al., 1992). Cross-species mixing between different kinds of bats has facilitated a maintenance of less discriminatory viruses capable of infecting a broader variety of hosts. Bats are thus a carrier of a pool of viruses able to perform inter-species transmission, which has been a reason for concern long before the COVID-19 outbreak (Calisher et al., 2006). SARS-CoV2 is a pneumotropic virus that mainly spreads through respiratory secretions like coughing and sneezing. The transmission may also occur *via* contaminated surfaces where the virus can

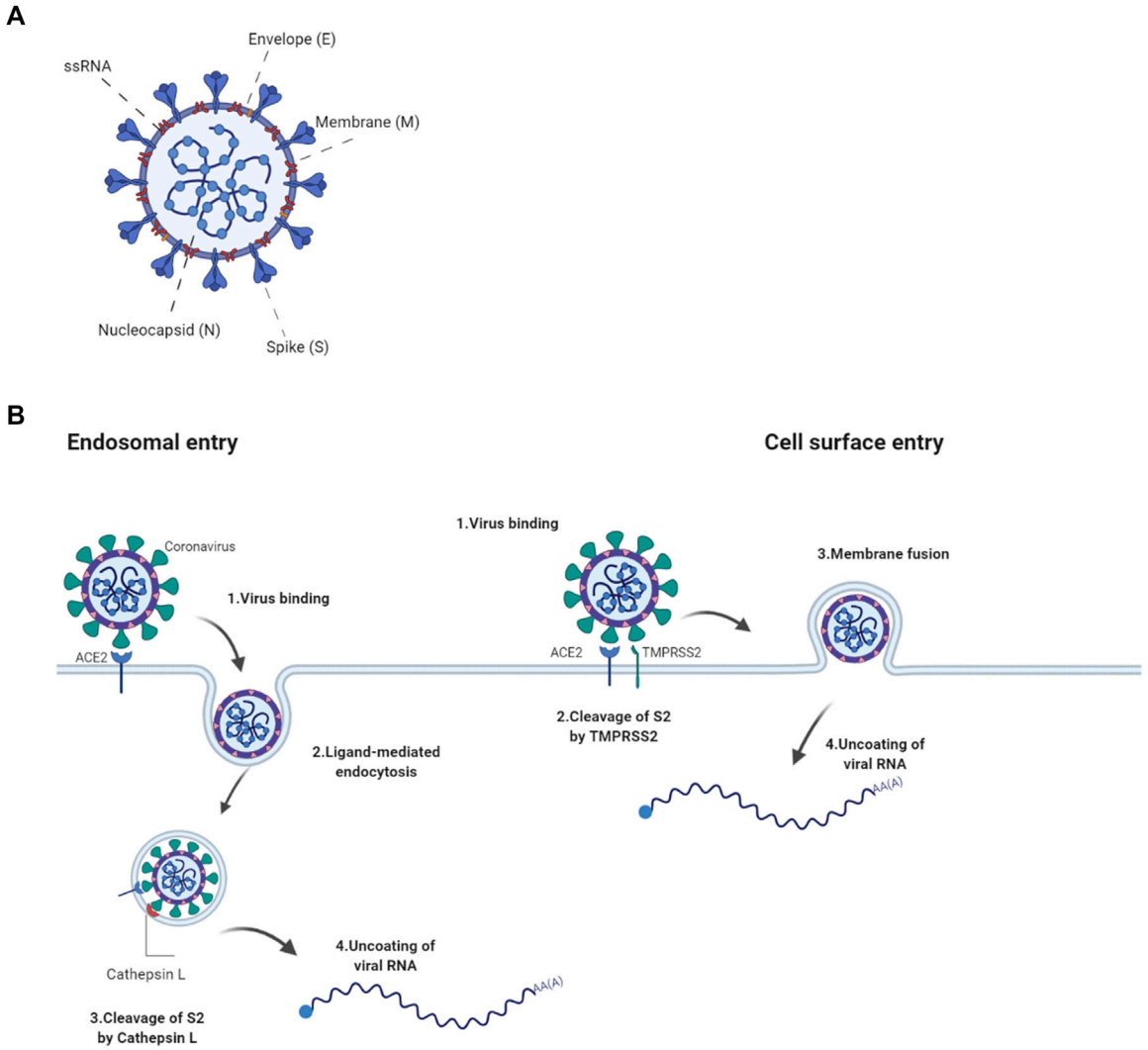


FIGURE 1 | ACE2-mediated entry of SARS-COV2 into a cell **(A)** The SARS-CoV2 virion consists of structural proteins, namely spike (S), envelope (E), membrane (M), nucleocapsid (N) The positive-sense, single-stranded RNA genome (+ssRNA) is bound by N in a beads-on-a-string formation. **(B)** SARS-CoV2 binds to the cell *via* S interaction with the host's ACE2 receptor. Entry to the host cell either goes through receptor-mediated endocytosis where fusion is potentiated by the cleavage of S2 by Cathepsin L or by cell surface fusion *via* the TMPRSS2 serine protease. Following fusion, the virion is uncoated and the viral genome released. Created with Biorender.com.

survive up to 6 days, making preventive measures such as surface disinfection, hand hygiene and masks important in combating transmission (Leclerc et al., 2020).

Once infected, COVID-19 manifests in a large variety of symptoms however, the most common ones include fever, sore throat, fatigue, cough, dyspnoea and immune system dysregulation, often ending with cytokine storm (Ragab et al., 2020). SARS-CoV2 is not only capable of affecting the respiratory system by pulmonary infiltration and inflammation but can spread to multiple organ systems. For the majority of people the disease symptoms are mild and the infection runs its course without any medical intervention but for approximately 5–10%, it severely affects the fitness of the individual and for another 2% it has a mortal outcome (Gavriatopoulou et al., 2021).

The SARS-CoV2 consists of approximately 29.9kB of single-stranded, non-segmented, positive-sense RNA.(Triggle et al., 2021). The genome is composed by 13–15 open reading frames largely resembling the make-up of MERS-CoV and SARS-CoV. The genome contains 11 protein coding genes with ultimate expression of 12 expressed proteins (Lu et al., 2020).

Structurally, it consists of four proteins namely, spike (S), envelope (E), membrane (M) and nucleocapsid (N) (**Figure 1A**). These proteins play important parts in entry, fusion and replication in the host cells. The non-structural have roles imperative to viral pathogenesis by regulating early transcription, helicase activity, gene transactivation and countering antiviral response (J Alsaadi and Jones, 2019; Tang et al., 2020).

The spike glycoprotein plays a pivotal role in the pathogenesis of SARS-CoV2 as it pivotal for the entry into the host cell. It is assembled as a homotrimer and inserted in multiple copies into the virus membrane, giving the virus a crown-like appearance, thus its name coronavirus (Jackson et al., 2022). It consists of two functional subunits, S1 and S2, that both part take in the entry of the virus. The S1 subunit has a receptor-binding domain (RBD) and is responsible for anchoring the host cell upon binding between the RBD and the human angiotensin-converting enzyme 2 (hACE2), thus stabilizing the virus (Hoffmann et al., 2020). Once the RBD region of the S1 subunit binds to the hACE2, the virus enters the host's endosomes *via* ligand-mediated endocytosis or membrane-fusion. Once bound to the ACE2, the S protein undergoes conformational changes, which are important to therapeutically limit its infection cycle (Wrapp et al., 2020). Although several mutations have been found in the RBD of the S1 subunit, its affinity to and interaction with the hACE2 is preserved in most species however not in mouse (Chan et al., 2020; Wrapp et al., 2020). The S2 subunit functions as a fusion protein between the virus and the host cell membrane. The S2 exhibit three different conformational changes during the process namely, i) native state before fusion, ii) intermediate state and iii) post fusion hairpin state (Qing and Gallagher, 2020; Walls et al., 2020). Finally the S protein is cleaved either by the host cell surface serine protease TMPRSS or by host's Cathepsin L in the endosomal compartment at the S2′ cleavage site (**Figure 1B**; Simmons et al., 2005). The cleavage releases a fusion peptide, which initiates the fusion pore formation. Once the pore expands and the cell membranes of both the virion and the host are combined, the viral genome can be released in to the cytoplasm. The cell membrane or endosomal fusion, represent the two different modes of entry for the viral genome to be released.

The N protein, composed by two separate domains, is present in the nucleocapsid complex that tightly binds the RNA genome of the virus. Both the N-terminal and C-terminal domain can bind to RNA but is more efficient when both bind simultaneously (Chang et al., 2006). The N protein bind the viral RNA genome in a beads-on-a-string conformation. The ribonucleotide protein (RNP) complex is subsequently packaged in to viral particles enveloped by a fatty lipid bi-layer (Fehr and Perlman, 2015).

The envelope protein is a relatively small protein that plays a substantial role in viral assembly. The protein assemble in to the host membrane forming protein-lipid pores referred to as viroporins. The envelope protein is highly conserved between SARS-CoV and SARS-CoV2 (Fehr and Perlman, 2015).

SARS-CoV2's membrane protein is the most abundant structural protein and is a transmembrane with a short NH2 terminal on the outside and a long cytoplasmic COOH terminus. Completion of viral assembly is potentiated partly by the binding between M proteins and N proteins leading to a stabilization of the N-Protein and RNA complex internally (Thomas, 2020).

2 INFECTION ROUTE AND ACE2 FUNCTION

The primary route of entry for the SARS-CoV2 is the upper respiratory tract. The virus gains access to the host cells by binding to the ACE2 receptors and subsequently introduced in to the cytoplasm *via* receptor-mediated endocytosis. The virus particles then goes through uncoating. The RNA and proteins needed for translation are released followed by transcription and assembly, finally the viral loads are shed thus completing the viral replication cycle (Jiang et al., 2020). As the virus sheds, the newly replicated and released particles bind upon another host cell and the cycle starts again. The ACE2 receptor is a carboxypeptidase consisting of 805 amino acids that removes a single amino acid from the C terminus of its substrates (Turner and Hooper, 2002). The ACE2 receptors are expressed in alveolar epithelial cells and capillary endothelial cells that are abundant in organs such as the lungs, kidneys, brain and gut hence explaining the multisystem infection found in a substantial amount of patients (Samavati and Uhal, 2020). The physiological role of ACE2 in humans is to convert angiotensin I and II to angiotensin 1–9 and angiotensin 1–7, respectively. This is one of the steps making up the Renin Angiotensin Aldosterone System (RAAS) a system, which functions to elevate blood volume and arterial tone *via* sodium and water reabsorption and vascular tone (Nehme et al., 2019). Infection results in a decrease of physiologically available ACE2 receptors thus disrupts the RAAS system, leading to potential downstream complications such as inflammation and circulatory dysfunction (Guo et al., 2020).

3 TRANSLATIONAL STUDIES: IMPORTANCE OF MOUSE MODELS

Since the start of the COVID-19 pandemic, we have gained substantial knowledge about the SARS-CoV2 virus in terms of its genetic make-up, transmission, infection and pathogenesis. This allows us to develop therapeutic agents to combat it. However, to perform scientifically sound and reliable research it is of the utmost importance to work with an appropriate model organism for *in vivo* study. The laboratory mouse is the most used animal in medical research as they are inexpensive, easy to handle, are genetically very similar to humans and can be genetically modified relatively easy (Sellers, 2017). They are often present as inbred strains, making it a highly controlled system, which is desirable in medical research. The mouse as an organism for translational research in COVID-19 medical research is however not well suited for COVID-19 as the ACE2 receptor of the mouse is not efficiently bound by the SARS-CoV2 virus, thus rendering the mouse immune to severe infection. This seemingly huge barrier has been surpassed by the generation of various modified mouse models capable of infection (Jia et al., 2020), as exemplified in the text below.

The COVID-19 outbreak pointed out a desperate need for relevant animal models for SARS-CoV2 research. As mentioned above wild type mouse cells and tissues are not very susceptible to SARS-CoV2 due to lack of human ACE2 specifically. Basically, mouse Ace2 does not bind the virus efficiently enough to mediate cell entry. To overcome this obstacle and study COVID-19 in mouse models, researchers have developed several approaches such as "*murinisation*" of SARS-CoV2 (Dinnon et al., 2020; Gu

TABLE 1 | Overview of COVID-19 mouse models and their characteristics.

Transgenic mouse model/background	Promoter/tissue	SARS-CoV2 dose/Most affected tissues/Symptoms/Lethality (intranasal route-IN, intravascular-IV)	References
Krt18-hACE2 (C57BL/6	Epithelial cell cytokeratin-18 promoter, epithelial cells	2.5×10^4 PFU (IN)/lung, kidney, brain, heart, spleen/severe interstitial pneumonia/lethal	McCray et al. (2007), Winkler et al., (2020)
HFH4-hACE2 (C3H, C57BL/6)	HFH4/FOXJ1 - lung ciliated epithelial cell-specific promoter, predominantly expressed in lung (also detected in brain, liver, kidney and gut)	3×10^4 $TCID_{50}$ (IN)/lung, heart, eye, brain/ severe interstitial pneumonia/lethal	Menachery et al. (2016), Jiang et al. (2020)
pCAGGS-hACE2 (C57BL/6 or BALB/c)	Cytomegalovirus enhancer with chicken β-actin promoter/universal expression	2×10^5 or 10^3 $TCID_{50}$ (IN) of SARS-CoV/ lungs, brain/acute wasting syndrome/lethal	Tseng et al. (2007)
Ace2-hACE2 Ace2-hACE2-IRES-tdTomato (ICR,C57Bl/6	Murine angiotensin converting enzyme 2/ intestine, brain, heart, kidney	10^5 $TCID_{50}$ (IN)/lung, intestine, brain/ moderate interstitial pneumonia/non-lethal	Bao et al. (2020a), Sun S.-H. et al. (2020), Yang et al. (2007b)
hACE2(LoxP-STOP) (C57Bl/6J)	Cytomegalovirus enhancer with chicken β-actin promoter/conditioned expression of hACE2-IRES-eGFP cassette	4.5 l g FFU (IN)/lung, brain/dramatic weight loss and rapid mortality/lethal (ubiquitous expression)	Bruter et al. (2021), Dolskiy et al. (2022)
Rosa26-chACE2 (C57Bl/6N)	Cytomegalovirus enhancer with chicken β-actin promoter/conditioned expression	2×10^3 PFU (IN)/not characterized/weight loss and rapid mortality/lethal (ubiquitous expression)	Czech Centre for Phenogenomics (2021)
Sensitised mouse models	**Promoter/tissue**	**SARS-CoV2 dose/Most affected tissues/Symptoms/Lethality (intranasal route-IN, intravascular-IV)**	**References**
AdV-hACE2/AdV-hACE2-GFP (BALB/c; C57BL/6J; Rag1$^{-/-}$ C57BL/6, Stat1$^{-/-}$ C57BL/6; DBA/2J; AG129)	Cytomegalovirus promoter, lung	10^5 FFU (IN); 10^5 PFU(IN, IV)/lung, heart, brain, liver, spleen/weight loss/non-lethal	Hassan et al. (2020), Sun J. et al. (2020)
AAV-hACE2 (C57BL/6J,B6(Cg) Ifnar1tm1.2Ees/J(Ifnar1$^{-/-}$); C57BL/6NCrl	Cytomegalovirus promoter/lung*	3×10^7 PFU/ml (IN); 1×10^4 PFU (IN)/lung (other organs not characterized)/weight loss/ non-lethal (*note: Localization of AAV-hACE2 expression is dependent on route of AAV application and used AAV serotype.)	Israelow et al. (2020), De Gasparo et al. (2021)
Lenti-hACE2 (C57BL/6J, IFNAR−/−)	Elongation factor 1 alpha promoter/lung	2×10^5 pfu (IN); 10^5 $CCID_{50}$ per mouse (IN)/ lung (inflammatory response)/mild symptoms of the COVID-19 disease, weight loss/non-lethal	Rawle et al. (2021), Katzman et al. (2022)

et al., 2020) or humanisation of mouse models (McCray et al., 2007; Tseng et al., 2007; Menachery et al., 2016). Alternatively they used different animal models which are sensitive to known SARS-CoV2 variants, such as hamsters, ferrets or non-human primates (Enkirch and von Messling, 2015; Finch et al., 2020; Munster et al., 2020; Rockx et al., 2020; Gruber et al., 2021).

Several transgenic mouse models have been developed and used in COVID-19 research to overcome limits of mouse Ace2 and generate inexpensive models with high-throughput study potential. The models are based on ubiquitous or cell/tissue specific expression of human ACE2, a protein well-known for its importance in SARS-CoV2 entry in the cell.

3.1 K18-hACE2 Model

This mouse model expresses hACE2 under control of the epithelial cell cytokeratin-18 promoter, expressed in subset of epithelial cells (**Table 1**). Even though the model has been generated to study SARS-CoV during 2003 epidemics, it remains relevant for SARS-CoV2 research too (McCray et al., 2007). K18-hACE2 model is susceptible to both strains of SARS-CoV, one and 2. In the context of COVID-19 research, the model responds to the infection by progressive weight loss, high viral titres in the lung at the beginning of infection, and with progressing infection increasing viral titres in the brain and gut. Other less severely affected organs are heart, kidney and spleen (**Figure 2**). (McCray et al., 2007; Rathnasinghe et al., 2020).

3.2 HFH4-hACE2 Model

The model expresses hACE2 under lung ciliated epithelial cell-specific promoter (HFH4/FOXJ1), which was supposed to drive lung-specific hACE2 expression. However, detailed characterization of the model revealed a moderate hACE2 expression in other tissues such as the brain, eye, heart, liver, kidney and gut (**Figure 2**). The model is highly responsive to SARS-CoV2 infection with main replication of the virus in lungs, eyes, heart and brain accompanied with severe symptoms such as interstitial pneumonia sometimes succumbed to lethal encephalitis (**Table 1**) (Menachery et al., 2016; Jiang et al., 2020).

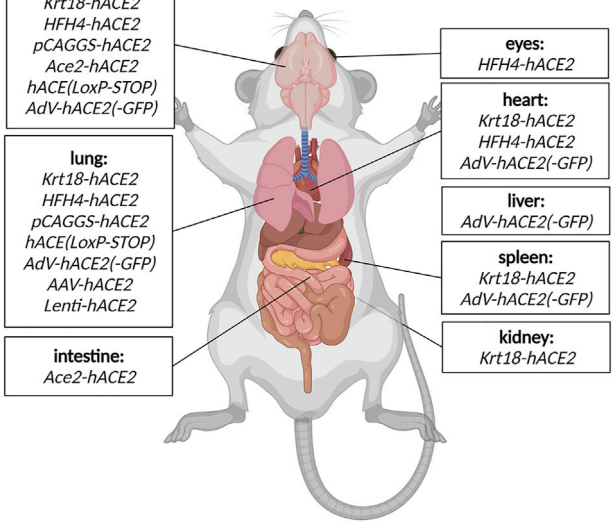


FIGURE 2 | Detected SARS-CoV2 replication in COVID-19 mouse models. The organs where viral replication has been detected in specific COVID mouse models are depicted schematically. Created with Biorender.com.

3.3 pCAGG-hACE2 Model

A model generated with multiple random integrations of pCAG-hACE2 cassette throughout the genome. Cytomegalovirus enhancer with chicken β-actin promoter (CAG) allows ubiquitous and constant expression of hACE2 in all tissues but mainly in lung, brain, heart and kidney (**Table 1**). This model is highly susceptible to SARS-CoV1 and 2 after intranasal application. The infection starts with initial exponential growth of viral titre in lungs and continues with gradual transmission to the brain. Slight presence of the virus was also observed in heart, kidney, spleen and small intestine (**Figure 2**). Of note, the lethal titre of the virus for the pCAGG-hACE2 model (2×10^2 to 2×10^4 $TCID_{50}$) is lower than in case of K18-hACE2 model (10^4 to 10^5 $TCID_{50}$) This fact might be connected to multiple insertion of pCAGG-hACE2 cassette in the genome in combination with ubiquitous and strong expression of hACE2 (Tseng et al., 2007; Asaka et al., 2021).

3.4 Ace2-hACE2 Model

Two major Ace2-hACE2 models have been generated to more closely mimic expression pattern of Ace2. The first model by was generated by random integration of a hACE2 cDNA under control of Ace2 promoter (Yang X. H. et al., 2007). The model indeed recapitulates endogenous expression of Ace2 in tissues such as lung, kidney, heart and intestines; the model is responsive to SARS-CoV2 infection with the major impact on lung tissue (Bao et al., 2020b).

The second Ace2-hACE2 model, generated by Sun S.-H. et al. (2020), is based on replacement of Ace2 coding sequence with hACE2 and tdTomato cDNA (**Table 1**). Therefore, the expression of transgenic cassette hACE2-IRES-tdTomato is under control of endogenous Ace2 promoter and present only in one or two copies depending on zygosity. Despite the lack of clinical symptoms or elevated mortality, this model responds to SARS-CoV2 infection by interstitial pneumonia of distinct scale depending on age. Sun's group also points out different abundance of hACE2 throughout-tissues in human (kidney, heart, oesophagus, bladder, ileum) and hACE2 in their model (liver, spleen, small intestine, ovary, and brain), however without further explanation. Furthermore, the group identified brain, lung and trachea as the main tissues of SARS-CoV2 replication (**Figure 2**; Sun S.-H. et al., 2020).

3.5 hACE2(LoxP-STOP) Model

hACE2 (LoxP-STOP) also termed TgCAGLoxPStopACE2GFP is a model generated by random integration of a loxP-CRE dependent cassette under the CAG promoter (**Table 1**). In the presence of Cre recombinase, the STOP cassette is removed and expression of hACE2 cDNA and eGFP is turned on. This model allows for conditioned, tissue-specific and traceable expression of hACE2-IRES-GFP transgene (Bruter et al., 2021). Dolskiy and collective have tested two inducible and ubiquitously expressed Cre-ERT2 drivers (UBC-ACE2 and Rosa26-ACE2) to promote conditioned hACE2 expression. In this case, the most severely affected organs were lung and brain (**Figure 2**). Their results further suggested that severity and infection progress is dependent on the particular Cre driver, more specifically on its expression potency. Furthermore, relatively recent changes in renin-angiotensin system due to hACE2 overexpression can be another factor influencing response to the infection (Dolskiy et al., 2022).

3.6 Rosa26-chACE2

A model similar to the previous one, but a CAG-LoxP-STOP-LoxP-hACE2 cassette is inserted in Rosa26 locus in a site-specific manner (**Table 1**). Therefore, transgene copy number depends on zygosity. The model has not been validated yet through SARS-CoV2 infection. It is available at Czech Centre for Phenogenomics and will be soon available *via* European Mouse Mutant Archive (EMMA) (Czech Centre for Phenogenomics, 2021).

Of note, transgenic models have an important role in SARS-CoV2 research. However, their ectopic expression of ACE2 protein, specifically in case of K18-hACE2, HFH4-hACE2, and pCAGG-hACE2 models may lead to different response, development and impact of the infection. This fact to some extend limits translatability of gathered data to clinical practise (Shou et al., 2021). Therefore, ACE2 under control of endogenous Ace2 promoter or conditional expression might provide more precise understanding of systemic or tissue-specific importance of ACE2 in the context of COVID-19.

3.7 Sensitised Mouse Models

In order to circumvent the desperate need for COVID-19 mouse models in the peak of pandemics, researchers focused on development of alternative SARS-CoV2- sensitive mouse models. Paradoxically, a rapid generation of such models was mediated by viruses. Inhalation or intranasal application of a viral

vector carrying hACE2 gene under strong promoter may lead to humanisation of upper and lower respiratory tracts. This approach allows fast, affordable and versatile generation of a sensitive model in various mouse strains and genetic backgrounds. It has been shown that the most suitable viral vectors for rapid humanisation happen to be adeno-associated virus, adenovirus, and lentivirus.

3.8 AAV-hACE2

Two independent groups have used Adeno-Associated vector of serotype 9 to deliver a cassette with hACE2 under control of CMV to the lung (**Table 1**; Israelow et al. (2020) have used commercially available AAV-CMV-hACE2 plasmid for AAV production and applied the vector virus *via* injection into the trachea. The De Gasparo's group assembled the AAV-CMV-hACE2 plasmid by subcloning hACE2 cDNA isolated form HEK293 cells and the vector was administered with forced inhalation into the lung and upper respiratory system. Both groups confirmed functionality of AAV-mediated humanisation where treated mice became susceptible to SARS-CoV2 infection accompanied with progressive inflammatory immune response in lung (**Figure 1**; Israelow et al., 2020; De Gasparo et al., 2021). In addition to establishing a new sensitized model, Israelow and collective focused on deciphering the role of type I interferon during SARS-CoV2 infection. Whereas, De Gasparo and collective tested bispecific antibodies that reduced SARS-CoV2 infection and weight loss associated with ongoing virus infection. Humanisation with AAV offers rapid, adaptable mouse model with long-term transgene expression and low immunogenicity which is crucial for immunological studies (De Gasparo et al., 2021; Kovacech et al., 2022).

3.9 AdV-hACE2

Replication defective Adenovirus encoding hACE2 (AdV-hACE2) was used to humanise several mouse strains in order to overcome unavailability of transgenic models (**Table 1**). The vector is delivered intranasally and it is capable to sensitise lung tissue for SARS-CoV2 entry and replication. In other organs, low levels of SARS-CoV2 replication was also identified, such as heart, spleen, brain and liver (**Figure 2**). Sensitised models suffer from weight loss, develop lung pathologies and respond positively to treatment with neutralising antibodies. However, the model has limitations in the form of bronchial inflammation associated with AdV delivery (Hassan et al., 2020).

3.10 Lenti-hACE2

Lentiviral vectors can be also used for sensitising a mouse to SARS-CoV2. Two independent publications describe utility of a lentiviral vector encoding hACE2 and its ability to avoid significant immune response in lung tissue before SARS-CoV2 exposure (**Table 1**). The advantage of lentiviral systems is their integrative character, with possibly stable long-term expression allowing re-infection studies in the sensitised mice. Both publications emphasize the role of IFNAR1 depletion and its impact on SARS-CoV2 progression in sensitised models. However, the collectives also point out the presence of mild COVID-19 symptoms in the models, probably due to relatively low expression of hACE2 by lentivirus (Rawle et al., 2021; Katzman et al., 2022).

Transgenic mouse models, expressing hACE2, represent convenient systems for large-scale, rapid (compared to other animal models), and relatively inexpensive SARS-CoV2 research. However, their availability during pandemics has been limited and their expansion in larger cohorts is time-consuming and expensive. Furthermore, distinct transgenic models differ in their response to infection, some suffer from lethal neuroinvasion, some show only mild symptoms. In general, variability of these models is significant, and no universal transgenic model has been established yet (Yang XH. et al., 2007; Yang XH. et al., 2007; McCray et al., 2007; Tseng et al., 2007; Menachery et al., 2016; Jiang et al., 2020; Sun J. et al. (2020) Bruter et al., 2021; Dolskiy et al., 2022).

In contrast with transgenic models stand virus-sensitised models, which can be generated on wide variety of genetic backgrounds and genotypes in relatively large scale and short-time. Sensitised models often do not develop severe disease mainly due to absence of neuroinvasion, but their symptoms and impact on lung tissue resembles pathology in COVID-19 patients. Moreover, the distribution and scale of hACE2 expression varies with tropism of a used viral vector or promoter. Importantly, use of viral vectors may be associated with a risk of potential inflammation leading to interference with subsequent SARS-CoV2 infection (Hassan et al., 2020; Israelow et al., 2020; De Gasparo et al., 2021; Rawle et al., 2021; Katzman et al., 2022).

3.11 Other Animal Models

Alternatives to mouse models are other animals that are naturally susceptible to SARS-CoV2, such as hamsters (*Mesocricetus auratus*, *Phodopus roborovskii*, *Cricetulus griseus*), ferrets (*Mustela putorius furo*) minks (*Neovison vison*) (Shuai et al., 2021)and non-human primates (*Macaca mulatta*, *Macaca fascicularis*, *Chlorocebus aethiops*) (Enkirch and von Messling, 2015; Finch et al., 2020; Gruber et al., 2021; Munster et al., 2020; Rockx et al., 2020). In these models there is no need for genetic modifications in order to study COVID-19 progression. Nevertheless, the models are less frequently used either due to lack of research tools, limited availability, high costs, complex husbandry or associated ethical concerns.

3.12 Murinised SARS-CoV2

While most efforts have been made in generating mouse models humanising the ACE2 to potentiate study of entry and infection *in vivo*, efforts have also been made in murinising the SARS-CoV2 virus itself. In a study by Muruato et al. (2021), they used a reverse genetic system and *in vivo* adaptation to successfully generate SARS-CoV2 strains capable of infecting mice (Muruato et al., 2021). Following infection of the murinised SARS-CoV2 strain the mouse lung exhibited substantial damage manifested with inflammation, immune infiltration, and pneumonia. The infection with the adapted virus was however only exhibited in the upper respiratory tract, thus is inappropriate for studies focusing on multisystem infection. It is worth mentioning that the novel adaptation of the virus was shown to keep its ability to

infect human airway cells (Muruato et al., 2021). This system, with a murinised SARS-CoV and a standard wild type laboratory mouse, overcomes tropism leading to encephalitis seen in infected transgenic mouse models whilst offering a system applicable for both *in vivo* mouse studies and *in vitro* studies on human primary cells (McCray et al., 2007; Winkler et al., 2020). In a study from 2020 the investigators had also produced a murinised SARS-CoV2 *via* reverse genetics to remodel the interaction between the mouse ACE2 and the virus which resulted in a recombinant virus able to infect the BALB/c mice. It was able replicate in both young and old mice however leading to more severe disease in older mice and exhibiting more clinically relevant phenotypes as compared to the disease presentation between non-modified SARS-CoV2 and transgenic mouse models (Dinnon et al., 2020). This gives the murinised ACE2 system better face validity, however the construct validity is decreased.

4 RECENT TRANSLATIONAL APPLICATIONS OF RODENT MODELS SUSCEPTIBLE TO SARS-COV2

The transgenic and transiently sensitised, humanised mouse models of SARS-CoV2 infection have gifted scientists the opportunity to study the potential destruction this, so far, relentless virus can cause to its host *in vivo*. Towards the beginning of the pandemic, initial studies using these models focused on the mechanisms in which viral entry can occur as well as their points of entry, the tissues primarily affected and the pathology of those tissues. These ongoing attempts to recreate infection have assisted our understanding of the infection timeline and has provided a guide to possible symptoms to be aware of in COVID-19 patients. Discussed here are animal studies performed in order to obtain risk assessments of new variants and evaluate the efficacy and safety of candidate anti-viral drugs for treatment in COVID-19 patients.

4.1 Risk Assessments of Variants of Concern

Particular mutations in the RBD have been key to identifying variants of concern. N501Y is one substitution that is characteristic of the Alpha (B.1.1.7) variant but is also found in the Beta (B.1.351), Gamma (B.1.1.28) and Omicron (B.1.1.529) variants (European Centre for Disease Prevention and Control, 2021; He et al., 2021). This means that N501Y is present in all but one variant of concern. *In silico* models predicted that this substitution occurs at a key residue for the RBD that is directly responsible for its strengthened affinity for ACE2 (Shahhosseini et al., 2021). The influence of N501Y was proved using a hamster model, where both donors and recipients inoculated with virus carrying N501Y showed significantly increased viral load in nasal washes and lung and trachea homogenates at 1–4 dpi compared to virus carrying a predecessor 'wild-type' spike protein. Substitutions S982A and D1118H were also shown to decrease viral fitness (Liu et al., 2022). Interestingly, N501Y increases the infectivity of hosts expressing both either hACE2 or mACE2 (Pan et al., 2021), as it has been revealed that variants with this substitution possess an 8-fold higher affinity for the receptor (Bayarri-Olmos et al., 2021). While this demonstrates a key application of using animals in order to evaluate the potential potency of infection with rapidly evolving variants, we need more studies that apply these principles in the established transgenic and sensitised animal models expressing hACE2. This is because, ultimately, we will require data on how mutations in SARS-CoV2 will affect transmission between, and the health of, humans in the future.

Studies of this nature have been carried out. K18-hACE2 mice infected with B.1.1.7 show increased weight loss and hyperthermia earlier compared to mice exposed to B.1.351 or the initial WA-1 variant of concern. However, B.1.1.7 and B.1.351 infected mice display more severe clinical manifestations overall compared to WA-1 in a viral dose-dependent manner, with WA-1 infected mice displaying a 50% lower mortality rate at a dose of 10^3 pfu (Horspool et al., 2021). Again in K18-hACE2 mice, whilst both WA-1 and B.1.1.7 inoculated intranasally caused COVID-19-like disease in the mice, a lower dose of B.1.1.7 was required to cause a severe disease state (Bayarri-Olmos et al., 2021). In contrast, C57Bl/6J hACE2 knock-in mice display reduced viral load in lung and nasal turbinate, and a more minor lung pathology and inflammatory response on exposure to WA-1, B.1.1.7 or B.1.351 variants compared to the K18-hACE2 model, where viral RNA is concentrated at the epithelia of larger airways (Winkler et al., 2022). This is most likely attributed to the difference in approach of hACE2 expression in these two mouse lines and highlights the benefits of multiple rodent models of infection, chosen depending on the study focus, but also shows how different models could be affected when exposed to differing strains. Here, studies using models that more accurately recreate human infection to novel strains will possess increased extrapolative power.

The appearance of the B.1.1.529 (Omicron) variant in late 2021 came with heightened suspicions whether the current vaccines and therapies in progress would still provide suitable protection against a variant with >30 mutations in the RBD compared to variants described so far (Hodcroft, 2021). Halfmann et al. found that K18-hACE2 mice inoculated with B.1.351 showed significantly increased viral load in nasal turbinate and lung tissue homogenates at 3 dpi, and greater weight loss at 6 dpi compared to B.1.1.529 infected mice (Halfmann et al., 2022), suggesting reduced severity in viral manifestation on infection with the Omicron lineage in comparison to Beta lineage. These studies show the potential of exploiting the current animal models available in order to screen variants with specific mutations to assess their risk to humans, and for practitioners and governments to make appropriate decisions regarding patient care and infection control strategies.

4.2 Screening the Efficacy of Anti-Viral Therapies

The current pandemic has called for the development of anti-viral drugs in order to reduce or eliminate viral infection in, especially,

hospitalised COVID-19 patients. Due to the haste in which these drugs are required to ease the pressure of the pandemic on the world, drug development processes for SARS-CoV2 may be accelerated straight to clinical trials in humans, bypassing preclinical animal safety and efficacy studies.

Anti-viral molecules have been tested in a mouse setting though. PF-07304814 is a phosphate prodrug that on administration is processed into its active form PF-00835231, a potent cysteine protease inhibitor of coronavirus $3CL^{pro}$, that was originally considered as a treatment for the 2002 SARS-CoV epidemic in 2003 (Hoffman et al., 2020). PF-00835231 is effective against alpha, beta, and gamma coronaviruses by preventing viral replication through inhibition of essential proteolysis by $3CL^{pro}$. BALB/C mice infected with SARS-CoV2 MA10 display no weight loss and complete viral elimination when PF-00835231 is administered subcutaneously twice per day at a dose of 300 mg/kg. Initial weight loss is observed in mice receiving 30–100 mg/kg doses, which recovered to the starting weight at 4 dpi with viral load decreasing in dose-dependent manner. Significant decreases in viral load were also measured in SARS-COV2 exposed mice expressing hACE2 on treatment with PF-00835231. Additionally, this trend is also obtained even when treatment was delayed by 1dpi (Boras et al., 2021), highlighting the importance of identifying infection early, especially in high-risk patients. Despite hACE2 being expressed under a CMV promoter, which may not accurately follow the expected human expression of ACE2, this work shows the power of this inhibitor to prevent viral replication and poses a good option for further development into human clinics.

PF-07321332 (Nirmatrelvir) is another $3CL^{pro}$ inhibitor, which is the active component of the Pfizer-produced PAXLOVID™ (Pfizer, 2021), that gained approval in the UK (Medicines & Healthcare products Regulatory Agency, 2021a) and the United States (U.S. Food and Drug Administration, 2021a) at the end of 2021, and in the EU in January 2022 for treatment of COVID-19 (European Medicines Agency, 2022). An efficacy study in mice investigated the anti-viral activity of PF-07321332 in BALB/C mice infected with mouse-adapted SARS-CoV2 MA10. Mice treated *via* oral administration were protected from weight loss, had significantly reduced lung viral titre at 4 dpi and showed markedly decreased nucleocaspid presence in lung sections (Owen et al., 2021). Syrian hamsters have been shown to be protected from severe B.1.351 infection when treated with PF-07321332. Significant dose-dependent reductions in viral lung titre and improved weight retention at 4 dpi, as well as lung anatomy closely resembling uninfected hamsters was observed in those treated with PF-07321332. Hamsters were also completely protected from infection when co-housed for 2 days with a B.1.617.2 (Delta) variant positive cage mate when treated with PF-07321332 compared to those not (Abdelnabi et al., 2022). These rodent models support the continuing development and protective ability of PAXLOVID™ use in COVID-19 patients against multiple variants of concern, including the benefits of easy oral administration. Yet, further validation in humanised ACE2 rodent models, such as the hACE2 model used in Bao et al. (2020a) may be required for increased value *in vivo,* as the mentioned studies comprised of mouse adapted SARS-CoV2 infection and wild-type Syrian hamsters as part of their models. These studies could also be extended to examine potential side effects or long term ramifications for patients prescribed this anti-viral treatment. Synthesis and study of additional $3CL^{pro}$ inhibitors with favourable oral, intraperitoneal, and intravenous bioavailability have been reported and trialled in Sprague-Dawley rats and a CRISPR/Cas9 generated hACE2 expressing mice model (Qiao et al., 2021). However more work is required in this area, and PF-07321332 seems to have won the race for clinical trial approval.

Molnupiravir is another anti-viral drug that instead enforces a high mutagenesis rate *via* integration of its active form, β-D-N4-hydroxycytidine triphosphate (NHC), into viral RNA in the place of cytidine or uridine (Sheahan et al., 2020; Kabinger et al., 2021). It has so far gained approval for at-risk and hospitalised patients in the United States (U.S. Food and Drug Administration, 2021b) and the UK (Medicines & Healthcare products Regulatory Agency, 2021b). NHC shows potent viral inhibition and significantly reduces viral load in cell culture (Zhou et al., 2021) and diminishes weight loss, indicators of lung haemorrhage and lung viral titre at 500 mg/kg dosage in C57Bl/6 mice infected with either mouse adapted SARS-MA15 or MERS-CoV. Importantly, initiating NHC treatment before 24 h post infection showed to be crucial to maintaining reductions in weight loss, lung haemorrhaging, viral lung titre and lung and alveolar injury scores (Sheahan et al., 2020). However, a warning of mutagenic toxicity to host DNA during NHC treatment has been given, where mutations in a reporter gene increased in a dose-dependent manner with NHC. It has been suggested that the possible conversion of NHC to dNHC (2'-deoxyribose form of NHC) could be the cause of this increased mutational rate in the host genome (Zhou et al., 2021), and should be investigated further in an *in vivo* model focusing on tissues with natural proliferative tendency.

One study utilised immunodeficient mice with hACE2-and hTMPRSS2- expressing human lung tissue implanted in the animals' backs. This *in vivo* tissue model was susceptible to SARS-CoV, MERS-CoV and SARS-CoV2 infection, showed histopathological symptoms echoing viral damage and a 1000-fold increase in proinflammatory cytokines. Beginning NHC treatment at 12- and 24-h post-infection was extremely effective at reducing viral load, however if treatment started 12 h prior to infection, viral titre in the implanted human lung tissue was measured at >100,000-fold lower than the vehicle control, bestowing the protective potential of Molnupiravir in high-risk patients (Wahl et al., 2021). Molunipiravir-derived inhibitors have also shown to be effective at impeding SARS-CoV2 transmission in ferrets (Cox et al., 2021), and reducing viral replication and its associated lung pathologies in SARS-CoV2-susceptible Syrian hamsters, with amplified viral RNA mutations detected in hamsters that started treatment 12 h pre-infection compared to 12 h post-infection or vehicle control (Rosenke et al., 2021). When used in combination with Favipiravir, another anti-viral drug that acts through lethal mutagenesis but requires higher doses for optimal SARS-CoV2 suppression (Kaptein et al., 2020), hamsters treated with sub-optimal doses of a Molnupiravir/Favipiravir cocktail displayed lower viral loads

than hamsters treated with only one alone, with an implied additional transmission protection from cage-mates (Abdelnabi et al., 2021b).

These examples show that animal models can serve effectively in the screening of anti-virals in the current and future pandemics, and could assist in the recommendation of single or combinational therapies to complement human clinical trials. Finally, Molnupiravir has also shown its high protective ability against the B.1.1.7 and B.1.351 variants in Syrian hamsters (Abdelnabi et al., 2021a) and emphasises NHC's potent anti-viral mechanism is not dependent on specific sequences in the viral genome which may be mutated in future variants of concern, such is the case with e.g. monoclonal antibody treatment.

5 FUTURE RESEARCH DIRECTIONS

5.1 Risk Factors Suitable for Rodent Research

This pandemic has revealed that certain individuals are risk of developing severe COVID-19 illness or death. Factors such as age, male sex, and ethnicity have been attributed to a tendency to suffer from severe symptoms (Ebinger et al., 2020; Mughal et al., 2020; Williamson et al., 2020), as well as patients with comorbidities such as diabetes, hypertension and obesity (Alguwaihes et al., 2020; Ebinger et al., 2020; Huang et al., 2020; Li X. et al., 2020; Mughal et al., 2020; Williamson et al., 2020; Goyal et al., 2022). Whereas asthma may actually be protective (Avdeev et al., 2020; Skevaki et al., 2020; Zhu et al., 2020). Genetic or induced mouse models of these disease states are already well established, and the opportunity to combine transgenic and sensitised SARS-CoV2 models with models of human conditions potentially vulnerable to COVID-19 is waiting to be seized. This will allow us to further study comorbidities that may aggravate SARS-CoV2 transmission and pathophysiology, or contribute to any long-term damaging effects in humans. The human population is genetically and culturally diverse, but isolating comorbidities or genetic traits for study in a controlled environment will be vital.

5.1.1 Age

A report from early in the pandemic described that every additional 10 years of age associates with a 1.5-fold increased chance of requiring a higher level of hospitalised care during COVID-19 infection (Ebinger et al., 2020). SARS-COV2-related deaths peak in those aged 80+, who possess more than a 20-fold higher chance of death than those aged 50–59 (Williamson et al., 2020). This most likely attributed to an increase in comorbidities with age, even if yet to be detected. Rodents experience a much shorter life span than humans, making them an excellent model for studying age-related changes in COVID-19 research. ACE2 receptor expression has been described to both increase (Baker et al., 2021; Wark et al., 2021) and decrease (Chen et al., 2020; J.; Gu et al., 2021; Xudong et al., 2006; Yoon et al., 2016) with age in humans and rodents. However, Berni Canani et al. (2021) observed no significant differences in ACE2 expression between children <10 years old and adults 20–80 years old, and Li M.-Y. et al. (2020) detected this same trend when comparing expression across multiple tissues in adults above or below 49 years of age. These contradicting reports suggest that ACE2 expression alone may not be a robust marker for identifying severe risk of SARS-CoV2 infection, and other factors in combination with ACE2 receptor expression must possess a decisive role. Comprehensive studies encompassing widespread tissue analysis of ACE2 expression in multiple age groups could well be accomplished to solve this, surely context-dependent, matter in rodent models of infection.

5.1.2 Diabetes

Diabetic patients are at increased risk of hospitalisation and mortality on infection with SARS-CoV2 (Ebinger et al., 2020), and are significantly more likely to require oxygen, intubation, antibiotics or dexamethasone on admission to hospital than non-diabetic patients (Alguwaihes et al., 2020). This is not entirely surprising considering increased cellular glucose levels assists in supporting viral replication (Codo et al., 2020). Overexpression of hACE2 boosts glucose tolerance and pancreatic β-cell function in diabetic mice (Bindom et al., 2010), whilst $ACE2^{-/y}$ knockout mice display impaired glucose tolerance alongside hepatic steatosis (Cao et al., 2016). Infection-induced downregulation of ACE2, and the resulting angiotensin II excess, therefore intensifies an already unbalanced glucose homeostasis. For these reasons, a bi-directional relationship between diabetes and COVID-19 infection has been proposed (Muniangi-Muhitu et al., 2020).

HFD-induced diabetic $DPP4^{H/M}$ male C57Bl/6 mice have been shown to be more vulnerable to severe signs of disease on infection with MERS-CoV when compared to lean controls, displaying prolonged weight loss and lung inflammation up to 21dpi (Kulcsar et al., 2019). Ma et al. (2021) however is the only study we found to date that has addressed the effect of the current SARS-CoV2 in a hACE2 expressing mouse model of diabetes. Ob/ob mice showed greater weight loss and increased lung immune infiltration when compared to non-diabetic mice at 5 dpi. Interestingly, this study also observed higher fasting blood glucose levels in both wild type and ob/ob mice infected with SARS-CoV2, compared to non-infected. Insulin tolerance was also non-significantly reduced in infected ob/ob mice (Ma et al., 2021). This is an alarming observation, and shows the potency of COVID-19 infection to disturb glucose homeostasis, not only in diabetic patients. Given that genetically-, chemically- or diet-induced rodent models of type 1 and type 2 diabetes are well established (King, 2012) in scientific literature, more research utilising hACE2-expressing rodents combined with these diabetic models will be extremely beneficial to understanding the risk posed on diabetic and non-diabetic people, both during and after contracting COVID-19.

5.1.3 Obesity

Obesity is another major risk factor for severe COVID-19 symptoms (Alguwaihes et al., 2020; Goyal et al., 2022) and COVID-19-related hospitalisation and mortality (Popkin et al., 2020). Its involvement in instigating this is likely intertwined with other comorbidities such as diabetes and hypertension. ACE2 is

expressed in subcutaneous and visceral adipose tissue (Al-Benna, 2020), and SARS-CoV2 nucleocapsids were detected in up to 5% of adipocytes in a small cohort of deceased COVID-19 patients (Basolo et al., 2022). Consequently, more adipose tissue will lead to surges in viral penetration and illness.

C57Bl/6 male mice fed a high-fat diet (HFD) display higher ACE2 expression in the lungs and trachea but reduced Tmprss2 expression in the oesophagus, whereas obese females display reduced ACE2 expression in the oesophagus and trachea with no differences in lung tissue (Sarver and Wong, 2021). This, at least in rodents, shows how obesity affects the expression of key SARS-CoV2 entry proteins differently in the two sexes, and should be investigated further in order to understand whether human patients should be treated according to sex. Further, HFD-fed rats see a 3.8- and 6-fold increase in lung Ace2 expression, and a 5.1- and 3.4-fold increase in Tmprss2 expression compared to standard and ketogenic diet fed rats, respectively. AT_1R and AT_2R levels were also significantly increased in HFD fed rats. Interestingly though, mice fed a ketogenic diet saw reduced AT_1R expression in pulmonary tissue compared to rats fed standard chow (da Eira et al., 2021), and this type of diet may help to safeguard diabetic or hypertensive humans. It would be meaningful to see further studies into the potential protective effects of certain diets on SARS-CoV2 infected rodent models.

The obesity-prone C57Bl/6N strain can provide valuable information in support of increased weight and diet on disease advancement and severity in SARS-CoV2 infected rodent models. Zhang et al., utilising leptin receptor dysfunctional C57BL/KsJ-db/db mice, observed a maintained 10% weight loss and more severe pulmonary pathology and inflammation in the obese model compared to db/+ controls inoculated with a mouse-adapted SARS-CoV2. Viral load was also significantly higher in obese lungs, nasal turbinates and trachea (Zhang et al., 2021). HFD-fed C57Bl/6N mice transduced with AdV-hACE2 also display more severe lung pathology than lean mice at 10 days post SARS-CoV2 infection, however a more comprehensive inflammatory profile should be included when studying models such as these (Rai et al., 2021).

This presented evidence further supports the role of obesity in severe COVID-19 patients, and in a way embodies the fusion of two pandemics. Researchers may now also look towards rodent models, preferably expressing hACE2 under its namesake promoter, to develop treatments to ease symptoms and reduce mortality in these patients in the short term. Patients may then turn to improve their diet and lifestyle habits post-recovery.

5.1.4 Hypertension

As the ACE2 receptor is responsible for initial SARS-CoV2 cell entry, it is logical that hypertension was among the top clinical presentations in patients suffering from severe COVID-19 (Huang et al., 2020; Li X. et al., 2020), through viral disruption of RAAS. ACE2 usually acts a negative regulator of RAAS, lowering blood pressure with anti-inflammatory effects. A number of RAAS modulators have been tested on rat primary *in vitro* cultures that principally act to increase or decrease ACE2 mRNA or protein levels (Hu et al., 2021), which in regards to a COVID-19 patient may either encourage additional viral penetration or further increase blood pressure, respectively. ACE inhibitors or ARB drugs however, seem to display a protective effect against SARS-CoV2 infection and in-hospital mortality (Hippisley-Cox et al., 2020). Nevertheless, these types of studies mainly take into account hospital admissions and must account for a large number of comorbidities and variables.

Diet-induced obese C57Bl/6J mice display weight loss, improved glucose tolerance and reduced expression of inflammatory cytokines when treated with ACE inhibitors (Premaratna et al., 2012). $ACE^{-/-}$ mice show a similar trend (Jayasooriya et al., 2008), displaying the potential for this treatment to ease multiple COVID-19-related risk factors at once. There are a high number of inbred, outbred and transgenic rodent models used for hypertension research (Lerman et al., 2019). Jiang et al. (2022) recently published that SARS-CoV2 viral load in the lungs is higher in transgenic hACE2-expressing mice that have been induced into hypertension compared to normotensive hACE2 mice. Further, AT_1R blocker treatment improved lung pathology, reduced blood pressure and downregulated IL-6 and TNF-α expression in hypertensive hACE2 mice. This signifies that treatment provided protection to the organs on SARS-CoV2 infection overall, despite increased viral penetration in the heart and kidneys initially at early infection (Jiang et al., 2022).

A recent preprint article reported that the ACE inhibitor Lisinopril can raise the ACE2 expression landscape in the lungs, small intestine, kidney and brain of healthy mice, an effect that persists to at least 21 days post-termination of treatment (Brooks et al., 2022). Captopril, which also acts as an ACE inhibitor, appears to improve lung pathology and reduce inflammation during SARS-CoV2 infection in an angiotensin II-induced hypertensive and hACE2-expressing mouse model, without any detectable effect on viral load (Gao et al., 2021). These reports reinforce the potential but need for further clarification on RAAS modulators in COVID-19 research, but studies focusing specifically on hypertension in rodents on infection with the SARS-CoV2 virus are lacking. Nonetheless, with blood pressure measurements by techniques such as tail-cuff plethysmography and radiotelemetry readily available for use in rodents (Burger et al., 2014) and with a number of hypertension remedies on the market, future COVID-19 animal research focused on hypertension risk or the efficacy of RAAS modulators would benefit from integrating these methodologies into their study design for a greater *in vivo* view of hypertension in the current pandemic.

5.2 Insights Into Long-COVID

Post-acute COVID-19 sequelae or 'long-COVID' is a condition in which patients continue to suffer multiple COVID-19-related symptoms weeks or even months after testing negative for the virus, and can come in continuous or relapsing forms. The mechanisms behind symptom persistence are still unclear as presentation varies from patient to patient. Large scale studies from around the globe have witnessed exhaustive lists of symptoms (Davis et al., 2021; Hossain et al., 2021; C. Huang

et al., 2021; Pérez-González et al., 2022), with those who were hospitalised or required intensive care during primary infection especially at risk (Xie et al., 2022).

Rodent models of post-acute COVID-19 syndrome have been close to non-existent so far. This is likely due to subsided infections, or death, of animals in the models currently available, and the incorporation of early terminal analysis into experiment design. To more accurately study the long-term effects of SARS-CoV2 infection we require models that are even more 'comprehensively human' than those presented in **Table 1**, which more closely mimic aspects such as our own immune system. Researching viral infections by utilising non-human primates is an attractive option, due to marked similarities in physiology and immune responses to antigens with humans with the possibility for longitudinal studies in controlled environments (Estes et al., 2018). The rhesus macaque, African green monkey and pigtail macaque are susceptible to SARS-CoV2 infection and show mild-moderate COVID-19-associated lung pathologies (Clancy et al., 2021). Further, Böszörményi et al. (2021) observed that infected macaques show worsening lung lesions in CT scans, increases in specific cytokines in plasma, mild to moderate histopathological signs of pneumonia and the presence of viral RNA levels in a myriad of tissues up to 38 dpi, despite all subjects testing negative for SARS-CoV2 after 14 dpi. This suggests that these non-human primates are also susceptible to post-acute COVID-19 in a similar way to humans (Böszörményi et al., 2021).

For many researchers however, rodents are a preferred model based on their lower maintenance costs, shorter gestation period and the wealth of tools for transgenic manipulation. A promising example of a mouse model with a humanised immune system is MISTRG6. These immunodeficient mice express seven human cytokine genes knocked into their respective locus in the mouse's genome, and tolerate human hematopoietic stem cell engraftment (Rongvaux et al., 2014). MISTRG6 mice that transiently express hACE2 sustain prolonged viral titres and RNA, more severe lung pathology, and immune cell signatures to at least 35dpi of SARS-CoV2 compared to controls, emulating severe COVID-19 disease in humans. Convalescent plasma therapy showed a protective effect in these mice in regards to weight loss and viral clearance, however only prophylactic monoclonal antibody treatment improved prevention of T cell lung infiltration (Sefik et al., 2021). This again highlights the importance of early diagnosis in high risk patients, and it will be interesting to see more therapies tested on this model over longer time periods.

Finally, a recent preprint article has described their tracking of 10-weeks- and 1-year-old BALB/C mice for 120 days post infection with mouse adapted SARS-CoV2 MA10. Younger mice cleared infection twice as fast as older mice, with cytokine responses enduring in the latter until 30 dpi. Interestingly though, mice in the younger age group displayed a greater capability for tissue repair, and Molnupiravir was also effective at reducing disease prevalence in the older age group (Dinnon et al., 2022). Although this study is yet to be peer-reviewed, long-term mouse studies such as this will prove valuable in the fight against post-acute COVID-19 syndrome.

6 CONCLUDING REMARKS

The COVID-19 pandemic prompted a global scientific effort to produce a number of diverse animal models that mimic SARS-CoV2 infection. Mice have been and continue to be the preferred model organism used in scientific research due to their easy manipulation, short breeding time and genetic similarity to humans. However, the SARS-CoV2 virus binds inefficiently to the ACE2 receptor in mice thus preventing severe infection. This seemingly large barrier has been surmounted by the generation of transgenic and humanised mouse models. Additionally, efforts have been placed in reverse engineering the virus itself to increase its affinity to the mouse ACE2 receptor and causing COVID-19 symptoms. The aim of this review was to highlight individual COVID-19 mouse models and the tissue-specific replication of the virus and pathophysiology upon infection. We substantiate the review with examples of how these models have been used in regards to risk assessment of novel strains, developing therapeutics and elucidating the mechanisms of risk factors such as old age, diabetes, obesity and hypertension. We believe that this review can be used as a comprised guide for investigators researching which mouse model or which strategy to employ in regards to future COVID-19 research.

AUTHOR CONTRIBUTIONS

PN, MR, and LS wrote the article with equal contributions. RS supervised.

REFERENCES

Abdelnabi, R., Foo, C. S., de Jonghe, S., Maes, P., Weynand, B., and Neyts, J. (2021a). Molnupiravir Inhibits Replication of the Emerging SARS-CoV-2 Variants of Concern in a Hamster Infection Model. *J. Infect. Dis.* 224 (5), 749–753. doi:10.1093/infdis/jiab361

Abdelnabi, R., Foo, C. S., Jochmans, D., Vangeel, L., de Jonghe, S., Augustijns, P., et al. (2022). The Oral Protease Inhibitor (PF-07321332) Protects Syrian Hamsters against Infection with SARS-CoV-2 Variants of Concern. *Nat. Commun.* 13 (1). doi:10.1038/s41467-022-28354-0

Abdelnabi, R., Foo, C. S., Kaptein, S. J. F., Zhang, X., Do, T. N. D., Langendries, L., et al. (2021b). The Combined Treatment of Molnupiravir and Favipiravir Results in a Potentiation of Antiviral Efficacy in a SARS-CoV-2 Hamster Infection Model. *EBioMedicine* 72, 103595. doi:10.1016/j.ebiom.2021.103595

Al-Benna, S. (2020). Association of High Level Gene Expression of ACE2 in Adipose Tissue with Mortality of COVID-19 Infection in Obese Patients. *Obes. Med.* 19, 100283. doi:10.1016/j.obmed.2020.100283

Alguwaihes, A. M., Al-Sofiani, M. E., Megdad, M., Albader, S. S., Alsari, M. H., Alelayan, A., et al. (2020). Diabetes and Covid-19 Among Hospitalized Patients in Saudi Arabia: a Single-centre Retrospective Study. *Cardiovasc. Diabetol.* 19 (1), 205. doi:10.1186/s12933-020-01184-4

Alsaadi, E. J. A., and Jones, I. M. (2019). Membrane Binding Proteins of Coronaviruses. *Future Virol.* 14 (4), 275–286. doi:10.2217/fvl-2018-0144

Asaka, M. N., Utsumi, D., Kamada, H., Nagata, S., Nakachi, Y., Yamaguchi, T., et al. (2021). Highly Susceptible SARS-CoV-2 Model in CAG Promoter-Driven

hACE2-Transgenic Mice. *JCI Insight* 6 (19), e152529. doi:10.1172/JCI.INSIGHT.152529

Avdeev, S., Moiseev, S., Brovko, M., Yavorovskiy, A., Umbetova, K., Akulkina, L., et al. (2020). Low Prevalence of Bronchial Asthma and Chronic Obstructive Lung Disease Among Intensive Care Unit Patients with COVID-19. *Allergy* 75 (10), 2703–2704. doi:10.1111/all.14420

Baker, S. A., Kwok, S., Berry, G. J., and Montine, T. J. (2021). Angiotensin-converting Enzyme 2 (ACE2) Expression Increases with Age in Patients Requiring Mechanical Ventilation. *PLoS ONE* 16 (2 February), e0247060. doi:10.1371/journal.pone.0247060

Bao, L., Deng, W., Huang, B., Gao, H., Liu, J., Ren, L., et al. (2020a). The Pathogenicity of SARS-CoV-2 in hACE2 Transgenic Mice. *Nature* 583 (7818), 830–833. doi:10.1038/s41586-020-2312-y

Bao, L., Gao, H., Deng, W., Lv, Q., Yu, H., Liu, M., et al. (2020b). Transmission of Severe Acute Respiratory Syndrome Coronavirus 2 via Close Contact and Respiratory Droplets Among Human Angiotensin-Converting Enzyme 2 Mice. *J. Infect. Dis.* 222 (4), 551–555. doi:10.1093/INFDIS/JIAA281

Basolo, A., Poma, A. M., Bonuccelli, D., Proietti, A., Macerola, E., Ugolini, C., et al. (2022). Adipose Tissue in COVID-19: Detection of SARS-CoV-2 in Adipocytes and Activation of the Interferon-Alpha Response. *J. Endocrinol. Invest.* 45, 1021–1029. doi:10.1007/s40618-022-01742-5

Bayarri-Olmos, R., Johnsen, L. B., Idorn, M., Reinert, L. S., Rosbjerg, A., Vang, S., et al. (2021). The alpha/B.1.1.7 SARS-CoV-2 Variant Exhibits Significantly Higher Affinity for ACE-2 and Requires Lower Inoculation Doses to Cause Disease in K18-hACE2 Mice. *ELife* 10, e70002. doi:10.7554/eLife.70002

Berni Canani, R., Comegna, M., Paparo, L., Cernera, G., Bruno, C., Strisciuglio, C., et al. (2021). Age-Related Differences in the Expression of Most Relevant Mediators of SARS-CoV-2 Infection in Human Respiratory and Gastrointestinal Tract. *Front. Pediatr.* 9. doi:10.3389/fped.2021.697390

Bindom, S. M., Hans, C. P., Xia, H., Boulares, A. H., and Lazartigues, E. (2010). Angiotensin I-Converting Enzyme Type 2 (ACE2) Gene Therapy Improves Glycemic Control in Diabetic Mice. *Diabetes* 59 (10), 2540–2548. doi:10.2337/db09-0782

Boras, B., Jones, R. M., Anson, B. J., Arenson, D., Aschenbrenner, L., Bakowski, M. A., et al. (2021). Preclinical Characterization of an Intravenous Coronavirus 3CL Protease Inhibitor for the Potential Treatment of COVID19. *Nat. Commun.* 12 (1), 6055. doi:10.1038/s41467-021-26239-2

Böszörményi, K. P., Stammes, M. A., Fagrouch, Z. C., Kiemenyi-Kayere, G., Niphuis, H., Mortier, D., et al. (2021). The post-acute Phase of Sars-Cov-2 Infection in Two Macaque Species Is Associated with Signs of Ongoing Virus Replication and Pathology in Pulmonary and Extrapulmonary Tissues. *Viruses* 13 (8), 1673. doi:10.3390/v13081673

Brooks, S. D., Smith, R. L., Moreira, A. S., and Ackerman, H. C. (2022). Oral Lisinopril Raises Tissue Levels of ACE2, The SARS-CoV-2 Receptor, in Healthy Male and Female Mice. *Front. Pharmacol.* 13, 798349. doi:10.3389/fphar.2022.798349

Bruter, A. V., Korshunova, D. S., Kubekina, M. V., Sergiev, P. V., Kalinina, A. A., Ilchuk, L. A., et al. (2021). Novel Transgenic Mice with Cre-dependent Co-expression of GFP and Human ACE2: a Safe Tool for Study of COVID-19 Pathogenesis. *Transgenic Res.* 30 (3), 289–301. doi:10.1007/S11248-021-00249-8/TABLES/1

Burger, D., Reudelhuber, T. L., Mahajan, A., Chibale, K., Sturrock, E. D., and Touyz, R. M. (2014). Effects of a Domain-Selective ACE Inhibitor in a Mouse Model of Chronic Angiotensin II-dependent Hypertension. *Clin. Sci.* 127 (1), 57–63. doi:10.1042/CS20130808

Calisher, C. H., Childs, J. E., Field, H. E., Holmes, K. V., and Schountz, T. (2006). Bats: Important Reservoir Hosts of Emerging Viruses. *Clin. Microbiol. Rev.* 19 (3), 531–545. doi:10.1128/CMR.00017-06

Cao, X., Yang, F., Shi, T., Yuan, M., Xin, Z., Xie, R., et al. (2016). Angiotensin-converting Enzyme 2/angiotensin-(1-7)/Mas axis Activates Akt Signaling to Ameliorate Hepatic Steatosis. *Sci. Rep.* 6, 21592. doi:10.1038/srep21592

Chan, J. F.-W., Yip, C. C.-Y., To, K. K.-W., Tang, T. H.-C., Wong, S. C.-Y., Leung, K.-H., et al. (2020). Improved Molecular Diagnosis of COVID-19 by the Novel, Highly Sensitive and Specific COVID-19-RdRp/Hel Real-Time Reverse Transcription-PCR Assay Validated *In Vitro* and with Clinical Specimens. *J. Clin. Microbiol.* 58 (5), e00310–20. doi:10.1128/JCM.00310-20

Chang, C.-k., Sue, S.-C., Yu, T.-h., Hsieh, C.-M., Tsai, C.-K., Chiang, Y.-C., et al. (2006). Modular Organization of SARS Coronavirus Nucleocapsid Protein. *J. Biomed. Sci.* 13 (1), 59–72. doi:10.1007/s11373-005-9035-9

Chen, J., Jiang, Q., Xia, X., Liu, K., Yu, Z., Tao, W., et al. (2020). Individual Variation of the SARS-CoV-2 Receptor ACE2 Gene Expression and Regulation. *Aging Cell* 19 (7), e13168. doi:10.1111/acel.13168

Clancy, C. S., Shaia, C., Munster, V., de Wit, E., Hawman, D., Okumura, A., et al. (2021). Histologic Pulmonary Lesions of SARS-CoV-2 in 4 Nonhuman Primate Species: An Institutional Comparative Review. *Vet. Pathol.*, 3009858211067468. doi:10.1177/03009858211067468

Codo, A. C., Davanzo, G. G., Monteiro, L. d. B., de Souza, G. F., Muraro, S. P., Virgilio-da-Silva, J. V., et al. (2020). Elevated Glucose Levels Favor SARS-CoV-2 Infection and Monocyte Response through a HIF-1α/Glycolysis-dependent Axis. *Cel Metab.* 32 (3), 437–446. e5. doi:10.1016/j.cmet.2020.07.007

Cox, R. M., Wolf, J. D., and Plemper, R. K. (2021). Therapeutically Administered Ribonucleoside Analogue MK-4482/EIDD-2801 Blocks SARS-CoV-2 Transmission in Ferrets. *Nat. Microbiol.* 6 (1), 11–18. doi:10.1038/s41564-020-00835-2

Czech Centre for Phenogenomics (2021). *Covid-19 Mouse Models Available at CCP - Czech Centre for Phenogenomics*. Vestec, Czechia. https://www.phenogenomics.cz/2021/09/covid-19-mouse-models-available-at-ccp/.

da Eira, D., Jani, S., and Ceddia, R. B. (2021). Obesogenic and Ketogenic Diets Distinctly Regulate the SARS-CoV-2 Entry Proteins ACE2 and TMPRSS2 and the Renin-Angiotensin System in Rat Lung and Heart Tissues. *Nutrients* 13 (10), 3357. doi:10.3390/nu13103357

Davis, H. E., Assaf, G. S., McCorkell, L., Wei, H., Low, R. J., Re'em, Y., et al. (2021). Characterizing Long COVID in an International Cohort: 7 Months of Symptoms and Their Impact. *EClinicalMedicine* 38, 101019. doi:10.1016/j.eclinm.2021.101019

De Gasparo, R., Pedotti, M., Simonelli, L., Nickl, P., Muecksch, F., Cassaniti, I., et al. (2021). Bispecific IgG Neutralizes SARS-CoV-2 Variants and Prevents Escape in Mice. *Nature* 593 (7859), 424–428. doi:10.1038/s41586-021-03461-y

Dinnon, K. H., III, Leist, S. R., Okuda, K., Dang, H., Fritch, E. J., Gully, K. L., et al. (2022). A Model of Persistent post SARS-CoV-2 Induced Lung Disease for Target Identification and Testing of Therapeutic Strategies. *BioRxiv*. doi:10.1101/2022.02.15.480515

Dinnon, K. H., Leist, S. R., Schäfer, A., Edwards, C. E., Martinez, D. R., Montgomery, S. A., et al. (2020). A Mouse-Adapted Model of SARS-CoV-2 to Test COVID-19 Countermeasures. *Nature* 586 (7830), 560–566. doi:10.1038/s41586-020-2708-8

Dolskiy, A. A., Gudymo, A. S., Taranov, O. S., Grishchenko, I. V., Shitik, E. M., Prokopov, D. Y., et al. (2022). The Tissue Distribution of SARS-CoV-2 in Transgenic Mice with Inducible Ubiquitous Expression of hACE2. *Front. Mol. Biosciences* 8, 1339. doi:10.3389/FMOLB.2021.821506/BIBTEX

Ebinger, J. E., Achamallah, N., Ji, H., Claggett, B. L., Sun, N., Botting, P., et al. (2020). Pre-existing Traits Associated with Covid-19 Illness Severity. *PLoS ONE* 15 (7 July), e0236240. doi:10.1371/journal.pone.0236240

Enkirch, T., and von Messling, V. (2015). Ferret Models of Viral Pathogenesis. *Virology* 479-480, 259–270. doi:10.1016/J.VIROL.2015.03.017

Estes, J. D., Wong, S. W., and Brenchley, J. M. (2018). Nonhuman Primate Models of Human Viral Infections. *Nat. Rev. Immunol.* 18 (6), 390–404. doi:10.1038/s41577-018-0005-7

European Centre for Disease Prevention and Control (2021). *Assessment of the Further Emergence of the SARS-CoV-2 Omicron VOC in the Context of the Ongoing Delta VOC Transmission in the EU/EEA, 18th Update*. Stockholm, Sweden: ECDC. Available at: https://www.ecdc.europa.eu/en/publications-data/covid-19-assessment-further-emergence-omicron-18th-risk-assessment (Accessed February 25, 2022).

European Medicines Agency (2021). *EMA Issues Advice on Use of Lagevrio (Molnupiravir) for the Treatment of COVID-19*. Amsterdam, Netherlands: European Medicines Agency. Available at: https://www.ema.europa.eu/en/news/ema-issues-advice-use-lagevrio-molnupiravir-treatment-covid-19 (Accessed February 10, 2022).

European Medicines Agency (2022). *Paxlovid*. European Medicines Agency. Available at: https://www.ema.europa.eu/en/medicines/human/EPAR/paxlovid (Accessed February 11, 2022).

Fehr, A. R., and Perlman, S. (2015). Coronaviruses: an Overview of Their Replication and Pathogenesis. *Methods Mol. Biol.* 1282, 1–23. doi:10.1007/978-1-4939-2438-7_1

Finch, C. L., Crozier, I., Lee, J. H., Byrum, R., Cooper, T. K., Liang, J., et al. (2020). Characteristic and Quantifiable COVID-19-like Abnormalities in CT- and PET/CT-imaged Lungs of SARS-CoV-2-Infected Crab-Eating Macaques (*Macaca fascicularis*). *BioRxiv*. doi:10.1101/2020.05.14.096727

Forni, D., Cagliani, R., Clerici, M., and Sironi, M. (2017). Molecular Evolution of Human Coronavirus Genomes. *Trends Microbiol.* 25 (1), 35–48. doi:10.1016/j.tim.2016.09.001

Gao, W.-C., Ma, X., Wang, P., Ma, X., Wang, P., He, X.-Y., et al. (2021). Captopril Alleviates Lung Inflammation in SARS-CoV-2-Infected Hypertensive Mice. *Zoolog. Res.* 42 (5), 633–636. doi:10.24272/J.ISSN.2095-8137.2021.206

Gavriatopoulou, M., Ntanasis-Stathopoulos, I., Korompoki, E., Fotiou, D., Migkou, M., Tzanninis, I.-G., et al. (2021). Emerging Treatment Strategies for COVID-19 Infection. *Clin. Exp. Med.* 21 (2), 167–179. doi:10.1007/s10238-020-00671-y

Goyal, A., Gupta, Y., Kalaivani, M., Praveen, P. A., Ambekar, S., and Tandon, N. (2022). SARS-CoV-2 Seroprevalence in Individuals with Type 1 and Type 2 Diabetes Compared with Controls. *Endocr. Pract.* 28 (2), 191–198. doi:10.1016/j.eprac.2021.12.009

Gruber, A. D., Firsching, T. C., Trimpert, J., and Dietert, K. (2021). Hamster Models of COVID-19 Pneumonia Reviewed: How Human Can They Be? *Vet. Pathol.*, 030098582110571. doi:10.1177/03009858211057197

Gu, H., Chen, Q., Yang, G., He, L., Fan, H., Deng, Y.-Q., et al. (2020). Rapid Adaptation of SARS-CoV-2 in BALB/c Mice: Novel Mouse Model for Vaccine Efficacy. *BioRxiv*. doi:10.1101/2020.05.02.073411

Gu, J., Yin, J., Zhang, M., Li, J., Wu, Y., Chen, J., et al. (2021). Study on the Clinical Significance of Ace2 and its Age-Related Expression. *Jir* Vol. 14, 2873–2882. doi:10.2147/JIR.S315981

Guo, T., Fan, Y., Chen, M., Wu, X., Zhang, L., He, T., et al. (2020). Cardiovascular Implications of Fatal Outcomes of Patients with Coronavirus Disease 2019 (COVID-19). *JAMA Cardiol.* 5 (7), 811–818. doi:10.1001/jamacardio.2020.1017

Halfmann, P. J., Iida, S., Iwatsuki-Horimoto, K., Maemura, T., Kiso, M., Scheaffer, S. M., et al. (2022). SARS-CoV-2 Omicron Virus Causes Attenuated Disease in Mice and Hamsters. *Nature* 603, 687–692. doi:10.1038/s41586-022-04441-6

Hassan, A. O., Case, J. B., Winkler, E. S., Thackray, L. B., Kafai, N. M., Bailey, A. L., et al. (2020). A SARS-CoV-2 Infection Model in Mice Demonstrates Protection by Neutralizing Antibodies. *Cell* 182 (3), 744–753. doi:10.1016/J.CELL.2020.06.011

He, X., Hong, W., Pan, X., Lu, G., and Wei, X. (2021). SARS-CoV-2 Omicron Variant: Characteristics and Prevention. *MedComm* 2 (4), 838–845. doi:10.1002/mco2.110

Hippisley-Cox, J., Young, D., Coupland, C., Channon, K. M., Tan, P. S., Harrison, D. A., et al. (2020). Risk of Severe COVID-19 Disease with ACE Inhibitors and Angiotensin Receptor Blockers: Cohort Study Including 8.3 Million People. *Heart* 106 (19), 1503–1511. doi:10.1136/heartjnl-2020-317393

Hodcroft, E. B. (2021). *CoVariants: SARS-CoV-2 Mutations and Variants of Interest*. Available at https://covariants.org/ (Accessed February 10, 2022).

Hoffman, R. L., Kania, R. S., Brothers, M. A., Davies, J. F., Ferre, R. A., Gajiwala, K. S., et al. (2020). Discovery of Ketone-Based Covalent Inhibitors of Coronavirus 3CL Proteases for the Potential Therapeutic Treatment of COVID-19. *J. Med. Chem.* 63 (21), 12725–12747. doi:10.1021/acs.jmedchem.0c01063

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181 (2), 271–280. e8. doi:10.1016/j.cell.2020.02.052

Horspool, A. M., Ye, C., Wong, T. Y., Russ, B. P., Lee, K. S., Winters, M. T., et al. (2021). SARS-CoV-2 B.1.1.7 and B.1.351 Variants of Concern Induce Lethal Disease in K18-hACE2 Transgenic Mice Despite Convalescent Plasma Therapy. *BioRxiv*. doi:10.1101/2021.05.05.442784

Hossain, M. A., Hossain, K. M. A., Saunders, K., Uddin, Z., Walton, L. M., Raigangar, V., et al. (2021). Prevalence of Long COVID Symptoms in Bangladesh: A Prospective Inception Cohort Study of COVID-19 Survivors. *BMJ Glob. Health* 6 (12), e006838. doi:10.1136/bmjgh-2021-006838

Hu, Y., Liu, L., and Lu, X. (2021). Regulation of Angiotensin-Converting Enzyme 2: A Potential Target to Prevent COVID-19? *Front. Endocrinol.* 12. doi:10.3389/fendo.2021.725967

Huang, C., Huang, L., Wang, Y., Li, X., Ren, L., Gu, X., et al. (2021). 6-month Consequences of COVID-19 in Patients Discharged from Hospital: a Cohort Study. *The Lancet* 397 (10270), 220–232. doi:10.1016/S0140-6736(20)32656-8

Huang, S., Wang, J., Liu, F., Liu, J., Cao, G., Yang, C., et al. (2020). COVID-19 Patients with Hypertension Have More Severe Disease: a Multicenter Retrospective Observational Study. *Hypertens. Res.* 43 (8), 824–831. doi:10.1038/s41440-020-0485-2

Israelow, B., Song, E., Mao, T., Lu, P., Meir, A., Liu, F., et al. (2020). Mouse Model of SARS-CoV-2 Reveals Inflammatory Role of Type I Interferon Signaling. *J. Exp. Med.* 217 (12). doi:10.1084/JEM.20201241

Jackson, C. B., Farzan, M., Chen, B., and Choe, H. (2022). Mechanisms of SARS-CoV-2 Entry into Cells. *Nat. Rev. Mol. Cel Biol* 23 (1), 3–20. doi:10.1038/s41580-021-00418-x

Jayasooriya, A. P., Mathai, M. L., Walker, L. L., Begg, D. P., Denton, D. A., Cameron-Smith, D., et al. (2008). Mice Lacking Angiotensin-Converting Enzyme Have Increased Energy Expenditure, with Reduced Fat Mass and Improved Glucose Clearance. *Proc. Natl. Acad. Sci. U.S.A.* 105 (18), 6531–6536. www.pnas.orgcgi. doi:10.1073/pnas.0802690105

Jia, H., Yue, X., and Lazartigues, E. (2020). ACE2 Mouse Models: a Toolbox for Cardiovascular and Pulmonary Research. *Nat. Commun.* 11 (1), 5165. doi:10.1038/s41467-020-18880-0

Jiang, R.-D., Di, Liu, M.-Q., Liu, Y., Shan, C., Zhou, Y.-W., Shen, X.-R., et al. (2020). Pathogenesis of SARS-CoV-2 in Transgenic Mice Expressing Human Angiotensin-Converting Enzyme 2. *Cell* 182 (1), 50–58. e8. doi:10.1016/J.CELL.2020.05.027

Jiang, X., Li, H., Liu, Y., Bao, L., Zhan, L., Gao, H., et al. (2022). The Effects of ATIR Blocker on the Severity of COVID-19 in Hypertensive Inpatients and Virulence of SARS-CoV-2 in Hypertensive hACE2 Transgenic Mice. *J. Cardiovasc. Trans. Res.* 15, 38–48. doi:10.1007/s12265-021-10147-3

Kabinger, F., Stiller, C., Schmitzová, J., Dienemann, C., Kokic, G., Hillen, H. S., et al. (2021). Mechanism of Molnupiravir-Induced SARS-CoV-2 Mutagenesis. *Nat. Struct. Mol. Biol.* 28 (9), 740–746. doi:10.1038/s41594-021-00651-0

Kaptein, S. J. F., Jacobs, S., Langendries, L., Seldeslachts, L., ter Horst, S., Liesenborghs, L., et al. (2020). Favipiravir at High Doses Has Potent Antiviral Activity in SARS-CoV-2–infected Hamsters, whereas Hydroxychloroquine Lacks Activity. *Proc. Natl. Acad. Sci. U.S.A.* 117 (43), 26955–26965. doi:10.1073/pnas.2014441117/-/DCSupplemental

Katzman, C., Israely, T., Melamed, S., Politi, B., Sittner, A., Yahalom-Ronen, Y., et al. (2022). Modeling Sars-Cov-2 Infection in Mice Using Lentiviral Hace2 Vectors Infers Two Modes of Immune Responses to Sars-Cov-2 Infection. *Viruses* 14 (1), 11. doi:10.3390/V14010011/S1

King, A. J. (2012). The Use of Animal Models in Diabetes Research. *Br. J. Pharamacology* 166, 877–894. doi:10.1111/j.1476-5381.2012.01911.x

Kovacech, B., Fialova, L., Filipcik, P., Skrabana, R., Zilkova, M., Paulenka-Ivanovova, N., et al. (2022). Monoclonal Antibodies Targeting Two Immunodominant Epitopes on the Spike Protein Neutralize Emerging SARS-CoV-2 Variants of Concern. *EBioMedicine* 76, 103818. doi:10.1016/j10.1016/j.ebiom.2022.103818

Kulcsar, K. A., Coleman, C. M., Beck, S. E., and Frieman, M. B. (2019). Comorbid Diabetes Results in Immune Dysregulation and Enhanced Disease Severity Following MERS-CoV Infection. *JCI Insight* 4 (20), e131774. doi:10.1172/jci.insight.131774

Leclerc, Q. J., Fuller, N. M., Knight, L. E., Funk, S., Knight, G. M., and Knight, G. M. (2020). What Settings Have Been Linked to SARS-CoV-2 Transmission Clusters? *Wellcome Open Res.* 5, 83. doi:10.12688/wellcomeopenres.15889.1

Lerman, L. O., Kurtz, T. W., Touyz, R. M., Ellison, D. H., Chade, A. R., Crowley, S. D., et al. (2019). Animal Models of Hypertension: A Scientific Statement from the American Heart Association. *Hypertension* 73 (6), e87–e120. doi:10.1161/HYP.0000000000000090

Li, M.-Y., Li, L., Zhang, Y., and Wang, X.-S. (2020). Expression of the SARS-CoV-2 Cell Receptor Gene ACE2 in a Wide Variety of Human Tissues. *Infect. Dis. Poverty* 9 (1). doi:10.1186/s40249-020-00662-x

Li, X., Xu, S., Yu, M., Wang, K., Tao, Y., Zhou, Y., et al. (2020). Risk Factors for Severity and Mortality in Adult COVID-19 Inpatients in Wuhan. *J. Allergy Clin. Immunol.* 146 (1), 110–118. doi:10.1016/j.jaci.2020.04.006

Liu, Y., Liu, J., Plante, K. S., Plante, J. A., Xie, X., Zhang, X., et al. (2022). The N501Y Spike Substitution Enhances SARS-CoV-2 Infection and Transmission. *Nature* 602 (7896), 294–299. doi:10.1038/s41586-021-04245-0

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., et al. (2020). Genomic Characterisation and Epidemiology of 2019 Novel Coronavirus: Implications for Virus Origins and Receptor Binding. *The Lancet* 395 (10224), 565–574. doi:10.1016/s0140-6736(20)30251-8

Ma, Y., Lu, D., Bao, L., Qu, Y., Liu, J., Qi, X., et al. (2021). SARS-CoV-2 Infection Aggravates Chronic Comorbidities of Cardiovascular Diseases and Diabetes in Mice. *Anim. Models Exp. Med.* 4 (1), 2–15. doi:10.1002/ame2.12155

McCray, P. B., Pewe, L., Wohlford-Lenane, C., Hickey, M., Manzel, L., Shi, L., et al. (2007). Lethal Infection of K18- hACE2 Mice Infected with Severe Acute Respiratory Syndrome Coronavirus. *J. Virol.* 81 (2), 813–821. doi:10.1128/JVI.02012-06

Medicines & Healthcare products Regulatory Agency (2021b). *First Oral Antiviral for COVID-19, Lagevrio (Molnupiravir), Approved by MHRA. [Press release]* London, United Kingdom. Available at: https://www.gov.uk/government/news/first-oral-antiviral-for-covid-19-lagevrio-molnupiravir-approved-by-mhra (Accessed February 24, 2022).

Medicines & Healthcare products Regulatory Agency (2021a). *Oral COVID-19 Antiviral, Paxlovid, Approved by UK regulator. [Press release]*. London, United Kingdom (Available at: https://www.gov.uk/government/news/oral-covid-19-antiviral-paxlovid-approved-by-uk-regulator (Accessed February 24, 2022).

Menachery, V. D., Yount, B. L., Sims, A. C., Debbink, K., Agnihothram, S. S., Gralinski, L. E., et al. (2016). SARS-like WIV1-CoV Poised for Human Emergence. *Proc. Natl. Acad. Sci. U.S.A.* 113 (11), 3048–3053. doi:10.1073/PNAS.1517719113

Mughal, M. S., Kaur, I. P., Jaffery, A. R., Dalmacion, D. L., Wang, C., Koyoda, S., et al. (2020). COVID-19 Patients in a Tertiary US Hospital: Assessment of Clinical Course and Predictors of the Disease Severity. *Respir. Med.* 172, 106130. doi:10.1016/j.rmed.2020.106130

Muniangi-Muhitu, H., Akalestou, E., Salem, V., Misra, S., Oliver, N. S., and Rutter, G. A. (2020). Covid-19 and Diabetes: A Complex Bidirectional Relationship. *Front. Endocrinol.* 11. doi:10.3389/fendo.2020.582936

Munster, V. J., Feldmann, F., Williamson, B. N., van Doremalen, N., Pérez-Pérez, L., Schulz, J., et al. (2020). Respiratory Disease in Rhesus Macaques Inoculated with SARS-CoV-2. *Nature* 585 (7824), 268–272. doi:10.1038/S41586-020-2324-7

Muruato, A., Vu, M. N., Johnson, B. A., Davis-Gardner, M. E., Vanderheiden, A., Lokugamage, K., et al. (2021). Mouse-adapted SARS-CoV-2 Protects Animals from Lethal SARS-CoV challenge. *Plos Biol.* 19 (11), e3001284. doi:10.1371/journal.pbio.3001284

Nehme, A., Zouein, F. A., Zayeri, Z. D., and Zibara, K. (2019). An Update on the Tissue Renin Angiotensin System and its Role in Physiology and Pathology. *Jcdd* 6 (2), 14. doi:10.3390/jcdd6020014

Owen, D. R., Allerton, C. M. N., Anderson, A. S., Aschenbrenner, L., Avery, M., Berritt, S., et al. (2021). An Oral SARS-CoV-2 M Pro Inhibitor Clinical Candidate for the Treatment of COVID-19. *Science* 374 (6575), 1586–1593. https://www.science.org. doi:10.1126/science.abl4784

Pan, T., Chen, R., He, X., Yuan, Y., Deng, X., Li, R., et al. (2021). Infection of Wild-type Mice by SARS-CoV-2 B.1.351 Variant Indicates a Possible Novel Cross-Species Transmission Route. *Sig Transduct Target. Ther.* 6 (1), 420. doi:10.1038/s41392-021-00848-1

Pérez-González, A., Araújo-Ameijeiras, A., Fernández-Villar, A., Crespo, M., Poveda, E., Cabrera, J. J., et al. (2022). Long COVID in Hospitalized and Non-hospitalized Patients in a Large Cohort in Northwest Spain, a Prospective Cohort Study. *Sci. Rep.* 12 (1), 3369. doi:10.1038/s41598-022-07414-x

Popkin, B. M., Du, S., Green, W. D., Beck, M. A., Algaith, T., Herbst, C. H., et al. (2020). Individuals with Obesity and COVID-19: A Global Perspective on the Epidemiology and Biological Relationships. *Obes. Rev.* 21 (11), e13128. doi:10.1111/obr.13128

Premaratna, S. D., Manickam, E., Begg, D. P., Rayment, D. J., Hafandi, A., Jois, M., et al. (2012). Angiotensin-converting Enzyme Inhibition Reverses Diet-Induced Obesity, Insulin Resistance and Inflammation in C57BL/6J Mice. *Int. J. Obes.* 36 (2), 233–243. doi:10.1038/ijo.2011.95

Qiao, J., Li, Y.-S., Zeng, R., Liu, F.-L., Luo, R.-H., Huang, C., et al. (2021). SARS-CoV-2 M Pro Inhibitors with Antiviral Activity in a Transgenic Mouse Model. *Science* 371 (6536), 1374–1378. https://pymol.org. doi:10.1126/science.abf1611

Qing, E., and Gallagher, T. (2020). SARS Coronavirus Redux. *Trends Immunol.* 41 (4), 271–273. doi:10.1016/j.it.2020.02.007

Ragab, D., Salah Eldin, H., Taeimah, M., Khattab, R., and Salem, R. (2020). The COVID-19 Cytokine Storm; what We Know So Far. *Front. Immunol.* 11. doi:10.3389/fimmu.2020.01446

Rai, P., Chuong, C., LeRoith, T., Smyth, J. W., Panov, J., Levi, M., et al. (2021). Adenovirus Transduction to Express Human ACE2 Causes Obesity-specific Morbidity in Mice, Impeding Studies on the Effect of Host Nutritional Status on SARS-CoV-2 Pathogenesis. *Virology* 563, 98–106. doi:10.1016/j.virol.2021.08.014

Rathnasinghe, R., Strohmeier, S., Amanat, F., Gillespie, V. L., Krammer, F., García-Sastre, A., et al. (2020). Comparison of Transgenic and Adenovirus hACE2 Mouse Models for SARS-CoV-2 Infection. *BioRxiv*. doi:10.1101/2020.07.06.190066

Rawle, D. J., Le, T. T., Dumenil, T., Yan, K., Tang, B., Nguyen, W., et al. (2021). ACE2-lentiviral Transduction Enables Mouse SARS-CoV-2 Infection and Mapping of Receptor Interactions. *Plos Pathog.* 17 (7), e1009723. doi:10.1371/JOURNAL.PPAT.1009723

Rockx, B., Kuiken, T., Herfst, S., Bestebroer, T., Lamers, M. M., Oude Munnink, B. B., et al. (2020). Comparative Pathogenesis of COVID-19, MERS, and SARS in a Nonhuman Primate Model. *Science* 368 (6494), 1012–1015. doi:10.1126/SCIENCE.ABB7314

Rongvaux, A., Willinger, T., Martinek, J., Strowig, T., Gearty, S. v., Teichmann, L. L., et al. (2014). Development and Function of Human Innate Immune Cells in a Humanized Mouse Model. *Nat. Biotechnol.* 32 (4), 364–372. doi:10.1038/nbt.2858

Rosenke, K., Hansen, F., Schwarz, B., Feldmann, F., Haddock, E., Rosenke, R., et al. (2021). Orally Delivered MK-4482 Inhibits SARS-CoV-2 Replication in the Syrian Hamster Model. *Nat. Commun.* 12 (1). doi:10.1038/s41467-021-22580-8

Samavati, L., and Uhal, B. D. (2020). ACE2, Much More Than Just a Receptor for SARS-COV-2. *Front. Cell Infect. Microbiol.* 10. doi:10.3389/fcimb.2020.00317

Sarver, D. C., and Wong, G. W. (2021). Obesity Alters Ace2 and Tmprss2 Expression in Lung, Trachea, and Esophagus in a Sex-dependent Manner: Implications for COVID-19. *Biochem. Biophysical Res. Commun.* 538, 92–96. doi:10.1016/j.bbrc.2020.10.066

Sefik, E., Israelow, B., Mirza, H., Zhao, J., Qu, R., Kaffe, E., et al. (2021). A Humanized Mouse Model of Chronic COVID-19. *Nat. Biotechnol*. doi:10.1038/s41587-021-01155-4

Sellers, R. S. (2017). Translating Mouse Models. *Toxicol. Pathol.* 45 (1), 134–145. doi:10.1177/0192623316675767

Shahhosseini, N., Babuadze, G., Wong, G., and Kobinger, G. P. (2021). Mutation Signatures and In Silico Docking of Novel Sars-Cov-2 Variants of Concern. *Microorganisms* 9 (5), 926. doi:10.3390/microorganisms9050926

Sheahan, T. P., Sims, A. C., Zhou, S., Graham, R. L., Pruijssers, A. J., Agostini, M. L., et al. (2020). An Orally Bioavailable Broad-Spectrum Antiviral Inhibits SARS-CoV-2 in Human Airway Epithelial Cell Cultures and Multiple Coronaviruses in Mice. *Sci. Transl. Med.* 12 (541). doi:10.1126/SCITRANSLMED.ABB5883

Shou, S., Liu, M., Yang, Y., Kang, N., Song, Y., Tan, D., et al. (2021). Animal Models for COVID-19: Hamsters, Mouse, Ferret, Mink, Tree Shrew, and Non-human Primates. *Front. Microbiol.* 12. doi:10.3389/fmicb.2021.626553

Shuai, L., Zhong, G., Yuan, Q., Wen, Z., Wang, C., He, X., et al. (2021). Replication, Pathogenicity, and Transmission of SARS-CoV-2 in Minks. *Natl. Sci. Rev.* 8 (3), 2021. doi:10.1093/NSR/NWAA291

Simmons, G., Gosalia, D. N., Rennekamp, A. J., Reeves, J. D., Diamond, S. L., and Bates, P. (2005). Inhibitors of Cathepsin L Prevent Severe Acute Respiratory Syndrome Coronavirus Entry. *Proc. Natl. Acad. Sci. U.S.A.* 102 (33), 11876–11881. doi:10.1073/pnas.0505577102

Skevaki, C., Karsonova, A., Karaulov, A., Xie, M., and Renz, H. (2020). Asthma-associated Risk for COVID-19 Development. *J. Allergy Clin. Immunol.* 146 (6), 1295–1301. doi:10.1016/j.jaci.2020.09.017

Su, Y., Yuan, D., Chen, D. G., Ng, R. H., Wang, K., Choi, J., et al. (2022). Multiple Early Factors Anticipate post-acute COVID-19 Sequelae. *Cell* 185, 881–895. doi:10.1016/j.cell.2022.01.014

Sun, J., Zhuang, Z., Zheng, J., Li, K., Wong, R. L.-Y., Liu, D., et al. (2020). Generation of a Broadly Useful Model for COVID-19 Pathogenesis, Vaccination, and Treatment. *Cell* 182 (3), 734–743. e5. doi:10.1016/j.cell.2020.06.010

Sun, S.-H., Chen, Q., Gu, H.-J., Yang, G., Wang, Y.-X., Huang, X.-Y., et al. (2020). A Mouse Model of SARS-CoV-2 Infection and Pathogenesis. *Cell Host and Microbe* 28 (1), 124–133. e4. doi:10.1016/J.CHOM.2020.05.020/ATTACHMENT/34A485A8-CAD9-409E-AA5B-0EF7B1B54781/MMC1.PDF

Tang, C., Deng, Z., Li, X., Yang, M., Tian, Z., Chen, Z., et al. (2020). Helicase of Type 2 Porcine Reproductive and Respiratory Syndrome Virus Strain HV Reveals a Unique Structure. *Viruses* 12 (2), 215. doi:10.3390/v12020215

Thomas, S. (2020). The Structure of the Membrane Protein of SARS-CoV-2 Resembles the Sugar Transporter SemiSWEET. *Pai* 5 (1), 342–363. doi:10.20411/pai.v5i1.377

Triggle, C. R., Bansal, D., Ding, H., Islam, M. M., Farag, E. A. B. A., Hadi, H. A., et al. (2021). A Comprehensive Review of Viral Characteristics, Transmission, Pathophysiology, Immune Response, and Management of SARS-CoV-2 and COVID-19 as a Basis for Controlling the Pandemic. *Front. Immunol.* 12, 631139. doi:10.3389/fimmu.2021.631139

Tseng, C.-T. K., Huang, C., Newman, P., Wang, N., Narayanan, K., Watts, D. M., et al. (2007). Severe Acute Respiratory Syndrome Coronavirus Infection of Mice Transgenic for the Human Angiotensin-Converting Enzyme 2 Virus Receptor. *J. Virol.* 81 (3), 1162–1173. doi:10.1128/JVI.01702-06/ASSET/DACA1C30-425C-4D1C-8B34-1CE2B664B6C9/ASSETS/GRAPHIC/ZJV0030787060008.JPEG

Turner, A. J., and Hooper, N. M. (2002). The Angiotensin-Converting Enzyme Gene Family: Genomics and Pharmacology. *Trends Pharmacol. Sci.* 23 (4), 177–183. doi:10.1016/s0165-6147(00)01994-5

U.S. Food and Drug Administration (2021b). *Coronavirus (COVID-19) Update: FDA Authorizes Additional Oral Antiviral for Treatment of COVID-19 in Certain Adults*. Silver Spring, MD: FDA. Available at: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-additional-oral-antiviral-treatment-covid-19-certain (Accessed February 11, 2022).

U.S. Food and Drug Administration (2021a). *Coronavirus (COVID-19) Update: FDA Authorizes First Oral Antiviral for Treatment of COVID-19*. Silver Spring, MD: FDA. Available at: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-oral-antiviral-treatment-covid-19 (Accessed February 10, 2022).

Wahl, A., Gralinski, L. E., Johnson, C. E., Yao, W., Kovarova, M., Dinnon, K. H., et al. (2021). SARS-CoV-2 Infection Is Effectively Treated and Prevented by EIDD-2801. *Nature* 591 (7850), 451–457. doi:10.1038/s41586-021-03312-w

Walls, A. C., Park, Y.-J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* 181 (2), 281–292. e6. doi:10.1016/j.cell.2020.02.058

Wark, P. A. B., Pathinayake, P. S., Kaiko, G., Nichol, K., Ali, A., Chen, L., et al. (2021). ACE2 Expression Is Elevated in Airway Epithelial Cells from Older and Male Healthy Individuals but Reduced in Asthma. *Respirology* 26 (5), 442–451. doi:10.1111/resp.14003

Williamson, E. J., Walker, A. J., Bhaskaran, K., Bacon, S., Bates, C., Morton, C. E., et al. (2020). Factors Associated with COVID-19-Related Death Using OpenSAFELY. *Nature* 584 (7821), 430–436. doi:10.1038/s41586-020-2521-4

Winkler, E. S., Chen, R. E., Alam, F., Yildiz, S., Case, J. B., Uccellini, M. B., et al. (2022). SARS-CoV-2 Causes Lung Infection without Severe Disease in Human ACE2 Knock-In Mice. *J. Virol.* 96 (1), e0151121. doi:10.1128/JVI10.1128/JVI.01511-21

Winkler, E. S., Bailey, A. L., Kafai, N. M., Nair, S., McCune, B. T., Yu, J., et al. (2020). SARS-CoV-2 Infection of Human ACE2-Transgenic Mice Causes Severe Lung Inflammation and Impaired Function. *Nat. Immunol.* 21 (11), 1327–1335. doi:10.1038/s41590-020-0778-2

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C.-L., Abiona, O., et al. (2020). Cryo-EM Structure of the 2019-nCoV Spike in the Prefusion Conformation. *Science* 367 (6483), 1260–1263. doi:10.1126/science.abb2507

Xie, Y., Xu, E., Bowe, B., and Al-Aly, Z. (2022). Long-term Cardiovascular Outcomes of COVID-19. *Nat. Med.* 28, 583–590. doi:10.1038/s41591-022-01689-3

Xudong, X., Junzhu, C., Xingxiang, W., Furong, Z., and Yanrong, L. (2006). Age- and Gender-Related Difference of ACE2 Expression in Rat Lung. *Life Sci.* 78 (19), 2166–2171. doi:10.1016/j.lfs.2005.09.038

Yang, X. H., Deng, W., Tong, Z., Liu, Y. X., Zhang, L. F., Zhu, H., et al. (2007b). Mice Transgenic for Human Angiotensin-Converting Enzyme 2 Provide a Model for SARS Coronavirus Infection. *Comp. Med.* 57 (5), 450–459.

Yang, X. H., Deng, W., Tong, Z., Liu, Y.-x., Zhang, L., and Zhu, H. (2007a). Mice Transgenic for Human Angiotensin-Converting Enzyme 2 Provide a Model for SARS Coronavirus Infection. *Comp. Med.* 57, 450–459. *Ingenta Connect*. from https://www.ingentaconnect.com/content/aalas/cm/2007/00000057/00000005/art00003# (Retrieved March 6, 2022).

Yong, S. J. (2021). Long COVID or post-COVID-19 Syndrome: Putative Pathophysiology, Risk Factors, and Treatments. *Infect. Dis.* 53 (Issue 10), 737–754. doi:10.1080/23744235.2021.1924397

Yoon, H. E., Kim, E. N., Kim, M. Y., Lim, J. H., Jang, I.-A., Ban, T. H., et al. (2016). Age-Associated Changes in the Vascular Renin-Angiotensin System in Mice. *Oxidative Med. Cell Longevity* 2016, 1–14. doi:10.1155/2016/6731093

Zhang, X., Kousoulas, K. G., and Storz, J. (1992). The Hemagglutinin/esterase Gene of Human Coronavirus Strain OC43: Phylogenetic Relationships to Bovine and Murine Coronaviruses and Influenza C Virus. *Virology* 186 (1), 318–323. doi:10.1016/0042-6822(92)90089-8

Zhang, Y.-N., Zhang, Z.-R., Zhang, H.-Q., Li, X.-D., Li, J.-Q., Zhang, Q.-Y., et al. (2021). Increased Morbidity of Obese Mice Infected with Mouse-Adapted SARS-CoV-2. *Cell Discov* 7 (Issue 1), 74. doi:10.1038/s41421-021-00305-x

Zhou, S., Hill, C. S., Sarkar, S., Tse, L. v., Woodburn, B. M. D., Schinazi, R. F., et al. (2021). β-d-N4-hydroxycytidine Inhibits SARS-CoV-2 through Lethal Mutagenesis but Is Also Mutagenic to Mammalian Cells. *J. Infect. Dis.* 224 (3), 415–419. doi:10.1093/infdis/jiab247

Zhu, Z., Hasegawa, K., Ma, B., Fujiogi, M., Camargo, C. A., Jr., and Liang, L. (2020). Association of Asthma and its Genetic Predisposition with the Risk of Severe COVID-19. *J. Allergy Clin. Immunol.* 146 (2), 327–e4. doi:10.1016/j.jaci.2020.05.02110.1016/j.jaci.2020.06.001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

MINI REVIEW
published: 10 May 2022
doi: 10.3389/fddsv.2022.899239

Furin and COVID-19: Structure, Function and Chemoinformatic Analysis of Representative Active Site Inhibitors

Bruno O. Villoutreix[1,2]*, *Iker Badiola*[3] *and Abdel-Majid Khatib*[4,5]*

[1]Université Paris Cité, Inserm UMR 1141 NeuroDiderot, Robert-Debré Hospital, Paris, France, [2]Akttyva Therapeutics, Inc., Mansfield, MA, United States, [3]Department of Cell Biology and Histology, Faculty of Medicine and Nursing, University of Basque Country (UPV/EHU), Leioa, Spain, [4]RYTME, Bordeaux Institute of Oncology (BRIC)- UMR1312 Inserm, University of Bordeaux, Pessac, France, [5]Institut Bergonié, Bordeaux, France

Furin is involved in the endoproteolytic processing of various protein precursors implicated in many diseases such as diabetes, obesity, atherosclerosis, cancer, Alzheimer's disease and viral infection including COVID-19. Recently, cell entry of SARS-CoV-2 was found to require sequential cleavage of the viral spike glycoprotein (S protein) at the S1/S2 and the S2′ cleavage sites. The S1/S2 site (PRRAR) can be cleaved by the proprotein convertase furin that facilitates membrane fusion and viral spread. Here we summarized the recent findings on furin and S protein structures, the role of S protein cleavage by furin during SARS-CoV-2 infection. We analyzed 12 diverse representative inhibitors of furin using a chemoinformatic approach starting from a list of 628 compounds downloaded from the ChEMBL database. Among those, only 76 survived a soft rule of five filtering step. Structural alerts are present on most of these molecules while some compounds are also predicted to act on toxicity targets. No clinical trials are presently listed at the ClinicalTrials.gov website regarding small molecule inhibitors of furin.

Keywords: furin, COVID-19, small molecules, drug discovery, S protein

OPEN ACCESS

Edited by:
Ho Leung Ng,
Kansas State University, United States

Reviewed by:
José J. Naveja,
Johannes Gutenberg University Mainz, Germany

***Correspondence:**
Bruno O. Villoutreix
Bruno.villoutreix@inserm.fr
Abdel-Majid Khatib
majid.khatib@inserm.fr

Specialty section:
This article was submitted to In Silico Methods and Artificial Intelligence for Drug Discovery, a section of the journal Frontiers in Drug Discovery

Received: *18 March 2022*
Accepted: *25 April 2022*
Published: *10 May 2022*

Citation:
Villoutreix BO, Badiola I and Khatib A-M (2022) Furin and COVID-19: Structure, Function and Chemoinformatic Analysis of Representative Active Site Inhibitors. Front. Drug. Discov. 2:899239. doi: 10.3389/fddsv.2022.899239

SARS-COV-2 INFECTION AND FURIN

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the pandemic disease COVID-19, is a vigorous infection that has affected millions of people worldwide. This contagious respiratory infection provokes flu-like symptoms such as cough with or without fever that spreads more easily than flu. Of the infected people, up to 20% seemed to develop respiratory difficulties including pneumonia (Hu et al., 2021).

Like with other coronaviruses (CoVs), SARS-CoV-2 presents spike glycoproteins (S protein) on their viral envelope used by the virus to bind host receptors and fuse with cell membrane, a molecular event crucial for the viral infection. The S proteins are type I transmembrane proteins composed of two subunits S1 and S2. The S1 subunit is responsible for receptor binding while the S2 subunit is involved in membrane fusion (**Figure 1**). The S1 domain contains an N-terminal domain (NTD) and a receptor-binding domain (RBD) used for binding the SARS-CoV-2 receptor: the angiotensin-converting enzyme 2 (ACE2) and heparan sulfate (Clausen et al., 2020; Walls et al., 2020). To facilitate fusion, the S protein that is synthesized as immature protein precursor, is proteolyticaly cleaved at the S1/S2 boundary and at the S2′ site located close to the S2 fusion peptide (FP)

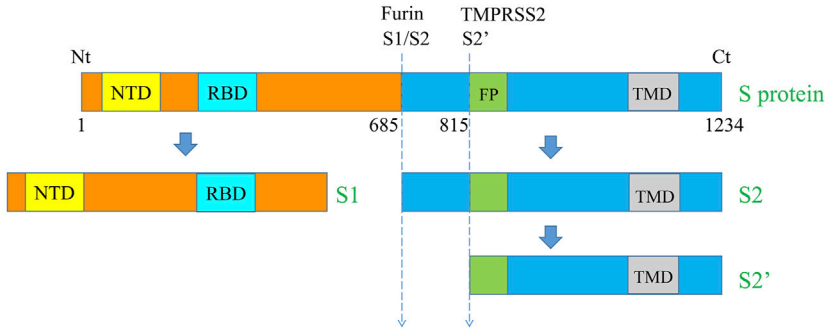


FIGURE 1 | Schematic representation of the major SARS-CoV-2 S protein domains and the cleavage sites. The S protein of SARS-CoV-2 is a transmembrane protein that contains various domains including NTD, RBD, FP, and TMD. The total aa sequence length (1,273 aa) and the position of the cleavage sites S1/S2 (685/686) and S2′ (815/816) are also indicated. NTD, N-terminal domain, RBD, receptor-binding domain, FP, Fusion peptide, TMD, transmembrane domain, Nt, N-terminal and Ct, C-terminal.

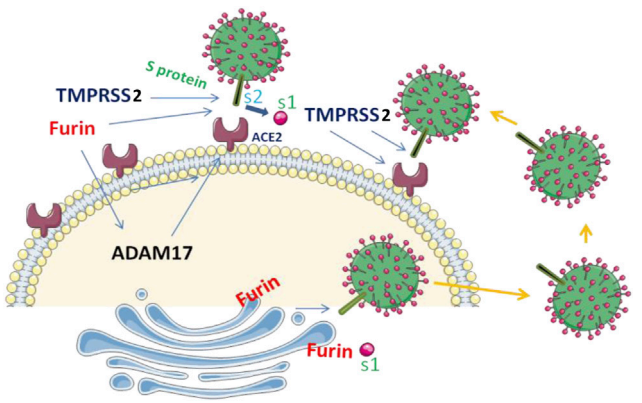


FIGURE 2 | S protein cleavage and SARS-CoV-2 infection. SARS-CoV-2 entry into the cells is mediated by the binding of the viral spike (S) protein to ACE2 and the cleavage of S protein by furin and TMPRSS2 at the S1/S2 site. ACE2 can also be cleaved by TMPRSS2 and ADAM17 and ADAM17 precursor involves furin for its cleavage and activation.

(**Figure 1**). Indeed, upon cleavage at the S1/S2 site, and engagement of ACE2 with the spike RBD domain, the S2′ site is also cleaved by serine proteases, leading to S2 fusion peptide generation that allows viral–host membrane fusion initiation (**Figure 2**).

Earlier studies revealed that SARS-CoV-2 has superior affinity for the ACE2 receptor compared to SARS-CoV (Shang et al., 2020; Lemmin et al., 2020) and was associated to S protein cleavage by furin, a proprotein convertase member (also known as dibasic-processing enzyme) that is ubiquitously expressed and involved in the proteolytic activation of various protein precursors (Siegfried et al., 2020; Soulet et al., 2020; Scamuffa et al., 2014). Indeed, cell entry of SARS-CoV-2 depends on binding of the viral S protein to the cellular receptor ACE2 and on S protein priming by furin and TMPRSS2. TMPRSS2 also cleaves ACE2 that augment viral infectivity (**Figure 2**) (Heurich et al., 2014; Hoffmann et al., 2020). In addition, TMPRSS2 competes with the ADAM17 for ACE2 cleavage (Haga et al., 2008; Haga et al., 2010; Wong et al., 2015). Like various MMPs, ADAM17 precursor also requires furin for its activation (Wong et al., 2015). Thereby, the high expression of furin as found in lung could be exploited by the SARS-CoV-2 for the cleavage of S protein and ACE2 leading to enhanced spread of the virus. However, although furin is the main protease responsible for the cleavage at the site S1/S2, this proteolytic cleavage may also happen in the absence of furin, suggesting the involvement of other proprotein convertases in these processes (Jaimes et al., 2020; Wrobel et al., 2020).

In comparison to all known SARS-CoVs, the protein of SARS-CoV-2 possesses a unique four-residue PRRA insertion at the cleavage site S1/S2 (**Figure 1**). Studies analyzing the effect of the mutation of this site in various cell lines and animal models revealed that this deletion significantly reduced viral infection. On the other hand, the cleavage at the S1/S2 site was found not to be required for infection and replication in Vero E6 cells (Lau et al., 2020; Johnson et al., 2021; Peacock et al., 2021). Lately, using infected hamster models, Lau et al. reported that compared to the wild-type, SARS-CoV-2 with mutated S1/S2 site replicated weakly in tracheal and lung tissues (Lau et al., 2020). Accordingly, deletion of this site was confirmed to reduce by up to 10-fold the replication in human cell lines, and reduce the severity of COVID-19 in hamsters and K18-hACE2 transgenic mice (Johnson et al., 2021). Taken together, these findings indicate the potential use of furin inhibitors to modulate SARS-CoV-2 infection.

FURIN 3D STRUCTURE, SUBSTRATES AND INHIBITORS

Furin 3D Structure

Twenty-four crystal structures of furin are available at the Protein Data Bank (Burley et al., 2021) at the time of writing. Furin is a 794 amino acid calcium-dependent multidomain enzyme. The structure of furin in complex with meta-guanidinomethyl-Phac-RVR-Amba (PDB file 5JXH) at 2.0 Angstrom resolution (Dahms et al., 2014) is shown as an example (**Figure 3A**). Only the

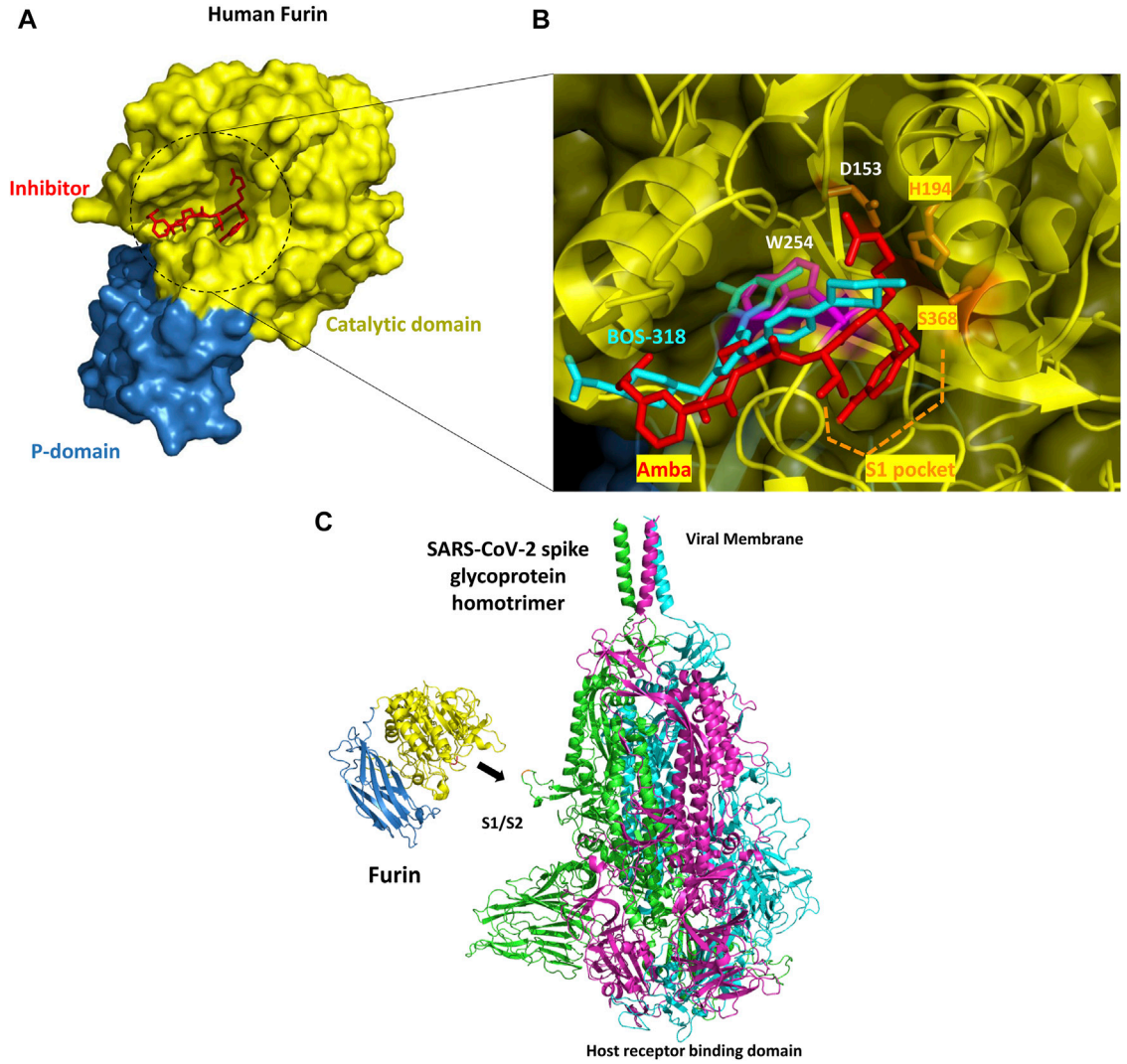


FIGURE 3 | (A) Overall structure of human furin. The molecular surface is shown (the catalytic domain and the P-domain are in yellow and blue, respectively) with a view down the active site (PDB file 5JXH). A small inhibitor (meta-guanidinomethyl-Phac-RVR-Amba or Amba compound) co-crystallized in the catalytic site is shown in red **(B)** Zoom into the catalytic site. The experimental structure of BOS-318 in complex with furin (PDB file 7LCU) was superimposed onto furin co-crystallized with an Amba compound (PDB file 5JXH). Amba compounds and in general all known furin inhibitors have a P1 residue inserted into the S1 pocket and they make contacts with the catalytic triad residues. By contrast, BOS-318 does not directly engage with the catalytic triad, does not interact with the S1 pocket and seems to induce the flip of furin W254 (magenta, side chain present in 5JXH), thereby creating novel binding subpocket that allows the binding of the dichlorophenyl moiety of BOS-318 (without such change in the orientation of the W side chain, major steric clashes would occur and the binding of BOS-318 would not be possible) **(C)** Overview of the SARS-CoV-2 spike glycoprotein homotrimer - human furin complex. Furin was manually positioned nearby the S1/S2 region cleavage site. The three monomers of the SARS-CoV-2 S protein are colored green, magenta, and cyan while furin is showed in blue and yellow. The catalytic site of the enzyme is next to the S protein PRRAR cleavage site loop (in red). There seems to be enough room to accommodate three furin molecules on the S protein trimer at the same time.

catalytic and P domains are present in these experimental 3D structures (i.e., the other domains: the cysteine-rich domain, the transmembrane domain and the C-terminal cytoplasmic domain are not visible). On the N-terminal side, the protein also contains a so-called prodomain that acts as an intramolecular chaperone, and seems important to assist proper folding of the enzyme. This prodomain was reported to regulate the furin activity *in vitro* and *in vivo* (López et al., 2021; Descarpentrie et al., 2022). The catalytic domain is similar to the one of subtilisin, it consists of a twisted beta-sheet composed of seven parallel and one antiparallel beta-stands flanked by five adjacent and two peripheral helices (Henrich et al., 2003). Yet, the active site cleft of furin differs from that of subtilisins with respect to depth, shape and charges (i.e., the furin active site is mainly electronegative with at least six negatively charged residues directly surrounding the Ser368-His194-Asp153 active site triad). Furin preferentially recognizes/cleaves the sequence motif RXR/KR-X (the "-" indicates the scissile peptide bond) found in various protein precursors (Siegfried et al., 2005; Scamuffa et al., 2008; He et al., 2020a). While the catalytic

activity of furin is strictly calcium-dependent, the three calcium ions found in the catalytic domain are not directly involved in the catalytic reaction, they are indirectly important by forming the right geometry of the active site. The adjacent P domain is organized as a separate eight-stranded β-sandwich and is thought to be important for the catalytic activity of the enzyme.

New information about the catalytic site have been recently reported with the experimental structure of furin in complex with a small inhibitor named BOS-318 (Douglas et al., 2022). Furin inhibitors usually make significant contacts with the catalytic triad residues and have a P1 residue inserted into the S1 binding pocket (**Figure 3B**). This is the case for instance with the Amba family of compounds. But the selective BOS-318 inhibitor does not make such contacts (PDB file 7LCU). Surprisingly, BOS-318 seems to induce a flip of furin W254 which in turn creates a novel binding pocket that is filled by the dichlorophenyl moiety of BOS-318. This novel mode of binding found in that study provides novel insights to rationally design novel inhibitors of furin.

Spike Substrate

Furin have been linked to various pathologies including Alzheimer's disease, tumorigenesis, and infections (Thomas, 2002). In all these diseases, furin activity was found to mediate the proteolytic activation of various protein precursors directly linked to the initiation and/or progression of these pathologies. The cleavage of the furin substrates can occurs inside the cell at the Golgi apparatus and on the cell surface membrane where furin is located (Molloy et al., 1998). Indeed, furin is predominantly localized to the TGN can reach the plasma membrane and internalized back to the TGN (Molloy et al., 1998). In Alzheimer's disease, furin mediates the cleavage of α- and β-secretase precursors, involved in the accumulation of amyloid-β (Aβ), the principal constituent of senile plaques (He et al., 2020a). The implication of furin in tumor progression was revealed based on changes in several biological functions that directly affect the malignant and the metastatic potential of tumor cells after furin inhibition (Basak et al., 2010; He et al., 2020a; Siegfried et al., 2020). This through repressed processing and activation of many molecules involved in cell proliferation, adhesion, invasion and survival (Scamuffa et al., 2008; Basak et al., 2009; López et al., 2021). The protein precursors include metalloproteases, adhesion molecules, growth factors, and growth factor receptors (He et al., 2020b; Siegfried et al., 2020). The role of furin in various viral infection is also well established ((Jaimes et al., 2020)– (Burley et al., 2021)). The acquisition of the infectious capacity and/or cell-cell spread of various viruses require the maturation of the viral cell surface glycoproteins by furin. These include HIV-1, Ebola virus, Hong Kong influenza virus, the severe acute respiratory syndrome coronavirus (Anderson et al., 1993; Basak et al., 2001; Thomas, 2002). Inhibition of processing of some of these viral surface glycoproteins by furin inhibitors was found to abrogate the virus-induced cellular cytopathicity (Anderson et al., 1993; Basak et al., 2001; Thomas, 2002).

In the context of COVID-19, it was suggested that furin could cleave the SARS-CoV-2 S protein. The experimental structure of furin is seen oriented toward a cleavage site, located between the S1/S2 subunits of the S protein and present on a solvent exposed loop structure (**Figure 3C**). This loop should accommodate into the deep groove located in the catalytic domain of the protease and should make numerous hydrophobic, polar and electrostatic interactions with the enzyme. The S proteins assemble as a trimer and are in part inserted into the viral membrane. The molecule system is formed of three identical protomers that strongly binds to ACE2 receptor upon activation, a receptor found on the surface of many cell types. The S protein is here a theoretical 3D model structure derived from several cryo-EM structures (Fertig et al., 2022) including PDB files 6VSB (Wrapp et al., 2020) and 6XR8 (Mendez et al., 2019). The model was developed because some loops were missing in the experimental structures. The binding of the S protein to the ACE2 receptor requires several proteolytic cleavages and conformational changes. One such cleavage on the S protein (cleavage motif PRRAR) is performed by furin, at least on one site located at the S1/S2 boundary. This cleavage is expected to promote disordering of the S protein and then exposure of a domain (the RBD domain) critical for ACE2 binding. Yet, as mentioned above, S protein processing can occur independently of furin although the presence of this protease significantly increases cleavage.

Small Molecules Inhibitors

It has been suggested that furin inhibitors would be very valuable to reduce but not completely abolish viral spread. Several small molecules have been shown to inhibit furin. Yet, most of the time, these involve peptides and peptide-like structures that are often not well suited for human administration due to short half-life, instability, degradation by proteases or difficulties to administrate via oral route. In fact, it seems that most furin preclinical candidates have been stopped thus far due to various absorption, distribution, metabolism, excretion and toxicity problems (ADME-Tox, e.g., cellular toxicity, low cell permeability, chemical instability, etc.) (see below). A search with furin as keyword at the https://www.clinicaltrials.gov/ website did not output ongoing clinical trials involving direct small molecule inhibitors. To investigate previously reported furin inhibitors, we mined the ChEMBL database (Mendez et al., 2019). We downloaded 628 compounds that have been tested on furin and removed molecules that are too far away from the rule of 5. This rule was introduced in 1997, and suggests that to administrate a small molecule by oral route, the molecular weight (MW) of the compound should be less than 500 daltons, the lipophilicity (evaluated via computed logP) cLogP should not exceed 5, the H-bond donor count (sum of OH + NH groups) should be less than 5 and the H-bond acceptor count (sum of O + N atoms) should be less than 10 (Lipinski, 2000). As some of the compounds available in ChEMBL for furin have not been extensively optimized toward clinical candidates, we initially only applied a soft filter on MW and selected molecules with MW ~600 or less. This simple filtering step led to the selection of 76 molecules. Then we focused on activity characterized by the so-called pChEMBL value. As defined at the ChEMBL website, the pChEMBL value allows a number of roughly comparable measures of half-maximal response concentration/potency/affinity to be compared on a negative logarithmic scale. For

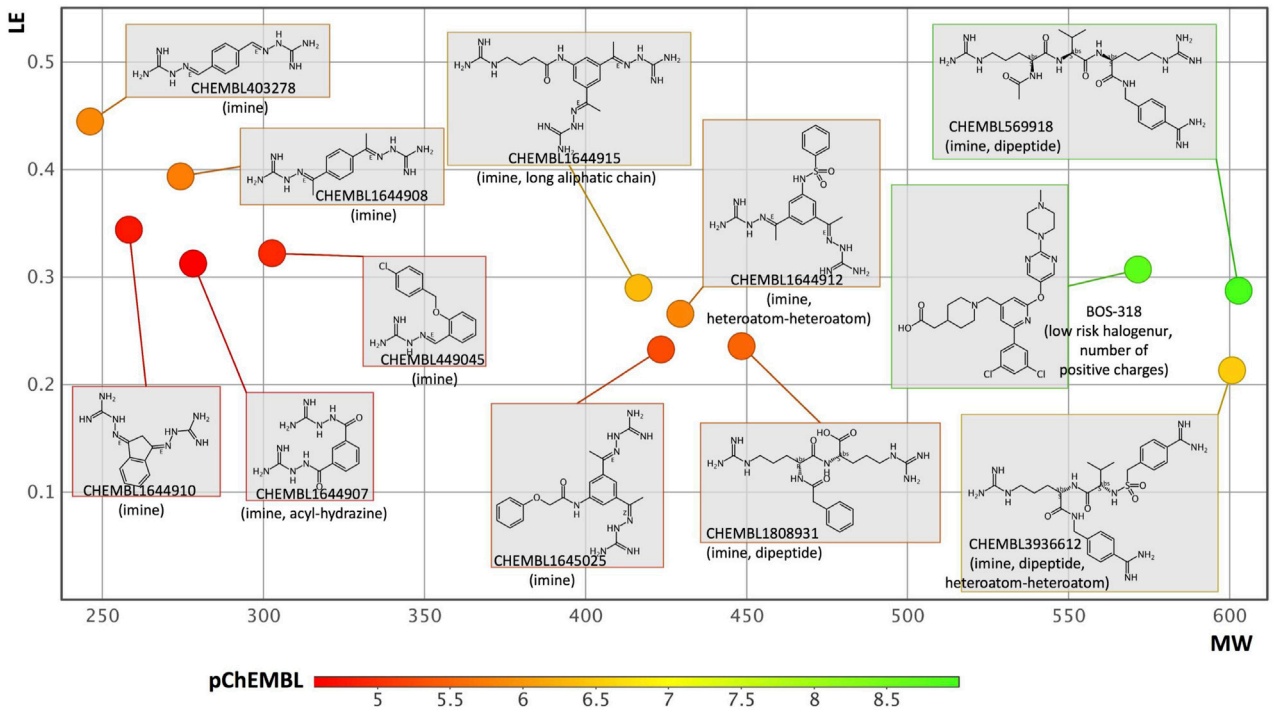


FIGURE 4 | Analysis of furin inhibitors present in the ChEMBL database. After applying simple filtering rules (see text), removing duplicates and clustering the molecules based upon structural similarities, 11 molecules were selected. To these compounds, a recently co-crystallized selective furin inhibitor named BOS-318 was added. The plot considers MW and LE values while the compounds are color coded according to the their pChEMBL values (i.e., green means more potent). Next to each compound, the ChEMBL-ID (or name) is written. Some structural alerts and physico-chemical problems that may impede development are noted between parentheses. These data were generated with the FAF-Drugs server. More specific endpoints were computed with the interpretable-ADMET server, for some compounds no information could be obtained as outside the applicability domain of the method, for the others, the main warnings are as follow: compound CHEMBL403278, putative inhibition of hERG (cardiotoxicity), and inhibition of OATP1B3 organic anion transporting polypeptides (a critical transporter); CHEMBL1644908 or CHEMBL1644915, putative inhibitor of hERG; CHEMBL1644910 or CHEMBL449045, putative inhibitor of hERG and OATP1B3; CHEMBL1645025, putative hERG and Pgp-inhibitor and possible respiratory toxicity; CHEMBL1808931, putative inhibitor of OATP1B3, modulator of nuclear receptors and possible respiratory toxicity; CHEMBL1644912 putative inhibitor of hERG and modulator of nuclear receptors; CHEMBL569918, putative inhibitor of OATP1B3; BOS-318, CHEMBL3936612 and CHEMBL1644907, tend to be outside the applicability domain of the method and further computations will be required.

example, an IC50 measurement of 1 nM would have a pChEMBL value of 9. This value is defined as: -log(molar IC50, XC50, EC50, AC50, Ki, Kd or Potency). It was possible to obtain a pChEMBL value for only 42 molecules. Then, removing duplicate compounds and clustering the molecules by structural similarities, we ended up with 11 drug-like molecules that inhibit human furin (**Figure 4**). To these compounds we manually added a molecule missing in the ChEMBL database named BOS-318 (**Figure 3B**), this compound has been recently co-crystallized with furin and was shown to be selective and cell-permeable (Douglas et al., 2022).

Analysis of the molecules was performed with our software FAF-Drugs (Lagorce et al., 2017) and the visualization carried out with DataWarrior (Sander et al., 2015). While potency is an important parameter for drug discovery, other metrics can be used to evaluate small molecules and help to select compounds for further developments. One such metric is called ligand efficiency or LE. This value normalizes the experimental activity to molecular weight, heavy atom count, or other molecular size quantifier (i.e., an acceptable level for LE is around ≥ 0.3) (Hopkins et al., 2004). LE metrics are thus considered as valuable indicator of compound quality in the early stage of drug discovery as they help focus on molecules that are relatively small and yet that have a significant target activity, suggesting that optimization of the molecules should be easier. Indeed, in most cases, compound optimization usually deteriorates parameters such as MW and log P (e.g., increasing these two parameters often lead to increased interaction with off-targets and anti-targets, render oral administration more difficult. . .). In **Figure 4**, we plotted LE as a function of MW (e.g., to for instance see if compounds could grow in size) while color coding the molecules with the computed pChEMBL value. We note for instance a peptide-like molecule (CHEMBL569918) with a high MW that shows high potency with a reasonable starting LE, yet such molecule would require significant optimization before becoming a clinical candidate. Some molecules with a relatively small MW could be more interesting to optimize as they have a favorable LE but the starting chemistry is eventually not optimal. Indeed, some structural alerts (i.e., toxicophores, simple means to flag potential chemical hazards and problematic compounds) are often noticed in these molecules (please see details about

major structural alerts at the FAF-Drugs website). Such chemical groups often impede further developments (e.g., many reactive warheads react with off-targets) and/or have been found to interfere with the reading of high-throughput screening experiments and/or can be promiscuous aggregators. Next to more global physical-chemistry properties and structural alerts mentioned above, more specific ADMET endpoints can also be predicted. We used the recently reported Interpretable-ADMET web service (Wei et al., 2022) and found that several compounds could be inhibitors of important toxicity targets such as hERG (cardiotoxicity) (see legend of **Figure 4**). Overall, this analysis suggests that important medicinal chemistry efforts will be needed to develop furin inhibitors that could enter clinical trials. Yet, the available compounds together with several experimental structures are highly valuable and should definitively facilitate the design of novel, more ADME-Tox friendly inhibitors exploiting novel furin subpockets.

CONCLUSION

Since its discovery in the 1980s, furin has acquired the status of therapeutic target for the treatment of various diseases including cancer (Siegfried et al., 2020; He et al., 2022). The discovery of a furin cleavage site in the S protein of SARS-CoV-2 virus further stimulates research on this enzyme for the treatment of infectious diseases. The availability of several high-resolution experimental 3D structures in complex with different types of inhibitors (peptide-like and non-peptide like) should facilitate the design of novel high-quality inhibitors (Osman et al., 2022). However, furin is ubiquitous and mediates the activation of various constitutively secreted substrates involved in numerous physiological processes such as angiogenesis, extracellular matrix remodeling and others (Scamuffa et al., 2006; Lahlil et al., 2009). Thereby the use of furin inhibitors as therapeutic approach may affect these biological functions and induces several side effects. In addition, the ability of furin to control the expression and/or activity of TMPRSS2, and ADAM17 (Haga et al., 2008; Haga et al., 2010; Heurich et al., 2014; Wong et al., 2015; Hoffmann et al., 2020), suggests that furin inhibitors can also interfere with the biological function mediated by these proteases. Thereby, prior any application in clinical setting, the side effect of the developed furin inhibitors should be evaluated and more effort should be oriented toward the development of reversible furin inhibitors, of which the action could be stopped when necessary to prevent any potential side effects. Taken together, while it is clear that furin is a challenging therapeutic target, it definitively deserves further investigation.

AUTHOR CONTRIBUTIONS

BV, IB and A-MK wrote and drafted the manuscript. All authors contributed to the article and approved the final version.

FUNDING

This work was supported by SPARK 2020 (University of Bordeaux) to A-MK and grants from INCA and ARC (BV). This work was supported by the INSERM institute.

REFERENCES

Anderson, E. D., Thomas, L., Hayflick, J. S., and Thomas, G. (1993). Inhibition of HIV-1 Gp160-dependent Membrane Fusion by a Furin-Directed Alpha 1-antitrypsin Variant. *J. Biol. Chem.* 268, 24887. doi:10.1016/s0021-9258(19)74548-7

Basak, A., Chen, A., Scamuffa, N., Mohottalage, D., Basak, S., and Khatib, A.-M. (2010). Blockade of Furin Activity and Furin-Induced Tumor Cells Malignant Phenotypes by the Chemically Synthesized Human Furin Prodomain. *Curr. Med. Chem.* 17, 2214–2221. doi:10.2174/092986710791331040

Basak, A., Khatib, A.-M., Mohottalage, D., Basak, S., Kolajova, M., Bag, S. S., et al. (2009). A Novel Enediynyl Peptide Inhibitor of Furin that Blocks Processing of proPDGF-A, B and proVEGF-C. *PLoS ONE* 4, e7700. doi:10.1371/journal.pone.0007700

Basak, A., Zhong, M., Munzer, J. S., Chrétien, M., and Seidah, N. G. (2001). Implication of the Proprotein Convertases Furin, PC5 and PC7 in the Cleavage of Surface Glycoproteins of Hong Kong, Ebola and Respiratory Syncytial Viruses: a Comparative Analysis with Fluorogenic Peptides. *Biochem. J.* 353, 537–545. doi:10.1042/0264-6021:3530537

Burley, S. K., Bhikadiya, C., Bi, C., Bittrich, S., Chen, L., Crichlow, G. V., et al. (2021). RCSB Protein Data Bank: Powerful New Tools for Exploring 3D Structures of Biological Macromolecules for Basic and Applied Research and Education in Fundamental Biology, Biomedicine, Biotechnology, Bioengineering and Energy Sciences. *Nucleic Acids Res.* 49, D437–D451. doi:10.1093/nar/gkaa1038

Clausen, T. M., Sandoval, D. R., Spliid, C. B., Pihl, J., Perrett, H. R., Painter, C. D., et al. (2020). SARS-CoV-2 Infection Depends on Cellular Heparan Sulfate and ACE2. *Cell* 183, 1043–1057. doi:10.1016/j.cell.2020.09.033

Dahms, S. O., Hardes, K., Becker, G. L., Steinmetzer, T., Brandstetter, H., and Than, M. E. (2014). X-ray Structures of Human Furin in Complex with Competitive Inhibitors. *ACS Chem. Biol.* 9, 1113–1118. doi:10.1021/cb500087x

Descarpentrie, J., Araúzo-Bravo, M. J., He, Z., François, A., González, Á., Garcia-Gallastegi, P., et al. (2022). Role of Furin in Colon Cancer Stem Cells Malignant Phenotype and Expression of LGR5 and NANOG in KRAS and BRAF-Mutated Colon Tumors. *Cancers* 14, 1195. doi:10.3390/cancers14051195

Douglas, L. E. J., Reihill, J. A., Ho, M. W. Y., Axten, J. M., Campobasso, N., Schneck, J. L., et al. (2022). A Highly Selective, Cell-Permeable Furin Inhibitor BOS-318 Rescues Key Features of Cystic Fibrosis Airway Disease. *Cell Chem. Biol.* (22), S2451. doi:10.1016/j.chembiol.2022.02.001

Fertig, T. E., Chitoiu, L., Terinte-Balcan, G., Peteu, V. E., Marta, D., and Gherghiceanu, M. (2022). The Atomic Portrait of SARS-CoV-2 as Captured by Cryo-electron Microscopy. *J. Cell. Mol. Medi* 26, 25–34. doi:10.1111/jcmm.17103

Haga, S., Nagata, N., Okamura, T., Yamamoto, N., Sata, T., Yamamoto, N., et al. (2010). TACE Antagonists Blocking ACE2 Shedding Caused by the Spike Protein of SARS-CoV Are Candidate Antiviral Compounds. *Antivir. Res.* 85, 551–555. doi:10.1016/j.antiviral.2009.12.001

Haga, S., Yamamoto, N., Nakai-Murakami, C., Osawa, Y., Tokunaga, K., Sata, T., et al. (2008). Modulation of TNF-α-Converting Enzyme by the Spike Protein of SARS-CoV and ACE2 Induces TNF-α Production and Facilitates Viral Entry. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7809–7814. doi:10.1073/pnas.0711241105

He, Z., Khatib, A.-M., and Creemers, J. W. M. (2020). Loss of the Proprotein Convertase Furin in T Cells Represses Mammary Tumorigenesis in Oncogene-Driven Triple Negative Breast Cancer. *Cancer Lett.* 484, 40–49. doi:10.1016/j.canlet.2020.05.001

He, Z., Khatib, A.-M., and Creemers, J. W. M. (2022). The Proprotein Convertase Furin in Cancer: More Than an Oncogene. *Oncogene* 41, 1252–1262. doi:10.1038/s41388-021-02175-9

He, Z., Thorrez, L., Siegfried, G., Meulemans, S., Evrard, S., Tejpar, S., et al. (2020). The Proprotein Convertase Furin Is a Pro-oncogenic Driver in KRAS and BRAF Driven Colorectal Cancer. *Oncogene* 39, 3571–3587. doi:10.1038/s41388-020-1238-z

Henrich, S., Cameron, A., Bourenkov, G. P., Kiefersauer, R., Huber, R., Lindberg, I., et al. (2003). The Crystal Structure of the Proprotein Processing Proteinase Furin Explains its Stringent Specificity. *Nat. Struct. Mol. Biol.* 10, 520–526. doi:10.1038/nsb941

Heurich, A., Hofmann-Winkler, H., Gierer, S., Liepold, T., Jahn, O., and Pöhlmann, S. (2014). TMPRSS2 and ADAM17 Cleave ACE2 Differentially and Only Proteolysis by TMPRSS2 Augments Entry Driven by the Severe Acute Respiratory Syndrome Coronavirus Spike Protein. *J. Virol.* 88, 1293–1307. doi:10.1128/JVI.02202-13

Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* 181, 271–280. doi:10.1016/j.cell.2020.02.052

Hopkins, A. L., Groom, C. R., and Alex, A. (2004). Ligand Efficiency: a Useful Metric for Lead Selection. *Drug Discov. Today* 9, 430–431. doi:10.1016/S1359-6446(04)03069-7

Hu, B., Guo, H., Zhou, P., and Shi, Z.-L. (2021). Characteristics of SARS-CoV-2 and COVID-19. *Nat. Rev. Microbiol.* 19, 141–154. doi:10.1038/s41579-020-00459-7

Jaimes, J. A., Millet, J. K., and Whittaker, G. R. (2020). Proteolytic Cleavage of the SARS-CoV-2 Spike Protein and the Role of the Novel S1/S2 Site. *iScience* 23, 101212. doi:10.1016/j.isci.2020.101212

Johnson, B. A., Xie, X., Bailey, A. L., Kalveram, B., Lokugamage, K. G., Muruato, A., et al. (2021). Loss of Furin Cleavage Site Attenuates SARS-CoV-2 Pathogenesis. *Nature* 591, 293–299. doi:10.1038/s41586-021-03237-4

Lagorce, D., Bouslama, L., Becot, J., Miteva, M. A., and Villoutreix, B. O. (2017). FAF-Drugs4: Free ADME-Tox Filtering Computations for Chemical Biology and Early Stages Drug Discovery. *Bioinformatics* 33, 3658–3660. doi:10.1093/bioinformatics/btx491

Lahlil, R., Calvo, F., and Khatib, A.-M. (2009). The Potential Anti-tumorigenic and Anti-metastatic Side of the Proprotein Convertases Inhibitors. *Recent. Pat Anticancer. Drug. Discov* 4, 83–91. doi:10.2174/157489209787002470

Lau, S.-Y., Wang, P., Mok, B. W.-Y., Zhang, A. J., Chu, H., Lee, A. C.-Y., et al. (2020). Attenuated SARS-CoV-2 Variants with Deletions at the S1/S2 Junction. *Emerg. Microbes Infect.* 9, 837–842. doi:10.1080/22221751.2020.1756700

Lemmin, T., Kalbermatter, D., Harder, D., Plattet, P., and Fotiadis, D. (2020). Structures and Dynamics of the Novel S1/S2 Protease Cleavage Site Loop of the SARS-CoV-2 Spike Glycoprotein. *J. Struct. Biol. X* 4, 100038. doi:10.1016/j.yjsbx.2020.100038

Lipinski, C. A. (2000). Drug-like Properties and the Causes of Poor Solubility and Poor Permeability. *J. Pharmacol. Toxicol. Methods* 44, 235–249. doi:10.1016/s1056-8719(00)00107-6

López, J. J., Siegfried, G., Cantonero, C., Soulet, F., Descarpentrie, J., Smani, T., et al. (2021). Furin Prodomain ppFurin Enhances $Ca2^{+}$ Entry through Orai and TRPC6 Channels' Activation in Breast Cancer Cells. *Cancers* 13, 1670. doi:10.3390/cancers13071670

Mendez, D., Gaulton, A., Bento, A. P., Chambers, J., De Veij, M., Félix, E., et al. (2019). ChEMBL: towards Direct Deposition of Bioassay Data. *Nucleic Acids Res.* 47, D930–D940. doi:10.1093/nar/gky1075

Molloy, S. S., Thomas, L., Kamibayashi, C., Mumby, M. C., and Thomas, G. (1998). Regulation of Endosome Sorting by a Specific PP2A Isoform. *J. Cell Biol.* 142, 1399–1411. doi:10.1083/jcb.142.6.1399

Osman, E. E. A., Rehemtulla, A., and Neamati, N. (2022). Why All the Fury over Furin? *J. Med. Chem.* 65, 2747–2784. doi:10.1021/acs.jmedchem.1c00518

Peacock, T. P., Goldhill, D. H., Zhou, J., Baillon, L., Frise, R., Swann, O. C., et al. (2021). The Furin Cleavage Site in the SARS-CoV-2 Spike Protein Is Required for Transmission in Ferrets. *Nat. Microbiol.* 6, 899–909. doi:10.1038/s41564-021-00908-w

Sander, T., Freyss, J., von Korff, M., and Rufener, C. (2015). DataWarrior: an Open-Source Program for Chemistry Aware Data Visualization and Analysis. *J. Chem. Inf. Model.* 55, 460–473. doi:10.1021/ci500588j

Scamuffa, N., Calvo, F., Chrétien, M., Seidah, N. G., and Khatib, A. M. (2006). Proprotein Convertases: Lessons from Knockouts. *FASEB J.* 20, 1954–1963. doi:10.1096/fj.05-5491rev

Scamuffa, N., Sfaxi, F., Ma, J., Lalou, C., Seidah, N., Calvo, F., et al. (2014). Prodomain of the Proprotein Convertase Subtilisin/kexin Furin (ppFurin) Protects from Tumor Progression and Metastasis. *Carcinogenesis* 35, 528–536. doi:10.1093/carcin/bgt345

Scamuffa, N., Siegfried, G., Bontemps, Y., Ma, L., Basak, A., Cherel, G., et al. (2008). Selective Inhibition of Proprotein Convertases Represses the Metastatic Potential of Human Colorectal Tumor Cells. *J. Clin. Invest.* 118, 352–363. doi:10.1172/JCI32040

Shang, J., Ye, G., Shi, K., Wan, Y., Luo, C., Aihara, H., et al. (2020). Structural Basis of Receptor Recognition by SARS-CoV-2. *Nature* 581, 221–224. doi:10.1038/s41586-020-2179-y

Siegfried, G., Basak, A., Prichett-Pejic, W., Scamuffa, N., Ma, L., Benjannet, S., et al. (2005). Regulation of the Stepwise Proteolytic Cleavage and Secretion of PDGF-B by the Proprotein Convertases. *Oncogene* 24, 6925–6935. doi:10.1038/sj.onc.1208838

Siegfried, G., Descarpentrie, J., Evrard, S., and Khatib, A.-M. (2020). Proprotein Convertases: Key Players in Inflammation-Related Malignancies and Metastasis. *Cancer Lett.* 473, 50–61. doi:10.1016/j.canlet.2019.12.027

Soulet, F., Bodineau, C., Hooks, K. B., Descarpentrie, J., Alves, I., Dubreuil, M., et al. (2020). ELA/APELA Precursor Cleaved by Furin Displays Tumor Suppressor Function in Renal Cell Carcinoma through mTORC1 Activation. *JCI Insight* 5, 129070. doi:10.1172/jci.insight.129070

Thomas, G. (2002). Furin at the Cutting Edge: from Protein Traffic to Embryogenesis and Disease. *Nat. Rev. Mol. Cell Biol.* 3, 753–766. doi:10.1038/nrm934

Walls, A. C., Park, Y.-J., Tortorici, M. A., Wall, A., McGuire, A. T., and Veesler, D. (2020). Structure, Function, and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell* 181, 281–292. doi:10.1016/j.cell.2020.02.058

Wei, Y., Li, S., Li, Z., Wan, Z., and Lin, J. (2022). Interpretable-ADMET: a Web Service for ADMET Prediction and Optimization Based on Deep Neural Representation. *Bioinformatics*. btac192. doi:10.1093/bioinformatics/btac192

Wong, E., Maretzky, T., Peleg, Y., Blobel, C. P., and Sagi, I. (2015). The Functional Maturation of A Disintegrin and Metalloproteinase (ADAM) 9, 10, and 17 Requires Processing at a Newly Identified Proprotein Convertase (PC) Cleavage Site. *J. Biol. Chem.* 290, 12135. doi:10.1074/jbc.M114.624072

Wrapp, D., Wang, N., Corbett, K. S., Goldsmith, J. A., Hsieh, C.-L., Abiona, O., et al. (2020). Cryo-EM Structure of the 2019-nCoV Spike in the Prefusion Conformation. *Science* 367, 1260–1263. doi:10.1126/science.abb2507

Wrobel, A. G., Benton, D. J., Xu, P., Roustan, C., Martin, S. R., Rosenthal, P. B., et al. (2020). SARS-CoV-2 and Bat RaTG13 Spike Glycoprotein Structures Inform on Virus Evolution and Furin-Cleavage Effects. *Nat. Struct. Mol. Biol.* 27, 763–767. doi:10.1038/s41594-020-0468-7

Conflict of Interest: Author BV was employed by the company Akttyva Therapeutics, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

REVIEW
published: 10 May 2022
doi: 10.3389/fddsv.2022.899477

Peptide-Based Strategies Against SARS-CoV-2 Attack: An Updated *In Silico* Perspective

*G. Moroy and P. Tuffery**

Université Paris Cité, CNRS, INSERM, Unité de Biologie Fonctionnelle et Adaptative, Paris, France

Because of its scale and suddenness, the SARS-CoV-2 pandemic has created an unprecedented challenge in terms of drug development. Apart from being natural candidates for vaccine design, peptides are a class of compounds well suited to target protein-protein interactions, and peptide drug development benefits from the progress of *in silico* protocols that have emerged within the last decade. Here, we review the different strategies that have been considered for the development of peptide drugs against SARS-CoV-2. Thanks to progress in experimental structure determination, structural information has rapidly become available for most of the proteins encoded by the virus, easing *in silico* analyses to develop drugs or vaccines. The repurposing of antiviral/antibacterial peptide drugs has not been successful so far. The most promising results, but not the only ones, have been obtained targeting the interaction between SARS-CoV-2 spike protein and the Angiotensin-Converting Enzyme 2, which triggers cellular infection by the virus and its replication. Within months, structure-based peptide design has identified competing for picomolar candidates for the interaction, proving that the development of peptide drugs targeting protein-protein interactions is maturing. Although no drug specifically designed against SARS-CoV-2 has yet reached the market, lessons from peptide drug development against SARS-CoV-2 suggest that peptide development is now a plausible alternative to small compounds.

Keywords: SARS-CoV-2, peptide, in silico, protein-protein interaction, synthetic vaccine

OPEN ACCESS

Edited by:
Bruno Villoutreix, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Ashvinder Raina, Post Graduate Institute of Medical Education and Research (PGIMER), India
Claudio N. Cavasotto, Universidad Austral, Argentina

****Correspondence:***
P. Tuffery
pierre.tuffery@univ-paris-diderot.fr
pierre.tuffery@inserm.fr

Specialty section:
This article was submitted to In silico Methods and Artificial Intelligence for Drug Discovery, a section of the journal Frontiers in Drug Discovery

Received: *18 March 2022*
Accepted: *21 April 2022*
Published: *10 May 2022*

Citation:
Moroy G and Tuffery P (2022) Peptide-Based Strategies Against SARS-CoV-2 Attack: An Updated In Silico Perspective. Front. Drug. Discov. 2:899477. doi: 10.3389/fddsv.2022.899477

1 INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is responsible for COVID-19 (coronavirus disease 2019) and spread rapidly following its emergence in Wuhan in December 2019. While the majority of COVID-19 infections are relatively mild, with most patients recovering in 2–3 weeks, a significant number of patients can develop a severe respiratory illness. Due to the instability of its genome, numerous mutations have appeared in the SARS-CoV-2. Some of them have induced traits considered beneficial for viral adaptation (Pachetti et al., 2020), such as an increase of its transmissibility, an enhancement of its ability to evade natural immunity or a decreasing susceptibility to neutralizing antibodies. Currently, the World Health Organization (WHO) has declared five variants of concern: Alpha, Beta, Delta, Gamma, and Omicron variants (Karim and Karim, 2021; Starr et al., 2021; Tegally et al., 2021; Xie et al., 2021), but the list keeps growing.

Since the end of 2020, several COVID-19 vaccines are available, enabling the reduction of the spread, severity, and death caused worldwide. A monoclonal antibody (sotrovimab) and two

combinations of two monoclonal antibodies (casirivimab/imdevimab and bamlanivimab/etesevimab) have been approved as drugs. However, their manufacturing costs are high and they are not convenient for patients since they are administered by intravenous injection. Currently, four non-biologic drugs have been approved. In October 2020, Remdesivir was the first small molecule approved by Food and Drug Administration (FDA). It is a broad-spectrum antiviral medication, which is administrated by intravenous injection. Remdesivir can be also administrated in combination with baricitinib, a drug approved for the treatment of rheumatoid arthritis. Since July 2021, the FDA authorized the use of baricitinib without remdesivir for patients requiring supplemental oxygen. Interestingly, in March 2022, the RECOVERY (Randomised Evaluation of COVID-19 Therapy) trial showed that baricitinib alone is able to reduce mortality by about 20 percent. In December 2021, Molnupiravir has been approved by the FDA for certain patients for whom other treatments are not possible. Molnupiravir is an anti-viral drug, which acts by preventing RNA virus replication. Since the end of December 2021, a fourth drug, Paxlovid, has been available for people who are at high risk of having severe COVID-19. Paxlovid contains the antiviral medications nirmatrelvir and ritonavir. Baricitinib, Molnupiravir, and Paxlovis are administrated orally to the patient.

Although vaccines and the four available drugs are great therapeutic advances to cure COVID-19, it is still necessary to develop new treatments, which are more convenient or less limiting for the patients (Fenton and Keam, 2022). Even if the small molecules are usually more easily manufactured, more stable, and less costly than biologics (Makurvet, 2021), it is important not to just focus on small molecules to design new drugs. In particular, peptide-based drugs have significant advantages such as low toxicity and better specificity.

Peptides have in recent years gained increased interest as candidate therapeutics, with presently over 80 peptide drugs on the market, more than 150 peptides in clinical development, and over 400 undergoing preclinical studies (Muttenthaler et al., 2021). Peptides are easy to develop both in terms of time and technology and are cost-effective, which makes them good candidates for the development of hits or probes up to the proof of concept. Compared to small compounds, despite they have the advantage of low intrinsic toxicity and better specificity, the main limitations of peptides have long been the mode of delivery, mostly parenteral, and the renal clearance that reduces quickly the bioavailability of the peptide drugs (Muttenthaler et al., 2021). However, recent developments have led to progress on those limitations: new modes of formulation and protection, to cite some, have resulted in improved bioavailability, biostability, and biodelivery. Some peptide drugs on the market such as the Semaglutide or plecanatide, can be administered orally (Zhang & Chen, 2021; Lewis et al., 2022). In addition, progress in cell-penetrating peptides now makes it likely to design peptides tissue, cell, or organelle-specific (Xu et al., 2019), enlarging the landscape of possible applications of peptides targeting PPIs to almost any kind of interaction. Apart from synthetic hormones (Vlieghe et al., 2010) and peptides targeting GPCRs (Davenport et al., 2020), peptides are especially well suited to target protein-protein interactions (Nevola and Giralt, 2015; Wójcik and Berlicki, 2016; Bruzzoni-Giovanelli et al., 2018). Compared to small compounds, peptides have higher molecular weights and can bind to a larger surface, which usually results in binding targets with higher specificity and affinity.

The development of peptide drugs also benefits from the progress of structural biology and structural bioinformatics. SARS-CoV-2 illustrates very well the reactivity of structural biology. Thanks to X-ray diffraction and electron microscopy, many SARS-CoV-2 protein structures have been available as soon as 2020, and most structures have been available not later than mid-2021, with some exceptions, though, as illustrated from the compendium available at RCSB or EBI (https://www.rcsb.org/news/feature/5e74d55d2d410731e9944f52; https://www.ebi.ac.uk/pdbe/covid-19). Furthermore, the structure of some protein complexes contributing to viral infection has also been solved, particularly that of the SARS-CoV-2 Spike protein receptor-binding domain (RBD) in interaction with the Angiotensin-Converting Enzyme 2 (ACE2) [PDB: 6M0J (Wang et al., 2020)]. Although not all molecular mechanisms related to SARS-CoV-2 viral infection are fully understood, this structural information has revealed precious to propose peptide candidates combating the viral attack. Concomitantly, the progress of *in silico* approaches for the prediction of peptide structure, protein-peptide, and protein-protein interactions, as well as progress in conformational sampling, including molecular dynamics (MD) simulations and docking can in principle make *in silico* protocols starting from the structure of the targets effective to identify peptide candidates.

Here, we review the various results obtained so far that exploit *in silico* protocols for peptide development in the context of the SARS-CoV-2 pandemic. We describe shortly efforts to identify immunogenic peptides from the available structures as well as efforts to identify candidate peptide drugs targeting viral infection, and we discuss their expected impact on the road to SARS-CoV-2 drug development.

2 THE DESIGN OF VACCINE BASED ON SYNTHETIC PEPTIDES

T cells have a crucial role in the immune response to viral infections like COVID-19. The presentation of short viral peptides by the human leukocyte antigen (HLA) complex is the first step in the development of T-cell immunity. Thus, the viral peptides presented by HLA class I molecules and HLA class II molecules can activate CD8 T cells and CD4 T cells, respectively. Once activated, CD8 T cells can recognize and kill virus-infected cells. Activated CD4 T cells act by the release of cytokines, which stimulate other immune cells to trigger the appropriate immune response.

At the beginning of 2020, understanding how the immune system reacts to the SARS-CoV-2 infection is critical to the development of vaccines. In this aim, the T cell memory in 42 patients who recovered from SARS-CoV-2 infection and 16

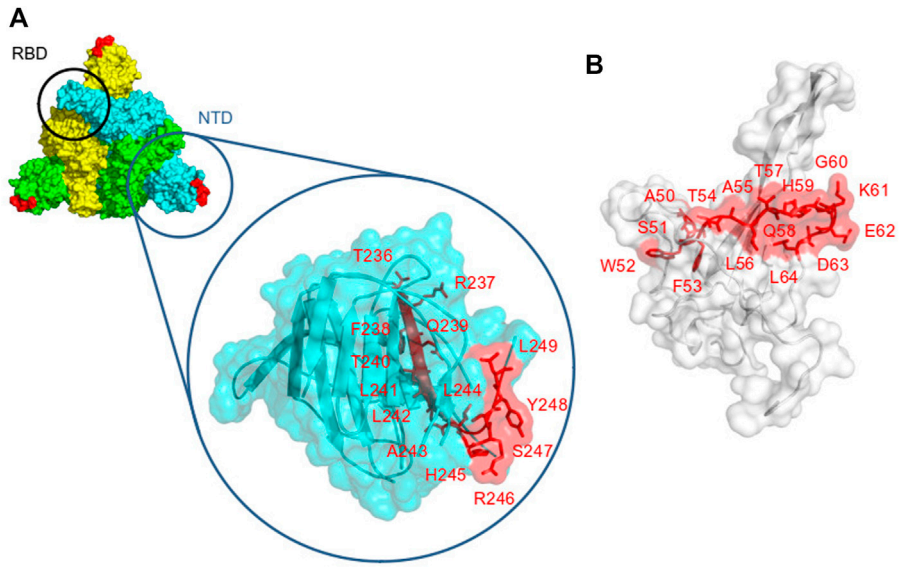


FIGURE 1 | Localization of the epitopes that are contained in CoVac-1 on the viral protein's surfaces. **(A)** spike protein surface [PDB id: 6XLU (Zhou et al., 2020)], spike monomers are colored in green, yellow, and cyan, respectively. The Receptor Binding Domain (RBD) and the N Terminal Domain (NTD) are indicated. The epitope (T_{236}RFQTLLALHRSYL_{249}) is in red. **(B)** nucleocapsid protein surface in white (PDB id: 7CR5 (Kang et al., 2021)), the epitope (A_{50}SWFTALTQHGKEDL$_{64}$) is displayed in red.

unexposed donors has been studied by experimental assays using peptides spanning SARS-CoV-2 (Peng et al., 2020). 41 peptides containing $CD4^+$ and/or $CD8^+$ epitopes have been identified. Among these peptides, three peptides deriving from spike protein, two from membrane protein, and one from nucleocapsid protein were frequently targeted by T cells, suggesting that they can trigger an immune response.

A systematic vaccine-informatics approach has been applied to the spike protein to identify antigenic peptides, which could be used to design a novel vaccine candidate (Alam et al., 2021). Based on the spike protein sequence, they applied several bioinformatics tools to highlight potential immunogenic peptides and assess their autoimmune, allergic, and toxic response. Thus, 12 antigenic peptides have been identified. They have 80%–90% identity with experimentally identified epitopes of SARS-CoV. They are predicted as nontoxic, nonallergenic, and highly antigenic. Moreover, the authors performed docking computations of eight peptides on the surface of the HLA to understand how the peptides interact with HLA. Although the authors are confident in the ability of these peptides to trigger an effective immune response against the SARS-CoV-2, no experimental confirmation supports their conclusions.

Recently, a peptide vaccine candidate, named CoVac-1, completed a phase I clinical trial (Heitmann et al., 2022). CoVac-1 is a peptide composed of multiple SARS-CoV-2 epitopes derived from various viral proteins (**Figure 1**), such as spike, nucleocapsid, membrane, envelope, and open reading frame 8. It contains a synthetic toll-like receptor 1/2 ligand, which acts as an adjuvant to help the vaccine produce a better immune response. From 28 November 2020 to 1 April 2021, 36 healthy adults were enrolled and received one dose of CoVac-1. The participants have been followed up until 56 days after the CoVac-1 injection. 56 adverse effects have been observed, but they were predominately mild (headache, fatigue, nausea . . .) indicating that CoVac-1 has a favorable safety profile. Moreover, T cell responses were persistent 3 months following vaccination and were not affected by mutation of Alpha and Beta variants. CoVac-1 is currently in phase II clinical trial.

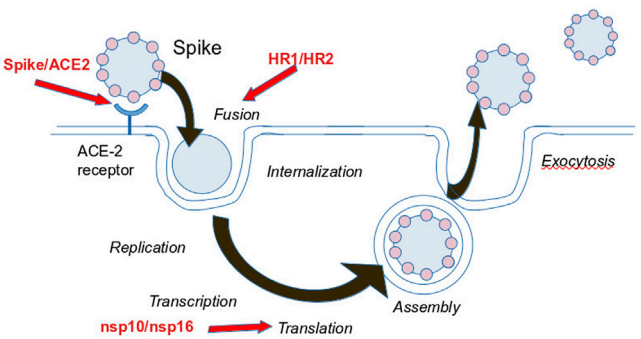


FIGURE 2 | Foreseen strategies to block SARS-CoV-2 infection. Red Arrows indicate the targets contributing to the reproduction cycle considered for peptide development.

3 THE SEARCH FOR DRUGS COMBATING SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 ATTACK

3.1 Structure Based Peptide Design

Figure 2 summarizes the different strategies that have been explored to block SARS-CoV-2 proliferation. The main

TABLE 1 | Candidate peptides. For each, we report its sequence, the rationale underlying its development, the target, a brief summary of the methods employed, the status about its experimental validation (- means none) and the reference related to the peptide identification.

Best peptide(s)	Rationale	Target	Methods	Validation	References
FLDKFNHEAEDLFYQSSL	ACE2 fragment binding RBD	ACE2: RBD	Docking (PyDock, HADDOCK, ZDOCK) MD refinement (50 ns, Gromacs—conditions not detailed) Toxicity prediction (ToxinPred)	None	Baig et al. (2020)
VPEQLYCLLQKFNGEAEMLFSRS TETQAKTFLDKFNHSAEDLFYQS IFEQAKTFTAQFNHEKEDLFYQS IFEQAKTFTAQFNHEKEDLFYQS	ACE2 evolved fragment binding RBD	ACE2: RBD	MD (100 ns, NAMD2.13—CHARMM36 force field) MMGB-SA free energies Adaptative evolution based on MD	None	Chaturvedi et al. (2020)
EQEERIQQDKRKNEQEDKRYQRYGRGKGHQP	Evolved ACE2 fragment	ACE2: RBD	EvoDesign (1000 independent design trajectories)	None	Huang et al. (2020b)
GSHMGDAQDKLKYLVKQLERALRELKKSLDELERSLEELEKNPSEDALVENNRLNVENNKIIVEVLRIILELAKASAKLA	Evolved ACE2 fragment	ACE2: RBD	Structural homology Docking (HADDOCK) K_D prediction (PRODIGY) MD: (100 ns, Gromacs 2020.2)	None	Jaiswal and Kumar, (2020)
EDLFYQ	ACE2 fragment	ACE2: RBD	Structural analysis	IC_{50}: 1.9 mM Infection reduction rate: −70%	Larue et al. (2020)
QAKTFLDKFNHEAEDLFYQSSLA	ACE2 fragment binding RBD	ACE2: RBD	Fragment identification: Rosetta/ PeptiDerive Local docking: FlexPepDock/ Rosetta (300 models) Single point mutational scan: Rosetta/backrub	Infection reduction rate: 60%	Chatterjee et al. (2020)
DKEWILQKIYEIMRLLDELGHAEASMRVSDLIYEFMKKGD ERLLEEAERLLEEVER	Mini-protein binding RBD	ACE2: RBD	Template based design: Rosetta-fragment assembly Docking: Rosetta-RifDock + *de novo* scaffold library	IC_{50}: 23 p.m.	Cao et al. (2020)
EEQAKTFLDKFNHEAEDLFYQSS EEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITEE EEQAKTFLDKFNHEAEDLFYQSS-G-LGKGDFR	ACE2 fragment	ACE2: RBD	MD: Gromacs 5.1.4. Structure Based Model/Go-model, WHAM.	None	Freitas et al. (2021)
SALEEQYKTFLDKFMHELEDLLYQLAL-nh2	ACE2 fragment	ACE2: RBD	User expertise based optimization	IC_{50}: 42 nM K_D: 0.03 nM	Karoyan et al. (2021)
QAKTFLDKFNHEAEDLFYQ	ACE2 fragment	ACE2: RBD	Docking perturbation upon amino acid substitution (Autodock-vina, 108 peptides) MD (Gromacs 4.6.1—conditions not detailed)	IC_{50}: 0.7 µM	Kuznetsov et al. (2022)
GARAHANSIVQQLVSEGADLVQTYVALVAALNGLEVNFSR VEQNIFRQHFPNMPMHGISAEDKLAFALAGALERATRQ GHIEHANSIVQQLVSEGADISRTLRLLFAALRGIEVRFSR VEQNIFRQHFPNMPMHGISSRDKLALALLGALAEALVN GAEAHANSIVQQLVSEGADLARTYALLLAATNGDRVNFSR VEQNIFRQHFPNMPMHGISAEDELAIALLGALERADRQ	ACE2 optimized fragment	ACE2: RBD	Scaffold library Scaffold docking (patchdock, 2000 models per scaffold) Interface design: Rosetta-fastdesign	None	Etemadi et al., 2022

(Continued on following page)

TABLE 1 | (*Continued*) Candidate peptides. For each, we report its sequence, the rationale underlying its development, the target, a brief summary of the methods employed, the status about its experimental validation (- means none) and the reference related to the peptide identification.

Best peptide(s)	Rationale	Target	Methods	Validation	References
			MD (20/50 ns, Gromacs, AMBER99SB-ILDN force field, TIP3P water) Binders docking (patchdock, ClusPro)		
Ace-TIEEQ-Z-KTFLDK-X-NHEAEDLFYQ-X-SLA-X-WN-nh2 X,Z: stapling residues	ACE2 optimized fragment	ACE2: RBD	Stapling	K_D: 2.22 μM IC_{50}: 2.8 μM Serum stability	Curelli et al. (2020)
IEEQAKTFLDKFNHEKEDLEYQSSLASWNYNTNIT Bold: stapling residues	ACE2 fragment	ACE2: RBD	Stapling	K_D: 2.1 μM IC_{50}: 3.6 μM	Maas et al. (2021)
NQKLIANQFNSAIGKIQDSLSSTASALGKLQDVVNQNAQALNTLVKQLVPRGSGGSGGSGGLEVLFQGPGINASVVNIQK EIDRLNEVAKNLNESLIDL Bold: linker	Spike fragment	Fusion	Homology modeling (swissmodel) Docking (GRAMM-X) MD umbrella sampling binding energy (17 ns, Gromacs 2020; SPC water)	None	Ling et al. (2020)
VAPGTAVLRQWLPTGTLLVDSDLNDFVSDADSTLIG KGIMMNVAKYTQLCQYLNTLTLAVPYNDKGVAPGTAVLRQ WLPTGTLLVDSDLNDFVSDADSTLIG	Nsp10 fragments binding nsp16	nsp10: nsp16	Stable binding to nsp16 (MD) (150 ns, Gromacs, Charmm36 force field, TIP3P water)	None	Dutta and Iype, (2021)

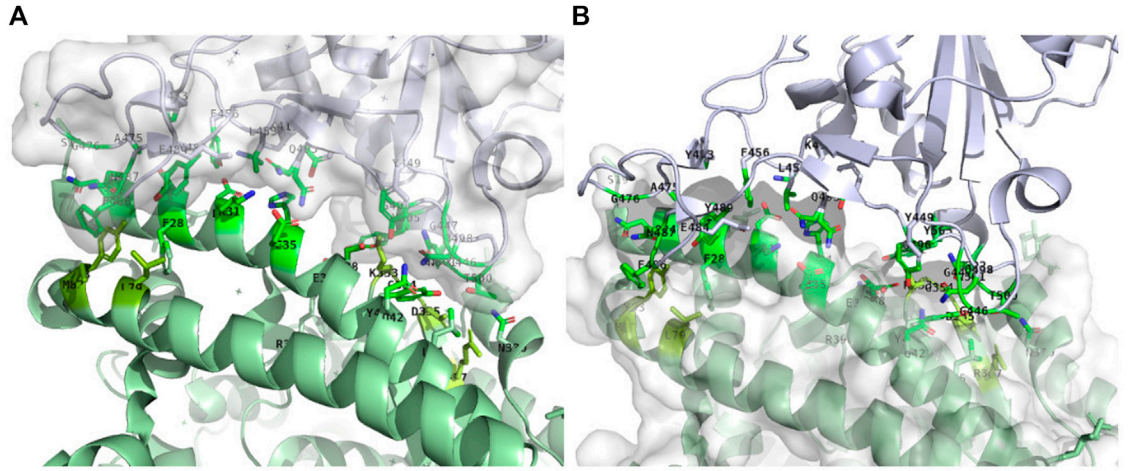


FIGURE 3 | Receptor binding domain of spike (within surface) in interaction with the ACE2 receptor (green). Only the side chains of the residues at the interface are displayed. **(A)**: ACE2-receptor (ACE2) residues interacting with the Receptor Binding Domain (RBD) of spike. The bright green residues correspond to the α_1-helix (residues 24–53), pale green residues below the α_1-helix to residues 79–83 and pale green residues in the back to residues 353–357. **(B)**: Residues of the RBD interacting with ACE2. PDB structure: 6m0j, image generated using PyMOL. Identification of the residues at the ACE2/RBD interface was performed using a distance cut-off of 5 Å.

strategies are to prevent virus entry into the cell and to block its replication. We discuss these in the next section. A summary of the identified peptides, the methods to identify them, and their experimental properties if measured, is provided **Table 1**.

3.1.1 Preventing Virus Internalization by Inhibiting the Spike-Angiotensin-Converting Enzyme 2 Interaction

SARS-CoV-22 enters cells through the interaction of the spike glycoprotein with the ACE2 human receptor (Gheblawi et al., 2020; Papageorgiou and Mohsin, 2020), making the spike/ACE2 interaction a preferential target to prevent cell infection. A structure obtained by electron microscopy at 2.9 Å resolution has been reported at the beginning of 2020 (Yan et al., 2020). **Figure 3A** shows that on the ACE2 side, the interaction involves mostly the N terminal helix—α_1-helix (residues 24–53, sequence: QAKTFLDKFNHEAEDLFYQSSLASWNYNTN), and to a lesser extent residues 79–83 (sequence: LAQMY) and 353–357 (sequence: KGDFR). On the spike protein side, contacts with residues from the RBD, involve mostly the stretch encompassing residues 480–501, and to a lesser extent the stretch encompassing residues 448–454 (**Figure 3B**). More precisely Barh et al. (2020), have suggested that effective peptides must bind to key positions of the RBD (G485, F486, N487, Q493, and Q498, T500, N501) and that F486, Q493, and N501 are critical residues.

The dominant strategy has been to design peptides directly inspired by the ACE2 peptidase, binding the RBD and thus competing with the interaction of the RBD with ACE2, preventing downstream cell penetration.

Predominantly, special attention has been brought to the 30 amino acid long α_1-helix critical for the RBD-ACE2 binding. From the analysis of the structures of SARS-CoV-2 and SARS-CoV in interaction with ACE2, Larue et al. (2020) have identified two ACE2-derived peptides able to bind Spike RBD in affinity precipitation assays (Larue et al., 2020). Those peptides have shown the ability to inhibit Spike-mediated infection with IC_{50} values in the low millimolar range. Starting from the fragment between the residues 21 and 45 of the α_1-helix, Sitthiyotha and Chunsrivirot (2020) have used computational protein design and molecular dynamics (MD) simulations to design peptides with enhanced theoretical affinity for SARS-CoV-2 RBD (Sitthiyotha and Chunsrivirot, 2020). During this iterative process, the design focus was on positions not reported to form favorable interactions with SARS-CoV-2-RBD, thus avoiding perturbing those corresponding to existing favorable interactions. Finally, Karoyan et al. (2021) have started with a peptide mimicking the α_1-helix of hACE2, to end with the best peptide-mimics able to block SARS-CoV-2 human pulmonary cell infection with an inhibitory concentration (IC_{50}) in the nanomolar range upon binding to the virus spike protein (Karoyan et al., 2021).

Such studies were based on the implicit hypothesis that the ACE2 fragments would adopt a conformation similar to that observed in the structure of ACE2 and that the binding of the peptide alone would result in a pose similar to that observed in the complex structure. Several early docking experiments have supported this hypothesis. Jaiswal and Kumar (2020) have for instance reported that the fragments of the α_1-helix are found to bind at the α_1-helix/RBD interface (Jaiswal and Kumar, 2020). In addition, Baig et al. (2020) have reported that some peptides they designed starting from 23 amino acids of the N-terminal helix, using alanine scanning to identify critical binding positions, can maintain their secondary structure during MD simulations and provide a highly specific and stable binding to SARS-CoV-2 (Baig et al., 2020). However, things might not be so straightforward in terms of structural behavior. Freitas et al. (2021) have also focused on the α_1-helix and explored the binding and folding dynamics of the natural and designed ACE2-based peptides by MD simulations using coarse-grained representations (Freitas et al., 2021). Their results show a difference in the folding mechanisms

of the modified peptides (a two-state folding mechanism) binding the RBD, as opposed to the naturally occurring α1 helix peptides, suggesting that amino acid substitution on the alpha-helical sequences can result in subtle changes in dynamic properties compared to the wild-type sequence. Moreover, Kuznetsov et al. (2022) have observed experimentally that a peptide comprising positions 24 to 42 of the ACE2 α1 helix can inhibit the formation of the S1-ACE2 complex in a manner dependent on the peptide concentration (Kuznetsov et al. 2022). They also observed the formation of a ternary complex suggesting that the peptide could bind to other sites besides that observed in the structure of the complex. The consequences of this observation on design are so far unknown. In summary, although effective, the peptides derived from the α_1-helix, validated *in vitro* and *in cellulo* by the different groups could exert a functional effect through mechanisms that are not necessarily those expected.

Attempts to combine different fragments of ACE2 distant in the sequence have also been considered. Barh et al. (2020) have for instance considered the residues of ACE2 interacting with RBD, searched databases of known activity for peptides blocking nCoV-RBD, and proposed chimeric peptides combining several candidates. Huang et al. (2020a) have designed peptides by grafting fragments from ACE2. The initial design of a peptide combining two segments of ACE2 (a.a. 22–44 and 351–357) was followed by an iterative redesign to enhance the binding to the RBD, using an in-house effective force-field, evoEF2, to drive the optimization (Huang et al., 2020a). The effectiveness of the designed peptides has however not yet been confirmed experimentally. Chatterjee et al. (2020) have, for their part, extended the strategy of ACE2 derived peptide design to target both the RBD and recruit E3 ubiquitin ligases for subsequent intracellular degradation of SARS-CoV-2 in the proteasome (Chatterjee et al., 2020). The design was performed using a protocol relying on the Rosetta program (Rohl et al., 2004), and *in cellulo* tests showed that one peptide is able to reduce the infection rate by −60%. Jaiswal and Kumar (2020) have extended the peptide up to a two helix bundle, using a protocol combining docking and MD simulations to identify stabilizing substitutions (Jaiswal and Kumar, 2020). The peptides showed predicted kD values on the order of 1 to a few nM, but experimental confirmation is missing. More recently, however, Zhou et al. (2021) have performed a thorough investigation of a strategy considering the two alpha-helices of hACE (Zhou et al., 2021). They concluded that the two helices cannot bind Spike when split from the ACE protein, the two peptides showing a propensity for disorder when out of ACE. They further concluded that stapling could be a relevant way to reduce the entropic cost upon binding of peptides containing one or two alpha-helices of ACE. Curreli et al. (2020); Maas et al. (2021) have reported, for lactam-stapled hACE2 peptides, experimental inhibition of the spike protein RBD-hACE2 complex formation, for concentrations on the order of 1–10 μM (Curreli et al., 2020; Maas et al., 2021).

Finally, the *de novo* design of peptides binding the RBD has also been explored. Chaturvedi et al. (2020) performed the *de novo* design of peptides targeting the RBD (Chaturvedi et al., 2020). Starting from selected ACE2 segments, natural RBD binder, the templates have been gradually modified by random mutations, while retaining those mutations that maximize their RBD-binding free energies. In this adaptive evolution, atomistic molecular dynamics simulations of the template-RBD complexes were iteratively perturbed by the peptide mutations, which were retained under favorable Monte Carlo decisions. The best candidate peptides remain however to be tested experimentally. Cao et al. (2020), have successfully designed alpha-helix bundle miniproteins encompassing the α_1-helix, with median inhibitory concentration (IC_{50}) values between 24 p.m. and 35 nM (Cao et al., 2020). The most potent exhibits a median inhibitory concentration (IC_{50}) of close to 0.16 ng ml^{-1}. The experimentally determined structure of the interaction between the peptides and the RBD is in excellent agreement with the computational models.

Of note, the interaction between RBD and ACE2 might be more complex than anticipated and involve more partners. Recently, another direction has been proposed by Beddingfield et al. (2021). It does not target directly the RBD ACE2 interface, but instead the spike protein and ACE2 interactions with the α5β1 integrin. Using docking, they identified three candidate binding sites for a candidate peptide active *in vitro* with an IC_{50} value of 3.16 μM. These results however require more explorations.

3.1.2 Preventing Virus Internalization by Preventing the Formation of the Fusion Core

The SARS-CoV-2 spike proteins consist of two subunits named S1, which contains the RBD, and S2 (**Figure 4A**). S2 is highly conserved among SARS-Like Coronaviruses and the mechanism responsible for the virus internalization is expected to be common to those viruses. After interacting with ACE2, the spike protein is cleaved into S1 and S2, and S2 undergoes a conformational rearrangement mediating viral fusion and cell entry. S2 is composed of a fusion peptide (FP), two heptad repeats (HR1 and HR2), a transmembrane domain (TM), and a cytoplasmic domain fusion (CP). After cleavage, the FP is inserted into the target cell membrane, which results in HR1 and HR2 forming a 6-helix bundle (**Figure 4B**). The formation of this bundle brings the cellular and viral lipid bilayers into proximity, which initiates the membrane fusion process (Belouzard et al., 2012). Preventing the formation of the helix bundle has been described as a possible strategy to block membrane fusion and prevent the entry of the virus into cells. Ling et al. (2020) have explored the design of peptides mimicking HR2 and binding HR1, to block the fusion process (Ling et al., 2020). After modeling the structure of the 6 helix bundle using homology with other viruses of the family, they investigated the binding energy of HR1 to HR2 and conversely concluded that HR2-derived peptides are probably more efficient than HR1-derived peptides to prevent the formation of the 6-helix bundle and viral infection. Efaz et al. (2021) analyzed 17 SARS-CoV-1 HR2-derived fusion inhibitor peptides known to show effective antiviral activity against the HR1 of SARS-CoV-1 and SARS-CoV-2 (Efaz et al., 2021). Using MD simulations and monitoring the free energy landscape of their binding with HR1, they identified the two best candidates. Experimental validation is however not provided at this time.

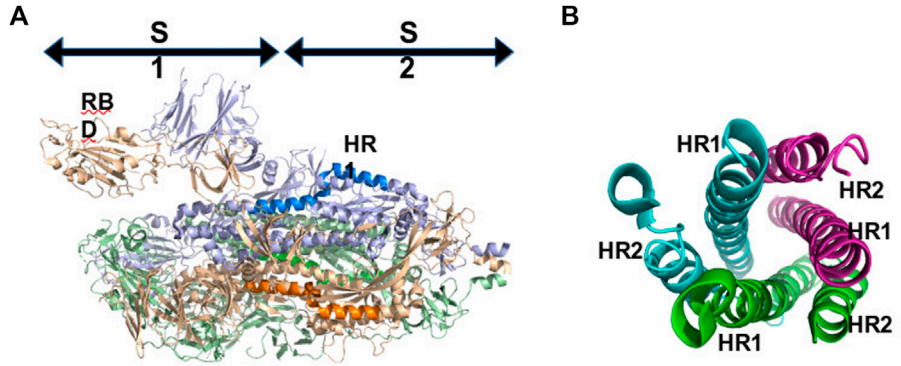


FIGURE 4 | (A): Localization of subdomains S1 and S2 of SPIKE in the pre-fusion conformation. HR1 residues are in blue. **(A)**: Conformation of the 6 helix bundle (fusion conformation).

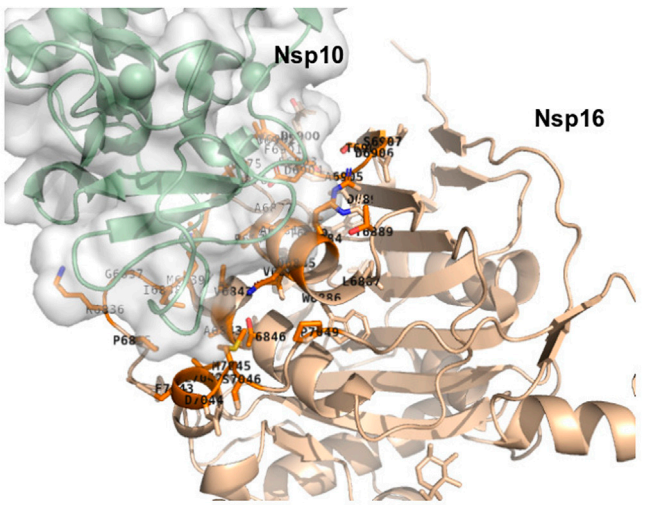


FIGURE 5 | Interaction between nsp10 in green and nsp16 in wheat (PDB id: 6w4h) generated using PyMOL. Only the side chains of the residues at the interface are displayed. Identification of the residues at the nsp10/nsp16 interface was performed using a distance cut-off of 5 Å.

3.1.3 Towards Targeting Intra-cellular Interactions

Targeting cell penetration of the virus is not the only strategy that could reveal effectiveness. Once the virus has entered the cell, other protein-protein interactions have been considered of potential interest.

One of these is the interaction between nsp10 and nsp16 (**Figure 5**). The virus replicates in the cytoplasm, and cannot access the capping machinery of the host located in the nucleus. To compensate the virus encodes its own capping enzymes, and several nsp such as nsp14 and nsp16, which are involved in viral RNA capping. Nsp16 has a binding pocket for S-adenosyl-L-methionine (SAM) which acts as a methyl group donor for the 2′-O-methylation reaction. This pocket is stabilized by the interaction with another nsp, nsp10 and consequently, the inhibition of the nsp16/nsp10 interaction is a possible strategy to prevent virus replication. Dutta and Iype (2021) have analyzed the binding interface of the nsp10/nsp16 complex [PDB id: 6W4H (Rosas-Lemus et al., 2020)] to identify peptides of nsp16 blocking the interaction between nsp10 and nsp16 (Dutta and Iype, 2021). Combined docking with MD simulations, they prospectively analyzed the binding of several candidates and concluded that two of them two and five were stable and able to bind to the nsp16 interacting region of nsp10, thus potentially preventing the interaction between the two proteins. Again, experimental confirmation is still required.

Another interaction is that of the PDZ binding domain of the envelope protein (E-protein) of SARS-CoV-2 with PALS1. The presence of PDZ binding motifs (PBM) that bind specific cellular PDZ domain proteins is frequent in viruses, leading to pathogenic dysfunctions of these proteins. The E-protein of SARS-CoV-2 has such a PBM known to interact with PALS1. Despite the PBM/PDZ interactions being usually weak, Toto et al. (2020) have proposed to design peptides mimicking SARS-CoV-2 E-protein targeting the PDZ domain of PALS1 (Toto et al., 2020). PALS1 participates in the maintenance of epithelial polarity, and it has been suggested that E-protein/PALS1 interaction is involved in the degradation of the integrity of the lung epithelia, resulting in dramatically increased viral dissemination. Analyzing the structure of the E-protein, they identified peptide mimics of the E-protein, assessed their relative affinity for PALS1 compared to the equivalent peptides of SARS-CoV-2, and concluded an increased affinity of the E-protein of SARS-CoV-2 for PALS1. However, no successful inhibitors have been obtained to date.

3.2 Searching for Natural Peptides Active Against Severe Acute Respiratory Syndrome Coronavirus 2 Infection

Not considering the structural information available, a direction that has repeatedly proven effective is the search among natural peptides known to have biological activities. The urgency to respond to the SARS-CoV-2 pandemic has non surprisingly stimulated the search for such candidates, although in some cases no clear rationale underlying the search existed, leading

to mostly conceptual studies. It is for instance the case for the search for active peptides in the colostrum and milk. Çakır et al. (2021) have considered peptides from the goat milk whey fraction obtained by enzymatic digestion and assessed their potential combining *in silico* data-based prediction and docking against ACE2 and DPP-4 enzymes (Çakır et al., 2021). Pradeep et al. (2021) have reported a study in the same orientation, starting from peptides previously identified from Buffalo colostrum and milk, targeting entry points such as ACE2, Spike, TMPRSS, Cathepsin-L, or Furin, the endosomal maturation components such as AAK1, GAK, PIKfyve or TPC2, the replication transcription complex (PLpro, clpro, nsp12, nsp13) and Virion Assembly (N Protein) combining docking with MD simulations, assessing the stability of the binding of the peptide with the target (Pradeep et al., 2021). Although both studies identified some candidates of interest, these remain to be further assessed *in vitro* and *in vivo*. Yu et al. (2021) have prospectively analyzed the potential of peptides resulting from the *in silico* digestion of Tuna myosin to block ACE2 (Yu et al., 2021).

Several studies have considered targeting side effects of the virus infection, instead of directly addressing it. For instance, the antioxidant and anti-inflammatory effects of grehlin, have been considered to reduce the complications of the SARS-CoV-2 (Jafari et al., 2021). As well, since cardiovascular diseases are strong negative prognostic factors since they exacerbate the effects of the viral infection and lead to worse outcomes, it has been suggested that natriuretic peptides could exert a key protective role toward the virus infection whereas an impairment of NPs release contributes to the virus deleterious effects (Rubattu et al., 2021).

Anti-Microbial Peptides (AMPs) are another class of peptides with potential interest. Indeed, among the close to 3,200 AMPs discovered, close to 200 have also been reported to have antiviral activities (Wang, 2020; Mousavi Maleki et al., 2021). However, these peptides seem to have varied mechanisms of action in varied contexts. The interaction of Nisin, a food-grade antimicrobial peptide produced by lactic acid bacteria, with ACE2, has been assessed using modeling and docking. The results suggest that Nisin could act as a competitor of the RBD to bind ACE2 (Bhattacharya et al., 2021).

Finally suppressing the activity of the PLpro enzyme by using potential plant-derived protease inhibitor peptides has also been considered. (Moradi et al., 2022) have tested 11 plant-derived peptides selected from the literature that could potentially inhibit protease activity. Docking experiments suggest that VcTI from Veronica hederifolia provides effective molecular interactions at both the liable Zn site and the classic active site of PLpro. These results remain to be confirmed experimentally.

Overall, however, the exploration of natural peptides has so far led to few promises, if any.

4 DISCUSSION

In the context of the SARS-CoV-2 pandemic, the urgent need to identify means to fight against the SARS-CoV-2 attack has raised an unprecedented effort, based on a wide panel of strategies. These encompass drug and vaccine development, or drug repurposing. Among these, peptide-based development was a possible direction to consider. Here, we have reviewed how *in silico* protocols have contributed to such structure-based development. It is obvious that peptide drug development does not require, *per se*, *in silico* approaches. It could proceed for instance by developing and screening experimentally peptide libraries. For instance, Rathod et al. (2020) have searched peptides from the AntiViral Peptide Database (AVPdb), with a repurposing perspective (Rathod et al., 2020). As well, cyclic peptides targeting the RBD have been experimentally designed using mRNA display (Norman et al., 2021), and both linear and cyclic peptides targeting the M^{pro}protease have been identified using *in vitro* screening (Pisarchik, 2021). As summarized in **Table 1**, it is however striking that the vast majority of studies have consisted of structure-based *in silico* design.

Indeed, the knowledge of the structure of the RBD in interaction with ACE2 has revealed extremely valuable to the design of candidate peptide drugs. *In silico* protocols and particularly MD simulations have made it possible to analyze in detail the interaction between the RBD and ACE2 to identify the key residues of the interaction. Their use has in turn led to the development of peptides validated *in vitro* as binders of the RBD, just using the expertise of some researchers focusing on substitutions at not essential sites for the interaction (Karoyan et al., 2021), or using stapling to optimize the binding (Curelli et al., 2020; Maas et al., 2021). More sophisticated studies have combined the use of docking and MD simulations to explore the stability of the binding of evolved peptides to the RBD. For the docking, peptides traditionally pose specific problems compared to small compounds due to their larger flexibility. To address this issue, it is striking to note that various, mostly flexible, docking approaches have been considered, often complemented by MD/refinement protocols to sample the conformational flexibility of the poses (**Table 1**). Probably here, the helical conformation of the ACE2 fragment helps making docking easier. Varied MD protocols have also been employed, using explicit or implicit solvent models, and sometimes sophisticated protocols such as umbrella sampling (Ling et al., 2020) or WHAM (Freitas et al., 2021). Finally, it is noticeable that the use of the Rosetta software (Leaver-Fay et al., 2011) has led to the effective design of several peptides binding the RBD with very low IC_{50} values, on the order of a few tens of pM (Cao et al., 2020), which is remarkable. The size of the peptides matters however, longer peptides tend to have lower IC_{50} values. Larue et al. (2020) reported that a 6 amino acid ACE2 fragment with a mM order IC_{50} is able to reduce cell infection by approximately 70% (Larue et al., 2020), while Chatterjee et al. (2020) reported 23 amino-acid peptides able to reduce the infection rate by 60% (Chatterjee et al., 2020), Kuznetsov et al. (2022) reported that a 19 amino acid peptide has an IC close to μM (Kuznetsov et al., 2022), and Karoyan et al. (2021) described a 27 amino acid with IC_{50} on the order of nM (Karoyan et al., 2021). The miniproteins designed by Cao et al. (2020) have much longer sizes, with over 55 amino

acids for the best ones (Cao et al., 2020). Finally, strategies to stabilize the peptides using stapling end with peptides having IC_{50} values on the order of a few μM.

The identification of peptides able to compete with the RBD/ACE2 interaction, and even to slow down viral infection reduction in cellular assays does not mean however that peptide drugs are close to getting on the market. The more divergent the sequences are from the natural sequence, and the longer they are, the more likely they could become associated with adverse effects, particularly in terms of the immune response. Finally, the longer they are, the more costly they become, which could become an obstacle to their development. To a lesser extent, studies are now escaping the RBD-ACE2 interaction to tackle other interactions that occur internally in the cells. These studies also benefit from the 3D information available, although not all the structures of the proteins in interaction are known. These developments have started later on and will be confronted with harder challenges for cell internalization.

As for synthetic vaccine development, many studies have proposed candidate immunogenic peptides, that for their vast majority, lack experimental validation. It is nevertheless seizing that *in silico* approaches now address a wide range of considerations, including MHC I, MHC II as well as CD4 or CD8 immune response. Nevertheless, the use of peptides as a vaccine for SARS-CoV-2 treatment seems to be promising. Thus, CoVac-1, which is a combination of viral proteins is in phase II clinical trials. To note, EpiVacCorona, a peptide-based vaccine is already accepted as medicine in Russia since December 2020. Because questions arose about how the peptides were selected and what is its real immunogenicity, EpiVacCoron is used almost exclusively in Russia.

Another point to consider is the emergence of variants. Drugs and vaccines that have been developed for the wild-type SARS-CoV-2 may become less effective. For example, all the variants of concern have mutations in the spike protein that enable to partially escape the immune response and/or to increase the interaction with ACE2. The efficacy of drugs that target spike protein could be therefore drastically affected by the mutations. In this case, the use of therapeutic peptides is particularly relevant to face these issues, since the peptides of interest can be adapted for a given variant with appropriate changes in the peptide sequence.

To summarize, the SARS-CoV-2 pandemic has highlighted the reactivity of the actors of drug development. Vaccine development has proven very effective and fast, whereas synthetic peptide vaccines are still under development. For chemical drug development, drug repurposing has been so far the more effective strategy. Peptide drug development assisted by *in silico* analyzes has proven very reactive, able to identify promising candidates within a few months. It keeps progressing on new targets (Chan et al., 2021). The same responsiveness has been observed to search for small compounds, although through much higher investment. So far, none has been able to propose convincing enough drugs able to reach the market. It will be interesting to reconsider, hopefully in a few months, lessons from drug development against SARS-CoV-2 in terms of drug development strategy.

AUTHOR CONTRIBUTIONS

GM and PT contributed to conception of this review. GM and PT wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version. GM and PT contributed equally to this work.

FUNDING

This work was supported, in part, by IdEX Université Paris Cité ANR-18-IDEX-0001 (HS-PS project, No. IdEx-2021-I-053).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support of the Université Paris Cité, the CNRS institute, and the INSERM institute.

REFERENCES

Alam, A., Khan, A., Imam, N., Siddiqui, M. F., Waseem, M., Malik, M. Z., et al. (2021). Design of an Epitope-Based Peptide Vaccine against the SARS-CoV-2: a Vaccine-Informatics Approach. *Briefings Bioinforma.* 22 (2), 1309–1323. doi:10.1093/bib/bbaa340

Baig, M. S., Rajpoot, S., Saqib, U., and Saqib, U. (2020). Identification of a Potential Peptide Inhibitor of SARS-CoV-2 Targeting its Entry into the Host Cells. *Drugs R. D.* 20 (3), 161–169. doi:10.1007/s40268-020-00312-5

Barh, D., Tiwari, S., Silva Andrade, B., Giovanetti, M., Almeida Costa, E., Kumavath, R., et al. (2020). Potential Chimeric Peptides to Block the SARS-CoV-2 Spike Receptor-Binding Domain. *F1000Res* 9, 576. doi:10.12688/f1000research.24074.1

Beddingfield, B. J., Iwanaga, N., Chapagain, P. P., Zheng, W., Roy, C. J., Hu, T. Y., et al. (2021). The Integrin Binding Peptide, ATN-161, as a Novel Therapy for SARS-CoV-2 Infection. *JACC Basic Transl. Sci.* 6 (1), 1–8. doi:10.1016/j.jacbts.2020.10.003

Belouzard, S., Millet, J. K., Licitra, B. N., and Whittaker, G. R. (2012). Mechanisms of Coronavirus Cell Entry Mediated by the Viral Spike Protein. *Viruses* 4 (6), 1011–1033. doi:10.3390/v4061011

Bhattacharya, R., Gupta, A. M., Mitra, S., Mandal, S., and Biswas, S. R. (2021). A Natural Food Preservative Peptide Nisin Can Interact with the SARS-CoV-2 Spike Protein Receptor Human ACE2. *Virology* 552, 107–111. doi:10.1016/j.virol.2020.10.002

Bruzzoni-Giovanelli, H., Alezra, V., Wolff, N., Dong, C.-Z., Tuffery, P., and Rebollo, A. (2018). Interfering Peptides Targeting Protein-Protein Interactions: the Next Generation of Drugs? *Drug Discov. Today* 23 (2), 272–285. doi:10.1016/j.drudis.2017.10.016

Çakır, B., Okuyan, B., Şener, G., and Tunali-Akbay, T. (2021). Investigation of Beta-Lactoglobulin Derived Bioactive Peptides against SARS-CoV-2 (COVID-19): In Silico Analysis. *Eur. J. Pharmacol.* 891, 173781. doi:10.1016/j.ejphar.2020.173781

Cao, L., Goreshnik, I., Coventry, B., Case, J. B., Miller, L., Kozodoy, L., et al. (2020). De Novo design of Picomolar SARS-CoV-2 Miniprotein Inhibitors. *Science* 370 (6515), 426–431. doi:10.1126/science.abd9909

Chan, H. H., Moesser, M. A., Walters, R. K., Malla, T. R., Twidale, R. M., John, T., et al. (2021). Discovery of SARS-CoV-2 M Pro Peptide Inhibitors From

Modelling Substrate and Ligand Binding. *Chemical Sci.* 12 (41), 13686–13703. doi:10.1039/d1sc03628a

Chatterjee, P., Ponnapati, M., Kramme, C., Plesa, A. M., Church, G. M., and Jacobson, J. M. (2020). Targeted Intracellular Degradation of SARS-CoV-2 *via* Computationally Optimized Peptide Fusions. *Commun. Biol.* 3 (1), 715–718. doi:10.1038/s42003-020-01470-7

Chaturvedi, P., Han, Y., Král, P., and Vuković, L. (2020). Adaptive Evolution of Peptide Inhibitors for Mutating SARS-CoV-2. *Adv. Theory Simul.* 3 (12), 2000156. doi:10.1002/adts.202000156

Curreli, F., Victor, S. M. B., Ahmed, S., Drelich, A., Tong, X., Tseng, C. K., et al. (2020). Stapled Peptides Based on Human Angiotensin-Converting Enzyme 2 (ACE2) Potently Inhibit SARS-CoV-2 Infection *In Vitro*. *Mbio* 11 (6), e02451–20. doi:10.1128/mBio.02451-20

Davenport, A. P., Scully, C. C. G., de Graaf, C., Brown, A. J. H., and Maguire, J. J. (2020). Advances in Therapeutic Peptides Targeting G Protein-Coupled Receptors. *Nat. Rev. Drug Discov.* 19 (6), 389–413. doi:10.1038/s41573-020-0062-z

Dutta, M., and Iype, E. (2021). Peptide Inhibitors against SARS-CoV-2 2′-O-Methyltransferase Involved in RNA Capping: A Computational Approach. *Biochem. Biophysics Rep.* 27, 101069. doi:10.1016/j.bbrep.2021.101069

Efaz, F. M., Islam, S., Talukder, S. A., Akter, S., Tashrif, M. Z., Ali, M. A., et al. (2021). Repurposing Fusion Inhibitor Peptide against SARS-CoV -2. *J. Comput. Chem.* 42 (32), 2283–2293. doi:10.1002/jcc.26758

Fenton, C., and Keam, S. J. (2022). Emerging Small Molecule Antivirals May Fit Neatly into COVID-19 Treatment. *Drugs & Ther. Perspect.* 2022, 1–15. doi:10.1007/s40267-022-00897-8

Freitas, F. C., Ferreira, P. H. B., Favaro, D. C., and Oliveira, R. J. D. (2021). Shedding Light on the Inhibitory Mechanisms of SARS-CoV-1/CoV-2 Spike Proteins by ACE2-Designed Peptides. *J. Chem. Inf. Model.* 61 (3), 1226–1243. doi:10.1021/acs.jcim.0c01320

Gheblawi, M., Wang, K., Viveiros, A., Nguyen, Q., Zhong, J.-C., Turner, A. J., et al. (2020). Angiotensin-Converting Enzyme 2: SARS-CoV-2 Receptor and Regulator of the Renin-Angiotensin System. *Circ. Res.* 126 (10), 1456–1474. doi:10.1161/circresaha.120.317015

Heitmann, J. S., Bilich, T., Tandler, C., Nelde, A., Maringer, Y., Marconato, M., et al. (2022). A COVID-19 Peptide Vaccine for the Induction of SARS-CoV-2 T Cell Immunity. *Nature* 601 (7894), 617–622. doi:10.1038/s41586-021-04232-5

Huang, X., Pearce, R., and Zhang, Y. (2020a). De Novo design of Protein Peptides to Block Association of the SARS-CoV-2 Spike Protein with Human ACE2. *Aging* 12 (12), 11263–11276. doi:10.18632/aging.103416

Huang, X., Pearce, R., and Zhang, Y. (2020b). EvoEF2: Accurate and Fast Energy Function for Computational Protein Design. *Bioinformatics* 36 (4), 1135–1142. doi:10.1093/bioinformatics/btz740

Jafari, A., Sadeghpour, S., Ghasemnejad-Berenji, H., Pashapour, S., and Ghasemnejad-Berenji, M. (2021). Potential Antioxidative, Anti-inflammatory and Immunomodulatory Effects of Ghrelin, an Endogenous Peptide from the Stomach in SARS-CoV2 Infection. *Int. J. Pept. Res. Ther.* 27 (3), 1875–1883. doi:10.1007/s10989-021-10217-9

Jaiswal, G., and Kumar, V. (2020). In-silico Design of a Potential Inhibitor of SARS-CoV-2 S Protein. *PLoS One* 15 (10), e0240004. doi:10.1371/journal.pone.0240004

Kang, S., Yang, M., He, S., Wang, Y., Chen, X., Chen, Y. Q., et al. (2021). A SARS-CoV-2 Antibody Curbs Viral Nucleocapsid Protein-Induced Complement Hyperactivation. *Nat. Commun.* 12 (1), 1–11. doi:10.1038/s41467-021-23036-9

Karim, S. S. A., and Karim, Q. A. (2021). Omicron SARS-CoV-2 Variant: a New Chapter in the COVID-19 Pandemic. *Lancet* 398 (10317), 2126–2128. doi:10.1016/s0140-6736(21)02758-6

Karoyan, P., Vieillard, V., Gómez-Morales, L., Odile, E., Guihot, A., Luyt, C. E., et al. (2021). Human ACE2 Peptide-Mimics Block SARS-CoV-2 Pulmonary Cells Infection. *Commun. Biol.* 4 (1), 197–199. doi:10.1038/s42003-021-01736-8

Kuznetsov, A., Arukuusk, P., Härk, H., Juronen, E., Ustav, M., Langel, Ü., et al. (2022). ACE2 Peptide Fragment Interaction with Different S1 Protein Sites. *Int. J. Pept. Res. Ther.* 28 (1), 7. doi:10.1007/s10989-021-10324-7

Larue, R. C., Xing, E., Kenney, A. D., Zhang, Y., Tuazon, J. A., Li, J., et al. (2020). Rationally Designed ACE2-Derived Peptides Inhibit SARS-CoV-2. *Bioconjugate Chem.* 32 (1), 215–223. doi:10.1021/acs.bioconjchem.0c00664

Leaver-Fay, A., Tyka, M., Lewis, S. M., Lange, O. F., Thompson, J., Jacak, R., et al. (2011). Rosetta3. *Methods Enzym.* 487, 545–574. doi:10.1016/b978-0-12-381270-4.00019-6

Lewis, A. L., McEntee, N., Holland, J., and Patel, A. (2022). Development and Approval of Rybelsus (Oral Semaglutide): Ushering in a New Era in Peptide Delivery. *Drug Deliv. Transl. Res.* 12 (1), 1–6. doi:10.1007/s13346-021-01000-w

Ling, R., Dai, Y., Huang, B., Huang, W., Yu, J., Lu, X., et al. (2020). In Silico design of Antiviral Peptides Targeting the Spike Protein of SARS-CoV-2. *Peptides* 130, 170328. doi:10.1016/j.peptides.2020.170328

Maas, M. N., Hintzen, J. C. J., Löffler, P. M. G., and Mecinović, J. (2021). Targeting SARS-CoV-2 Spike Protein by Stapled hACE2 Peptides. *Chem. Commun.* 57 (26), 3283–3286. doi:10.1039/d0cc08387a

Makurvet, F. D. (2021). Biologics vs. Small Molecules: Drug Costs and Patient Access. *Med. Drug Discov.* 9, 100075. doi:10.1016/j.medidd.2020.100075

Moradi, M., Golmohammadi, R., Najafi, A., Moosazadeh Moghaddam, M., Fasihi-Ramandi, M., and Mirnejad, R. (2022). In Silico Analysis of Inhibiting Papain-like Protease from SARS-CoV-2 by Using Plant-Derived Peptides. *Int. J. Pept. Res. Ther.* 28 (1), 24–11. doi:10.1007/s10989-021-10331-8

Mousavi Maleki, M. S., Rostamian, M., and Madanchi, H. (2021). Antimicrobial Peptides and Other Peptide-like Therapeutics as Promising Candidates to Combat SARS-CoV-2. *Expert Rev. anti-infective Ther.* 19 (10), 1205–1217. doi:10.1080/14787210.2021.1912593

Muttenthaler, M., King, G. F., Adams, D. J., and Alewood, P. F. (2021). Trends in Peptide Drug Discovery. *Nat. Rev. Drug Discov.* 20 (4), 309–325. doi:10.1038/s41573-020-00135-8

Nevola, L., and Giralt, E. (2015). Modulating Protein-Protein Interactions: the Potential of Peptides. *Chem. Commun.* 51 (16), 3302–3315. doi:10.1039/c4cc08565e

Norman, A., Franck, C., Christie, M., Hawkins, P. M. E., Patel, K., Ashhurst, A. S., et al. (2021). Discovery of Cyclic Peptide Ligands to the SARS-CoV-2 Spike Protein Using mRNA Display. *ACS Cent. Sci.* 7 (6), 1001–1008. doi:10.1021/acscentsci.0c01708

Pachetti, M., Marini, B., Benedetti, F., Giudici, F., Mauro, E., Storici, P., et al. (2020). Emerging SARS-CoV-2 Mutation Hot Spots Include a Novel RNA-Dependent-RNA Polymerase Variant. *J. Transl. Med.* 18 (1), 179–9. doi:10.1186/s12967-020-02344-6

Papageorgiou, A. C., and Mohsin, I. (2020). The SARS-CoV-2 Spike Glycoprotein as a Drug and Vaccine Target: Structural Insights into its Complexes with ACE2 and Antibodies. *Cells* 9 (11), 2343. doi:10.3390/cells9112343

Peng, Y., Mentzer, A. J., Liu, G., Yao, X., Yin, Z., Dong, D., et al. (2020). Broad and Strong Memory CD4+ and CD8+ T Cells Induced by SARS-CoV-2 in UK Convalescent Individuals Following COVID-19. *Nat. Immunol.* 21 (11), 1336–1345. doi:10.1038/s41590-020-0782-6

Pisarchik, A. (2021). A Novel Approach for Selecting Potent Peptide Inhibitors of the SARS-CoV2-Mpro Protease. Available at Research Square [doi: doi:10.21203/rs.3.rs-396336/v1

Pradeep, H., Najma, U., and Aparna, H. S. (2021). Milk Peptides as Novel Multi-Targeted Therapeutic Candidates for SARS-CoV2. *Protein J.* 40 (3), 310–327. doi:10.1007/s10930-021-09983-8

Rathod, S. B., Prajapati, P. B., Punjabi, L. B., Prajapati, K. N., Chauhan, N., and Mansuri, M. F. (2020). Peptide Modelling and Screening against Human ACE2 and Spike Glycoprotein RBD of SARS-CoV-2. *Silico Pharmacol* 8 (1), 3–9. doi:10.1007/s40203-020-00055-w

Rohl, C. A., Strauss, C. E. M., Misura, K. M. S., and Baker, D. (2004). Protein Structure Prediction Using Rosetta. *Methods Enzym.* 383, 66–93. Academic Press. doi:10.1016/s0076-6879(04)83004-0

Rosas-Lemus, M., Minasov, G., Shuvalova, L., Inniss, N. L., Kiryukhina, O., Brunzelle, J., et al. (2020). High-resolution Structures of the SARS-CoV-2 2'-O-Methyltransferase Reveal Strategies for Structure-Based Inhibitor Design. *Sci. Signal* 13 (651), eabe1202. doi:10.1126/scisignal.abe1202

Rubattu, S., Gallo, G., and Volpe, M. (2021). A Contemporary View of Natriuretic Peptides in the SARS-CoV-2 Era. *Front. Physiol.* 12, 643721. doi:10.3389/fphys.2021.643721

Sitthiyotha, T., and Chunsrivirot, S. (2020). Computational Design of 25-mer Peptide Binders of SARS-CoV-2. *J. Phys. Chem. B* 124 (48), 10930–10942. doi:10.1021/acs.jpcb.0c07890

Starr, T. N., Greaney, A. J., Dingens, A. S., and Bloom, J. D. (2021). Complete Map of SARS-CoV-2 RBD Mutations that Escape the Monoclonal Antibody LY-CoV555 and its Cocktail with LY-CoV016. *Cell Rep. Med.* 2 (4), 100255. doi:10.1016/j.xcrm.2021.100255

Tegally, H., Wilkinson, E., Giovanetti, M., Iranzadeh, A., Fonseca, V., Giandhari, J., et al. (2021). Detection of a SARS-CoV-2 Variant of Concern in South Africa. *Nature* 592 (7854), 438–443. doi:10.1038/s41586-021-03402-9

Toto, A., Ma, S., Malagrinò, F., Visconti, L., Pagano, L., Stromgaard, K., et al. (2020). Comparing the Binding Properties of Peptides Mimicking the Envelope Protein of SARS-CoV and SARS-CoV -2 to the PDZ Domain of the Tight Junction-associated PALS1 Protein. *Protein Sci.* 29 (10), 2038–2042. doi:10.1002/pro.3936

Vlieghe, P., Lisowski, V., Martinez, J., and Khrestchatisky, M. (2010). Synthetic Therapeutic Peptides: Science and Market. *Drug Discov. Today* 15 (1-2), 40–56. doi:10.1016/j.drudis.2009.10.009

Wang, G. (2020). The Antimicrobial Peptide Database Provides a Platform for Decoding the Design Principles of Naturally Occurring Antimicrobial Peptides. *Protein Sci.* 29 (1), 8–18. doi:10.1002/pro.3702

Wang, X., Lan, J., Ge, J., Yu, J., and Shan, S. (2020). Crystal Structure of SARS-CoV-2 Spike Receptor-Binding Domain Bound with ACE2 Receptor. *Nature* 581 (7807), 215–220.

Wójcik, P., and Berlicki, Ł. (2016). Peptide-based Inhibitors of Protein–Protein Interactions. *Bioorg. Med. Chem. Lett.* 26 (3), 707–713.

Xie, X., Liu, Y., Liu, J., Zhang, X., Zou, J., Fontes-Garfias, C. R., et al. (2021). Neutralization of SARS-CoV-2 Spike 69/70 Deletion, E484K and N501Y Variants by BNT162b2 Vaccine-Elicited Sera. *Nat. Med.* 27 (4), 620–621. doi:10.1038/s41591-021-01270-4

Xu, J., Khan, A. R., Fu, M., Wang, R., Ji, J., and Zhai, G. (2019). Cell-penetrating Peptide: a Means of Breaking through the Physiological Barriers of Different Tissues and Organs. *J. Control. Release* 309, 106–124. doi:10.1016/j.jconrel.2019.07.020

Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., and Zhou, Q. (2020). Structural Basis for the Recognition of SARS-CoV-2 by Full-Length Human ACE2. *Science* 367 (6485), 1444–1448. doi:10.1126/science.abb2762

Yu, Z., Kan, R., Ji, H., Wu, S., Zhao, W., Shuian, D., et al. (2021). Identification of Tuna Protein-Derived Peptides as Potent SARS-CoV-2 Inhibitors *via* Molecular Docking and Molecular Dynamic Simulation. *Food Chem.* 342, 128366. doi:10.1016/j.foodchem.2020.128366

Zhang, H., and Chen, S. (2022). Cyclic Peptide Drugs Approved in the Last Two Decades (2001-2021). *RSC Chem. Biol.* 3 (1), 18–31. doi:10.1039/d1cb00154j

Zhou, P., Wang, H., Chen, Z., and Liu, Q. (2021). Context Contribution to the Intermolecular Recognition of Human ACE2-Derived Peptides by SARS-CoV-2 Spike Protein: Implications for Improving the Peptide Affinity but Not Altering the Peptide Specificity by Optimizing Indirect Readout. *Mol. Omics* 17 (1), 86–94. doi:10.1039/d0mo00103a

Zhou, T., Tsybovsky, Y., Gorman, J., Rapp, M., Cerutti, G., Chuang, G.-Y., et al. (2020). Cryo-EM Structures of SARS-CoV-2 Spike without and with ACE2 Reveal a pH-dependent Switch to Mediate Endosomal Positioning of Receptor-Binding Domains. *Cell host microbe* 28 (6), 867–879. doi:10.1016/j.chom.2020.11.004

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

MINI REVIEW
published: 26 May 2022
doi: 10.3389/fddsv.2022.898654

Overcoming Market Failures in Pandemic Drug Discovery Through Open Science: A Canadian Solution

E. Richard Gold[1*†] *and Aled M. Edwards*[2†]

[1]*Faculty of Law and Faculty of Medicine and Health Sciences, McGill University, Montreal, QC, Canada,* [2]*Structural Genomics Consortium, University of Toronto, Toronto, ON, Canada*

Among the lessons learned from the COVID-19 pandemic is the need to develop antiviral drugs poised to treat the next pandemic. Unfortunately, traditional drug development economic models, centered principally on patents, are ineffective to induce private sector investment due to unpredictable timing and cause of the next pandemic. As a result, illustrated by the COVID-19 pandemic, it is the public and philanthropic sectors sectors that overwhelmingly fund the development of innovative vaccines and therapies. To meet the need for proactive antiviral medicines in advance of the next pandemic, new models of drug development are needed. Open science partnerships (OSPs) show promise in this regard. Rather than rely principally on patents and private investment, OSPs combine a variety of academic, philanthropic, governmental, and private sector incentives to share knowledge and develop and test antiviral drugs. Private sector investments are, within an OSP, not only leveraged against investments by other actors, but predicated on gaining regulatory data exclusivity, a known and secure form of commercial advantage. Building on domestic expertise in OSPs, Canadian leaders created the Viral Interruption Medicines Initiative, a not-for-profit OSP, to develop pandemic ready-antivirals and address other areas of market failure.

Keywords: pandemic, antimicrobial resistance, patents, open science, partnerships, regulatory data protection

OPEN ACCESS

Edited by:
Bruno Villoutreix, Institut National de la Santé et de la Recherche Médicale (INSERM), France

Reviewed by:
Juan Fernandez-Recio, Institute of Vine and Wine Sciences, Spain
Pierre Tuffery, Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
E. Richard Gold
richard.gold2@mcgill.ca

†These authors have contributed equally to this work

Specialty section:
This article was submitted to In silico Methods and Artificial Intelligence for Drug Discovery, a section of the journal Frontiers in Drug Discovery

Received: *17 March 2022*
Accepted: *20 April 2022*
Published: *26 May 2022*

Citation:
Gold ER and Edwards AM (2022) Overcoming Market Failures in Pandemic Drug Discovery Through Open Science: A Canadian Solution. Front. Drug. Discov. 2:898654. doi: 10.3389/fddsv.2022.898654

INTRODUCTION

The COVID-19 pandemic showcased both the best and worst of science. Collectively, the scientific world quickly shared the SARS-CoV-2 sequence, created vaccines, put those vaccines through clinical trials and delivered them in record time. At the same time, scientists promoted false treatments such as vitamin D, hydroxychloroquine, and firms exercised their proprietary positions over vaccines and drugs to leave low- and lower-middle income countries greatly under-vaccinated.

One important strand of the pandemic story is that, as of the date of writing, only three anti-viral pills have been approved for use—one new and two repurposed. Pfizer's Paxlovid is a derivative of a shelved drug lead developed 20 years ago for the original SARS virus while the other two—remdesivir and molnupiravir—are repurposed from legacy programs targeting other viruses. Many firms and university labs attempted to repurpose other types of drugs to identify those with antiviral activity, but none of these efforts proved fruitful (Edwards 2020). There are many antiviral antibody drugs approved, and dozens in development, although these must be administered in hospitals and are priced beyond the reach of most people. Broadly, the world simply had no advance plan to proactively develop simple drugs to treat pandemics—despite several epidemics since the Millennium (Edwards et al., 2022).

As night follows day, there will be another viral pandemic, for which we must prepare now. Unfortunately, existing models of drug discovery have not proven themselves suitable. Pandemic preparedness is an example of market failure where reliance on traditional forms of intellectual property simply do not provide the incentive to develop the vaccines and simple drugs needed (Outterson et al., 2007; Eccleston-Turner 2016; Jacobs 2019).

In this review, we investigate a novel intellectual property approach to drug development that promises to develop pandemic-ready drugs quickly and accessibly before the next pandemic hits. This approach, an open science partnership, has been put into practice for 2 decades in the health sciences. Specifically, we summarize how a Canadian not-for-profit corporation, the Virus Interruption Medicines Initiative (VIMI) and its international partners aim to develop an open science drug discovery ecosystem to develop pandemic-ready drugs without the use of patents.

MARKET FAILURES IN DRUG DISCOVERY

While debate continues, there is growing evidence that drug discovery is facing productivity declines (Gold 2021). Drug development costs increase and drugs are less novel, while patient costs increase and accessibility decreases. This tendency is exacerbated in addressing pandemics given the large uncertainty as to drug target and timing of the pandemic (Eccleston-Turner 2016, 583). In this field, the incentives provided by patents, relied on heavily by the pharmaceutical industry in relation to other disease areas (Hall 2022), become less effective. Eccleston-Turner (2016) notes, for example, that patents did not play a significant role in developing a vaccine against the 2009 H1N1 influenza pandemic. Sherkow et al. (2021) go further in respect of vaccine development against SARS-CoV-2:

> Patents are instead—surprisingly—something of a sideshow. To the extent that innovation policymakers are trying to figure out where to focus their efforts in improving vaccine development and distribution, they shouldn't focus on patents.

Despite the lack of significant incentive effect of patents, firms and universities have gone on a patenting extravaganza during the COVID-19 pandemic. The World Intellectual Property Organization reports that firms and universities filed over 5,000 patents in the period ending in September 2021 (World Intellectual Property Organization. 2022). This marks a significant increase over the 500 patents relating to influenza vaccines from 1941 to 2011. Around 80% of patents on drug candidates related to repurposed drugs (World Intellectual Property Organization. 2022).

The Pandemic Market Is Broken

The problem is that, while we know we will face future pandemics, we know neither its timing nor the virus involved. This uncertainty leads firms to underinvest in research and development prior to an outbreak (Eccleston-Turner 2016). Once a pandemic hits, it is government and philanthropic investments that dominate due to the enormous social and economic losses suffered by the public. These outweigh whatever profits the private sector would earn through ordinary market forces. Data to the end of June 2021 show that governments, philanthropies and international organizations invested the overwhelming portion of the over $45 billion put into vaccine research and delivery (Devex 2021). Private sector investments, while significant—Pfizer says it spent $2 billion beyond the German government's direct investments and government contracts—paled in comparison to these public investments (Lalani et al., 2022).

Without the pull of a known market, patents are insufficient to provide the incentive to invest. It is not surprising, for example, that it was the public and philanthropic sectors that not only developed but tested the Ebola vaccine while industry remained on the sidelines (Herder et al., 2020). The key components of the mRNA vaccines were developed through public funds, largely at universities and government laboratories (Herder et al., 2022; Lalani et al., 2022).

Although government spending dominated vaccine and antiviral drug markets, this did not prevent rent seeking behavior. As noted above, universities heavily invested in patenting everything they could even though government grants and spending, not patents, provided the incentive for development and commercialization. Both universities and firms sought to capitalize on government largesse to extract maximum individual benefit.

This rent-seeking behavior came with a substantial cost. According to former International President of Doctors without Borders, Dr. Joanne Liu, by March 2022, 23 countries had not been able to vaccinate even 10% of their population and 73 countries had not reached the 40% vaccination mark (Meloche-Holubowski 2022). The British Medical Journal reported how, when the World Health Organization set up a vaccine hub in Africa to address vaccine inequality, vaccine patent holders threatened patent infringement (Davies 2022). Two Canadian companies associated with a university—where researchers had conducted the basic research using public funds—did not share their critical lipid nanoparticle technology with the hub (Herder et al., 2022). While Afrigen Biologics developed its COVID-19 mRNA vaccine using publicly available information—delayed by the lack of active sharing—it still faces hurdles in getting to market because of a continued failure to share.

Because of these failures, developing countries brought forward proposals to temporarily waive compliance with intellectual property rules built into international trade agreements and to require active sharing. The United States and France joined in the call to waive compliance with trade agreements. Pressure mounted and soon after the British Medical Journal report, both Pfizer and Moderna, holding rights to the two approved mRNA vaccines, moved to build facilities in Africa (Khemlani 2022) and, in the case of Moderna, to permanently waive enforcement of its patents (Moderna Inc. 2022).

Unfortunately, none of the firms involved is sharing the know-how and data needed for Afrigen to bring its vaccine to market.

The inefficiencies caused by market failures in the vaccine and pandemic antiviral drug markets thus result in three interrelated problems: 1) underinvestment before health crises, leading to delays and increased costs; 2) lack of access to vaccines and drugs (Drugs for Neglected Diseases initiative 2022) in much of the world, leading to a prolongation of the health crisis and an environment hospitable to the emergence of variants and resistance; and 3) rent-seeking behavior not related, in any significant manner, to incentives.

OPEN SCIENCE DRUG DISCOVERY

Acknowledging the inefficiencies in the pandemic market, global public health experts called for a "reboot" of the global health research system. Critical among their recommendations was the need for "a change of rules and incentives", particularly around intellectual property, "to secure the rapid, open sharing of inputs, processes and outputs." (Swaminathan et al., 2022).

In fact, the innovation literature has increasing focused on, and found evidence for, inefficiencies in research and development system (Bloom et al., 2020; Gold 2021), including in the pharmaceutical industry. The combination of decreasing research and development productivity with market failure, calls for new approaches to pandemic preparedness.

The Efficiency of Open Science Partnerships

A new innovation model that seeks to increase the efficiency of innovation has been gaining attention: the open science partnership (OSP) (Gold 2021). These multi-sectoral partnerships leverage the incentives within academic, governmental, philanthropic, and industrial sectors to accelerate innovation from early to late stages of commercialization. OSPs build on the differential expertise of the various partners, with generally academic and governmental partners taking on a larger role in the earlier stages and firms leading in the later stages of product development, manufacturing, and distribution.

A defining feature of OSPs is their adherence to various forms of sharing within the partnership: open results, open publications (including pre-prints), open data, open tools, open materials and the lack of intellectual property—most particularly, patents—that limit these (Ali-Khan et al., 2018). Partners are free to use the results of the OSP to improve or develop their own proprietary products, but within the OSP sharing is paramount in order to decrease duplication, lower transaction costs, and facilitate knowledge development (Gold 2021).

In fields facing market failure, OSPs offer the greatest promise. These fields include pandemic and antimicrobial resistance (AMR) drug development (Rand Europe 2021), due to uncertainty in terms of timing and target, and rare and pediatric diseases, because of their small market sizes. In these fields, as noted, patents do not provide a significant or sufficient incentive to invest. Firms underinvest or, as the demise of two leading AMR drug firms illustrates, go under (Jacobs 2019).

OSPs offer four strategic advantages for these areas of market failure.

First, they rely on incentives other than patents to overcome uncertainties. Academic inducements, such as publishing in a novel area, philanthropic motivations, such as addressing a critical health need, government interest in preventing large-scale economic losses, and private sector market advantages combine to provide a broader and deeper set of incentives to engage in drug research and development.

Second, free-riding is not only not a concern, but a feature. The goal of the majority of actors investing in areas of market failure—university researchers, philanthropists, and governments—do so to encourage production of drugs in general rather than a single, proprietary drug. Thus, if their investment induces firms to jump in, all the better. In particular, governments do not seek, through their investments, any profit on the pandemic drugs developed, leaving these to the private sector.

Third, there remain strong private sector incentives to invest. Because of the breadth of actors in the OSP ecosystem, private firm investments are heavily leveraged by investments by governments and philanthropies, greatly reducing their risk and enabling them to hold off on the majority of funding until after proof of concept. Further, firms benefit from regulatory data exclusivity that, given the leverage of other investments, is sufficient to attract them (Morgan et al., 2018). In fact, because this exclusivity is not subject to the level of validity attacks as exist for patents and that it begins later in the life of drug development, it is often more attractive than are patents.

Fourth, OSPs accelerate information, data, and material flow, avoiding duplication of effort and investments but also speeding up drug development itself (Gold 2016). In one example, an open science partnership between the Structural Genomics Consortium (SGC), the Ontario Institute for Cancer Research (OICR), and academic laboratories around the world resulted in the largest pre-clinical deal in Canadian history when OICR's commercialization partner licensed a leukemia drug to Cellgene for $40 million upfront and up to $1 billion if the drug proved successful (Gold and Morgan 2019).

The Viral Interruption Medicines Initiative

In response to the COVID-19 pandemic, leaders in open science partnerships in Canada created an OSP focused on proactively developing simple molecule drugs for the next pandemic and beyond. Drawing on the experience of the SGC's largest campus in Toronto (Morgan Jones et al., 2014) and the open science policy of the 60-some laboratories at the Montreal Neurological Institute and Hospital (the Neuro) (Gold 2016), these leaders created the Viral Interruption Medicines Initiative (VIMI). VIMI brings together universities, SMEs, large pharmaceutical firms, philanthropies, and government to solve two problems.

First, VIMI aims to develop, in conjunction with an international consortium of actors, a stock of antiviral drugs to treat each of the major viral families responsible for pandemics. VIMI and its partners will develop drugs to the end of Phase 1 trials and put them on the shelf until the next

pandemic. With no patents, any firm or laboratory around the world could test, manufacture, distribute or improve the drug. Further, by licensing the preclinical regulatory data package, VIMI and its partners would position firms to move quickly into Phase 2/3 trials, as well as launch combination studies. Second, VIMI will develop new antibiotics up to the end of preclinical work to place on the shelf should the need arise due to antimicrobial resistance.

VIMI takes an ecosystem approach to drug development, identifying global leaders in virology, microbiology, computational drug development, chemistry, and strategy to bring not only drugs to market but the ancillary products and services needed to support drug development. Combining funding from multiple sectors—large firms, SMEs, universities, and government—VIMI operates through four pillars.

The first pillar is computational drug discovery. Housing the Critical Assessment of Computational Hit-finding Experiments (CACHE) initiative (Ackloo et al., 2022), VIMI will establish competitions for computational drug discovery firms to validate and improve their algorithms based on drug targets that fall within VIMI's mandate. While the firms retain all intellectual property rights over their algorithms, all molecules submitted to the competition will be patent free. This model is similar to that used by the COVID Moonshot team, which is crowd-sourcing the development of a new SARS-CovV-2 protease inhibitor (Consortium. 2020). CACHE researchers will openly disseminate the results of laboratory analyses and make the molecules available.

The second pillar is open science drug development. VIMI will work with firms or other non-profit initiatives, such as READDI (www.READDI.org), to pursue the development of molecules consistent with its open science principles of sharing and absence of patent rights. VIMI will provide strategic and management expertise and co-fund development of drugs falling within its mandate.

The third pillar is open science strategic support, ranging from education, to open science commercialization strategy, to policy assistance. Firms or universities that have decided to pursue an open science strategy will access advice on how to establish and manage OSPs, develop novel open science tools, and develop and implement an open science strategy based on regulatory data exclusivity and other means.

The last pillar is the ability to collaborate with other public and private sector efforts, such as the COVID Moonshot initiative now being led by DNDI ("COVID Moonshot | DNDi" n.d.), as well as any other global initiatives that aim to develop new medicines with a focus on equitable access, including repositories such as bioRxiv and medRxiv, provided they continue after the COVID-19 pandemic.

DISCUSSION

The COVID-19 pandemic highlighted the need for governments, universities and firms to develop antiviral medicines against the major pandemic viral families in advance of the next pandemic. Traditional intellectual property strategies, based on patenting and exclusive licensing, do not provide sufficient incentives given the uncertainty as to the virus responsible for the next pandemic and its timing. This has led to market failures, taking the form of too few medicines available to combat the pandemic when needed, and too little access to those medicines that exist.

While the pharmaceutical sector normally relies heavily on patents, patents come at a cost. They restrict use of knowledge, increase transaction costs, and, as the COVID-19 pandemic illustrated, create global access problems. With increased costs and decreased access, viruses have more time and opportunity to evolve, creating new variants. The health, social and economic cost is enormous. Given that patents are relatively weak incentives, especially in advance of a pandemic, their costs become unacceptable.

OSPs provide an alternative model of drug development that is particularly apt in areas of market failure. Based on open sharing of results, tools, materials, and publications, OSPs remove transaction costs involved with negotiating about patents by eschewing them (Gold 2021). OSPs leverage the different incentives that motivate academic, philanthropic, governmental, and industrial partners to achieve agreed upon goals. Drawing on the different forms of expertise—with academic researchers strongest at early stages of commercial development and firms at later stages—OSPs move technology through to delivery.

Rather than invest billions of dollars after a pandemic or health crisis, VIMI aims to develop drugs in advance of the next crisis at a much, much lower cost. Bringing together the research and development expertise of a broad group of actors, identifying gaps in the ecosystem and filling them, and providing education and strategic advice, VIMI is able to accelerate development while reducing transaction costs in proactively developing pandemic-ready drugs. VIMI will also pursue drug development in other areas of market failure: AMR, rare diseases, and pediatric diseases. With its focus on open science drug discovery in various areas of market failure, VIMI is unique.

While the use of OSPs to prepare for pandemics and other health crises is new, VIMI draws on the expertise of the SGC and of the Neuro to enter into this field. With its network and reputation within the national and international research and industrial communities, the SGC is jump-starting VIMI's activities.

The VIMI project raises several uncertainties. The first is whether it will successfully identify drugs with pandemic treatment potential. Even with access to global experts, there is no guarantee that VIMI will develop a portfolio of drugs that will be positioned to successfully treat the next pandemic or AMR crisis. The second is whether its partners will sustain their investments in open science once the COVID-19 pandemic is far in the rearview mirror. Experience from the SGC suggests that they will, but governments and firms have lost interest in preparing for pandemics in the past (Sirleaf and Clark 2021). Third, there remain questions about how VIMI will operationalize its commercial strategy during the next pandemic. For example, VIMI will need to quickly enable actors to acquire rights to the regulatory data package, encourage firms to conduct Phase II/III clinical studies, and enable those actors to manufacture and distribute drugs globally.

Beyond areas of market failure, the OSP model may provide an additional commercialization strategy, particularly in respect of technology that requires multiple sets of knowledge and skills not normally housed within a single entity, where the technology faces a

high technological risk of failure, where benefits are widespread rather than being able to be captured by a single entity, and where global access is important. With experiments such as VIMI, policy-makers and firms can learn how best to deploy this strategy.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

REFERENCES

Ackloo, S., Al-awar, R., Amaro, R. E., Arrowsmith, C. H., Azevedo, H., Batey, R. A., et al. (2022). CACHE (Critical Assessment of Computational Hit-Finding Experiments): A Public-Private Partnership Benchmarking Initiative to Enable the Development of Computational Methods for Hit-Finding. *Nat. Rev. Chem.* 6, 287–295. doi:10.1038/s41570-022-00363-z

Ali-Khan, S. E., Jean, A., and Gold, E. R. (2018). Identifying the Challenges in Implementing Open Science. *MNI Open Res.* 2 (October), 5. doi:10.12688/mniopenres.12805.1

Bloom, N., Jones, C. I., Van Reenen, J., and Webb, M. (2020). Are Ideas Getting Harder to Find? *Am. Econ. Rev.* 110 (4), 1104–1144. doi:10.1257/aer.20180338

Consortium, T. C. M., Chodera, J., Lee, A., London, N., and Delft, F. v. (2020). COVID Moonshot: Open Science Discovery of SARS-CoV-2 Main Protease Inhibitors by Combining Crowdsourcing, High-Throughput Experiments, Computational Simulations, and Machine Learning. *biorxiv*. doi:10.26434/chemrxiv.13158218.v1

Davies, M. (2022). Covid-19: WHO Efforts to Bring Vaccine Manufacturing to Africa Are Undermined by the Drug Industry, Documents Show. *BMJ* 376 (February), o304. doi:10.1136/bmj.o304

Devex (2021). Funding COVID-19 Vaccines: A Timeline." Devex. AvaliavleAt: https://www.devex.com/news/sponsored/funding-covid-19-vaccines-a-timeline-97950 (Accessed June 27, 2021).

Drugs for Neglected Diseases initiative (2022). Pfizer Blocking Research to Generate Evidence on Optimal Use of Novel Antiviral for COVID-19 Patients in Low- and Middle-Income Countries | DNDi. Avaliable at: https://dndi.org/press-releases/2022/pfizer-blocking-research-to-generate-evidence-optimal-use-novel-antiviral-covid19-low-and-middle-income-countries/ (Accessed March 15, 2022).

Eccleston-Turner, M. (2016). The Economic Theory of Patent Protection and Pandemic Influenza Vaccines. *Am. J. Law. Med.* 42 (2–3), 572–597. doi:10.1177/0098858816658280

Edwards, A. M., Baric, R. S., Saphire, E. O., and Ulmer, J. B. (2022). Stopping Pandemics before They Start: Lessons Learned from SARS-CoV-2. *Science* 375, 1133–1139. doi:10.1126/science.abn1900

Edwards, A. (2020). What Are the Odds of Finding a COVID-19 Drug from a Lab Repurposing Screen? *J. Chem. Inf. Model.* 60 (12), 5727–5729. doi:10.1021/acs.jcim.0c00861

Gold, E. R., and Morgan, M. (2019). For Innovation, Open Science Means Open for Business. AvaliableAt: https://www.cigionline.org/articles/innovation-open-science-means-open-business (Accessed February 6, 2019).

Gold, E. R. (2021). The Fall of the Innovation Empire and its Possible Rise through Open Science. *Res. Policy* 13, 104226. doi:10.1016/j.respol.2021.104226

Gold, E. R. (2016). Accelerating Translational Research through Open Science: The Neuro Experiment. *PLoS Biol.* 14 (12), e2001259. doi:10.1371/journal.pbio.2001259

Hall, B. H. (2022). Patents, Innovation, and Development. *Int. Rev. Appl. Econ.* 1, 26. doi:10.1080/02692171.2021.2022295

Herder, M., Gold, E. R., and Murthy, S. (2022). University Technology Transfer Has Failed to Improve Access to Global Health Products during the COVID-19 Pandemic Healthcare Policy. AvaliableAt: https://www.longwoods.com/content/26724/healthcare-policy/university-technology-transfer-has-failed-to-improve-access-to-global-health-products-during-the-cov (Accessed March 10, 2022).

Herder, M., GrahamGraham, J. E., and Gold, R. (2020). From Discovery to Delivery: Public Sector Development of the RVSV-ZEBOV Ebola Vaccine. *J. Law Biosci.* 7, lsz019. doi:10.1093/jlb/lsz019

Jacobs, A. (2019). Crisis Looms in Antibiotics as Drug Makers Go Bankrupt. *N. Y. Times* 25, 19. Avaliable at: https://www.nytimes.com/2019/12/25/health/antibiotics-new-resistance.html (Accessed March 14, 2022).

Morgan Jones, M., Castle-Clarke, S., Brooker, D., Nason, E., Huzair, F., and Chataway, J. (2014). The Structural Genomics Consortium: A Knowledge Platform for Drug Discovery: A Summary. *Rand Health Q.* 4 (3), 19.

Khemlani, A. (2022). Pfizer's South Africa Vaccine Partnership Won't Impact Global Vaccination Goals." Yahoo! Finance. AvaliableAt: https://finance.yahoo.com/news/pfizers-south-africa-vaccine-partnership-wont-impact-global-vaccination-goals-190800148.html (Accessed March 4, 2022).

Lalani, H. S., Avorn, J., and Kesselheim, A. S. (2022). US Taxpayers Heavily Funded the Discovery of COVID-19 Vaccines. *Clin Pharma Ther.* 111 (3), 542–544. doi:10.1002/cpt.2344

Meloche-Holubowski, M. (2022). Les masques tombent, mais de nombreuses personnes demeurent vulnérables à la COVID | Coronavirus." Radio-Canada.ca. Radio-Canada.ca. AvaliableAt: https://ici.radio-canada.ca/nouvelle/1868151/pandemie-covid-coronavirus-vulnerables-immunosupprime-cancer-aines (Accessed March 11, 2022).

Moderna Inc (2022). Moderna Announces its Global Public Health Strategy. AvaliableAt: https://s29.q4cdn.com/745959723/files/doc_news/Moderna-Announces-Its-Global-Public-Health-Strategy-2022.pdf (Accessed March 11, 2022).

Morgan, M. R., RobertsRoberts, O. G., and Edwards, A. M. (2018). Ideation and Implementation of an Open Science Drug Discovery Business Model - M4K Pharma. *Wellcome Open Res.* 3 (December), 154. doi:10.12688/wellcomeopenres.14947.1

Outterson, K., Samora, J. B., and Keller-Cuda, K. (2007). Will Longer Antimicrobial Patents Improve Global Public Health? *Lancet Infect. Dis.* 7 (8), 559–566. doi:10.1016/S1473-3099(07)70188-3

Rand Europe (2021). *How Drug-Resistant Infections Are Undermining Modern Medicine – and Why More Research Is Needed Now*. London: Wellcome Trust. AvaliableAt: https://cms.wellcome.org/sites/default/files/2021-11/RAND-How-drug-resistant-infections-are-undermining-modern-medicine.pdf (Accessed April 17, 2022).

Sherkow, Jacob. S., Nicholson, Price., and Sachs, Rachel. (2021). Written Description: Are Patents the Cause Of—Or Solution To—COVID-19 Vaccine Innovation Problems? (No!)." *Written Description* (Blog). AvaliableAt: https://writtendescription.blogspot.com/2021/03/are-patents-cause-ofor-solution-tocovid.html (Accessed March 4, 2021).

Sirleaf, E. J., and Clark, H. (2021). Report of the Independent Panel for Pandemic Preparedness and Response: Making COVID-19 the Last Pandemic. *Lancet* 398 (10295), 101–103. doi:10.1016/S0140-6736(21)01095-3

Swaminathan, S., Pécoul, B., Abdullah, H., Christou, C., Gray, G., IJsselmuiden, C., et al. (2022). Reboot Biomedical R&D in the Global Public Interest. *Nature* 602 (7896), 207–210. doi:10.1038/d41586-022-00324-y

World Intellectual Property Organization (2022). *Patent Landscape Report: COVID-19-Related Vaccines and Therapeutics*. Geneva, Switzerland: World Intellectual Property Organization. doi:10.34667/TIND.45030

Conflict of Interest: EG and AE are unpaid members of the Board of Directors of the Virus Interruption Medicines Initiative and Edwards is its President. Edwards is also CEO of the Structural Genomics Consortium that engages in public, non-patented, research related to pandemic drug discovery.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

MINI REVIEW
published: 18 July 2022
doi: 10.3389/fddsv.2022.927164

Nanobodies: COVID-19 and Future Perspectives

Guillermo Valenzuela-Nieto[1], *Zaray Miranda-Chacon*[1,2], *Constanza Salinas-Rebolledo*[1], *Ronald Jara*[1,3], *Alexei Cuevas*[1], *Anne Berking*[4] *and Alejandro Rojas-Fernandez*[1,4,5]*

[1]Institute of Medicine, Faculty of Medicine, Universidad Austral de Chile, Valdivia, Chile, [2]School of Medicine, University of Costa, San José, Rica, [3]Institute of Biochemistry and Microbiology, Universidad Austral de Chile, Valdivia, Chile, [4]Berking Biotechnology, Valdivia, Chile, [5]Institute of Philosophy and Complexity Sciences, Santiago, Chile

The COVID-19 pandemic has driven biotechnological developments to provide new and more effective tools for prophylaxis, diagnosis, and therapy. Historically, monoclonal antibodies have been valuable tools; however, the pandemic has shown some weaknesses, such as production limitations at a global scale. An alternative to conventional monoclonal antibodies are nanobodies, recombinant fragments of the variable region of single-domain antibodies derived mainly from the Camelidae family. Nanobodies have multiple characteristic benefits: they are small (15 KDa) and have remarkable refolding capability and unlimited possibilities for modifications due to their recombinant nature. Here, we review the application of nanobodies in diagnosis and treatment of SARS-CoV-2 infection.

Keywords: nanobodies (VHH), SARS-CoV-2, COVID-19, virus, llama (*Lama glama*), alpaca (*Vicugna pacos*), VHH antibody fragment

OPEN ACCESS

Edited by:
Juan Fernandez-Recio, Institute of Vine and Wine Sciences (CSIC), Spain

Reviewed by:
Fabian Glaser, Technion Israel Institute of Technology, Israel

***Correspondence:**
Alejandro Rojas-Fernandez
alejandro.rojas@uach.cl

Specialty section:
This article was submitted to Anti-Infective Agents, a section of the journal Frontiers in Drug Discovery

Received: *23 April 2022*
Accepted: *17 June 2022*
Published: *18 July 2022*

Citation:
Valenzuela-Nieto G, Miranda-Chacon Z, Salinas-Rebolledo C, Jara R, Cuevas A, Berking A and Rojas-Fernandez A (2022) Nanobodies: COVID-19 and Future Perspectives. Front. Drug. Discov. 2:927164. doi: 10.3389/fddsv.2022.927164

INTRODUCTION

Heavy-chain-only antibodies (HCAbs) are found in camelids (e.g., dromedaries, camels, llamas, and alpacas) (Arbabi-Ghahroudi, 2017) and some cartilaginous fish species such as nurse shark (*Ginglymostoma cirratum*), wobbegong shark (*Orectolobus maculatus*), spiny dogfish (*Squalus acanthias*), and smooth dogfish (*Mustelus canis*) (Camacho-Villegas et al., 2013; Cheong et al., 2020; Dooley and Flajnik, 2006; Dooley et al., 2006; Feige et al., 2014; Ohtani et al., 2013; Roux et al., 1998; Stanfield et al., 2004; Nuttall et al., 2001). Within their immune system, type IgG2 and IgG3 lack the CH1 domain due to alternative splicing. Consequently, the light chains do not pair to the final antibody leading to the generation of HCAbs (Hamers-Casterman et al., 1993). IgG2 and IgG3 rely on heavy variable domains or VHH, a small domain of approximately 15 kDa to bind to specific antigens (Muyldermans et al., 1994; Desmyter et al., 1996; Arbabi Ghahroudi et al., 1997; Vu et al., 1997; Hassanzadeh GH et al., 1998; Lauwereys et al., 1998). The key difference between conventional monoclonal antibodies and HCAbs is that the latter are encoded from single genes (**Figure 1A**). Thus, the VHH can be amplified from cDNA, cloned into libraries of diverse natures, and later isolated using conventional molecular biology procedures. Once isolated, the recombinant VHH fragments of HCAbs are known as nanobodies (Muyldermans et al., 1994). Nanobodies conserve selective binding to specific antigens in the ~15 KDa minimal structure, 10 times smaller than conventional antibodies (Arbabi Ghahroudi et al., 1997). Nanobodies are soluble and highly stable to a wide range of pH and temperatures (Bond et al., 2003), along with an outstanding penetrability (Muyldermans, 2013) (**Figure 1B**). Some nanobodies are able to penetrate the blood-brain barrier which places them as promising tools for future diagnosis and therapy for neurodegenerative diseases. Among all the outstanding advantages of nanobodies, one of the

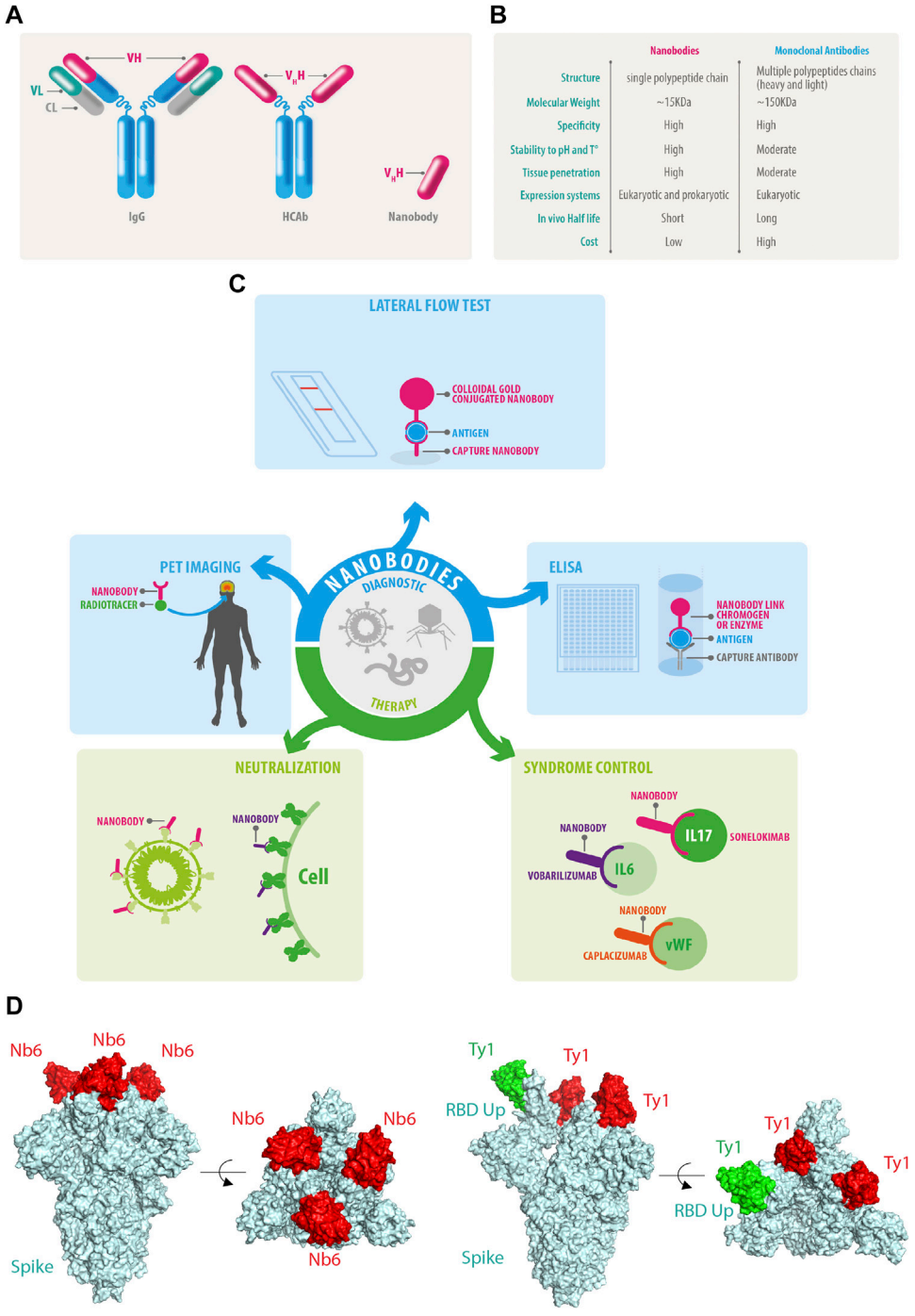


FIGURE 1 | Nanobody features and functions. **(A)** Conventional IgG antibodies contain heavy and light chains, HCAb does not associate with light chains allowing the isolation of the genetic fragments of the Vhh also known as nanobodies. **(B)** Comparative features of nanobodies versus monoclonal antibodies. **(C)** Current and future perspectives of nanobodies. **(D)** Nanobodies bind to the RBD of SARS-CoV-2 and neutralize the infection by different mechanisms: NB6 binds to the RBD of each Spike protomer in a down conformation (PDB ID: 7KKK), while Ty1 allows the upper conformation of one RBD of Spike in green (PDB ID: 6ZXN), in both examples, the nanobodies prevent the engagement of the human ACE2 receptor.

most attractive is the performance achieved through recombinant expression systems. Nanobodies can be produced efficiently in eukaryotic and prokaryotic systems (Arbabi-Ghahroudi et al., 2005; Liu and Huang, 2018; de Marco, 2020) and, because nanobody sequences are obtained during the selection process, the final molecule can be engineered to cope with several applications using conventional molecular biology tools. Moreover, open-source computational pipelines are available for nanobody humanization such as "Llamanade" (Sang et al., 2022).

Nanobodies are remarkable tools for diagnosing and treating various diseases, and the COVID-19 pandemic has driven the generation of several nanobodies against SARS-CoV-2. Here, we summarize some of the current applications and production strategies of nanobodies to help fight viral pathogens.

Nanobody Expression Systems

Nanobodies are compact structures and can be produced in high yields in several expression systems, such as bacteria, yeast, mammalian cells, and plants (Frenken et al., 2000; Arbabi-Ghahroudi et al., 2005; Ismaili et al., 2006).

Production in prokaryotes has a low cost and involves easy handling (Rosano et al., 2019). The traditional way to express recombinant proteins in bacteria is in the cytoplasm; however, the reducing cytoplasmic conditions can negatively affect the formation of disulfide bonds in some nanobodies, which are necessary for the correct folding of its tertiary structure (Govaert et al., 2012; Muyldermans, 2013; Hagihara and Saerens, 2014; Billen et al., 2017). Despite this, there are examples of nanobodies produced under cytoplasmatic conditions, which require the attachment of other proteins (Anderson et al., 2018), or expression in special bacterial strains with an oxidizing cytoplasmatic environment such as SHuffle T7 cells (Li et al., 2019) or co-expression with enzymes such as Erv1p sulfhydryl oxidase (Veggiani and de Marco, 2011; Shriver-Lake et al., 2017). High yields are also obtained when expressing proteins in classical inclusion bodies and further by denaturation in urea-mediated protein extraction (Maggi and Scotti, 2017). Also, there is an interesting alternative of expressing the nanobody coupled to a secretion pathway, such as the hemolysin secretion system (Günaydın et al., 2014; Ruano-Gallego et al., 2019).

Nowadays, the most convenient method for low-scale production of recombinant nanobodies is in *Escherichia coli* periplasm (Salema et al., 2013). Its oxidizing environment ensures correct folding and disulfide bond formation, and the periplasmic extracts enriched with the recombinant nanobodies facilitate subsequent purification (Conrath et al., 2001; Billen et al., 2017). The N-terminal pelB leader sequence drives the protein to the post-translational Sec pathway, the nanobodies are completely synthesized in the ribosome and then released to the Sec-translocase, which enables its carriage through the inner bacterial membrane and accumulates the nanobodies in the periplasm (Keen and Tamaki, 1986; Yoon et al., 2010; Billen et al., 2017). The recombinant proteins that accumulate in the periplasmic space are usually recovered after an osmotic shock which softly breaks the outer bacterial membrane, allowing the release of the proteins and preventing further contamination with *E. coli* cytoplasmatic proteins. Affinity purification can be applied directly after using various affinity matrices, such as hexahistidine (His6)-tag (Salema and Fernández, 2013), maltose-binding protein (MBP), or the Avi-tag, which allows *in vivo* biotinylation of the tagged protein (Hernot et al., 2012; Zhu et al., 2014; Noor et al., 2018; Sun et al., 2018; Du et al., 2019).

Nanobodies can also recognize linear peptides, an example of this is a nanobody called ALFA Selector, which recognizes the short ALFA-tag (SRLEEELRRRLTE) sequence, a very efficient tag for protein purification, including nanobodies themselves (Götzke et al., 2019; Kilisch et al., 2021).

Human or humanized monoclonal antibodies have been approved during the pandemic as emergency treatment measures. However, the production limitation led to the access of being almost exclusive to high-income countries in the northern hemisphere. The production of conventional antibodies occurs in mammalian cells, which is costly and time-consuming. In most cases, single clones of mammalian HEK293 or CHO cells must be isolated and expanded into cell banks, a process that takes several months. In contrast, therapeutic nanobodies and nanobodies fused to the Fc of conventional antibodies (Nb-Fc) can be produced in yeast, such as *Pichia pastoris,* in an endotoxin-free manner and at a very low cost. Interesting technologies based on CRISPR/Cas9 improve the glycosylation homogeneity of *P. pastoris,* which could allow efficient expression for therapeutic technologies using nanobodies (Krainer et al., 2013; Weninger et al., 2016; Schepens et al., 2021).

Nanobodies as Diagnostic Tools

Immunoaffinity techniques are the primary tools for rapid diagnosis, not only in infectious diseases but also in an extensive range of pathologies. Nanobodies are incipient in this field, and some technical difficulties regarding their small size and low retention on nitrocellulose strips remain to be solved. Serological tests have been implemented for parasitic infections such as *Taenia solium* cysticercosis (Deckers et al., 2009; Huang et al., 2010), *Trypanosoma spp* (Saerens et al., 2008a), and bacterial diseases, such as *S. aureus* (Stijlemans et al., 2004; Stijlemans et al., 2017). Indeed, this proof of concept opens up a large field of action for nanobodies in the upcoming years.

Nanobodies have been generated to capture several viral proteins of HIV (Gray et al., 2017); norovirus (Koromyslova and Hansman, 2017), dengue (Fatima et al., 2014) among others. When used for diagnosis, nanobodies can be classified in two groups: the primary nanobodies responsible for the recognition of a pathogen or molecule of interest and the secondary antibodies that bind to primary antibodies and unveil its presence by colorimetric or enzymatic reactions. Nanobodies as recombinant proteins can be modified to accomplish the function of primary and secondary antibodies simultaneously, for instance, nanobody fusion to enzymes such as horseradish peroxidase (HRP) has been used for the detection of anti-Newcastle disease virus (NDV) antibodies in chicken sera (Sheng et al., 2019).

Significant efforts have been made to develop diagnostic techniques for COVID-19, focusing mainly on speed and

accuracy. The first available tools were based on conventional monoclonal IgM and IgG lateral flow immunoassays (Yetisen et al., 2013; Goossens et al., 2017); however, the expression of IgM and IgG against SARS-CoV-2 antigens is only detectable in late disease stages. Therefore, the gold standard diagnostic test for SARS-CoV-2 diagnosis is the reverse-transcription quantitative polymerase chain reaction (RT-qPCR) from nasopharyngeal and oropharyngeal swab samples (Wang et al., 2020a; To et al., 2020). After vaccines were implemented, IgM and IgG lateral flow immunoassays became useful to determine the immune response against vaccines. Recently developed secondary nanobodies showed superior properties for cellular biology studies regarding penetrance, staining accuracy and, furthermore, secondary nanobodies can be premixed with primary antibodies to bypass the primary antibody animal-species limitations (Sograte-Idrissi et al., 2020). Nanobodies can be fused to the Fc of conventional immunoglobulins and produced recombinantly, which complements nanobody binding capabilities with several technologies already available for monoclonal antibodies (Bao et al., 2021; Girt et al., 2021; Valenzuela Nieto et al., 2021) (**Figure 1C**).

Nowadays nanobody-based lateral flow tests can rapidly detect recombinant human interferon α2b (Qin et al., 2021). They have also been used to detect active *Trypanosoma congolense* infections (Pinto Torres et al., 2018). In addition to rapid tests, nanobodies are efficient and sensitive tools for enzyme-linked immunosorbent assay (ELISA), a method allowing the capture and quantitative measure of antigens in the small absorbent surface. In short, nanobodies can be immobilized on the surface of ELISA plates to capture the molecule of interest, and further a second non-competitive nanobody associated to enzymatic activity can be applied to detect the already trapped molecule of interest. Nanobodies covalently coupled to HRP provide sensitive detection of SARS-CoV-2-specific full-length trimeric spike or RBD (Valenzuela Nieto et al., 2021).

In recent years, miniaturization of chips and sensing layers for biosensor equipment linked with microfluidic devices have been proposed as the best option to obtain the most sensitive detection level (Conroy et al., 2009). However, biosensor sensitivity depends on the physical properties of the molecule that binds its target (Saerens et al., 2008b). One of the advantages of nanobodies is their small size, allowing higher molecule density on a surface, and the possibility of easy directional immobilization, which translates into a higher ligand binding capacity leading to improved sensitivity for detecting low analyte concentrations (Huang et al., 2005). Another recently developed system is the use of nanobody-based organic electrochemical transistors (OECTs) which applies a conjugated polymer and a nanobody to detect SARS-CoV-2 spike protein (Guo et al., 2021).

In the field of non-infectious diseases, nanobodies outperform some conventional antibodies, for instance, in the detection of human prostate-specific antigen, an early marker of prostate cancer (Huang et al., 2005; Saerens et al., 2005). Nanobodies have also been conjugated with radioisotopes as modern diagnostic tools for personalized imaging medicine such as positron emission tomography (PET) to detect EGFR, a tyrosine kinase receptor that is highly expressed in most epithelial cancer cells, believed to contribute to tumor malignancy (Penault-Llorca et al., 2006; Gainkam et al., 2008; Tijink et al., 2008). PET imaging is a promising and emergent field for nanobody applications in *in vivo* imaging (**Figure 1C**). It requires the accumulation of radiolabeled nanobodies at the target tissue or tumor and rapid excretion of the remanent circulating nanobodies to distinguish between the real signal and background (Massoud and Gambhir, 2003). Most radioisotopes used for *in vivo* imaging have a short half-life; for instance, Fluorine ^{18}F decays by positron (β+) emission with a half-life of 109.7 min. Conventional antibodies persist in circulation, leading to a high background signal for some applications. In contrast, nanobodies are generally cleared rapidly suggesting a potential extended use for PET diagnostics (Tijink et al., 2008; Harmand et al., 2021). Nanobodies are expected to become important for cancer diagnosis: Molecules including monoclonal antibodies (Frigerio et al., 2021) used for PET diagnostics are useful for directed radiotherapies through the simple exchange of radioisotopes with, for instance, Lutetium ^{77}Lu. These radiotracers used for diagnostics and therapeutical applications are known as Theranostics (Debnath et al., 2022; Woźniak et al., 2022).

Nanobodies show plenty of advantages and overcome some of the problems observed with conventional antibodies, creating many opportunities for future diagnostic applications.

Immunotherapies Based on Nanobodies

Previous to the COVID-19 pandemic, nanobody-based therapeutic approaches were developed against viruses of global concern such as human immunodeficiency virus-1 (HIV-1) (Chen et al., 2008; Gong et al., 2012; McCoy et al., 2012; Matz et al., 2013), influenza viruses (Ashour et al., 2015; Schmidt et al., 2016), hepatitis C virus (HCV) (Tarr et al., 2013), respiratory syncytial virus (RSV) (Hultberg et al., 2011; Schepens et al., 2011), and enteric viruses (Wu et al., 2017). Antiviral nanobodies were also tested in clinical trials against rotavirus and the human respiratory syncytial virus (RSV). Remarkably, ALX-0171, a trivalent nanobody that neutralizes RSV, substantially decreased the viral load in children. Additionally, it is the first nanobody-based treatment delivered by nebulization through the airway (Palomo and Mas, 2016; Stohr and Palomo, 2016; Larios Mora and Gallup, 2018). Nanobodies have significant advantages when used as a therapy (Chakravarty et al., 2014; Jovčevska and Muyldermans, 2020). Several nanobodies were developed to modulate the immune and inflammatory responses. For example, vobarilizumab (ALX-0061) (Dörner et al., 2017) is a bispecific anti-IL6-R nanobody which has been engineered to extend its half-life targeting human serum albumin; secukinumab (ALX-0761) is a trivalent nanobody against IL-17A/F (Langley et al., 2014; De Munter et al., 2018; Svecova et al., 2019; Xie et al., 2019); caplacizumab (ALX-0081 or ALX-0681) is a bivalent humanized anti–von Willebrand Factor (vWF) nanobody (Abdelghany and Baggett, 2016; Peyvandi et al., 2016; Peyvandi et al., 2017; Scully et al., 2019), which has received approval from the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) in the United States, for treating patients with thrombotic thrombocytopenic purpura

TABLE 1 | Nanobodies and SARS-CoV-2.

Nanobodies	Source	Method	Target	References
2NSP23; 2NSP90	Llama	Phage display	Nsp9	Esposito et al., 2021 (Esposito et al., 2021)
6ID10 (5; 6; 16; 40; 70; 71; 75; 96; 99; 113)	Shark	Phage display	RBD	Gauhar et al., 2021 (Gauhar et al., 2021)
A8-G11-Fc	Llama (naïve library)	Phage display	ACE2	Lu et al., 2021 (Lu et al., 2021b)
aRBD-2–5; aRBD2-7	Alpaca	Phage display	RBD	Ma et al., 2021 (Ma et al., 2021)
C5; H3; C1; F2	Llama	Phage display	RBD	Huo et al., 2021 (Huo et al., 2021)
H11-D4; H11-H4	Llama (naïve library)	Phage display	RBD	Huo et al., 2020 (Huo et al., 2020)
k-874A	cDNA library	cDNA display	RBD	Haga et al., 2021 (Haga et al., 2021)
KA1; KC1; KC3	Synthetic library	Yeast display	RBD	Zupancic et al., 2021 (Zupancic et al., 2021)
MR3; MR17; SR4; SR31	Synthetic library	Ribosome and Phage display	RBD	Li et al., 2021; Yao et al., 2021 (Li et al., 2021b; Yao et al., 2021)
Nanosota-1	Llama and alpaca (naïve library)	Phage display	RBD	Ye et al., 2021 (Ye et al., 2021)
NB1A7; NB1B11	Camels	Phage display	RBD	Shi et al., 2022 (Shi et al., 2022)
NB1-Nb2-Fc	Synthetic library	Phage display	RBD	Chi et al., 2022 (Chi et al., 2022)
Nb11-59	Camels	Phage display	RBD	Gai et al., 2021 (Gai et al., 2021)
Nb15; Nb56; Nb12; Nb30	Llama; alpaca; dromedary, Bactrian camel	Phage display; Nanomouse	RBD	Xu et al., 2021 (Xu et al., 2021)
NB15; Nb22; Nb31	Alpaca	Phage display	RBD	Wu et al., 2021 (Wu et al., 2021b)
Nb6	Synthetic library	Yeast display	Spike	Schoof et al., 2020 (Schoof et al., 2020)
Nb91-Nb3-hFc	Camel (naïve library)	Phage display	RBD	Lu et al., 2021 (Lu et al., 2021a)
Nbs 89	Llama	MS proteomic	RBD	Nambulli et al., 2021; Sun et al., 2021; Xiang et al., 2020 (Xiang et al., 2020; Nambulli et al., 2021; Sun et al., 2021)
NIH-CoVnb-112	Llama	Phage display	Spike	Esparza et al., 2020 (Esparza et al., 2020)
NM1226; NM1230	Alpaca	Phage display	RBD	Wagner et al., 2021 (Wagner et al., 2021)
P2C5; P5F8; P2G1	Camel	Phage display	RBD	Favorskaya et al., 2022 (Favorskaya et al., 2022)
Re6H06; Re9B09; Re5D06; R28	Alpaca	Phage display	RBD	Güttler et al., 2021 (Güttler et al., 2021)
S1-49; S1-1; S1-23; S1-46; RBD-9; RBD-35; S2-10; S2-40	Llama	MS proteomic	RBD	Mast et al., 2021 (Mast et al., 2021)
S14	Alpaca	Phage display	RBD	Li et al., 2021 (Li et al., 2021c)
saRBD-1	Alpaca	Phage display	RBD	Weinstein et al., 2022 (Weinstein et al., 2022)
Sb14; Sb16; Sb45; Sb68	Synthetic library	Ribosome and Phage display	RBD	Ahmad et al., 2021 (Ahmad et al., 2021)
Sb23	Synthetic library	Phage display	RBD	Custódio et al., 2020 (Custódio et al., 2020)
SP1b4; SP1D9; SP3H4	Synthetic library	Phage display	RBD	Stefan et al., 2021 (Stefan et al., 2021)
SR6v15; Nb21; SR6	CeVICA	Ribosome display	RBD	Chen et al., 2021 (Chen et al., 2021)
TB202-1; TB202-3; TB202-63	Synthetic library	Phage display	RBD	Yuan et al., 2022 (Yuan et al., 2022)
Ty1; Fu2	Alpaca	Phage display	RBD	Hanke et al., 2020, 2022 (Hanke et al., 2020a; Hanke et al., 2022)
VHH-E; VHH-U; VHH-V; VHH-W	Alpaca and llama	Phage display	RBD	Koenig et al., 2021 (Koenig et al., 2021)
VHH-Fc	Llama (naïve library)	Phage display	Spike	Dong et al., 2020 (Dong et al., 2020a; Dong et al., 2020b)
VHH72*	Llama	Phage display	RBD	Schepens et al., 2021 (Schepens et al., 2021)
W25	Alpaca	Bacterial display	RBD	Valenzuela Nieto et al., 2021 (Valenzuela Nieto et al., 2021)
WNb2; WNb7; WNb10	Alpacas	Phage display	RBD	Pymm et al., 2021 (Pymm et al., 2021)

**An enhanced version of this Fc-linked nanobody in clinical study phase II is called XVR011. (www.clinicaltrials.gov).*

(Jovčevska and Muyldermans, 2020), becoming the first nanobody approved for clinical therapy of a chronic disease (**Figure 1C**).

The primary global response to the COVID-19 pandemic was the creation of efficient vaccines (Li et al., 2020; Li et al., 2021a; Awadasseid et al., 2021; Wu et al., 2021a; Baden et al., 2021; Russell et al., 2021). Nowadays, the global vaccination initiatives cover 56% of the world population with two doses. Global vaccination success was limited by inaccessibility to vaccines and refusal to be vaccinated for personal reasons. In addition, significant efforts were placed in the study of repurposing drugs that may limit mortality and ameliorate COVID-19 symptoms (Wang et al., 2020b; Canedo-Marroquín et al., 2020; Valle et al., 2020). There are more than 2000 clinical trials registered on www.clinicaltrials.gov with various topics ranging from contact tracing, dietary supplements, anti-viral therapies, and drugs that have been in the spotlight, such as chloroquine, hydroxychloroquine, and ivermectin.

The current development of antibodies against SARS-CoV-2 focuses on neutralizing antibodies against the spike protein (Chi et al., 2020a; Shi et al., 2020). In the early days of the first wave of the COVID-19 outbreak, plasmapheresis of convalescent SARS-CoV-2 patients was implemented to supplement antibodies to those at risk due to the lack of alternatives (Jiang et al., 2020; Liu et al., 2020; Longueira et al., 2021) (**Table 1**).

The first neutralizing nanobodies against SARS-CoV-2 targeted SARS-CoV-1 and MERS-CoV RBDs, but fortunately, they also exhibited a remarkable cross-reactivity and neutralization capability against SARS-CoV-2 (Wrapp et al., 2020). These were followed by nanobodies identified

from synthetic libraries, "sybodies", against SARS-CoV-2 (Dong et al., 2020a; Custódio et al., 2020; Walter et al., 2020). The framework regions of the synthetic library were partially humanized to decrease the immune response if administered to humans.

Other reported strategies were the generation of a platform to develop SARS-CoV-2-specific single-domain antibodies of human origin (Wu et al., 2020), humanizing the nanobody backbone, and reducing potential immune recognition. Other studies reported successful isolation of nanobodies and their fusion to the human IgG1-Fc region, improving their binding and neutralizing capabilities (Chi et al., 2020b; Xiaojie et al., 2020; Lu et al., 2021a; Valenzuela Nieto et al., 2021) (**Figure 1C**).

Also, another nanobody set was isolated by from yeast surface-displayed synthetic library against epitopes of the SARS-CoV-2 spike protein. The researchers used Nb6 to design bivalent and trivalent nanobodies resulting in a 2000-fold increase in inhibitory activity against both pseudo virus and live SARS-CoV-2 in infection assays (Schoof et al., 2020) (**Figure 1D**).

Another group reported the isolation and characterization of an alpaca-derived single domain antibody fragment, Ty1, against spike protein (Hanke et al., 2020a; Hanke et al., 2020b). In this study, Ty1 was fused to an Fc domain, increasing the neutralizing capabilities of the nanobody. Moreover, in contrast to Nb6 that binds RBD in the down conformation (**Figure 1D**), cryo-electron microscopy studies demonstrated that Ty1 binds to an epitope on the RBD, accessible in both the "up" and "down" conformations (**Figure 1E**). Glycosylation sites N165, N234, and N343 on the spike protein shield RBD from conventional antibodies, especially when the RBD is in a "down" conformation (Watanabe et al., 2020). In the RBD-down conformation, the glycan on N165 points towards the Ty1-binding epitope, likely not leaving sufficient space to accommodate a conventional antibody. This indicates that nanobodies most likely recognize more epitopes for SARS-CoV-2 neutralization than conventional antibodies. Interestingly, a group of multivalent nanobodies bind the RBD domain of spike and lock it in the "up" conformation, a state that is typically associated with receptor binding activation. The premature activation of the fusion machinery on virions enhances neutralization in a non-reversible manner (Koenig et al., 2021).

Not only alpacas and llamas have been contributing for the generation of SARS-CoV-2 neutralizing nanobodies, also nanobodies isolated from a semisynthetic shark-derived library have been shown to neutralize SARS-CoV-2 (Gauhar et al., 2021).

DISCUSSION

During the COVID-19 pandemic, new technologies were developed for the rapid isolation of nanobodies. Our team implemented a new procedure for fast, economical, and efficient selection of high affinity nanobodies based on bacterial display and density gradient (Valenzuela Nieto et al., 2021).

Unfortunately, new SARS-CoV-2 variants significantly scape the immune response raised by either vaccination or previous SARS-CoV-2 infections. The most dramatic example are the Omicron variants. Omicron BA1 has become the infectious virus in the history of humanity. Several mutations on the spike protein generated less efficient cleavage of the S1 domain by the furin protease TMPRSS2 at the cell membrane. Consequently, the infection predominantly affects the upper airway but is less severe in human and animal models (Chen et al., 2022; Wrenn et al., 2022). However, due to the substantial number of infected people, death rates increased worldwide. Furthermore, a second subvariant of Omicron, BA2, developed in parallel. BA2 preserves the immune evasion capabilities of BA1, but unfortunately, current reports indicate a higher lethality (Wolter et al., 2022). The mechanisms behind the high severity of Omicron BA2 infection are not well understood.

More than 2 years after the first COVID-19 outbreak, over six million succumbed to the disease. There were four infective waves worldwide, and currently, infection rates are rising again, suggesting we are entering another wave of COVID-19 caused by Omicron subvariants. Vaccines have been beneficial and saved millions of lives; however, we must take new complementary approaches due to immune escape.

Our immune responses as humans are determined by the way our immune system is organized, for instance, we cannot raise single chain antibodies as part of our antibody defense. The pandemic has challenged the human population with a virus that replicates, mutates, evades, and overcomes our immune response. Nanobodies differ from our own defense and provide new possibilities for the generation of effective neutralizing antibodies that we could never develop ourselves.

AUTHOR CONTRIBUTIONS

Conceptualization: GV-N and AR-F. All authors have contributed to writing, reviewing, and editing sections and have agreed to the published version of the manuscript.

FUNDING

This work was funded by FONDECYT, No. 1200427; the regional Council of the "Los Rios region" projects FICR19-20 and FICR21-01; the Bio and Medical Technology Development Program of the National Research Foundation (NRF) funded by the Korean government (MSIT) (NRF-2020M3A9H5112394) to AR-F; postdoctoral fellowship ANID 3220635 to GV-N; graduate fellowship ANID N° 21161365 to RJ; ANID N°21160481 to A.C; and ANID N° 22170632 to CS; ZM-C was funded by the academic graduate student fellowship of the University of Costa Rica of the academic mobility program, and AB was funded by CORFO Project 19 IRLR–110191.

REFERENCES

Abdelghany, M. T., and Baggett, M. V. (2016). Caplacizumab for Acquired Thrombotic Thrombocytopenic Purpura. *N. Engl. J. Med.* 374 (25), 2497–2498. doi:10.1056/NEJMc1603180

Ahmad, J., Jiang, J., Boyd, L. F., Zeher, A., Huang, R., Xia, D., et al. (2021). Structures of Synthetic Nanobody-SARS-CoV-2 Receptor-Binding Domain Complexes Reveal Distinct Sites of Interaction. *J. Biol. Chem.* 297 (4), 101202. doi:10.1016/j.jbc.2021.101202

Anderson, G., Shriver-Lake, L., Walper, S., Ashford, L., Zabetakis, D., Liu, J., et al. (2018). Genetic Fusion of an Anti-BclA Single-Domain Antibody with Beta Galactosidase. *Antibodies* 7 (4), 36. doi:10.3390/antib7040036

Arbabi Ghahroudi, M., Desmyter, A., Wyns, L., Hamers, R., and Muyldermans, S. (1997). Selection and Identification of Single Domain Antibody Fragments from Camel Heavy-Chain Antibodies. *FEBS Lett.* 414 (3), 521–526. doi:10.1016/s0014-5793(97)01062-4

Arbabi-Ghahroudi, M. (2017). Camelid Single-Domain Antibodies: Historical Perspective and Future Outlook. *Front. Immunol.* 8, 1589. doi:10.3389/fimmu.2017.01589

Arbabi-Ghahroudi, M., Tanha, J., and MacKenzie, R. (2005). Prokaryotic Expression of Antibodies. *Cancer Metastasis Rev.* 24 (4), 501–519. doi:10.1007/s10555-005-6193-1

Ashour, J., Schmidt, F. I., Hanke, L., Cragnolini, J., Cavallari, M., Altenburg, A., et al. (2015). Intracellular Expression of Camelid Single-Domain Antibodies Specific for Influenza Virus Nucleoprotein Uncovers Distinct Features of its Nuclear Localization. *J. Virol.* 89 (5), 2792–2800. doi:10.1128/JVI.02693-14

Awadasseid, A., Wu, Y., Tanaka, Y., and Zhang, W. (2021). Current Advances in the Development of SARS-CoV-2 Vaccines. *Int. J. Biol. Sci.* 17 (1), 8–19. doi:10.7150/ijbs.52569

Baden, L. R., El Sahly, H. M., Essink, B., Kotloff, K., Frey, S., Novak, R., et al. (2021). Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N. Engl. J. Med.* 384 (5), 403–416. doi:10.1056/NEJMoa2035389

Bao, K., Liu, X., Xu, Q., Su, B., Liu, Z., Cao, H., et al. (2021). Nanobody Multimerization Strategy to Enhance the Sensitivity of Competitive ELISA for Detection of Ochratoxin A in Coffee Samples. *Food control.* 127, 108167. doi:10.1016/j.foodcont.2021.108167

Billen, B., Vincke, C., Hansen, R., Devoogdt, N., Muyldermans, S., Adriaensens, P., et al. (2017). Cytoplasmic versus Periplasmic Expression of Site-Specifically and Bioorthogonally Functionalized Nanobodies Using Expressed Protein Ligation. *Protein Expr. Purif.* 133, 25–34. doi:10.1016/j.pep.2017.02.009

Bond, C. J., Marsters, J. C., and Sidhu, S. S. (2003). Contributions of CDR3 to VHH Domain Stability and the Design of Monobody Scaffolds for Naive Antibody Libraries. *J. Mol. Biol.* 332 (3), 643–655. doi:10.1016/s0022-2836(03)00967-7

Camacho-Villegas, T., Mata-Gonzalez, T., Paniagua-Solis, J., Sanchez, E., and Licea, A. (2013). Human TNF Cytokine Neutralization with a vNAR fromHeterodontus Francisci shark: A Potential Therapeutic Use. *MAbs* 5 (1), 80–85. doi:10.4161/mabs.22593

Canedo-Marroquín, G., Saavedra, F., Andrade, C. A., Berrios, R. V., Rodríguez-Guilarte, L., Opazo, M. C., et al. (2020). SARS-CoV-2: Immune Response Elicited by Infection and Development of Vaccines and Treatments. *Front. Immunol.* 11, 569760. doi:10.3389/fimmu.2020.569760

Chakravarty, R., Goel, S., and Cai, W. (2014). Nanobody: the "magic Bullet" for Molecular Imaging? *Theranostics* 4 (4), 386–398. doi:10.7150/thno.8006

Chen, F., Liu, Z., and Jiang, F. (2021). Prospects of Neutralizing Nanobodies against SARS-CoV-2. *Front. Immunol.* 12, 690742. doi:10.3389/fimmu.2021.690742

Chen, J., Wang, R., Gilby, N. B., and Wei, G.-W. (2022). Omicron Variant (B.1.1.529): Infectivity, Vaccine Breakthrough, and Antibody Resistance. *J. Chem. Inf. Model.* 62 (2), 412–422. doi:10.1021/acs.jcim.1c01451

Chen, W., Zhu, Z., Feng, Y., and Dimitrov, D. S. (2008). Human Domain Antibodies to Conserved Sterically Restricted Regions on Gp120 as Exceptionally Potent Cross-Reactive HIV-1 Neutralizers. *Proc. Natl. Acad. Sci. U.S.A.* 105 (44), 17121–17126. doi:10.1073/pnas.0805297105

Cheong, W. S., Leow, C. Y., Abdul Majeed, A. B., and Leow, C. H. (2020). Diagnostic and Therapeutic Potential of Shark Variable New Antigen Receptor (VNAR) Single Domain Antibody. *Int. J. Biol. Macromol.* 147, 369–375. doi:10.1016/j.ijbiomac.2020.01.039

Chi, X., Liu, X., Wang, C., Zhang, X., Li, X., Hou, J., et al. (2020). Humanized Single Domain Antibodies Neutralize SARS-CoV-2 by Targeting the Spike Receptor Binding Domain. *Nat. Commun.* 11 (1), 4528. doi:10.1038/s41467-020-18387-8

Chi, X., Yan, R., Zhang, J., Zhang, G., Zhang, Y., Hao, M., et al. (2020). A Neutralizing Human Antibody Binds to the N-Terminal Domain of the Spike Protein of SARS-CoV-2. *Science* 369 (6504), 650–655. doi:10.1126/science.abc6952

Chi, X., Zhang, X., Pan, S., Yu, Y., Shi, Y., Lin, T., et al. (2022). An Ultrapotent RBD-Targeted Biparatopic Nanobody Neutralizes Broad SARS-CoV-2 Variants. *Sig Transduct. Target Ther.* 7 (1), 44. doi:10.1038/s41392-022-00912-4

Conrath, K. E., Lauwereys, M., Galleni, M., Matagne, A., Frere, J.-M., Kinne, J., et al. (2001). β-Lactamase Inhibitors Derived from Single-Domain Antibody Fragments Elicited in the Camelidae. *Antimicrob. Agents Chemother.* 45 (10), 2807–2812. doi:10.1128/AAC.45.10.2807-2812.2001

Conroy, P. J., Hearty, S., Leonard, P., and O'Kennedy, R. J. (2009). Antibody Production, Design and Use for Biosensor-Based Applications. *Seminars Cell & Dev. Biol.* 20 (1), 10–26. doi:10.1016/j.semcdb.2009.01.010

Custódio, T. F., Das, H., Sheward, D. J., Hanke, L., Pazicky, S., Pieprzyk, J., et al. (2020). Selection, Biophysical and Structural Analysis of Synthetic Nanobodies that Effectively Neutralize SARS-CoV-2. *Nat. Commun.* 11 (1), 5588. doi:10.1038/s41467-020-19204-y

de Marco, A. (2020). Recombinant Expression of Nanobodies and Nanobody-Derived Immunoreagents. *Protein Expr. Purif.* 172, 105645. doi:10.1016/j.pep.2020.105645

De Munter, S., Ingels, J., Goetgeluk, G., Bonte, S., Pille, M., Weening, K., et al. (2018). Nanobody Based Dual Specific Cars. *Ijms* 19 (2), 403. doi:10.3390/ijms19020403

Debnath, S., Zhou, N., McLaughlin, M., Rice, S., Pillai, A. K., Hao, G., et al. (2022). PSMA-targeting Imaging and Theranostic Agents-Current Status and Future Perspective. *Ijms* 23 (3), 1158. doi:10.3390/ijms23031158

Deckers, N., Saerens, D., Kanobana, K., Conrath, K., Victor, B., Wernery, U., et al. (2009). Nanobodies, a Promising Tool for Species-specific Diagnosis of Taenia Solium Cysticercosis. *Int. J. Parasitol.* 39 (5), 625–633. doi:10.1016/j.ijpara.2008.10.012

Desmyter, A., Transue, T. R., Ghahroudi, M. A., Dao Thi, M.-H., Poortmans, F., Hamers, R., et al. (1996). Crystal Structure of a Camel Single-Domain VH Antibody Fragment in Complex with Lysozyme. *Nat. Struct. Mol. Biol.* 3 (9), 803–811. doi:10.1038/nsb0996-803

Dong, J., Huang, B., Jia, Z., Wang, B., Gallolu Kankanamalage, S., Titong, A., et al. (2020). Development of Multi-specific Humanized Llama Antibodies Blocking SARS-CoV-2/ace2 Interaction with High Affinity and Avidity. *Emerg. microbes Infect.* 9 (1), 1034–1036. doi:10.1080/22221751.2020.1768806

Dong, J., Huang, B., Wang, B., Titong, A., Gallolu Kankanamalage, S., Jia, Z., et al. (2020). Development of Humanized Tri-specific Nanobodies with Potent Neutralization for SARS-CoV-2. *Sci. Rep.* 10 (1), 17806. doi:10.1038/s41598-020-74761-y

Dooley, H., and Flajnik, M. F. (2006). Antibody Repertoire Development in Cartilaginous Fish. *Dev. Comp. Immunol.* 30 (1-2), 43–56. doi:10.1016/j.dci.2005.06.022

Dooley, H., Stanfield, R. L., Brady, R. A., and Flajnik, M. F. (2006). First Molecular and Biochemical Analysis of *In Vivo* Affinity Maturation in an Ectothermic Vertebrate. *Proc. Natl. Acad. Sci. U.S.A.* 103 (6), 1846–1851. doi:10.1073/pnas.0508341103

Dörner, T., Weinblatt, M., and Beneden, K. V. (2017). "FRI0239 Results of a Phase 2b Study of Vobarilizumab, an Anti-interleukin-6 Receptor Nanobody, as Monotherapy in Patients with Moderate to Severe Rheumatoid Arthritis," in *Poster Presentations* (BMJ Publishing Group Ltd and European League Against Rheumatism), 575, 2–575.

Du, T., Zhu, G., Wu, X., Fang, J., and Zhou, E.-M. (2019). Biotinylated Single-Domain Antibody-Based Blocking ELISA for Detection of Antibodies against Swine Influenza Virus. *Ijn* 14, 9337–9349. doi:10.2147/IJN.S218458

Esparza, T. J., Martin, N. P., Anderson, G. P., Goldman, E. R., and Brody, D. L. (2020). High Affinity Nanobodies Block SARS-CoV-2 Spike Receptor Binding Domain Interaction with Human Angiotensin Converting Enzyme. *Sci. Rep.* 10 (1), 22370. doi:10.1038/s41598-020-79036-0

Esposito, G., Hunashal, Y., Percipalle, M., Venit, T., Dieng, M. M., Fogolari, F., et al. (2021). NMR-Based Analysis of Nanobodies to SARS-CoV-2 Nsp9 Reveals a

Possible Antiviral Strategy against COVID-19. *Adv. Biol.* 5 (12), 2101113. doi:10.1002/adbi.202101113

Fatima, A., Wang, H., Kang, K., Xia, L., Wang, Y., Ye, W., et al. (2014). Development of VHH Antibodies against Dengue Virus Type 2 NS1 and Comparison with Monoclonal Antibodies for Use in Immunological Diagnosis. *Plos One* 9 (4), e95263. doi:10.1371/journal.pone.0095263

Favorskaya, I. A., Shcheblyakov, D. V., Esmagambetov, I. B., Dolzhikova, I. V., Alekseeva, I. A., Korobkova, A. I., et al. (2022). Single-Domain Antibodies Efficiently Neutralize SARS-CoV-2 Variants of Concern. *Front. Immunol.* 13, 822159. doi:10.3389/fimmu.2022.822159

Feige, M. J., Gräwert, M. A., Marcinowski, M., Hennig, J., Behnke, J., Ausländer, D., et al. (2014). The Structural Analysis of Shark IgNAR Antibodies Reveals Evolutionary Principles of Immunoglobulins. *Proc. Natl. Acad. Sci. U.S.A.* 111 (22), 8155–8160. doi:10.1073/pnas.1321502111

Frenken, L. G. J., van der Linden, R. H. J., Hermans, P. W. J. J., Bos, J. W., Ruuls, R. C., de Geus, B., et al. (2000). Isolation of Antigen Specific Llama VHH Antibody Fragments and Their High Level Secretion by *Saccharomyces cerevisiae*. *J. Biotechnol.* 78 (1), 11–21. doi:10.1016/S0168-1656(99)00228-X

Frigerio, B., Luison, E., Desideri, A., Iacovelli, F., Camisaschi, C., Seregni, E. C., et al. (2021). Validity of Anti-PSMA ScFvD2B as a Theranostic Tool: A Narrative-Focused Review. *Biomedicines* 9 (12), 1870. doi:10.3390/biomedicines9121870

Gai, J., Ma, L., Li, G., Zhu, M., Qiao, P., Li, X., et al. (2021). A Potent Neutralizing Nanobody against SARS-CoV-2 with Inhaled Delivery Potential. *MedComm* 2 (1), 101–113. doi:10.1002/mco2.60

Gainkam, L. O. T., Huang, L., Caveliers, V., Keyaerts, M., Hernot, S., Vaneycken, I., et al. (2008). Comparison of the Biodistribution and Tumor Targeting of Two 99mTc-Labeled Anti-EGFR Nanobodies in Mice, Using Pinhole SPECT/micro-CT. *J. Nucl. Med.* 49 (5), 788–795. doi:10.2967/jnumed.107.048538

Gauhar, A., Privezentzev, C. V., Demydchuk, M., Gerlza, T., Rieger, J., Kungl, A. J., et al. (2021). Single Domain Shark VNAR Antibodies Neutralize SARS-CoV-2 Infection *In Vitro*. *FASEB J.* 35 (11), e21970. doi:10.1096/fj.202100986RR

Girt, G. C., Lakshminarayanan, A., Huo, J., Dormon, J., Norman, C., Afrough, B., et al. (2021). The Use of Nanobodies in a Sensitive ELISA Test for SARS-CoV-2 Spike 1 Protein. *R. Soc. open Sci.* 8 (9), 211016. doi:10.1098/rsos.211016

Gong, R., Wang, Y., Ying, T., and Dimitrov, D. S. (2012). Bispecific Engineered Antibody Domains (Nanoantibodies) that Interact Noncompetitively with an HIV-1 Neutralizing Epitope and FcRn. *Plos One* 7 (8), e42288. doi:10.1371/journal.pone.0042288

Goossens, J., Sein, H., Lu, S., Radwanska, M., Muyldermans, S., Sterckx, Y. G.-J., et al. (2017). Functionalization of Gold Nanoparticles with Nanobodies through Physical Adsorption. *Anal. Methods* 9 (23), 3430–3440. doi:10.1039/C7AY00854F

Götzke, H., Kilisch, M., Martínez-Carranza, M., Sograte-Idrissi, S., Rajavel, A., Schlichthaerle, T., et al. (2019). The ALFA-Tag Is a Highly Versatile Tool for Nanobody-Based Bioscience Applications. *Nat. Commun.* 10 (1), 4403. doi:10.1038/s41467-019-12301-7

Govaert, J., Pellis, M., Deschacht, N., Vincke, C., Conrath, K., Muyldermans, S., et al. (2012). Dual Beneficial Effect of Interloop Disulfide Bond for Single Domain Antibody Fragments. *J. Biol. Chem.* 287 (3), 1970–1979. doi:10.1074/jbc.M111.242818

Gray, E. R., Brookes, J. C., Caillat, C., Turbé, V., Webb, B. L. J., Granger, L. A., et al. (2017). Unravelling the Molecular Basis of High Affinity Nanobodies against HIV P24: *In Vitro* Functional, Structural, and In Silico Insights. *ACS Infect. Dis.* 3 (7), 479–491. doi:10.1021/acsinfecdis.6b00189

Günaydın, G., Álvarez, B., Lin, Y., Hammarström, L., and Marcotte, H. (2014). Co-expression of Anti-rotavirus Proteins (Llama VHH Antibody Fragments) in Lactobacillus: Development and Functionality of Vectors Containing Two Expression Cassettes in Tandem. *Plos One* 9 (4), e96409. doi:10.1371/journal.pone.0096409

Guo, K., Wustoni, S., Koklu, A., Díaz-Galicia, E., Moser, M., Hama, A., et al. (2021). Rapid Single-Molecule Detection of COVID-19 and MERS Antigens via Nanobody-Functionalized Organic Electrochemical Transistors. *Nat. Biomed. Eng.* 5 (7), 666–677. doi:10.1038/s41551-021-00734-9

Güttler, T., Aksu, M., Dickmanns, A., Stegmann, K. M., Gregor, K., Rees, R., et al. (2021). Neutralization of SARS-CoV-2 by Highly Potent, Hyperthermostable, and Mutation-tolerant Nanobodies. *Embo J.* 40 (19), e107985. doi:10.15252/embj.2021107985

Haga, K., Takai-Todaka, R., Matsumura, Y., Song, C., Takano, T., Tojo, T., et al. (2021). Nasal Delivery of Single-Domain Antibody Improves Symptoms of SARS-CoV-2 Infection in an Animal Model. *PLoS Pathog.* 17 (10), e1009542. doi:10.1371/journal.ppat.1009542

Hagihara, Y., and Saerens, D. (2014). Engineering Disulfide Bonds within an Antibody. *Biochimica Biophysica Acta (BBA) - Proteins Proteomics* 1844 (11), 2016–2023. doi:10.1016/j.bbapap.2014.07.005

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hammers, C., Songa, E. B., et al. (1993). Naturally Occurring Antibodies Devoid of Light Chains. *Nature* 363 (6428), 446–448. doi:10.1038/363446a0

Hanke, L., Das, H., Sheward, D. J., Perez Vidakovics, L., Urgard, E., Moliner-Morro, A., et al. (2022). A Bispecific Monomeric Nanobody Induces Spike Trimer Dimers and Neutralizes SARS-CoV-2 *In Vivo*. *Nat. Commun.* 13 (1), 155. doi:10.1038/s41467-021-27610-z

Hanke, L., Perez, L. V., Sheward, D. J., Das, H., Schulte, T., Moliner-Morro, A., et al. (2020). An Alpaca Nanobody Neutralizes SARS-CoV-2 by Blocking Receptor Interaction. *BioRxiv*. doi:10.1101/2020.06.02.130161

Hanke, L., Vidakovics Perez, L., Sheward, D. J., Das, H., Schulte, T., Moliner-Morro, A., et al. (2020). An Alpaca Nanobody Neutralizes SARS-CoV-2 by Blocking Receptor Interaction. *Nat. Commun.* 11 (1), 4420. doi:10.1038/s41467-020-18174-5

Harmand, T. J., Islam, A., Pishesha, N., and Ploegh, H. L. (2021). Nanobodies as *In Vivo*, Non-invasive, Imaging Agents. *RSC Chem. Biol.* 2 (3), 685–701. doi:10.1039/d1cb00023c

Hassanzadeh Gh, G., De Silva, K. S., Dambly-Chaudière, C., Brys, L., Ghysen, A., Hamers, R., et al. (1998). Isolation and Characterization of Single-Chain Fv Genes Encoding Antibodies Specific for Drosophila Poxn Protein. *FEBS Lett.* 437 (1-2), 75–80. doi:10.1016/s0014-5793(98)01204-6

Hernot, S., Unnikrishnan, S., Du, Z., Shevchenko, T., Cosyns, B., Broisat, A., et al. (2012). Nanobody-coupled Microbubbles as Novel Molecular Tracer. *J. Control. Release* 158 (2), 346–353. doi:10.1016/j.jconrel.2011.12.007

Huang, L., Muyldermans, S., and Saerens, D. (2010). Nanobodies: Proficient Tools in Diagnostics. *Expert Rev. Mol. Diagnostics* 10 (6), 777–785. doi:10.1586/erm.10.62

Huang, L., Reekmans, G., Saerens, D., Friedt, J.-M., Frederix, F., Francis, L., et al. (2005). Prostate-specific Antigen Immunosensing Based on Mixed Self-Assembled Monolayers, Camel Antibodies and Colloidal Gold Enhanced Sandwich Assays. *Biosens. Bioelectron.* 21 (3), 483–490. doi:10.1016/j.bios.2004.11.016

Hultberg, A., Temperton, N. J., Rosseels, V., Koenders, M., Gonzalez-Pajuelo, M., Schepens, B., et al. (2011). Llama-derived Single Domain Antibodies to Build Multivalent, Superpotent and Broadened Neutralizing Anti-viral Molecules. *Plos One* 6 (4), e17665. doi:10.1371/journal.pone.0017665

Huo, J., Le Bas, A., Ruza, R. R., Duyvesteyn, H. M. E., Mikolajek, H., Malinauskas, T., et al. (2020). Neutralizing Nanobodies Bind SARS-CoV-2 Spike RBD and Block Interaction with ACE2. *Nat. Struct. Mol. Biol.* 27 (9), 846–854. doi:10.1038/s41594-020-0469-6

Huo, J., Mikolajek, H., Le Bas, A., Clark, J. J., Sharma, P., Kipar, A., et al. (2021). A Potent SARS-CoV-2 Neutralising Nanobody Shows Therapeutic Efficacy in the Syrian Golden Hamster Model of COVID-19. *Nat. Commun.* 12 (1), 5469. doi:10.1038/s41467-021-25480-z

Ismaili, A., Jalali Javaran, M., Rasaee, M. J., Rahbarizadeh, F., and Rajabi Memari, H. (2006). Cloning and Expression of Recombinant Camelid Single-Domain Antibody in Tobacco. *Iran. J. Biotechnol.*

Jiang, S., Hillyer, C., and Du, L. (2020). Neutralizing Antibodies against SARS-CoV-2 and Other Human Coronaviruses. *Trends Immunol.* 41 (5), 355–359. doi:10.1016/j.it.2020.03.007

Jovčevska, I., and Muyldermans, S. (2020). The Therapeutic Potential of Nanobodies. *BioDrugs* 34 (1), 11–26. doi:10.1007/s40259-019-00392-z

Keen, N. T., and Tamaki, S. (1986). Structure of Two Pectate Lyase Genes from Erwinia Chrysanthemi EC16 and Their High-Level Expression in *Escherichia coli*. *J. Bacteriol.* 168 (2), 595–606. doi:10.1128/jb.168.2.595-606.1986

Kilisch, M., Götzke, H., Gere-Becker, M., Crauel, A., Opazo, F., and Frey, S. (2021). Discovery and Characterization of an ALFA-tag-specific Affinity Resin Optimized for Protein Purification at Low Temperatures in Physiological Buffer. *Biomolecules* 11 (2), 269. doi:10.3390/biom11020269

Koenig, P.-A., Das, H., Liu, H., Kümmerer, B. M., Gohr, F. N., Jenster, L.-M., et al. (2021). Structure-guided Multivalent Nanobodies Block SARS-CoV-2 Infection and Suppress Mutational Escape. *Science* 371, 6530. doi:10.1126/science.abe6230

Koromyslova, A. D., and Hansman, G. S. (2017). Nanobodies Targeting Norovirus Capsid Reveal Functional Epitopes and Potential Mechanisms of Neutralization. *PLoS Pathog.* 13 (11), e1006636. doi:10.1371/journal.ppat.1006636

Krainer, F. W., Gmeiner, C., Neutsch, L., Windwarder, M., Pletzenauer, R., Herwig, C., et al. (2013). Knockout of an Endogenous Mannosyltransferase Increases the Homogeneity of Glycoproteins Produced in Pichia pastoris. *Sci. Rep.* 3, 3279. doi:10.1038/srep03279

Langley, R. G., Elewski, B. E., Lebwohl, M., Reich, K., Griffiths, C. E. M., Papp, K., et al. (2014). Secukinumab in Plaque Psoriasis - Results of Two Phase 3 Trials. *N. Engl. J. Med.* 371 (4), 326–338. doi:10.1056/NEJMoa1314258

Larios Mora, A., and Gallup, L. (2018). Delivery of ALX-0171 by Inhalation Greatly Reduces Respiratory Syncytial Virus Disease in Newborn Lambs. *MAbs* 10 (5), 778–795. doi:10.1080/19420862.2018.1470727

Lauwereys, M., Arbabi Ghahroudi, M., Desmyter, A., et al. (1998). Potent Enzyme Inhibitors Derived from Dromedary Heavy-Chain Antibodies. *EMBO J.* 17 (13), 3512–3520. doi:10.1093/emboj/17.13.3512

Li, D., Ji, F., Huang, C., and Jia, L. (2019). High Expression Achievement of Active and Robust Anti-β2 Microglobulin Nanobodies via E.Coli Hosts Selection. *Molecules* 24 (16), 2860. doi:10.3390/molecules24162860

Li, J.-F., He, L., Deng, Y.-Q., Qi, S.-H., Chen, Y.-H., Zhang, X.-L., et al. (2021). Generation and Characterization of a Nanobody against SARS-CoV. *Virol. Sin.* 36 (6), 1484–1491. doi:10.1007/s12250-021-00436-1

Li, L., Guo, P., Zhang, X., Yu, Z., Zhang, W., and Sun, H. (2021). SARS-CoV-2 Vaccine Candidates in Rapid Development. *Hum. vaccines Immunother.* 17 (3), 644–653. doi:10.1080/21645515.2020.1804777

Li, T., Cai, H., Yao, H., Zhou, B., Zhang, N., van Vlissingen, M. F., et al. (2021). A Synthetic Nanobody Targeting RBD Protects Hamsters from SARS-CoV-2 Infection. *Nat. Commun.* 12 (1), 4635. doi:10.1038/s41467-021-24905-z

Li, Y.-D., Chi, W.-Y., Su, J.-H., Ferrall, L., Hung, C.-F., and Wu, T.-C. (2020). Coronavirus Vaccine Development: from SARS and MERS to COVID-19. *J. Biomed. Sci.* 27 (1), 104. doi:10.1186/s12929-020-00695-2

Liu, S. T. H., Lin, H.-M., Baine, I., Wajnberg, A., Gumprecht, J. P., Rahman, F., et al. (2020). Convalescent Plasma Treatment of Severe COVID-19: a Propensity Score-Matched Control Study. *Nat. Med.* 26 (11), 1708–1713. doi:10.1038/s41591-020-1088-9

Liu, Y., and Huang, H. (2018). Expression of Single-Domain Antibody in Different Systems. *Appl. Microbiol. Biotechnol.* 102 (2), 539–551. doi:10.1007/s00253-017-8644-3

Longueira, Y., Polo, M. L., Turk, G., Laufer, N., and Laufer, N. (2021). Dynamics of SARS-CoV-2-specific Antibodies Among COVID19 Biobank Donors in Argentina. *Heliyon* 7 (10), e08140. doi:10.1016/j.heliyon.2021.e08140

Lu, Q., Liu, J., Zhao, S., Gomez Castro, M. F., Laurent-Rolle, M., Dong, J., et al. (2021). SARS-CoV-2 Exacerbates Proinflammatory Responses in Myeloid Cells through C-type Lectin Receptors and Tweety Family Member 2. *Immunity* 54 (6), 1304–1319. e9. doi:10.1016/j.immuni.2021.05.006

Lu, Q., Zhang, Z., Li, H., Zhong, K., Zhao, Q., Wang, Z., et al. (2021). Development of Multivalent Nanobodies Blocking SARS-CoV-2 Infection by Targeting RBD of Spike Protein. *J. Nanobiotechnol* 19 (1), 33. doi:10.1186/s12951-021-00768-w

Ma, H., Zeng, W., Meng, X., Huang, X., Yang, Y., Zhao, D., et al. (2021). Potent Neutralization of SARS-CoV-2 by Hetero-Bivalent Alpaca Nanobodies Targeting the Spike Receptor-Binding Domain. *J. Virol.* 95. doi:10.1128/JVI.02438-20

Maggi, M., and Scotti, C. (2017). Enhanced Expression and Purification of Camelid Single Domain VHH Antibodies from Classical Inclusion Bodies. *Protein Expr. Purif.* 136, 39–44. doi:10.1016/j.pep.2017.02.007

Massoud, T. F., and Gambhir, S. S. (2003). Molecular Imaging in Living Subjects: Seeing Fundamental Biological Processes in a New Light. *Genes Dev.* 17 (5), 545–580. doi:10.1101/gad.1047403

Mast, F. D., Fridy, P. C., Ketaren, N. E., Wang, J., Jacobs, E. Y., Olivier, J. P., et al. (2021). Highly Synergistic Combinations of Nanobodies that Target SARS-CoV-2 and Are Resistant to Escape. *eLife* 10, 73027. doi:10.7554/eLife.73027

Matz, J., Kessler, P., Bouchet, J., Combes, O., Ramos, O. H. P., Barin, F., et al. (2013). Straightforward Selection of Broadly Neutralizing Single-Domain Antibodies Targeting the Conserved CD4 and Coreceptor Binding Sites of HIV-1 Gp120. *J. Virol.* 87 (2), 1137–1149. doi:10.1128/JVI.00461-12

McCoy, L. E., Quigley, A. F., Strokappe, N. M., Bulmer-Thomas, B., Seaman, M. S., Mortier, D., et al. (2012). Potent and Broad Neutralization of HIV-1 by a Llama Antibody Elicited by Immunization. *J. Exp. Med.* 209 (6), 1091–1103. doi:10.1084/jem.20112655

Muyldermans, S., Atarhouch, T., Saldanha, J., Barbosa, J. A. R. G., and Hamers, R. (1994). Sequence and Structure of VH Domain from Naturally Occurring Camel Heavy Chain Immunoglobulins Lacking Light Chains. *Protein Eng. Des. Sel.* 7 (9), 1129–1135. doi:10.1093/protein/7.9.1129

Muyldermans, S. (2013). Nanobodies: Natural Single-Domain Antibodies. *Annu. Rev. Biochem.* 82, 775–797. doi:10.1146/annurev-biochem-063011-092449

Nambulli, S., Xiang, Y., Tilston-Lunel, N. L., Rennick, L. J., Sang, Z., Klimstra, W. B., et al. (2021). Inhalable Nanobody (PiN-21) Prevents and Treats SARS-CoV-2 Infections in Syrian Hamsters at Ultra-low Doses. *Sci. Adv.* 7 (22). doi:10.1126/sciadv.abh0319

Noor, A., Walser, G., Wesseling, M., Giron, P., Laffra, A.-M., Haddouchi, F., et al. (2018). Production of a Mono-Biotinylated EGFR Nanobody in the *E. coli* Periplasm Using the pET22b Vector. *BMC Res. Notes* 11 (1), 751. doi:10.1186/s13104-018-3852-1

Nuttall, S. D., Krishnan, U. V., Hattarki, M., De Gori, R., Irving, R. A., and Hudson, P. J. (2001). Isolation of the New Antigen Receptor from Wobbegong Sharks, and Use as a Scaffold for the Display of Protein Loop Libraries. *Mol. Immunol.* 38 (4), 313–326. doi:10.1016/s0161-5890(01)00057-8

Ohtani, M., Hikima, J.-i., Jung, T. S., Kondo, H., Hirono, I., and Aoki, T. (2013). Construction of an Artificially Randomized IgNAR Phage Display Library: Screening of Variable Regions that Bind to Hen Egg White Lysozyme. *Mar. Biotechnol.* 15 (1), 56–62. doi:10.1007/s10126-012-9456-1

Palomo, C., and Mas, V. (2016). Trivalency of a Nanobody Specific for the Human Respiratory Syncytial Virus Fusion Glycoprotein Drastically Enhances Virus Neutralization and Impacts Escape Mutant Selection. *Antimicrob. Agents Chemother.* 60 (11), 6498–6509. doi:10.1128/AAC.00842-16

Penault-Llorca, F., Cayre, A., Arnould, L., Bibeau, F., Bralet, M.-P., Rochaix, P., et al. (2006). Is There an Immunohistochemical Technique Definitively Valid in Epidermal Growth Factor Receptor Assessment? *Oncol. Rep.* 16 (6), 1173–1179. doi:10.3892/or.16.6.1173

Peyvandi, F., Scully, M., Kremer Hovinga, J. A., Cataland, S., Knöbl, P., Wu, H., et al. (2016). Caplacizumab for Acquired Thrombotic Thrombocytopenic Purpura. *N. Engl. J. Med.* 374 (6), 511–522. doi:10.1056/NEJMoa1505533

Peyvandi, F., Scully, M., Kremer Hovinga, J. A., Knöbl, P., Cataland, S., De Beuf, K., et al. (2017). Caplacizumab Reduces the Frequency of Major Thromboembolic Events, Exacerbations and Death in Patients with Acquired Thrombotic Thrombocytopenic Purpura. *J. Thromb. Haemost.* 15 (7), 1448–1452. doi:10.1111/jth.13716

Pinto Torres, J. E., Goossens, J., Ding, J., Li, Z., Lu, S., Vertommen, D., et al. (2018). Development of a Nanobody-Based Lateral Flow Assay to Detect Active Trypanosoma Congolense Infections. *Sci. Rep.* 8 (1), 9019. doi:10.1038/s41598-018-26732-7

Pymm, P., Adair, A., Chan, L.-J., Cooney, J. P., Mordant, F. L., Allison, C. C., et al. (2021). Nanobody Cocktails Potently Neutralize SARS-CoV-2 D614G N501Y Variant and Protect Mice. *Proc. Natl. Acad. Sci. U.S.A.* 118 (19), 118. doi:10.1073/pnas.2101918118

Qin, X., Duan, M., Pei, D., Lin, J., Wang, L., Zhou, P., et al. (2021). Development of Novel-Nanobody-Based Lateral-Flow Immunochromatographic Strip Test for Rapid Detection of Recombinant Human Interferon α2b. *J. Pharm. Analysis* 12, 308–316. doi:10.1016/j.jpha.2021.07.003

Rosano, G. L., Morales, E. S., and Ceccarelli, E. A. (2019). New Tools for Recombinant Protein Production in *Escherichia coli* : A 5-year Update. *Protein Sci.* 28 (8), 1412–1422. doi:10.1002/pro.3668

Roux, K. H., Greenberg, A. S., Greene, L., Strelets, L., Avila, D., McKinney, E. C., et al. (1998). Structural Analysis of the Nurse Shark (New) Antigen Receptor (NAR): Molecular Convergence of NAR and Unusual Mammalian Immunoglobulins. *Proc. Natl. Acad. Sci. U.S.A.* 95 (20), 11804–11809. doi:10.1073/pnas.95.20.11804

Ruano-Gallego, D., Fraile, S., Gutierrez, C., and Fernández, L. Á. (2019). Screening and Purification of Nanobodies from *E. coli* Culture Supernatants Using the Hemolysin Secretion System. *Microb. Cell Fact.* 18 (1), 47. doi:10.1186/s12934-019-1094-0

Russell, R. L., Pelka, P., and Mark, B. L. (2021). Frontrunners in the Race to Develop a SARS-CoV-2 Vaccine. *Can. J. Microbiol.* 67 (3), 189–212. doi:10.1139/cjm-2020-0465

Saerens, D., Frederix, F., Reekmans, G., Conrath, K., Jans, K., Brys, L., et al. (2005). Engineering Camel Single-Domain Antibodies and Immobilization Chemistry

for Human Prostate-specific Antigen Sensing. *Anal. Chem.* 77 (23), 7547–7555. doi:10.1021/ac051092j

Saerens, D., Huang, L., Bonroy, K., and Muyldermans, S. (2008). Antibody Fragments as Probe in Biosensor Development. *Sensors* 8 (8), 4669–4686. doi:10.3390/s8084669

Saerens, D., Stijlemans, B., Baral, T. N., Nguyen Thi, G. T., Wernery, U., Magez, S., et al. (2008). Parallel Selection of Multiple Anti-infectome Nanobodies without Access to Purified Antigens. *J. Immunol. Methods* 329 (1-2), 138–150. doi:10.1016/j.jim.2007.10.005

Salema, V., and Fernández, L. Á. (2013). High Yield Purification of Nanobodies from the Periplasm of *E. coli* as Fusions with the Maltose Binding Protein. *Protein Expr. Purif.* 91 (1), 42–48. doi:10.1016/j.pep.2013.07.001

Salema, V., Marín, E., Martínez-Arteaga, R., Ruano-Gallego, D., Fraile, S., Margolles, Y., et al. (2013). Selection of Single Domain Antibodies from Immune Libraries Displayed on the Surface of *E. coli* Cells with Two β-Domains of Opposite Topologies. *PLoS One* 8 (9), e75126. doi:10.1371/journal.pone.0075126

Sang, Z., Xiang, Y., Bahar, I., and Shi, Y. (2022). Llamanade: An Open-Source Computational Pipeline for Robust Nanobody Humanization. *Structure* 30 (3), 418–429. doi:10.1016/j.str.2021.11.006

Schepens, B., Ibañez, L. I., De Baets, S., Hultberg, A., Bogaert, P., De Bleser, P., et al. (2011). Nanobodies Specific for Respiratory Syncytial Virus Fusion Protein Protect against Infection by Inhibition of Fusion. *J. Infect. Dis.* 204 (11), 1692–1701. doi:10.1093/infdis/jir622

Schepens, B., van Schie, L., Nerinckx, W., Roose, K., Van Breedam, W., Fijalkowska, D., et al. (2021). An Affinity-Enhanced, Broadly Neutralizing Heavy Chain-Only Antibody Protects against SARS-CoV-2 Infection in Animal Models. *Sci. Transl. Med.* 13 (621), eabi7826. doi:10.1126/scitranslmed.abi7826

Schmidt, F. I., Hanke, L., Morin, B., Brewer, R., Brusic, V., Whelan, S. P. J., et al. (2016). Phenotypic Lentivirus Screens to Identify Functional Single Domain Antibodies. *Nat. Microbiol.* 1 (8), 16080. doi:10.1038/nmicrobiol.2016.80

Schoof, M., Faust, B., Saunders, R. A., Sangwan, S., Rezelj, V., Hoppe, N., et al. (2020). An Ultrapotent Synthetic Nanobody Neutralizes SARS-CoV-2 by Stabilizing Inactive Spike. *Science* 370 (6523), 1473–1479. doi:10.1126/science.abe3255

Scully, M., Cataland, S. R., Peyvandi, F., Coppo, P., Knöbl, P., Kremer Hovinga, J. A., et al. (2019). Caplacizumab Treatment for Acquired Thrombotic Thrombocytopenic Purpura. *N. Engl. J. Med.* 380 (4), 335–346. doi:10.1056/NEJMoa1806311

Sheng, Y., Wang, K., Lu, Q., Ji, P., Liu, B., Zhu, J., et al. (2019). Nanobody-horseradish Peroxidase Fusion Protein as an Ultrasensitive Probe to Detect Antibodies against Newcastle Disease Virus in the Immunoassay. *J. Nanobiotechnol* 17 (1), 35. doi:10.1186/s12951-019-0468-0

Shi, R., Shan, C., Duan, X., Chen, Z., Liu, P., Song, J., et al. (2020). A Human Neutralizing Antibody Targets the Receptor-Binding Site of SARS-CoV-2. *Nature* 584 (7819), 120–124. doi:10.1038/s41586-020-2381-y

Shi, Z., Li, X., Wang, L., Sun, Z., Zhang, H., Chen, X., et al. (2022). Structural Basis of Nanobodies Neutralizing SARS-CoV-2 Variants. *Structure* 30, 707–720. doi:10.1016/j.str.2022.02.011

Shriver-Lake, L. C., Goldman, E. R., Zabetakis, D., and Anderson, G. P. (2017). Improved Production of Single Domain Antibodies with Two Disulfide Bonds by Co-expression of Chaperone Proteins in the *Escherichia coli* Periplasm. *J. Immunol. Methods* 443, 64–67. doi:10.1016/j.jim.2017.01.007

Sograte-Idrissi, S., Schlichthaerle, T., Duque-Afonso, C. J., Alevra, M., Strauss, S., Moser, T., et al. (2020). Circumvention of Common Labelling Artefacts Using Secondary Nanobodies. *Nanoscale* 12 (18), 10226–10239. doi:10.1039/d0nr00227e

Stanfield, R. L., Dooley, H., Flajnik, M. F., and Wilson, I. A. (2004). Crystal Structure of a Shark Single-Domain Antibody V Region in Complex with Lysozyme. *Science* 305 (5691), 1770–1773. doi:10.1126/science.1101148

Stefan, M. A., Light, Y. K., Schwedler, J. L., McIlroy, P. R., Courtney, C. M., Saada, E. A., et al. (2021). Development of Potent and Effective Synthetic SARS-CoV-2 Neutralizing Nanobodies. *MAbs* 13 (1), 1958663. doi:10.1080/19420862.2021.1958663

Stijlemans, B., Conrath, K., Cortez-Retamozo, V., Van Xong, H., Wyns, L., Senter, P., et al. (2004). Efficient Targeting of Conserved Cryptic Epitopes of Infectious Agents by Single Domain Antibodies. *J. Biol. Chem.* 279 (2), 1256–1261. doi:10.1074/jbc.M307341200

Stijlemans, B., De Baetselier, P., Caljon, G., Van Den Abbeele, J., Van Ginderachter, J. A., and Magez, S. (2017). Nanobodies as Tools to Understand, Diagnose, and Treat African Trypanosomiasis. *Front. Immunol.* 8. doi:10.3389/fimmu.2017.00724

Stohr, L., and Palomo, T. (2016). Generation and Characterization of ALX-0171, a Potent Novel Therapeutic Nanobody for the Treatment of Respiratory Syncytial Virus Infection. *Antimicrob. Agents Chemother.* 60 (1), 6–13. doi:10.1128/AAC.01802-15

Sun, D., Sang, Z., Kim, Y. J., Xiang, Y., Cohen, T., Belford, A. K., et al. (2021). Potent Neutralizing Nanobodies Resist Convergent Circulating Variants of SARS-CoV-2 by Targeting Diverse and Conserved Epitopes. *Nat. Commun.* 12 (1), 4676. doi:10.1038/s41467-021-24963-3

Sun, Z., Lv, J., Liu, X., Tang, Z., Wang, X., Xu, Y., et al. (2018). Development of a Nanobody-AviTag Fusion Protein and its Application in a Streptavidin-Biotin-Amplified Enzyme-Linked Immunosorbent Assay for Ochratoxin A in Cereal. *Anal. Chem.* 90 (17), 10628–10634. doi:10.1021/acs.analchem.8b03085

Svecova, D., Lubell, M. W., Casset-Semanaz, F., Mackenzie, H., Grenningloh, R., and Krueger, J. G. (2019). A Randomized, Double-Blind, Placebo-Controlled Phase 1 Study of Multiple Ascending Doses of Subcutaneous M1095, an Anti-interleukin 17A/F Nanobody, in Moderate-To-Severe Psoriasis. *J. Am. Acad. Dermatology* 81 (1), 196–203. doi:10.1016/j.jaad.2019.03.056

Tarr, A. W., Lafaye, P., Meredith, L., Damier-Piolle, L., Urbanowicz, R. A., Meola, A., et al. (2013). An Alpaca Nanobody Inhibits Hepatitis C Virus Entry and Cell-To-Cell Transmission. *Hepatology* 58 (3), 932–939. doi:10.1002/hep.26430

Tijink, B. M., Laeremans, T., Budde, M., Walsum, M. S.-v., Dreier, T., de Haard, H. J., et al. (2008). Improved Tumor Targeting of Anti-epidermal Growth Factor Receptor Nanobodies through Albumin Binding: Taking Advantage of Modular Nanobody Technology. *Mol. Cancer Ther.* 7 (8), 2288–2297. doi:10.1158/1535-7163.MCT-07-2384

To, K. K.-W., Tsang, O. T.-Y., Yip, C. C.-Y., Chan, K.-H., Wu, T.-C., Chan, J. M.-C., et al. (2020). Consistent Detection of 2019 Novel Coronavirus in Saliva. *Clin. Infect. Dis.* 71 (15), 841–843. doi:10.1093/cid/ciaa149

Valenzuela Nieto, G., Jara, R., Watterson, D., Modhiran, N., Amarilla, A. A., Himelreichs, J., et al. (2021). Potent Neutralization of Clinical Isolates of SARS-CoV-2 D614 and G614 Variants by a Monomeric, Sub-nanomolar Affinity Nanobody. *Sci. Rep.* 11 (1), 3318. doi:10.1038/s41598-021-82833-w

Valle, C., Martin, B., Touret, F., Shannon, A., Canard, B., Guillemot, J. C., et al. (2020). Drugs against SARS-CoV -2: What Do We Know about Their Mode of Action? *Rev. Med. Virol.* 30 (6), 1–10. doi:10.1002/rmv.2143

Veggiani, G., and de Marco, A. (2011). Improved Quantitative and Qualitative Production of Single-Domain Intrabodies Mediated by the Co-expression of Erv1p Sulfhydryl Oxidase. *Protein Expr. Purif.* 79 (1), 111–114. doi:10.1016/j.pep.2011.03.005

Vu, K. B., Ghahroudi, M. A., Wyns, L., and Muyldermans, S. (1997). Comparison of Llama VH Sequences from Conventional and Heavy Chain Antibodies. *Mol. Immunol.* 34 (16-17), 1121–1131. doi:10.1016/s0161-5890(97)00146-6

Wagner, T. R., Ostertag, E., Kaiser, P. D., Gramlich, M., Ruetalo, N., Junker, D., et al. (2021). NeutrobodyPlex-monitoring SARS-CoV-2 Neutralizing Immune Responses Using Nanobodies. *EMBO Rep.* 22 (5), e52325. doi:10.15252/embr.202052325

Walter, J. D., Hutter, C. A. J., Zimmermann, I., Wyss, M., Egloff, P., Sorgenfrei, M., et al. (2020). Sybodies Targeting the SARS-CoV-2 Receptor-Binding Domain. *BioRxiv*. doi:10.1101/2020.04.16.045419

Wang, M.-Y., Zhao, R., Gao, L.-J., Gao, X.-F., Wang, D.-P., and Cao, J.-M. (2020). SARS-CoV-2: Structure, Biology, and Structure-Based Therapeutics Development. *Front. Cell. Infect. Microbiol.* 10, 587269. doi:10.3389/fcimb.2020.587269

Wang, W., Xu, Y., Gao, R., Lu, R., Han, K., Wu, G., et al. (2020). Detection of SARS-CoV-2 in Different Types of Clinical Specimens. *Jama* 323 (18), 1843–1844. doi:10.1001/jama.2020.3786

Watanabe, Y., Allen, J. D., Wrapp, D., McLellan, J. S., and Crispin, M. (2020). Site-specific Glycan Analysis of the SARS-CoV-2 Spike. *Science* 369 (6501), 330–333. doi:10.1126/science.abb9983

Weinstein, J. B., Bates, T. A., Leier, H. C., McBride, S. K., Barklis, E., and Tafesse, F. G. (2022). A Potent Alpaca-Derived Nanobody that Neutralizes SARS-CoV-2 Variants. *iScience* 25 (3), 103960. doi:10.1016/j.isci.2022.103960

Weninger, A., Hatzl, A.-M., Schmid, C., Vogl, T., and Glieder, A. (2016). Combinatorial Optimization of CRISPR/Cas9 Expression Enables Precision

Genome Engineering in the Methylotrophic Yeast Pichia pastoris. *J. Biotechnol.* 235, 139–149. doi:10.1016/j.jbiotec.2016.03.027

Wolter, N., Jassat, W., von Gottberg, A., Cohen, C., and Cohen, C. (2022). Clinical Severity of Omicron Sub-lineage BA.2 Compared to BA.1 in South Africa. *medRxiv*. doi:10.1101/2022.02.17.22271030

Woźniak, M., Płoska, A., Siekierzycka, A., Dobrucki, L. W., Kalinowski, L., and Dobrucki, I. T. (2022). Molecular Imaging and Nanotechnology-Emerging Tools in Diagnostics and Therapy. *Ijms* 23 (5), 2658. doi:10.3390/ijms23052658

Wrapp, D., De Vlieger, D., Corbett, K. S., Torres, G. M., Wang, N., Van Breedam, W., et al. (2020). Structural Basis for Potent Neutralization of Betacoronaviruses by Single-Domain Camelid Antibodies. *Cell* 181 (5), 1004–1015. e15. doi:10.1016/j.cell.2020.04.031

Wrenn, J. O., Pakala, S. B., Vestal, G., Shilts, M. H., Brown, H. M., Bowen, S. M., et al. (2022). COVID-19 Severity from Omicron and Delta SARS-CoV-2 Variants. *Influenza Resp. Viruses*. doi:10.1111/irv.12982

Wu, X., Cheng, L., Fu, M., Huang, B., Zhu, L., Xu, S., et al. (2021). A Potent Bispecific Nanobody Protects hACE2 Mice against SARS-CoV-2 Infection via Intranasal Administration. *Cell Rep.* 37 (3), 109869. doi:10.1016/j.celrep.2021.109869

Wu, Y., Jiang, S., and Ying, T. (2017). Single-Domain Antibodies as Therapeutics against Human Viral Diseases. *Front. Immunol.* 8, 1802. doi:10.3389/fimmu.2017.01802

Wu, Y., Li, C., Xia, S., Tian, X., Kong, Y., Wang, Z., et al. (2020). Identification of Human Single-Domain Antibodies against SARS-CoV-2. *Cell Host Microbe* 27 (6), 891–898. e5. doi:10.1016/j.chom.2020.04.023

Wu, Z., Hu, Y., Xu, M., Chen, Z., Yang, W., Jiang, Z., et al. (2021). Safety, Tolerability, and Immunogenicity of an Inactivated SARS-CoV-2 Vaccine (CoronaVac) in Healthy Adults Aged 60 Years and Older: a Randomised, Double-Blind, Placebo-Controlled, Phase 1/2 Clinical Trial. *Lancet Infect. Dis.* 21 (6), 803–812. doi:10.1016/S1473-3099(20)30987-7

Xiang, Y., Nambulli, S., Xiao, Z., Liu, H., Sang, Z., Duprex, W. P., et al. (2020). Versatile and Multivalent Nanobodies Efficiently Neutralize SARS-CoV-2. *Science* 370 (6523), 1479–1484. doi:10.1126/science.abe4747

Xiaojie, S., Yu, L., Lei, Y., Guang, Y., and Min, Q. (2020). Neutralizing Antibodies Targeting SARS-CoV-2 Spike Protein. *Stem Cell Res.* 50, 102125. doi:10.1016/j.scr.2020.102125

Xie, Y. J., Dougan, M., Jailkhani, N., Ingram, J., Fang, T., Kummer, L., et al. (2019). Nanobody-based CAR T Cells that Target the Tumor Microenvironment Inhibit the Growth of Solid Tumors in Immunocompetent Mice. *Proc. Natl. Acad. Sci. U.S.A.* 116 (16), 7624–7631. doi:10.1073/pnas.1817147116

Xu, J., Xu, K., Jung, S., Conte, A., Lieberman, J., Muecksch, F., et al. (2021). Nanobodies from Camelid Mice and Llamas Neutralize SARS-CoV-2 Variants. *Nature* 595 (7866), 278–282. doi:10.1038/s41586-021-03676-z

Yao, H., Cai, H., Li, T., Zhou, B., Qin, W., Lavillette, D., et al. (2021). A High-Affinity RBD-Targeting Nanobody Improves Fusion Partner's Potency against SARS-CoV-2. *PLoS Pathog.* 17 (3), e1009328. doi:10.1371/journal.ppat.1009328

Ye, G., Gallant, J., Zheng, J., Massey, C., Shi, K., Tai, W., et al. (2021). The Development of Nanosota-1 as Anti-SARS-CoV-2 Nanobody Drug Candidates. *eLife* 10. doi:10.7554/eLife.64815

Yetisen, A. K., Akram, M. S., and Lowe, C. R. (2013). Paper-based Microfluidic Point-Of-Care Diagnostic Devices. *Lab. Chip* 13 (12), 2210–2251. doi:10.1039/c3lc50169h

Yoon, S., Kim, S., and Kim, J. (2010). Secretory Production of Recombinant Proteins in *Escherichia coli*. *Biot* 4 (1), 23–29. doi:10.2174/187220810790069550

Yuan, T. Z., Garg, P., Wang, L., Willis, J. R., Kwan, E., Hernandez, A. G. L., et al. (2022). Rapid Discovery of Diverse Neutralizing SARS-CoV-2 Antibodies from Large-Scale Synthetic Phage Libraries. *MAbs* 14 (1), 2002236. doi:10.1080/19420862.2021.2002236

Zhu, M., Hu, Y., Li, G., Ou, W., Mao, P., Xin, S., et al. (2014). Combining Magnetic Nanoparticle with Biotinylated Nanobodies for Rapid and Sensitive Detection of Influenza H3N2. *Nanoscale Res. Lett.* 9 (1), 528. doi:10.1186/1556-276X-9-528

Zupancic, J. M., Desai, A. A., Schardt, J. S., Pornnoppadol, G., Makowski, E. K., Smith, M. D., et al. (2021). Directed Evolution of Potent Neutralizing Nanobodies against SARS-CoV-2 Using CDR-Swapping Mutagenesis. *Cell Chem. Biol.* 28 (9), 1379–1388. e7. doi:10.1016/j.chembiol.2021.05.019

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Drug Discovery

TYPE Review
PUBLISHED 26 August 2022
DOI 10.3389/fddsv.2022.925825

OPEN ACCESS

EDITED BY
Bruno Villoutreix,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

REVIEWED BY
Walaa A. Negm,
Tanta University, Egypt
Juan Fernandez-Recio,
Institute of Vine and Wine Sciences (CSIC), Spain

*CORRESPONDENCE
Emmanuel O. Ogbadoyi,
eogbadoyi@futminna.edu.ng

SPECIALTY SECTION
This article was submitted to Anti-Infective Agents,
a section of the journal
Frontiers in Drug Discovery

RECEIVED 21 April 2022
ACCEPTED 15 July 2022
PUBLISHED 26 August 2022

CITATION
Ogbadoyi EO and Umar N (2022), The challenges and opportunities for the development of COVID-19 therapeutics and preparing for the next pandemic. *Front. Drug. Discov.* 2:925825.
doi: 10.3389/fddsv.2022.925825

The challenges and opportunities for the development of COVID-19 therapeutics and preparing for the next pandemic

Emmanuel O Ogbadoyi[1,2,3]* and Ndagi Umar[1,2,4]

[1]Deparment of Biochemistry, School of Life Sciences, Federal University of Technology, Minna, Nigeria, [2]Africa Centre of Excellence for Mycotoxin and Food Safety (ACEMFS), West African Network for Infectious Diseases ACEs (WANIDA), Federal University of Technology, Minna, Nigeria, [3]Global Institute for Bioexploration, Centre for Genetic Engineering and Biotechnology, Federal University of Technology, Minna, Nigeria, [4]Trans-Sahara Disease Research Centre, Ibrahim Badamasi Babangida University, Lapai Niger State, Nigeria

The disease which is today known as COVID-19 is caused by severe acute respiratory. Syndrome coronavirus 2 (SARS-COV-2), was first reported in Wuhan, China in December 2019. The disease has claimed well over six million lives from over 500 million cases. Vaccine hesitancy militates against successful mass vaccination. There is the rapid emergence of new SARS-COV-2 variants, constituting a challenge to the effectiveness of vaccines. Moreover, none of the available vaccines offers 100% protection and even the protection offered is of short duration necessitating booster doses to be taken. Moving forward, the development of plant-based edible vaccines will be a remarkable strategic approach to overcome vaccine hesitancy and improve vaccine uptake. So far only about nine drugs for COVID-19 treatment have approvals by either or both the European Medicines Agency and the FDA. While drug repurposing to address the emerging need in the early period of the COVID-19 pandemic has been contextually very useful, investment in it remains relatively low for commercial reasons arising from patenting issues. Embarking on new drug discovery and development strategies targeting both the virus and host factors is a very appealing option. Targeting druggable targets that are present across viruses, particularly the coronaviruses, for drug discovery and development represents an important strategy for pandemic preparedness. Natural products are an important reservoir of chemical scaffolds with huge potential for the discovery of novel chemical entities for development of novel therapeutics. Phytopharming is an available technology that can be used for mass and accelerated production of therapeutic molecules that will be required within short periods of time as is the case in pandemic outbreaks. Nanotechnology provides excellent platforms for formulating multivalent vaccines and pan-viral medicines for the treatment of COVID-19. Taken together, this review discusses the potential for the development of therapeutics by using the tools of biocomputing, nanotechnology, and phytopharming for accelerated therapeutic development to achieve effective COVID-19 treatment and associated complications, including new and emerging variants of SARS-COV-2 and other viral pandemics that may emerge or re-emerge.

KEYWORDS
COVID-19, SARS-CoV-2, Drug discovery, Natural products, Computational methods

Introduction

SARS-CoV-2 (COVID-19)

The ongoing Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) which started in the city of Wuhan in Hubei province, China with the earliest onset of symptoms on 1 December 2019 (Liu et al., 2020) has today spread to about 226 countries and territories worldwide. The disease which was initially diagnosed as viral pneumonia (Huang et al., 2020; Zhu et al., 2020) was referred to as Wuhan pneumonia by the press (Liu et al., 2020).

The virus was initially termed 2019 novel coronavirus (2019-nCoV) on 12 January 2020 and a month later, the World Health Organization officially named the disease coronavirus disease 2019 (COVID-19) and went on to declare it a pandemic on 11 March 2020. According to the World Health Organization, globally there are 497,960,492 confirmed cases with 6,181,850 deaths as of 12 April 2022. Horton (2020) described COVID- 19 as a triple crisis–medical, economic, and psychological.

Control measures

Generally, diseases are controlled through the use of both pharmaceutical and non-pharmaceutical approaches. COVID-19 is not an exception to this generalization. COVID-19 prevention is through a combination of vaccination, prophylactic or preventive treatment, and non-pharmaceutical measures of social distancing, quarantine, isolating infected persons and patients, lockdowns, avoiding crowding, wearing protective face masks, regular washing of hands with soap, use of hand sanitizers, and not touching the face with hands, particularly the mouth, nose and the eyes which are ready and easy viral entry points into the body. Implementation of these were very challenging, particularly in low- and middle-income countries and among the unscientifically minded populations. This was most common with the use of face masks where: materials that will readily allow entry of the virus are used for the face masks; many people touched the masks that may have been infected with the virus with their bare hands thereby possibly carrying the viruses in their hands and then touching their faces with unwashed and unsanitized hands; inappropriate disposal of used face masks which become potential sources of spread of the virus within the population as children and even some adults pick up discarded used masks from roadsides and waste bins (this is common in poor resource settings and among individuals with a low level of education). The pharmaceutical approaches involve the use of therapeutics, which in a broad sense can be described as medications or remedies taken to prevent, treat, or remediate a health problem. Therapeutics include but are not limited to, vaccines, monoclonal antibodies, drugs, functional foods, and nutraceuticals.

Vaccine development: General approaches

The field of vaccinology came into being in 1796 with the discovery of the smallpox vaccine by Edward Jenner in which he used the whole live organism that causes smallpox, cowpox virus, to develop the vaccine. This was followed by the formulation of the polio vaccine using the killed or inactivated polio virus to develop the polio vaccine. Then there came the live attenuated vaccines in which the infectious agent is neither killed nor inactivated but rendered non-infective. Some examples include measles, mumps, and rubella vaccine (MMR). Following this are the toxoid vaccines which are based on inactivated toxins of pathogenic microorganisms such as tetanus and diphtheria. In more recent times, subunit vaccines (an example is the HPV vaccine), in which components of the pathogen antigen to which immune response is stimulated are used in developing the vaccines. The components of the pathogen antigen can also be produced as recombinants and used for vaccine production. Subunit vaccines have excellent safety profiles but are generally less immunogenic compared with inactivated or attenuated vaccines and require much stronger adjuvants for enhanced immunogenicity. Other forms of vaccine platforms include conjugate vaccines in which poorly immunogenic surface molecules, such as polysaccharides of many bacteria, are synthesized and conjugated to strongly immunogenic proteins and are then used for vaccine development. Some examples include HiB, meningitis C, and pneumococcal vaccines; the next generation vaccines: vectored vaccines which involve putting vaccine antigens inside replication-deficient microorganisms such as Adenoviruses and modified Vaccinia Ankara capable of triggering an immune response and the antigen is then vectored into the host cells; The genome-based approach (nucleic acid approach) which is based on the use of DNA or RNA to make desired antigens in the host thereby prompting an immune response; Reverse vaccinology in which vaccine candidates are selected on the basis of predicted immunogenicity emanating from sequence information obtained from the use of modern sequencing technology. This was successfully used in developing a

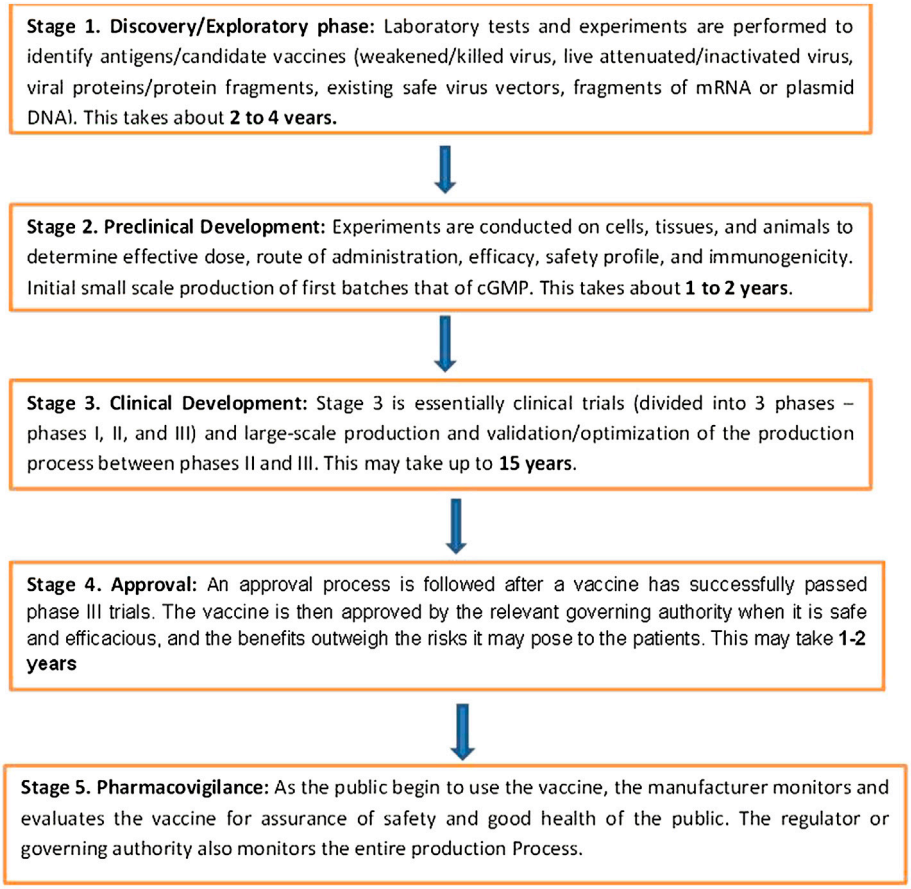


FIGURE 1
Stages of traditional vaccine discovery and devolopment process.

vaccine against meningococcus B, which, till then, was difficult to be achieved using conventional vaccine technologies (Rappuolia et al., 2021); Structural vaccinology (structure-based antigen design) which can also be considered as a variant of reverse vaccinology uses information on the structure of antigenic epitopes and protein conformation in the design of vaccines. Some examples include the design of a single meningococcal antigen containing the epitopes of three antigenic variants of the same molecule, and a host of others (reviewed by Rappuolia et al., 2021). The stages involved in traditional vaccine discovery and development are shown in Figure 1.

Structural vaccinology is novel and innovative and particularly potentially very useful in antiviral vaccine development to combat very challenging viruses that traditional approaches have failed or may fail to produce effective vaccines. Essentially, the process involves four stages: determining the atomic structure of the antigen or antigen-antibody complex; remodeling the antigen or the epitope by reverse molecular engineering; incorporating the re-engineered antigen or epitope into one of the vaccine platforms; and testing the safety and efficacy of the candidate vaccine *in vivo* (Anasir and Poh 2019).

In summarizing, three broad platforms can be used for the design of vaccines: the whole organism (virus or bacterium), the parts of the organism (subunits and recombinants), and the genetic material (DNA or RNA). These are illustrated in Figure 2.

Drug discovery and development: General approaches

Broadly, drug discovery involves the following approaches: Screening extracts of natural origin for bioactivity and then isolating bioactive compounds from potent extracts for drug development. Opium and taxol are a good testimony to this; Random synthesis and screening in which several organic compounds are synthesized and pharmacologically screened for therapeutic potentials. The efficiency of this approach has been greatly enhanced by automated high throughput screening

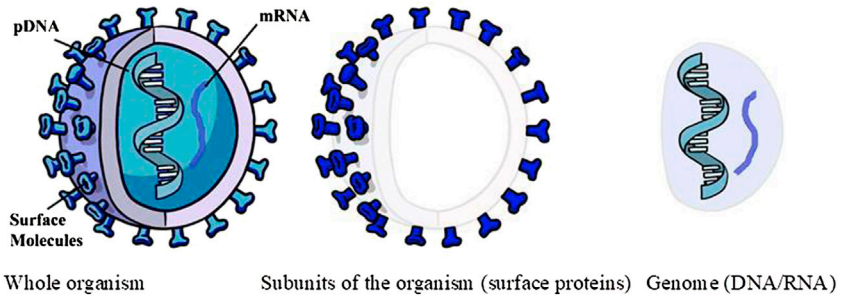


FIGURE 2
The three broad platforms for vaccine design and devolopment.

FIGURE 3
Summary of traditional drug discovery and devolopment process.

technology and combinatorial chemistry which has greatly accelerated synthetic methods, enabling the synthesis of a huge library of compounds that can then be screened for bioactivity. However, this approach is not a successful path to drug discovery; Rational drug design is dependent on several determinants such as the pathophysiology of the disease against which the drug is being developed. This uses the tools of genomics, structural biology, synthetic biology, medicinal

TABLE 1 Candidate vaccines in clinical development indicating platforms, number and percentage.

Platform	Candidate vaccines	
	Number	Percentage
Protein subunit (PS)	52	33
RNA	31	20
Viral Vector, non-replicating (VVnr)	21	13
Inactivated Virus (IV)	21	13
DNA	16	10
Virus Like Particle (VLP)	06	04
Viral Vector, replicating (VVr)	04	03
VVr + Antigen Presenting Cell (VVr + APC)	02	01
Live Attenuated Virus (LAV)	02	01
VVnr + Antigen Presenting Cell (VVnr + APC)	01	01
Bacterial antigen-spore expression Vector (BacAg-SpV)	01	01

Modified from WHO (2022) at https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines

chemistry, computational biology/bioinformatics, and cheminformatics. Rational drug design is a very efficient and successful approach as the innovative tools mentioned can significantly reduce the time for the discovery process. The steps involved in the drug discovery and development process are summarized in Figure 3.

Drug repurposing, though not drug discovery per se, is a useful approach to obtaining drugs for meeting unmet medical treatment needs.

Development of COVID-19 therapeutics and the challenges: The state-of-the-art

COVID-19 vaccine development

The world was unprepared to immediately and adequately mitigate the catastrophic consequences of the outbreak of COVID-19. Since there was no specific treatment for the disease at the onset, the most appropriate control method was to develop vaccines for mass immunization to prevent transmission and bring the disease under control. The response was massive as researchers in academia, research institutes, and industries worked together with manufacturing companies to produce various vaccines using eleven different platforms (Table 1). These platforms can be classified into conventional vaccines (live attenuated virus, inactivated virus, virus-like particle, and protein subunit) and next-generation vaccines (Gene-based vaccine platforms based on viral vectors, DNA, and RNA). The international coordinated efforts led to the unprecedented scientific achievement of getting a vaccine (Comirnaty vaccine of Pfizer/BioNTech) for use in just about a year of the pandemic outbreak and many others followed (Table 2). As of 20 May 2022, there were 157 vaccines in clinical development (Table 1) and 198 in preclinical development (World Health Organization, 2021). The percentage of vaccines in each platform is summarized in Figure 4. Two vaccine platforms, mRNA and viral vectors particularly stand out in the fight against COVID-19. Despite this remarkable progress, there are still serious challenges of limited efficacy (Table 2), waning protection over short periods of time, and rapid emergence of new variants that may further reduce the efficacy of existing vaccines. These inadequacies can be overcome by developing platforms for polyvalent vaccines using CRISPR-engineered viral vectors and/or nanomedicine. Furthermore, the availability of vaccines alone cannot prevent the disease unless people get vaccinated. However, vaccine hesitancy continues to remain a serious challenge militating against vaccine acceptance and uptake by the general population. The SAGE working group on vaccine hesitancy describes vaccine hesitancy as the "delay in acceptance or refusal of vaccination despite the availability of vaccination services" (MacDonald, 2015). Vaccine hesitancy, driven largely by misinformation and disinformation, has been described as one of the top ten global health threats (Scheres and Kuszewski, 2019). Although globally a total of 11,250,782,214 vaccine doses had been administered as of 5 April 2022, the dream of herd immunity is far from being realized. This is compounded by "vaccine nationalism and regionalism" and a lack of production capacity in many countries, particularly the low- and middle-income countries. For instance, there are only 24 countries out of the total of 235 countries and territories in the world that produce World Health Organization prequalified vaccines and none of the 24 countries is in Africa with twelve countries leading vaccine production as shown in Table 3. However, in February 2022, the

TABLE 2 Vaccines granted Emergency Use Listings as at16 March 2022.

Company	Vaccine	Platform	Date of EUL	Efficacy
Pfizer/BioNTech	Comirnaty vaccine	mRNA (nucleoside modified)	31 December 2020	95%
The SII/COVISHIELD and AstraZeneca	AZD1222 vaccines	Viral Vector (non-replicating)	16 February 2021	76%–100%
Johnson & Johnson	Janssen/Ad26.COV 2.S	Viral Vector (non- replicating	12 March 2021	85.4%–93.1%
Moderna	Moderna COVID-19 vaccine (mRNA 1273)	mRNA (nucleoside modified)	30 April 2021	94.1%
Sinopharm	Sinopharm COVID-19 vaccine	Inactivated virus	7 Ma y 2021	79%
Sinovac	CoronaVac	Inactivated virus	1 June 2021	51%–100%
Bharat Biotech	BBV152 COVAXIN vaccine	Inactivated virus	3 November 2021	78%–93%
Novavax-COVAX	Covovax (NVX-CoV2373) vaccine	Protein subunit	17 December2021	90%
Novavax	Nuvaxovid (NVX-CoV2373) vaccine		20 December 2021	90%–100%

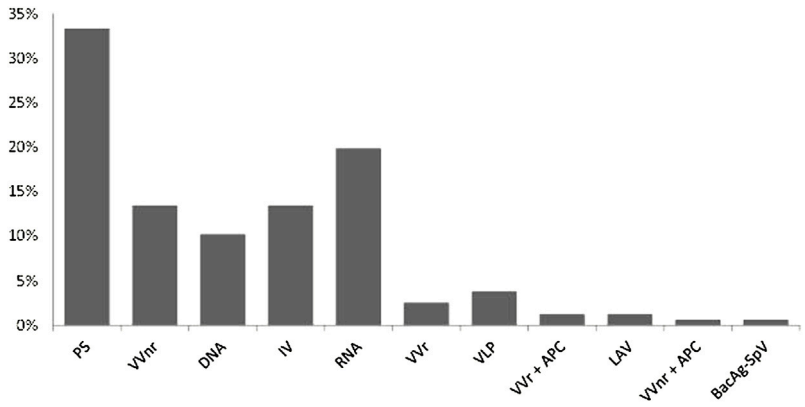


FIGURE 4
COVID-19 vaccines in clinical devolopment,the devolopmental platforms (*x* axis), and the percentage of vaccines in each devoloment platform (*y* axis). Key: PS=Protein subunit; VVnr= Viral Vector, non-replicating; DNA; IV= Inactivated virus; RNA; VVr = Viral Vector, replicating; VLP = Virus Like Particle; VVr + APC = VVr + Antigen Presenting Cell; LAV = Live Attenuated Virus; VVnr+ APC + VVnr + Antigen Presenting Cell; BacAg-SpV = Bacterial antigen-spore expression Vector. Based on data obtained from WHO (2022) at https://www.who.int/publications/m/item/draft-landscape-of covid-19-candidate-vaccines.

technology for the production of mRNA vaccines was given to six countries in Africa (Egypt, Kenya, Nigeria, Senegal, South Africa, and Tunisia) to enable the production of mRNA vaccines in these countries.

Drug development

Although the rollout of vaccines and non-pharmaceutical interventions are significantly helping to contain the COVID-19 pandemic, treatment remains a major control measure. However, it is an understatement to say that COVID-19 chemotherapeutic armory is grossly inadequate at the moment. There is huge potential to successfully address this inadequacy by providing solutions for COVID-19 treatment using drug repurposing and traditional drug discovery approaches.

TABLE 3 A total number of COVID-19 vaccine doses produced by the top 12 producing countries as of 03 March 2021.

Country	Number of doses
China	141,624,000
United States	103,000,000
Germany/Belgium	70,534,055
India	42,390,000
United Kingdom	12,200,000
Netherlands/Belgium	10,496,982
Russia	10,492,500
Switzerland	5,462,338
South Korea	1,617,000
Brazil	200,000
South Africa	160,000

https://www.statista.com/chart/24492/total-covid-19-vaccine-production-by-country/ (accessed 21 May 2022).

Drug repurposing

The outbreak of the COVID-19 pandemic can be said to have taken the world unaware and unprepared to contain it and there was more or less pandemonium at the onset as there was neither vaccine nor any specific treatment. While scientists and researchers immediately began efforts in collaborative vaccine development, intensified and concerted efforts were also made by scientists and researchers to evaluate pre-existing approved drugs for efficacy against COVID-19 so that they could be used for the treatment of the disease in what is known as drug repurposing (also referred to as repositioning, re-profiling, re-tasking, indication expansion, indication shift, or rescue of drugs). Drug repurposing has also been described as establishing new medical uses for already known drugs, including approved, discontinued, shelved, and experimental drugs (Talevi and Bellera, 2020). Therefore, in response to the immense pressure the disease placed on world health systems, the World Health Organization rationally established an international "Solidarity" clinical trial to accelerate the finding of an effective treatment for the disease (World Health Organization, 2020). What followed were clinical trials of pre-existing multiple antiviral medications that had been used for SARS-CoV, MERS-CoV, and antimalarials (Li and De Clercq, 2020). The initial repurposing efforts involved remdesivir, the malaria medications hydroxychloroquine and chloroquine, the combination of HIV drugs called Kaletra, consisting of lopinavir and ritonavir, and other combinations, including interferon beta-1a (Savi et al., 2020). Candidates for clinical trials came from the deployment of available screening techniques of current pharmacopoeia to reveal novel drug indications of already established drugs (Jarada et al., 2020).

The methodologies used for drug repurposing can be broadly categorized into three depending on available information in the context of pharmacological, toxicological, and biological activity. They include those based on the: drug, drug target, and disease/therapy. Three fundamental steps are involved before a potential drug for repurposing can be developed and marketed. These are: identifying the candidate drug; evaluating mechanistically, the drug effects in preclinical models; and evaluating the efficacy of candidate drugs in phase II clinical trials (Khataniar et al., 2022). There are two alternative but complementary approaches that can and have been used for drug repurposing: an experimental approach which is also referred to as the activity-based approach, and the computational methods (*in silico*) approach (Lipinski, 2011; Senanayake, 2020; Dhaneshwar and Bhasin, 2021; Naasani, 2021; Ng et al., 2021; Khataniar et al., 2022). Whichever approach is chosen, the process begins with the selection of the drug for repurposing followed by the identification of the drug target(s). From what is known about the biology of SARS-CoV-2, the drug targets frequently mentioned are: Spike (S) protein receptor binding domain (RBD), RNA-dependent RNA polymerases (RdRps), helicase; SARS-CoV Chymotrypsin-like cysteine protease, $3CL^{pro}$ (also known as M^{PRO}); papain-like cysteine protease (PL^{pro}), which are all important for viral replication and they are therefore viral-based targets. This has been reviewed in the literature (Jang et al., 2021; Nawaz, 2021; Yang and Rao, 2021; Khataniar et al., 2022) and will not be discussed in detail in the current review. There are also host factors that can be targeted for therapeutic intervention and they include, but are not limited to: transmembrane protease serine 2 (TMPRSS2) for viral entry; NF-κB, IL-1β, and IL-6, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and Janus kinase (JAK) for immunomodulation and cytokine storm prevention and control; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSPHx), reduced glutathione (GSH), glutathione reductase (GR) and lipid peroxidation (LPO) for mitigating against oxidative stress.

In the experimental approaches, either phenotypic or target-based screening can be done to identify potent drug candidates for validation, optimization, selection, and repositioning. Phenotypic screening is followed by target deconvolution (retrospective identification of molecular targets) using appropriate deconvolution strategies involving binding assays for identification of target interactions, in which techniques such as affinity chromatography and mass spectrometry, and other techniques such as three-hybrid systems, phage, and mRNA display, protein and "reverse transfected" cell microarrays, and biochemical suppression are frequently used. The targets can then be validated by functional studies that use methods such as RNA interference and protein overexpression.

Although drug repurposing has the advantages of saving time and cost (Pushpakom et al., 2018) and therefore a very compelling approach to drug discovery in emergencies as was the case in the early phase of COVID-19 drug discovery, it is not without shortcomings. The shortcomings include intellectual property and economic considerations (legal and commercial barriers); data and compound availability; and fear of exhausting the repurposing space (Talevi and Bellera, 2020). In spite of these challenges, remarkable success stories abound, including repurposed drugs for COVID-19 treatment (Remdesivir has got FDA approval) and some others have been granted emergency use authorization.

Computational methods are today used in both traditional drug discovery processes and drug repurposing and it is extensively discussed in another section of this review. Suffice it to mention here that the most common techniques used in computational methods include target-based, network or pathway-based, knowledge-based, signature-based, and artificial-intelligence-based techniques which rely largely on docking and molecular dynamics simulations.

Apart from drug repurposing, there have also been efforts in developing antibody therapies using blood samples obtained from recovered patients. These involve the isolation and identification of antibodies including neutralizing monoclonal antibodies and nanobodies for the

treatment of COVID-19 (Huo et al., 2020; Renn et al., 2020; Adamson et al., 2021).

Traditional drug discovery approach

The trajectories for drug repurposing and traditional drug discovery are essentially the same, differing mainly in speed and cost. While drug repurposing was going on, scientists across the globe were intensifying strategic basic research efforts to accumulate knowledge about SARS-CoV-2 and the pathophysiology of COVID-19 to allow for the commencement of the drug discovery process specifically for COVID-19. Technologies generally involved in drug discovery include various screening techniques such as phenotypic, CRISPR, and target-based screening; structure-based drug discovery; and Biocomputing/bioinformatics. Typically, the drug discovery and development process involve the following key steps: target (a biological molecule, usually a protein but can also be DNA or RNA or gene whose function is to be modulated to bring about a desired or beneficial therapeutic effect to the patient) discovery, identification, and validation; assay (investigative procedures for the qualitative evaluation of the effects of a compound on identified molecular, cellular, or biochemical targets) development; screening to obtain molecules or extracts with a promising activity which are referred to as hits; counter-screening to identify compounds that may interfere with the primary assay used in the primary screen and to eliminate cytotoxic compounds; hit-to-lead and lead optimization to obtain a compound with suitable properties that can be modified to produce a drug candidate. The drug candidate then goes into development and progresses into clinical trials.

Whatever the drug discovery approach used, challenges abound. A major factor militating against anti-SARS-CoV-2 drug discovery is the requirement for Category three containment level which is expensive and difficult to maintain and therefore not within the reach of many scientists, particularly in poor resource settings. Screening of compounds against live SARS-CoV-2 is therefore problematic. The alternatives are to use *ex vivo* systems such as pseudoviruses and/or perform inhibition studies against SARS-CoV-2 enzymes such as RNA-dependent RNA polymerases (RdRps) and proteases or block critical protein-protein interactions. SARS-CoV-2 is not an exception to drug resistance which is a major problem in antiviral therapy generally. This is particularly so with respect to RNA viruses as a consequence of the error-prone nature of RNA-dependent RNA polymerases (RdRps), the rapid rate of viral replication, and the high frequency of recombination events. The deployment of nanotechnology to formulate multi-component and multi-target nanoparticle cocktails will produce medicines to which resistance will be much less likely.

Natural products are compounds or substances produced by living organisms which include plants, animals, and microorganisms. The use of natural products for the treatment of diseases dates back to prehistoric times. Scientists are digging into nature to harness the "goldmine" of healing molecules to meet various unmet medical treatment needs. Natural products continue to be major sources of prototypes of antimicrobial and antiviral drugs (Adalja and Inglesby, 2019) and represent viable sources of therapeutic alternatives for many diseases (González-Maldonado et al., 2022). It is estimated that over 70% of all existing pharmaceutical products are of natural product origin (Wangchuk, 2018; Abd et al., 2019). In the United States, approximately 118 of the top 150 prescription drugs are based on natural sources (Chen et al., 2016). Several classes of natural compounds such as flavonoids, alkaloids, peptides, and others have been tested against COVID-19 (Antonio et al., 2020; Verma et al., 2020; Antonio et al., 2021; González-Maldonado et al., 2022) with promising outcomes.

Medicinal plants are globally recognized as valuable sources of new medicines with up to 80% of people in developing countries relying on herbal medicines for their primary health care needs. Additionally, more than 25% of medicines prescribed in developed countries are derived from wild plant species (reviewed by Chen et al., 2016). At the dawn of the 21st century, 11% of the 252 drugs considered basic and essential by the WHO were exclusive of flowering plant origin (Veeresham, 2012). Some plants known to have antiviral and immunomodulatory properties include *Glycyrrhiza glabra, Azadirachta indica, Andrographis paniculata, Calotropis gigantea, Ocimum sanctum, Curcuma longa, Withania somnifera, Zingiber officinale, Allium sativum, Tinospora cordifolia, Moringa oleifera* (Ganjhu et al., 2015; Tiwari et al., 2018). There are a number of plant-specific compounds such as lignans, saponins, alkaloids, flavonoids (kaempferol, luteolin, apigenin, baicalin, quercetin, catechins), and polysulphates (sulphated polysaccharides) that are known to destroy the nucleocapsid and genetic material, inhibit viral entry and inhibit the replication of viruses such as dengue, herpes simplex virus (HSV), hepatitis C virus (HCV), influenza, chikungunya, SARS, etc. (Dhama et al., 2018). Exploiting medicinal plants for the development of COVID-19 therapeutics is therefore a very attractive option.

In the early days of the heat of the COVID-19 pandemic when no specific treatment was available, many countries explored the potential of phytochemicals obtained from medicinal plants and herbs for treating COVID-19 patients (Aanouz et al., 2020; Divya et al., 2020; Jahan and Onay, 2020; Qamar et al., 2020; Xu and Zhang, 2020). Significant progress has already been made in COVID-19 drug discovery from medicinal plants. Polyphenols have been shown to inhibit coronaviruses (Mani et al., 2020) while virtual screening has demonstrated the strong binding potential of absinthin, quercetin 3-glucuronide-7-glucoside, and quercetin 3-

TABLE 4 Herbal medicines and efficacy levels as adjuvant symptomatic therapies in early COVID-19 (Silveira et al., 2020).

Plant	Efficacy
Althaea officinalis	Positive
Commiphora molmol	Positive
Glycyrrhiza glabra	Positive
Hedera helix	Positive
Sambucus nigra	Positive
Allium sativum	Promising
Andrographis paniculata	Promising
Echinacea angustifolia	Promising
Echinacea purpurea	Promising
Eucalyptus globulus essential oil	Promising
Justicia pectoralis	Promising
Magnolia officinalis	Promising
Mikania glomerata	Promising
Pelargonium sidoides	Promising
Pimpinella anisum	Promising
Salix sp	Promising
Zingiber officinale	Promising

vicianoside to SARS-CoV-2 main protease (Mpro) and angiotensin-converting enzyme 2 (Joshi et al., 2020). It is documented in the literature that 83 phytocompounds with anti-COVID-19 activity, the most potent being the alkaloid, lycorine, lignan, savinin, and a total of 39 herbal medicines (listed by WHO and European Medicines agency, EMA) which are indicated for "respiratory diseases" that were evaluated for efficacy as an adjuvant symptomatic therapy for COVID-19 the results of which are shown in Table 4 (Silveira et al., 2020). Thymoquinone obtained from natural *Nigella sativa* has also been documented to be potentially useful for the treatment of COVID-19 (Abdelrahim et al., 2022). Additionally, several plant-derived bioactive compounds from traditional herbal medicine include andrographolide, panduratin A, baicalein, digoxin, and digitoxin, have demonstrated potent SARS-CoV-2 antiviral activity comparable with some repurposed FDA-approved drugs (Liana and Phanumartwiwath, 2021). Methanolic extract of *Stachytarpheta cayennensis* remarkably inhibited SARS-CoV-2 entry (González-Maldonado et al., 2022). The Lamiaceae family members, *Zingiber officinale*, and *Glycyrrhiza* spp. are known to be particularly good as sources of medicines for the treatment of COVID-19.

While the descriptions of the huge potentials of natural products-derived anti-COVID-19 medicines are heartwarming, only Lianhuaqingwen, a Chinese herbal mixture of 11 medicinal species containing 61 compounds (Wang et al., 2016) has recommendation (by the Chinese National Health Commission) for clinical application to treat or manage COVID-19 (Yang et al., 2020). The herbal mixture inhibited SARS-CoV-2 replication in a dose-dependent manner with an IC50 of 411.2 μg/ml. Furthermore, the mixture was able to suppress the release of pro-inflammatory cytokines (TNF-a, IL-6, CCL-2/MCP-1, and CXCL-10/IP-10) in a dose-dependent manner (Runfeng et al., 2020).

Terpenoids, lectins, glycoproteins, lentinan, galactomannan, and polysaccharides from mushrooms (*Agaricus subrufescens* Peck, *Agaricus blazei* Murill, *Cordyceps sinensis* (Berk.) Sacc., *Ganoderma lucidum* (Curtis.) P. Karst., *Grifola frondosa* (Dicks.) Gray, *Hericium erinaceus* (Bull.) Pers., *Inonotus obliquus* (Arch. Ex Pers.) Pilát., *Lentinula edodes* (Berk.) Pegler, *Pleurotus ostreatus* (Jacq.) P. Kumm., *Poria cocos* F.A. Wolf, and *Trametes versicolor* (L.) Lloyd are promising prophylactic or therapeutic agents against COVID-19 (Arunachalam et al., 2022). The terpene, erylosides B from Red Sea invertebrate inhibits SARS-CoV-2 main protease (MPro) and identified as a promising anti-COVID-19 drug lead (Ibrahim et al., 2021).

Novavax vaccine consisting of proteins has been created in cultures of Fall armyworm (*Spodoptera frugiperda*) cells where the cells produce protein spikes that coat SARS-CoV-2 when infected with an engineered virus. The vaccine is achieving high success rates against all main variants of the virus and is entering authorization processes around the world. Peptides from animal venoms (snake, scorpion, frog, insect venoms) are rich sources of antiviral drugs.

Flavonoids, phlorotannins, alkaloids, terpenoids, peptides, lectins, polysaccharides, lipids and others substances of marine origin have been shown to exert desirable effects on coronaviruses penetration and entry into the cell, replication of the viral nucleic acid and release of the virion from the cell also can act on the host's cellular targets (Zaporozhets and Besednova, 2020). Similarly, several classes of compounds from various marine organisms (diverse sponges and algae and bacteria) have been shown to affect various virulence factors of SARS-CoV-2 and also induce the innate immune response and downregulate human ACE-2 (Geahchan et al., 2021).

As good and attractive as drug repurposing and other drug discovery processes are, drug discovery from natural sources is even more attractive and very compelling. This is because of the rich chemical diversity and the huge potential for the discovery of novel chemical scaffolds for discovery of novel drugs. The application of computational methods has made the discovery process much more robust and it is described in the section that follows.

BIOCOMPUTING/BIOINFORMATICS/ ARTIFICIAL intelligence

The availability of many untapped floral and faunal bioactive compounds (phytochemicals) in Africa and gradual understanding of the use of bioinformatics tools in drug discovery, drug design, and

computer applications in a genetic study is opening a new chapter of scientific understanding (gradual shift) from conventional laboratory investigation to *in silico* approach to drug discovery and molecular biology. In most African drug research centers, computer-aided drug design (CADD) is now being used to facilitate the process of drug discovery. Though the method is predictable the precision and its efficiency have been ascertained by various researchers. In the last decade, bioinformatics-based models of specific biomolecules have offered rapid and inexpensive methods for the discovery of effective viral therapies. In the presence of a target biomolecule, these models are capable of predicting inhibitor candidates in a structure-based manner. The inhibitors can also be used to predict the specific target (ligand-based) if enough data are presented to a model and it can aid the search for a drug or vaccine candidate by identifying patterns within the data. The availability of enormous data from African bioactive compounds (phytomedicines) provides a baseline for which bioinformatics tools can be used to explore inhibitors of various micro-organisms including viruses like SARS-CoV-2. Database, like phytochemical database, management system has been successfully developed and used in *in silico* drug design.

Phytochemicals database, a platform for virtual screening and computer-aided drug design

The phytomedicines derived from medicinal plants have proven to be a rich source of diverse chemical agents that have been used as drugs and supplements in the millennium (Mahmud et al., 2022). The dependence of Africans on phytomedicines as the first line in the treatment of disease conditions and perhaps the consumption of various vegetables with medicinal properties must have contributed to the low incidence of SARS-CoV-2 in most African countries relative to European countries despite a devastating prediction from World Health Organisation (WHO). It is therefore important to explore the efficacy of different African phytochemicals as inhibitors of SARS-CoV-2 using an already established phytochemical database and bioinformatics tools. In recent times, statistics of newly approved drugs by the United States Food and Drug Administration (FDA) shows that Phytomedicines account for a large number of the approved drugs used as general tonics, antioxidants, cell protectives, and immune stimulants (Kandeel et al., 2020) in the management of SARS-CoV-2, even in this era of combinatorial chemical drugs (Mahmud et al., 2022). Information relating to biological activity, molecular weight, and molecular structure of phytomedicines are deposited in the various databases. Few phytomedicine databases include:

Phytochemdb (Mahmud et al., 2022) is a database that is manually managed and compiles 525 lists of plants and their corresponding 8093 phytochemicals (Mahmud et al., 2022). It is a comprehensive database that gathers most of the information about medicinal plants in one platform, which is considered to be very beneficial to the work of researchers on medicinal plants. "Phytochemdb" is available for free *at* https://phytochemdb.com/.

TarNet

This is an evidence-based database for research on natural medicine. It is a cataloged database that provides information on traditional medicinal plants with natural compounds that includes potential bio-target information (Hu et al., 2016). Comprehensive information on a plant-compound-protein relationship can be accessed from the TarNet platform. TarNet is freely available at http://www.herbbol.org:8001/tarnet.

Ethiopian-Traditional Medicine Database

ETM-DB is the largest web-based integrated resource of Ethiopian traditional medicine (Bultum et al., 2019), freely accessible, and provides traditional herbal medicine entities and their relationships in well-structured forms including references (Bultum et al., 2019). The ETM-DB website interface is user-friendly and allows the users to search the entities using various options provided by the search menu. ETM-DB is expected to expedite the process of drug discovery and development and also promote *in silico* research from Ethiopian natural products leveraging information on the chemical composition and related human target gene/proteins. Phytochemicals from this database can be virtually screened against different targets of SARS-CoV-2. Therefore, this database is key in the discovery of antiviral drugs including RNA viruses such as SARS-Cov-2. The current version of ETM-DB is openly accessible at http://biosoft.kaist.ac.kr/etm.

African natural product database (AfroDb)

This is a database of selected highly potent and diverse natural product libraries from African medicinal plants (Ntie-Kang et al., 2013). AfroDb is said to represent the largest "drug-like" and diverse collection of 3D structures of natural products (NPs) covering the geographical region of the entire African continent (Ntie-Kang et al., 2013). The database is readily accessible and can be used in the integrated virtual screening program. The huge information on phytochemicals in this database can be leveraged in the discovery of antiviral drugs. This drug bank could serve as a reservoir of potent molecules active against most viral infections including SARS-CoV-2 and its variants. Since it is possible to predict the variants of SARS-CoV-2 by inducing mutation on SARS-CoV-2 main targets using *in silico* method, the targets from these variants can be used as reference targets for the virtual screening (target-based virtual screening) or the molecules from the database can be screened against targets (ligand-based virtual screening). AfroDb is therefore a drug bank in which artificial intelligence (bioinformatics tools) can be used to explore huge information on African phytochemicals to aid in drug

TABLE 5 Binding energies of some phytochemicals docked in the active sites of 3-chymotrypsin-like proteases of coronaviruses (Gyebi et al., 2021).

S/NO	Plant name	Compound	Binding energy (kcal/mol)
1	Vernonia amygdalina	Vernolide	−8.0
2	Vernonia amygdalina	Vernomygdin	−7.9
3	Occinum gratissimum	Chicoric acid	−7.7
4	Occinum gratissimum	Rosmarinic acid	−7.7
5	Vernonia amygdalina	Neoandrographolide	−7.7
6	Occinum gratissimum	Luteolin	−7.7
7	Vernonia amygdalina	Vernomenin	−7.7
8	Vernonia amygdalina	Isorhamnetin	−7.6

discovery and drug design, particularly in the pre and post pandemic period.

Computer-aided drug design and its application to COVID-19 drug discovery

Computer-aided drug design (CADD) has helped to facilitate the process of drug discovery and development by minimizing the cost and time (Gurung et al., 2021). The availability of a drug data bank (phytochemical database) also enhances the process of drug discovery. In the search for antiviral drugs, two important methods of computer-aided drug design (CADD) is key to the discovery of potent anti Covid-19 drugs (Gurung et al., 2021): the ligand-based and structure-based virtual screening. Molecular docking and molecular dynamic simulation are important techniques in structure-based drug design whereas ligand-based drug design includes pharmacophore modeling, quantitative structure-activity relationships (QSARs), and artificial intelligence (AI) (Gurung et al., 2021). The CADD plays a significant role in the design and discovery of promising drug candidates against various drug targets implicated in the pathogenesis of SARS-CoV-2 (Gurung et al., 2021).

Structure-based drug design

The availability of the three-dimensional crystal structure of the therapeutic target proteins and exploration of the binding site or active site residues forms the basis of structure-based drug design (SBDD) (Batool et al., 2019). This approach is highly selective and effectively fast in the identification of lead molecules and their optimization which has led to a better understanding of diseases at a molecular level (Lionta et al., 2014). Some of the common methods used in SBDD include structure-based virtual screening (SBVS), molecular docking, and molecular dynamics (MD) simulations (Gurung et al., 2021). A lot of information can be extracted from these methods, some of which include assessment of binding energetics, principal component analysis (PCA), dynamics cross-correlation, protein-ligand interactions, and conformational changes in the receptor upon ligand binding (Batool et al., 2019). SBDD is a computational technique has greatly helped in the discovery of several drugs available on the market today. For example, the discovery of Amprenavir as a potential inhibitor of the human immunodeficiency virus (HIV) protease using the crystallized protein model and molecular dynamics (MD) simulations (Adamson et al., 2009; Liao and Nicklaus, 2010). Others include thymidylate synthase inhibitor, an anticancer agent, and Raltitrexed implicated in the treatment of HIV-infected cancer patients using the SBDD approach (Anderson, 2003; Medina-Moreno et al., 2019). Norfloxacin a topoisomerases II and IV inhibitor was also discovered through SBVS (Batool *et al*., 2019). In recent time, SBVS conducted using different protein targets of SARS-CoV-2 and phytochemical database revealed high binding energy and very good interaction with the residues of the active site target protein such as 3-Chymotrypsin-like proteases ($3CL^{pro}$) of SARS-CoV-2 (Gyebi et al., 2021). Some of the phytochemical agents that show good binding energy and ligand-residue interaction are shown in Table 5.

These plants, native to most of the African countries, demonstrate the reservoir of phytochemicals in Africa with potent inhibitory activity against target proteins in SARS-CoV-2. The basic steps involved in Covid-19 drug discovery using bioinformatics tools (SBDD) consist of the preparation of target structure, identification of the ligand-binding site, compound library preparation, molecular docking and scoring functions, molecular dynamic simulation, and binding free energy calculation (Gurung et al., 2021).

Ligand-based drug design

Ligand-based drug design is a computer-aided drug design technique that is widely used in drug discovery and design. It is employed when the three-dimensional structure of the target receptor is not available. The information

obtained from a set of active compounds against a specific target receptor is useful in the identification of physicochemical and structural properties that are responsible for the specific biological activity which is based on the structure-activity relationship (Prathipati et al., 2007; Gurung et al., 2021). The commonest techniques used in the ligand-based virtual screening approach include pharmacophore modeling, quantitative structure-activity relationships (QSARs), and artificial intelligence (AI).

Artificial intelligence (AI) and prospects in Covid-19 drug discovery

Artificial intelligence (AI) is a machine learning intelligence that depends on the ability of computers to learn from existing data (Gurung et al., 2021). AI has been used in various computational modeling methods to predict the biological activities and toxicity profiles of drug molecules (Patel et al., 2014). In addition, AI has also been widely used in the prediction of protein folding, protein-protein interaction, virtual screening, Quantitative Structural Activity Relationship (QSAR), evaluation of absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of the drug molecule, and *de novo* drug design (Gurung et al., 2021).

There are two major methods of AI that are widely used in rational drug design: machine learning (ML) and deep learning (DL) (Cortes and Vapnik, 1995). A support vector machine (SVM) is an ML algorithm that has been extensively used in drug discovery (Cortes and Vapnik, 1995). Others include Random Forest (RF) (Breiman, 2001) and Naive Bayesian (NB) (Sammut and Webb, 2017). Deep learning methods include convolutional neural network (CNN), deep neural network (DNN), recurrent neural network (RNN), autoencoder, and restricted Boltzmann machine (RBN) (Gurung et al., 2021). In summary, artificial intelligence has been widely used in drug discovery and vaccine development (Arshadi et al., 2020). As has been documented (Keshavarzi et al., 2022), this advancement in therapeutics research is critical for the current situation of pandemic and urgent need for the SARS-COV-2 therapy discovery for the following reasons (1) deep learning has an automatic feature extraction ability that can support models with better accuracy and deliver good reliable results (2) deep learning models demonstrate the generative ability that can be utilized to create more druggable molecules and better epitope prediction, (3) reduced chances of failure in the drug pipeline, and (4) because of the novelty of the virus the data related to its therapies remain scarce, which is a suitable scenario for knowledge transfer while leveraging on the learned knowledge from previous tasks (e.g., TranscreenTM) (Salem et al., 2020). Transfer learning is very effective in the transferring of learned knowledge and parameters from a secondary task with big data available to the task at hand (Weiss et al., 2016). Therefore, the use of deep learning in the discovery of therapies for SARS-COV-2 is necessary for a timely and accurate response to the viral pandemic (Arshadi et al., 2020).

Strength and challenges of CADD in the discovery of COVID-19 drugs

With the increase in the number of confirmed positive and death cases from SARS-CoV-2 infection with related evidence of viral mutation, computer-aided drug design (CADD) is the most viable and reliable technique in drug and medicinal research because of its attributed time saving and cost reduction in the design of therapeutic agents (Gurung et al., 2021; Ojha et al., 2021). In addition, the high impact of the pandemic resulting from COVID-19 infection and the relative lack of approved drugs create room for drugs to be repurposed within a short period of time. The CADD enhances this method by facilitating the discovery of new drugs or repurposing FDA-approved drugs whose safety and adverse effects are already known (Basak et al., 2021; Gurung et al., 2021). Since SARS-CoV-2, an RNA virus, poses a high mutation rate, the genome may hinder disease prevention and treatment (Gurung et al., 2021). CADD can be used efficiently to induce mutation on the existing targets, allowing for *in silico* prediction of the possible SARS-CoV-2 variant and subsequently developing potent molecules against these variants that are likely to cause a future pandemic (Gurung et al., 2021; Sharma et al., 2021). This is one of the major advantages (strength) of CADD in the discovery of COVID-19 drugs. Therefore, CADD is an asset in the drug discovery and development process. However, the CADD method is limited by the inability to validate its lead compound through clinical trials before market approval (Ojha et al., 2021). The molecular understanding of the disease pathogenesis of COVID-19 are still been strengthened, and the existence of the limited data on variants of COVID-19 can have a major impact on the precision and accuracy of CADD methods such as artificial intelligence (Ojha et al., 2021).

COVID-19 and beyond

In spite of the odds, tremendous opportunities exist to overcome the challenges by integrating advances in science and technology such as Genomics (including CRISPR-Cas system) Phytopharming/Molecular farming, Biocomputing, and nanomedicine to develop novel and innovative antiviral therapies with broad-spectrum activities against human

pathogenic viruses of the present and those that may emerge or re-emerge in the future.

Phytopharming/plant molecular farming for increased and accelerated production of COVID-19 therapeutics

Phytopharming or plant molecular farming is the production of biopharmaceuticals in plants using the tools of plant metabolic engineering and plant biotechnology. It is intended to overcome the limitations of high operating cost, prolonged production time, low yield, chances of contamination with pathogenic microorganisms, and limited posttranslational modifications of the current platforms that use bacterial systems, microbial eukaryotes, insect cells, and mammalian cells (Shanmugaraj et al., 2020). The use of plants as a platform for the production of diagnostic reagents and pharmaceutical proteins has been around for well over 30 years (Schillberg et al., 2019; Fischer and Buyel, 2020). The Israeli biotech company Protalix and Pfizer got FDA approval for the first drug, Elelyso (taliglucerase alfa), developed in plant cells (cells from carrot) in 2012. In February 2022, the Canadian biotech company, Medicago, got approval for its two-dose COVID-19 vaccine, the first world's plant-based COVID-19 vaccine (The Pharmaeutical Journal, 2022). The rapidity with which this feat was achieved is outstanding, taking just over 2 weeks. This is what the executive vice president of Medicago, Marc-André D'Aoust said. "From the moment we had the sequence on our computer, to the moment we [had the] first purified product, it took 19 days". Comparing this speed with five to 6 months for a conventional egg-grown vaccine, the plant-based approach will be of great advantage in potential future pandemics. This is particularly useful in poor and developing countries where the cost of the infrastructure of conventional platforms for the production of biopharmaceuticals is out of reach. Living plants have therefore effectively become bioreactors for the production of biopharmaceuticals. It may well be that plants may offer the only platform that can be used to manufacture COVID-19 diagnostic reagents and therapeutics at scale in a timeframe of weeks, compared with months or even years for cell-based systems (Capell et al., 2020). Lico and other colleagues in Italy made a case for molecular farming to complement conventional methods of production for the rapid and scalable supply of protein antigens as reagents and vaccine candidates, antibodies for virus detection and passive immunotherapy, other therapeutic proteins, and virus-like particles as novel vaccine platforms to meet the urgent therapeutic needs imposed by COVID-19 (Lico et al., 2020).

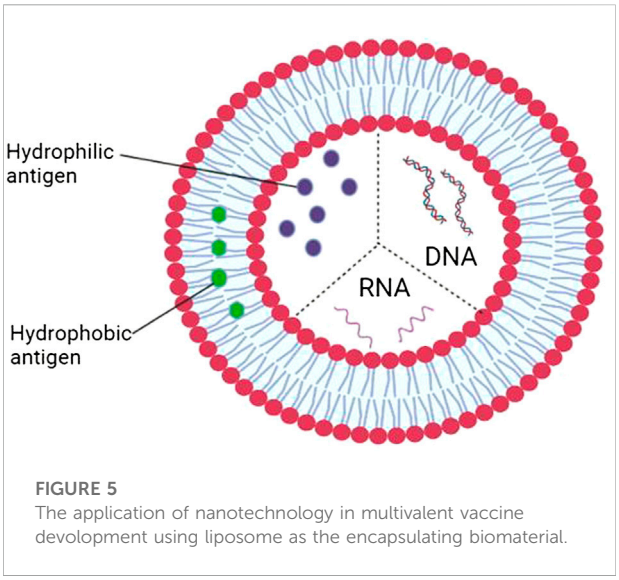


FIGURE 5
The application of nanotechnology in multivalent vaccine devolopment using liposome as the encapsulating biomaterial.

Edible vaccines in the battle against COVID-19

The concept is not different from molecular farming except that in this case the vaccine is produced in edible crops such as tomatoes, banana, potatoes, rice, carrot, corn, cucumber, lettuce, and spinach. Tomato has the advantages of having excellent biomass, being easy to transform and the whole plant can be generated within a short period. Tomato has therefore been described as a green vaccine factory (Sohrab, 2020). Sohrab and other colleagues listed the advantages of plant-based vaccines to include: oral use as fruits and vegetables; obtainable as capsules from dried leaf tissue powder; no requirement for adjuvants to enhance immune responses; mucosal immunity elicited by orally-introduced antigens; bulk production on site is easy and can be transported and stored at less cost and without cold chain requirement; not administered by injection and therefore no need for a trained medical person; easy to express, separate and purify; they can be stored as seeds and oils and dried tissue without any refrigeration; They do not have any risk of microbial contamination and disease spread; There is the possibility of enhanced compliance, especially in children (Sohrab et al., 2017). Significant progress has been made as scientists from the Centre for Genomics and Bioinformatics of the Academy of Sciences at Uzbekistan have developed a tomato-based vaccine against COVID-19 as of 2021. Seedlings in the laboratory are grown in the form of a vaccine from seedlings in the Centre after 2 months, and people who eat these tomatoes are expected to produce antibodies against the virus (Abdulkerimov, 2021).

Nanomedicine and enhancement of COVID-19 therapeutics

Nanomedicine is a branch of medicine that applies nanotechnology, which is essentially the manipulation and manufacture of materials and devices that are in the size range of 1–100 nm, to the diagnosis, prevention, treatment, and monitoring (follow-ups) of diseases and also for medical imaging and the repair and regeneration of biological systems (regenerative medicine). It uses the properties developed by a material at its nanometric scale which is different from those of the same material at a bigger scale in terms of physics, chemis,try or biology to achieve the many intended aims of nanomedicine applications. At the nano-scale, the surface-to-volume ratio is such that the surface properties become an intrinsic parameter of the potential actions of a particle or material. Therefore, coating the nanoparticles and functionalizing their surfaces enhance the biocompatibility of the particle and its circulation time in the blood, as well as ensuring a highly selective binding to the target of choice. The design and development of nanoparticles containing drug(s) (nanoparticulate nanomedicines) for drug delivery is an aspect of nanomedicine that has attracted tremendous attention. Nanoparticulate nanomedicines are designed and developed to deliver drugs through various mechanisms such as solubilization, passive targeting, active targeting, and triggered release which will ultimately increase therapeutic efficacy, decrease the therapeutically effective dose, and/or reduce the risk of systemic side effects (Hua et al., 2018). The nanomaterials of choice will include, but not limited to, liposomes (Figure 5), solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs), and polymeric nanoparticles. As of 2012, the global nanomedicine market stood at about 78 billion dollars (Viseu, 2022). In 2022, this stands at 159.43 billion dollars and this is expected to rise to $291.15 billion by 2027 (Market Data Forcast, 2022). This means that the market outlook for investors is bright and attractive and funding may not be a problem. Moreover, any investment in health is an economic investment because of the intricate relationship between health and the national economy.

Development of nanomedicine-based therapeutics against a variety of viral infections, including hepatitis B virus, human immunodeficiency virus, respiratory syncytial virus, influenza virus, and coronaviruses are already known. The attraction for the use of nanoparticles in nanomedicine is in their unique physicochemical properties: the small size which is also the scale of many biological mechanisms in the human body allows them to easily cross intracellular barriers/membranes to access new sites of delivery and interact with a variety of biological components of similar size, interacting with DNA or small proteins at different levels, in blood or within organs, tissues or cells; the surface polarity which can be modified by various functional groups to increase their binding efficacy and stability and reduce aggregation and precipitation (Dutta, 2022, ETPN, 2022).

Metallic nanoparticles such as iron-oxide, copper-oxide, and silver nanoparticles with antiviral properties can be used to entrap and inactivate SARS-CoV-2. These particles disrupt the cell membrane, damage proteins, and DNA, form free radicals, inhibit biofilm formation, and/or exert heavy metal toxicity in viruses thereby destroying the viruses. Iron-oxide nanoparticles have been shown to interact with the spike protein of SARS-CoV-2, altering its structural conformation. So have lipid nanoparticles loaded with SARS-CoV-2-specific small-interfering RNAs (siRNAs) for targeted delivery to the lungs to suppress viral replication and prevent the establishment of infection and disease. Poly lactic-co-glycolic acid polymer-based nanosponge coated with human lung epithelial cell and macrophage membrane to mimic the cellular physiology required for SARS-CoV-2 host cell entry is a good target for SARS-CoV-2. The binding of SARS-CoV-2 to this synthetic cellular nanosponge leads to the neutralization of the virus and infection of cells is blocked.

Apart from targeting SARS-CoV-2 directly, nanoparticulate nanomedicines can also be used to modulate the immune system in such a way that hyperinflammation and the consequent cytokine storm in COVID-19 patients can be prevented. Graphene-oxide nanoparticles are known to increase the levels of macrophages and T cells thereby enhancing adaptive immune response and viral clearance. Similarly nano-diamonds induce anti-inflammatory macrophages to reduce hyperinflammation. In the same way, carbon and graphene sheets can be modified to eliminate cytokines and interleukins (pro-inflammatory mediators) from the blood (reviewed by Dutta, 2022).

Concluding remarks and moving forward

Natural products represent a large reservoir of chemicals from which new chemical scaffolds can be obtained for the discovery of novel drugs, not only for COVID-19 but for other infectious diseases, including emerging and re-emerging diseases. Monotherapy in which one drug to one target approach is used is no longer a viable option. A strategic therapeutic approach in which a multicomponent and multi-target pan-viral therapy is developed for the treatment of COVID-19 and associated complications and future viral pandemics is a very compelling option and the drug discovery and development community should embrace it. Nanomedicine presents an excellent platform to achieve this as different drugs each with different targets can be loaded into a drugs-nanoparticle complex formulation as shown in Figure 5. This is also useful for the development of multivalent vaccines for activity against existing human pathogenic viruses and those that may emerge or re-emerge in the future. Plant molecular farming is undoubtedly a very useful technology for relatively cheap, fast, and large-scale production of biopharmaceuticals and relevant stakeholders should ensure that the required infrastructure for this is put in place in countries across the world for pandemic preparedness. After the “hype” about edible vaccines in the 90s,

it is now becoming a reality and should be vigorously pursued for COVID-19 vaccine production particularly in poor resource settings, and quick response in the future should there be an outbreak of a pandemic. Edible vaccines will be an important strategy to significantly reduce vaccine hesitancy. However, worries about GMO foods are a potential problem but strong advocacy may be able to remove or significantly reduce these worries. Finally, it is important to sustain and improve on the coordinated global effort by all stakeholders in the development of COVID-19 therapeutics so that the several decades of accumulated knowledge from previous epidemics and pandemics can be effectively combined with the tremendous advances in science and technology to develop therapeutics of the present and the future.

Author contributions

NU wrote the Biocomputing and Bioinformatics section while EO wrote the other portions of the manuscript.

Funding

Work in EO's laboratory is supported by the West African Network for Infectious Diseases ACEs (WANIDA) and the Africa Centre of Excellence for Mycotoxin and Food Safety (ACEMFS).

Acknowledgments

The authors are grateful to Israel Ogbadoyi for his assistance with the graphics.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors, and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Aanouz, I., Belhassan, A., El-Khatabi, K., Lakhlifi, T., El-Ldrissi, M., and Bouachrine, M. (2020). Moroccan Medicinal plants as inhibitors against SARS-CoV-2 main protease: Computational investigations. *J. Biomol. Struct. Dyn.* 39, 2971–2979. doi:10.1080/07391102.2020.1758790

Abd, E. N. H., Abd El-Aziz, F. A., Abouelmagd, S. A., Abd El-Hamid, B. N., and Hetta, H. F. (2019). Spidroin in carbopol-based gel promotes wound healing in earthworm's skin model. *Drug Dev. Res.* 80, 1051–1061. doi:10.1002/ddr.21583

Abdelrahim, M., Esmail, A., Al Saadi, N., Zsigmond, E., Al Najjar, E., Bugazia, D., et al. (2022). Thymoquinone's antiviral effects: It is time to be proven in the covid-19 pandemic era and its omicron variant surge. *Front. Pharmacol.* 13, 848676. doi:10.3389/fphar.2022.848676

Abdulkerimov, B. (2021). Uzbek Scientists working on edible vaccine against COVID-19. Available at: https://www.aa.com.tr/en/asia-pacific/uzbek-scientists-working-on-edible-vaccine-against-covid-19/2348236 (accessed 20 April 2022).

Adalja, A., and Inglesby, T. (2019). Broad-spectrum antiviral agents: a crucial pandemic tool. *Expert Rev. anti. Infect. Ther.* 17, 467–470. doi:10.1080/14787210.2019.1635009

Adamson, C. S., Chibale, K., Goss, R. J. M., Jaspars, M., Newman, D. J., and Dorrington, R. A. (2021). Antiviral drug discovery: preparing for the next pandemic. *Chem. Soc. Rev.* 50, 3647–3655. doi:10.1039/d0cs01118e

Adamson, C. S., Salzwedel, K., and Freed, E. O. (2009). Virus maturation as a new HIV-1 therapeutic target. *Expert Opin. Ther. Targets* 13, 895–908. doi:10.1517/14728220903039714

Anasir, M. I., and Poh, C. L. (2019). Structural vaccinology for viral vaccine design. *Front. Microbiol.* 10, 738. doi:10.3389/fmicb.2019.00738

Anderson, A. C. (2003). The process of structure-based drug design. *Chem. Biol.* 10, 787–797. doi:10.1016/j.chembiol.2003.09.002

Antonio, A. S., Wiedemann, L., and Veiga-Junior, V. (2020). Natural products' role against COVID-19. *RSC Adv.* 10, 23379–23393. doi:10.1039/d0ra03774e

Antonio, A. S., Wiedemann, L. S. M., Galante, E. B. F., Guimar~aes, A. C., Matharu, A. S., Veiga-Junior, V. F., et al. (2021). Efficacy and sustainability of natural products in COVID-19 treatment development: opportunities and challenges in using agro-industrial waste from citrus and apple. *Heliyon* 7, e07816. doi:10.1016/j.heliyon.2021.e07816

Arshadi, A. K., Webb, J., Salem, M., Cruz, E., Calad-Thomson, S., Ghadirian, N., et al. (2020). Artificial intelligence for COVID-19 drug discovery and vaccine development. *Front. Artif. Intell.* 3, 65. doi:10.3389/frai.2020.00065

Arunachalam, K., Sasidharan, S. P., and Yang, X. (2022). A concise review of mushrooms antiviral and immunomodulatory properties that may combat against COVID-19. *Food Chem. Adv.* 1, 100023.doi:10.1016/j.focha.2022.100023

Batool, M., Ahmad, B., and Choi, S. (2019). A structure-based drug discovery paradigm. *Int. J. Mol. Sci.* 20, 2783. doi:10.3390/ijms20112783

Breiman, L. (2001). Random forests. *Mach. Learn.* 45, 5–32. doi:10.1023/A:1010933404324

Bultum, L. E., Woyessa, A. M., and Lee, D. (2019). ETM-DB: Integrated Ethiopian traditional herbal medicine and phytochemicals database. *BMC Complement. Altern. Med.* 19, 212. doi:10.1186/s12906-019-2634-1

Capell, T., Twyman, R. M., Armario-Najera, V., Ma, J. K.-C., Schillberg, S., Christou, P., et al. (2020). Potential applications of plant biotechnology against SARS-CoV-2. *Trends Plant Sci.* 25 (7), 635–643. doi:10.1016/j.tplants.2020.04.009

Chen, S-L., Yu, H., Luo, H-M., Wu, Q., Li, C-F., Steinmetz, A., et al. (2016). Conservation and sustainable use of medicinal plants: problems, progress, and prospects. *Chin. Med.* 11, 37. doi:10.1186/s13020-016-0108-7

Cortes, C., and Vapnik, V. (1995). Support-vector networks. *Mach. Learn.* 20, 273–297. doi:10.1007/BF00994018

Dhama, K., Karthik, K., Khandia, R., Munjal, A., Tiwari, R., Rana, R., et al. (2018). Medicinal and therapeutic potential of herbs and plant metabolites/extracts countering viral pathogens—current knowledge and future prospects. *Curr. Drug Metab.* 19, 236–263. doi:10.2174/1389200219666180129145252

Dhaneshwar, S., and Bhasin, B. (2021). Drug repurposing: An emerging tool for drug reuse, recycling and discovery. *Curr. Drug Res. Rev.* 13, 101–119. doi:10.2174/2589977513666210211163711

Divya, M., Vijayakumar, S., Chen, J., Vaseeharan, B., and Durán-Lara, E. F. (2020). South Indian medicinal plant has the ability to combat against deadly viruses along with COVID-19? *Microb. Pathog.* 148, 104277.doi:10.1016/j.micpath.2020.104277

Dutta, S. S. (2022). Nanomedicine and COVID-19 news-medical. Available at: https://www.news-medical.net/health/Nanomedicine-and-COVID-19.aspx. (Accessed April 20, 2022)

Fears, J. R. (2004). The plague under Marcus Aurelius and the decline and fall of the Roman empire. *Infect. Dis. Clin. North Am.* 18 (1), 65–77. doi:10.1016/S0891-5520(03)00089-8

Fischer, R., and Buyel, J. F. (2020). Molecular farming – the slope of enlightenment. *Biotechnol. Adv.* 40, 107519. doi:10.1016/j.biotechadv.2020.107519

Ganjhu, R. K., Mudgal, P. P., Maity, H., Dowarha, D., Devadiga, S., Nag, S., et al. (2015). Herbal plants and plant preparations as remedial approach for viral diseases. *Virusdisease* 26, 225–236. doi:10.1007/s13337-015-0276-6

Geahchan, S., Ehrlich, H., and Rahman, M. A. (2021). The anti-viral applications of marine resources for COVID-19 treatment: An overview. *Mar. Drugs* 19, 409. doi:10.3390/md19080409

González-Maldonado, P., Alvarenga, N., Burgos-Edwards, A., Flores-Giubi, M. E., Barúa, J. E., Romero-Rodríguez, M. C., et al. (2022). Screening of natural products inhibitors of SARS-CoV-2 entry. *Molecules* 27, 1743. doi:10.3390/molecules27051743

Gurung, A. B., Ali, M. A., Lee, J., Farah, M. A., and Al-Anazi, K. M. (2021). An updated review of computer-aided drug design and its application to COVID-19. *Biomed. Res. Int.* 2021, 8853056. doi:10.1155/2021/8853056

Gyebi, G. A., Elfiky, A. A., Ogunyemi, O. M., Ibrahim, I. M., Adegunloye, A. P., Adebayo, J. O., et al. (2021). Structure-based virtual screening suggests inhibitors of 3-Chymotrypsin-Like Protease of SARS-CoV-2 from Vernonia amygdalina and Occinum gratissimum. *Comput. Biol. Med.* 136, 104671. doi:10.1016/j.compbiomed.2021.104671

Horton, Richard (2020). After COVID-19—Is an "alternate society" possible? *Lancet* 395 (10238), 1682. doi:10.1016/S0140-6736(20)31241-1

Hu, R., Ren, G., Sun, G., and Sun, X. (2016). TarNet: An evidence-based database for natural medicine research. *PLoS One* 11, e0157222. doi:10.1371/journal.pone.0157222

Hua, S., de Matos, M. B. C., Metselaar, J. M., and Storm, G. (2018). Current trends and challenges in the clinical translation of nanoparticulate nanomedicines: Pathways for translational development and commercialization. *Front. Pharmacol.* 9, 790. doi:10.3389/fphar.2018.00790

Huang, C., Wang, Y., Li, X., Ren, L., Zhao, J., Hu, Y., et al. (2020). Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 395, 497–506. doi:10.1016/S0140-6736(20)30183-5

Huo, J., Bas, A. L., Ruza, R. R., Duyvesteyn, H. M. E., Mikolajek, H., Malinauskas, T., et al. (2020). Neutralizing nanobodies bind SARS-CoV-2 spike RBD and block interaction with ACE2. *Nat. Struct. Mol. Biol.* 27, 846–854. doi:10.1038/s41594-020-0469-6

Ibrahim, M. A. A., Abdelrahman, A. H. M., Mohamed, T. A., Atia, M. A. M., Al-Hammady, M. A. M., Abdeljawaad, K. A. A., et al. (2021). *Silico Mining of Terpenes from Red-Sea Invertebrates for SARS-CoV-2*, 26, 2082. doi:10.3390/molecules26072082Main protease (mpro) inhibitors*Molecules*

Jahan, I., and Onay, A. (2020). Potentials of plant-based substance to inhabit and probable cure for the COVID-19. *Turk. J. Biol.* 44, 228–241. doi:10.3906/biy-2005-114

Jang, W. D., Jeon, S., Kim, S., and Lee, S. Y. (2021). Drugs repurposed for COVID-19 by virtual screening of 6218 drugs and cell-based assay. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2024302118. doi:10.1073/pnas.2024302118

Jarada, T. N., Rokne, J. G., and Alhajj, R. (2020). A review of computational drug repositioning: Strategies, approaches, opportunities, challenges, and directions. *J. Cheminform.* 12, 46. doi:10.1186/s13321-020-00450-7

Joshi, T., Joshi, T., Sharma, P., Mathpal, S., Pundir, H., Bhatt, V., et al. (2020). *In silico* screening of natural compounds against COVID-19 by targeting Mpro and ACE2 using molecular docking. *Eur. Rev. Med. Pharmacol. Sci.* 24, 4529–4536. doi:10.26355/eurrev_202004_21036

Kandeel, M., Kitade, Y., and Almubarak, A. (2020). Repurposing FDA-approved phytomedicines, natural products, antivirals and cell protectives against SARS-CoV-2 (COVID-19) RNA-dependent RNA polymerase. *PeerJ* 8, e10480. doi:10.7717/peerj.10480

Khataniar, A., Pathak, U., Rajkhowa, S., and Jha, A. N. (2022). A comprehensive review of drug repurposing strategies against known drug targets of COVID-19. *COVID* 2, 148–167. doi:10.3390/covid2020011

Li, G., and De Clercq, E. (2020). Therapeutic options for the 2019 novel coronavirus (2019-nCoV). *Nat. Rev. Drug Discov.* 19, 149. doi:10.1038/d41573-020-00016-0

Liana, D., and Phanumartwiwath, A. (2021). Leveraging knowledge of Asian herbal medicine and its active compounds as COVID-19 treatment and prevention. *J. Nat. Med.* 76, 20–37. doi:10.1007/s11418-021-01575-1

Liao, C., and Nicklaus, M. C. (2010). Computer tools in the discovery of HIV-1 integrase inhibitors. *Future Med. Chem.* 2, 1123–1140. doi:10.4155/fmc.10.193

Lico, C., Santi, L., Baschieri, S., Noris, E., Marusic, C., Donini, M., et al. (2020). Plant molecular farming as a strategy against COVID-19 – the Italian perspective. *Front. Plant Sci.* 11, 609910. doi:10.3389/fpls.2020.609910

Lionta, E., Spyrou, G., Vassilatis, D., and Cournia, Z. (2014). Structure-based virtual screening for drug discovery: Principles, applications and recent advances. *Curr. Top. Med. Chem.* 14, 1923–1938. doi:10.2174/1568026614666140929124445

Lipinski, C. (2011). "Drug repurposing," in Drug discovery today*: Therapeutic strategies* (Amsterdam, Netherlands: Elsevier).

Liu, Y-C., Kuo, R-L., and Shih, S-R. (2020). COVID-19: The first documented coronavirus pandemic in history. *Biomed. J.* 43, 328–333. doi:10.1016/j.bj.2020.04.007

MacDonald, N. E. (2015). Vaccine hesitancy: Definition, scope and determinants. *Vaccine* 33 (34), 4161–4164. doi:10.1016/j.vaccine.2015.04.036

Mahmud, S., Paul, G. K., Biswas, S., Kazi, T., Mahbub, S., Mita, M. A., et al. (2022). Phytochemdb: a platform for virtual screening and computer-aided drug designing. *Database.* 2022, baac002. doi:10.1093/database/baac002

Mani, J. S., Johnson, J. B., Steel, J. C., Broszczak, D. A., Neilsen, P. M., Walsh, K. B., et al. (2020). Natural product-derived phytochemicals as potential agents against coronaviruses: A review. *Virus Res.* 284, 197989. doi:10.1016/j.virusres.2020.197989

Market Data Forecast (2022). Nanomedicine market. Available at: https://www.marketdataforecast.com/market-reports/nanomedicine-market (accessed 18 April 2022).

Medina-Moreno, S., Zapata, J. C., Cottrell, M. L., Le, N. M., Tao, S., Bryant, J., et al. (2019). Disparate effects of cytotoxic chemotherapy on the antiviral activity of antiretroviral therapy: Implications for treatments of HIV-infected cancer patients. *Antivir. Ther.* 24, 177–186. doi:10.3851/IMP3285

Naasani, I. (2021). COMPARE analysis, a bioinformatic approach to accelerate drug repurposing against covid-19 and other emerging epidemics. *SLAS Discov.* 26, 345–351. doi:10.1177/2472555220975672

Nawaz, S. (2021). COVID-19, SARS-CoV-2, origin, transmission and treatment aspects, a brief review. *Infect. Disord. Drug Targets* 21, e270421186673. doi:10.2174/1871526520666201006163641

Ng, Y. L., Salim, C. K., and Chu, J. J. H. (2021). Drug repurposing for COVID-19: Approaches, challenges and promising candidates. *Pharmacol. Ther.* 228, 107930. doi:10.1016/j.pharmthera.2021.107930

Ntie-Kang, F., Zofou, D., Babiaka, S. B., Meudom, R., Scharfe, M., Lifongo, L. L., et al. (2013). AfroDb: a select highly potent and diverse natural product library from african medicinal plants. *PLoS One* 8, e78085. doi:10.1371/journal.pone.0078085

Patel, H. M., Noolvi, M. N., Sharma, P., Jaiswal, V., Bansal, S., Lohan, S., et al. (2014). Quantitative structure-activity relationship (QSAR) studies as strategic approach in drug discovery. *Med. Chem. Res.* 23, 4991–5007. doi:10.1007/s00044-014-1072-3

Prathipati, P., Dixit, A., and Saxena, A. (2007). Computer-aided drug design: Integration of structure-based and ligand-based approaches in drug design. *Curr. Comput. Aided-Drug Des.* 3, 133–148. doi:10.2174/157340907780809516

Pushpakom, S., Iorio, F., Eyers, P. A., Escott, K. J., Hopper, S., Wells, A., et al. (2018). Drug repurposing: progress, challenges and recommendations. *Nat. Rev. Drug Discov.* 18, 41–58. doi:10.1038/nrd.2018.168

Rappuolia, R., De Gregorioa, E., Del Giudicea, G., Phogata, S., Pecettaa, S., Pizzaa, M., et al. (2021). Vaccinology in the post-COVID-19 era. *Proc. Natl. Acad. Sci. U. S. A.* 118 (3), e2020368118. doi:10.1073/pnas.2020368118

Renn, A., Fu, Y., Hu, X., Hall, M. D., and Simeonov, A. (2020). Fruitful neutralizing antibody pipeline brings hope to defeat SARS-cov-2. *Trends Pharmacol. Sci.* 41, 815–829. doi:10.1016/j.tips.2020.07.004

Runfeng, L., Yunlong, H., Jicheng, H., Weiqi, P., Qinhai, M., Yongxia, S., et al. (2020). Lianhuaqingwen exerts anti-viral and anti-inflammatory activity against novel coronavirus (SARS-CoV-2). *Pharmacol. Res.* 156, 104761. doi:10.1016/j.phrs.2020.104761

Salem, M., Khormali, A., Arshadi, A. K., Webb, J., and Yuan, J. S. (2020). TranScreen: Transfer learning on graph-based anti-cancer virtual screening model. *Big Data Cogn. Comput.* 4, 16. doi:10.3390/bdcc4030016

Sammut, C., and Webb, G. I. (2017). *Encyclopedia of machine learning and data mining*. New York, NY: Springer.

Savi, C. D., Hughes, D. L., and Kvaerno, L. (2020). Quest for a COVID-19 cure by repurposing small-molecule drugs: Mechanism of action, clinical development, synthesis at scale, and outlook for supply. *Org. Process Res. Dev.* 24, 940–976. doi:10.1021/acs.oprd.0c00233

Scheres, J., and Kuszewski, K. (2019). The Ten Threats to Global Health in 2018 and 2019. A welcome and informative communication of WHO to everybody. *Zesz. Nauk. Ochr. Zdrowia. Zdr. Publiczne i Zarzadzanie* 17 (1), 2–8. doi:10.4467/20842627oz.19.001.11297

Schillberg, S., Raven, N., Spiegel, H., Rasche, S., and Buntru, M. (2019). Critical analysis of the commercial potential of plants for the production of recombinant Proteins.of plants for the production of recombinant proteins. *Front. Plant Sci.* 10, 720. doi:10.3389/fpls.2019.00720

Senanayake, S. L. (2020). Drug repurposing strategies for COVID-19. *Future Drug Discov.* 2, 2. doi:10.4155/fdd-2020-0010

Shanmugaraj, B., Bulaon, C. J. I., and Phoolcharoen, W. (2020). Plant molecular farming: A viable platform for recombinant biopharmaceutical production. *Plants* 9, 842. doi:10.3390/plants9070842

Sharma, T., Abohashrh, M., Baig, M. H., Dong, J. J., Alam, M. M., Ahmad, I., et al. (2021). Screening of drug databank against WT and mutant main protease of SARS-CoV-2: Towards finding potential compound for repurposing against COVID-19. *Saudi J. Biol. Sci.* 28, 3152–3159. doi:10.1016/j.sjbs.2021.02.059

Silveira, D., Prieto-Garcia, J. M., Boylan, F., Estrada, O., Fonseca-Bazzo, Y. M., Jamal, C. M., et al. (2020). COVID-19: Is there evidence for the use of herbal medicines as adjuvant symptomatic therapy? *Front. Pharmacol.* 11, 581840. doi:10.3389/fphar.2020.581840

Sohrab, S. S. (2020). An edible vaccine development for coronavirus disease 2019: the concept. *Clin. Exp. Vaccine Res.* 9, 164–168. doi:10.7774/cevr.2020.9.2.164

Sohrab, S. S., Suhail, M., Kamal, M. A., Husen, A., and Azhar, E. I. (2017). Recent development and future prospects of plant-based vaccines. *Curr. Drug Metab.* 18, 831–841. doi:10.2174/1389200218666170711121810

Talevi, A., and Bellera, C. L. (2020). Challenges and opportunities with drug repurposing: Finding strategies to find alternative uses of therapeutics. *Expert Opin. Drug Discov.* 15, 397–401. doi:10.1080/17460441.2020.1704729

The Pharmaceutical Journal (2022). Going plant-based: how molecular farming could revolutionise drug development. *Pharm. J.* 308 (7959), 1–8. doi:10.1211/PJ.2022.1.132683

Tiwari, R., Latheef, S. K., Ahmed, I., Iqbal, H., Bule, M. H., Dhama, K., et al. (2018). Herbal immunomodulators—a remedial panacea for designing and developing effective drugs and medicines: Current scenario and future prospects. *Curr. Drug Metab.* 19, 264–301. doi:10.2174/1389200219666180129125436

Veeresham, C. (2012). Natural products derived from plants as a source of drugs. *J. Adv. Pharm. Technol. Res.* 3, 200–201. doi:10.4103/2231-4040.104709

Verma, S., Twilley, D., Esmear, T., Oosthuizen, C. B., Reid, A-M., Nel, M., et al. (2020). Anti-SARS-CoV natural products with the potential to inhibit SARS-CoV-2 (COVID-19). *Front. Pharmacol.* 11, 561334. doi:10.3389/fphar.2020.561334

Viseu, A. (2022). Nanomedicine. Available at: https://www.britannica.com/science/nanomedicine (Accessed 16 April 2022).

Wang, C. H., Zhong, Y., Zhang, Y., Liu, J. P., Wang, Y. F., Jia, W. N., et al. (2016). A network analysis of the Chinese medicine Lianhua-Qingwen formula to identify its main effective components. network analysis of the Chinese medicine Lianhua-Qingwen formula to identify its main effective components. *Mol. Biosyst.* 12 (2), 606–613. doi:10.1039/C5MB00448A

Wangchuk, P. (2018). Therapeutic applications of natural products in herbal medicines, biodiscovery programs, and biomedicine. *J. Biol. Act. Prod. Nat.* 8 (1), 1–20. doi:10.1080/22311866.2018.1426495

Weiss, K., Khoshgoftaar, T. M., and Wang, D. D. (2016). A survey of transfer learning. *J. Big Data* 3, 9. doi:10.1186/s40537-016-0043-6

World Health Organization (2021). HIV/AIDS, key facts. Available at: https://www.who.int/news-room/fact-sheets/detail/hiv-aids (accessed April 12, 2022).

World Health Organization (2020). "Solidarity" clinical trial for COVID-19 treatments. Available at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/global-research-on-novel-coronavirus-2019-ncov/solidarity-clinical-trial-for-covid-19-treatments (accessed 13 April 2022).

Xu, J., and Zhang, Y. (2020). Traditional Chinese medicine treatment of COVID-19. *Complement. Ther. Clin. Pract.* 39, 101165. doi:10.1016/j.ctcp.2020.101165

Yang, H. T., and Rao, Z. H. (2021). Structural biology of SARS-CoV-2 and implications for therapeutic development. *Nat. Rev. Microbiol.* 19, 685–700. doi:10.1038/s41579-021-00630-8

Yang, Y., Islam, M. S., Wang, J., Li, Y., and Chen, X. (2020). Traditional Chinese medicine in the treatment of patients infected with 2019-new coronavirus (SARS-CoV-2): A review and perspective. *Int. J. Biol. Sci.* 16 (10), 1708–1717. doi:10.7150/ijbs.45538

Zaporozhets, T. S., and Besednova, Nataliya N. (2020). Biologically active compounds from marine organisms in the strategies for combating coronaviruses. *AIMS Microbiol.* 6 (4), 470–494. doi:10.3934/microbiol.2020028

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., et al. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *N. Engl. J. Med.* 382, 727–733. doi:10.1056/NEJMoa2001017

frontiers | Frontiers in Drug Discovery

TYPE Original Research
PUBLISHED 31 August 2022
DOI 10.3389/fddsv.2022.962988

OPEN ACCESS

EDITED BY
Bruno Villoutreix,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

REVIEWED BY
Cesar Augusto Roque-Borda,
São Paulo State University, Brazil
Ying Hong Li,
Chongqing University of Posts and Telecommunications, China

*CORRESPONDENCE
Chris Kahlenborn,
Ckahlenborn@gmail.com

SPECIALTY SECTION
This article was submitted to Anti-Infective Agents,
a section of the journal
Frontiers in Drug Discovery

RECEIVED 07 June 2022
ACCEPTED 08 July 2022
PUBLISHED 31 August 2022

CITATION
Kahlenborn C, Severs WB and Nawab K (2022), Bismuth subsalicylate as potential treatment for Covid-19 pneumonia: A case series report. *Front. Drug. Discov.* 2:962988. doi: 10.3389/fddsv.2022.962988

Bismuth subsalicylate as potential treatment for Covid-19 pneumonia: A case series report

Chris Kahlenborn[1]*, Walter B. Severs[2] and Khalid Nawab[1]

[1]Department of Internal Medicine, Penn State Health Holy Spirit Medical Center, Camp Hill, PA, United States, [2]Professor Emeritus of Pharmacology and Neuroscience, Penn State College of Medicine, Hershey, PA, United States

Various literature cited suggests that bismuth may have usefulness against Covid-19 both *in vitro* and *in vivo*. During the course of caring for Covid-19 patients we administered bismuth subsalicylate to those who displayed diarrhea and/or gastric complaints. Using relatively conservative criteria, upon retrospective review, we noted marked improvement in oxygen requirements in most of the cases. This improvement was observed even when prior therapy with standard anti-Covid drugs had failed. Our overall impression is that these positive results support a detailed evaluation of bismuth as an adjunct treatment for the treatment of Covid-19.

KEYWORDS

COVID, bismuth subsalicylate, pneumonia, oxygen, remdesivir

Introduction

As of 1 July 2022, Covid-19 had infected half a billion people worldwide and over 6.35 million had died. (Dadax. 2022. https://www.worldometers.info/coronavirus/ (Accessed 1 July 2022). Preventative measures include isolation/masking and vaccination whereas treatment options include steroids, vitamins, and drugs such as remdesivir, tocilizumab and baricitinib and monoclonal antibodies. (Covid-19: Management in hospitalized Adults (2022) https://www.uptodate.com/ (Accessed 1 July 2022). In spite of these measures, Covid-19 infection remains a major public health concern and treatments have had limited efficacy: for example, a prominent study (Beigel *et al.*, 2020), showed that while remdesivir appeared to shorten recovery time, the mortality rate was not statistically different from placebo. Another study (Horby *et al.*, 2021) noted that whereas dexamethasone had some efficacy over placebo in reducing mortality rates in intubated patients (ie., 29% vs. 41%) it had no effect for non-ventilated patients over placebo (18% vs. 14%).

The emergence of an inexpensive and efficacious anti-Covid medication with few side effects would be an ideal solution to this conundrum. One potential solution may be bismuth subsalicylate (BiS), a common over-the-counter medication which is widely used for various gastrointestinal symptoms. When taken orally it reacts with hydrochloric acid forming salicylate and bismuth oxychloride and has anti-inflammatory, anti-secretory and anti-microbial activity (Pasricha, 2006). It inhibits two key enzymes (ie, NTPase and RNA helicase) (Yang *et al.*, 2007; Shu *et al.*, 2020) which are necessary for viral replication

which might explain its' efficacy against other viruses including rotavirus, reovirus, noravirus, echovirus, polio and herpes virus (Ward *et al.*, 1985; Waldum *et al.*, 1998; Koo *et al.*, 2010). *In vitro* studies have also demonstrated bismuth's anti-Covid effect (Yang *et al.*, 2007). In addition, researchers in Hong Kong (Yuan *et al.*, 2020) noted that bismuth medications had efficacy in the animal model; hamsters who received ranitidine bismuth citrate had a 1000-fold reduction in their Covid-19 viral loads and improvement in their pneumonias. A case in 2020 (Wolf *et al.*, 2020) noted an 85-year-old man with Crohn's whose Covid symptoms (both URI and diarrhea) improved rapidly after receiving BiS without receiving any other anti-Covid related medications. Finally, preliminary results from a recent study from the University of Louisville noted that Covid patients were noted to experience a decrease in Covid symptoms within 48 h after completing a course of BiS (Yacyshyn *et al.*, 2022).

Materials and methods

In light of the apparent virustatic and/or virucidal effect of bismuth on Covid-19 virus both *in vitro* and in the animal model, after review of Covid-positive patients who were treated with BiS for their diarrhea, we noted that bismuth containing compounds appeared to have an adjunctive role in the treatment of Covid pneumonia. We herein report eight cases where BiS was administered to control gastric-related complaints (ie, loose stools or diarrhea) in hospitalized patients with Covid pneumonia. Bismuth subsalicylate has long been an over-the-counter drug in the United States and in our review, was used solely to treat diarrhea symptoms in patients hospitalized with Covid-19. Data on each of the cases was extracted and reviewed from the charts independently by two different hospitalists (CK and KN). (Figures 1–8). All patients received BiS, two different manufacturers are used in our institution: Rugby Laboratories, a Division of the Harvard Drug Group, LLC; NDC: 00536-1286-36 or AmerisourceBergen Corporation NDC: 46122-0306-34.

Cases were only included if they had at least 5 days of high-oxygen requirements prior to the dispensation of BiS. Each participant had to receive at least 4 total doses of BiS within a 48-h period of time with a minimum total dose of at least 1096 mg. The efficacy of BiS was graded on an arbitrary scale noted in Table 1; zero (minimal), one (moderate) or two (strong) was assigned to each case based on the response to BiS. Scaling criteria was based on the premise that a positive score required a major reduction in oxygen requirements within days of finishing BiS therapy. Patients who had longer duration of high-oxygen requirements prior to the administration of BiS (ie., spanning 10-45 days) had longer recovery phase criteria. Scaling criteria, although arbitrary, was the best clinical estimate as to what would constitute a minimal, moderate or strong response to therapy. For example, if a patient required high dose oxygen for 5 days prior to the reception of BiS, a positive response would have to be seen within 5 days of the last dose of BiS. Patients who had longer periods of high oxygen requirements prior to administration of BiS (eg, patient #4 who had been ventilated for 6 weeks prior to receiving BiS) had to have a major reduction in oxygen requirements within 10 days of receiving their last dose of BiS. Comprehensive data for each patient are included in Figures 1–8 which contain data on daily timing and dosing of all Covid related medications, BiS administration, in addition to complete vital signs data including daily oxygen requirements and pulse ox and temperature readings; lab data on CRP (C-Reactive Protein) as well as D-Dimer values were provided if available. Of note, two patients (#3 and #4) transitioned from either BiPAP (ie, Bilevel Positive Airway Pressure) or the ventilator, to lower dose oxygen delivery methods (ie, either simple mask or nasal cannula). In these cases, we estimated that oxygen flow rates for ventilator and/or BiPAP patients to be equivalent to at least 50 L/min of high-flow oxygen (Shoukri, 2021; Beran *et al.*, 2022). Each patient gave informed consent to use his or her medical data for publication; no names or other non-medical information were revealed.

Case reports

Patient 1 (Case 1) is a 79-year-old man with PMH of hypertension and hyperlipidemia admitted in January 2022 who required high-flow oxygen (ie, 50 L/min) for 10 days prior to the last dose of BiS (Figure 1). During the initial 10 days of treatment, he was given remsedivir, baricitinib and dexamethsone with no effect on his oxygen requirements. Within 7 days of finishing a total of 3144 mg of BiS therapy, he required only 8 L/min of oxygen, consistent with a strong (+2) effect.

Patient 2 (Case 2) is a 67-year-old with a PMH of diabetes mellitus and a recent deep vein thrombosis admitted in February 2021, who developed acute respiratory failure due to Covid pneumonia who received remdesivir, baricitinib and dexamethasone during the first 5 days of his admission with no change in his high-dose oxygen requirements (Figure 2). After receiving 2096 mg of BiS, his oxygen requirements improved significantly going from 50 L/min to 6 L/min, however, per criteria in Table 1, this was categorized as a minimal response (ie, Grade 0) because the noted decrease in oxygen requirements occurred after the 5-day period of BiS administration. (Note: on the seventh day post-BiS administration, patient's oxygen requirement had decreased to 6 L/min).

Patient 3 is a 78 year-old African American, admitted in January 2021, with a PMH of obstructive sleep apnea and diabetes mellitus who developed Covid pneumonia (Figure 3). He had no major change in his high oxygen requirements for the first 17 days of his admission while receiving remdesivir,

TABLE 1 Bismuth efficacy scale.

MINIMAL IF ANY (0 Points)	High oxygen requirements ≥ 5 days prior to last day of bismuth therapy and no achievement of less than 50% of oxygen requirements within 5 days of stopping bismuth
MODERATE (1 point)	High oxygen requirements ≥ 5 to 9 days prior to last day of bismuth therapy and reduction to less than 50% of oxygen requirements within 5 days of starting bismuth
MODERATE (1 point)	High oxygen requirements ≥ 10 to 29 days prior to last day of bismuth therapy and reduction to less than 50% of oxygen requirements within 7 days of stopping bismuth
MODERATE (2 point)	High Oxygen Requirements for ≥ 30 days prior to last day of bismuth therapy and reduction to less than 50% of oxygen requirements within 10 days of stopping bismuth therapy
STRONG (2 points)	High Oxygen Requirements for ≥ 5 to 9 days to last day of bismuth therapy and reduction to less than 25% of oxygen requirements within 5 days of stopping bismuth
STRONG (2 points)	High Oxygen Requirements for ≥ 10 to 29 days prior to last day of bismuth therapy and reduction to less than 25% of oxygen requirements within 7 days of stopping bismuth
STRONG (2 points)	High Oxygen Requirements for > 30 days prior to last day of bismuth therapy and reduction to less than 25% of oxygen requirements within 10 days of stopping bismuth therapy

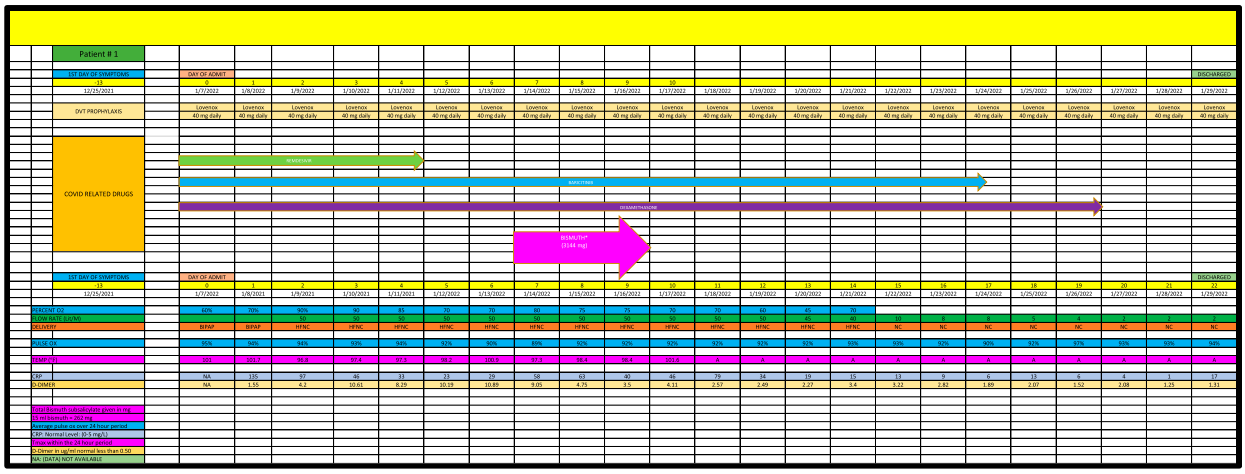


FIGURE 1
Medical Data for Patient #1.

baricitinib and dexamethasone and was given the option of hospice care on Day 17. Patient then received 1592 mg of BiS on Day 17 after developing loose stool and progressed to room air within 5 days after the last day of BiS, consistent with a strong (+2) response.

Patient 4 is a 70-year-old admitted in May 2021, with a PMH of esophageal dysphagia and prostate carcinoma with Covid pneumonia (Figure 4). He remained ventilated for almost 6 weeks despite receiving remdesivir, tocilizumab and dexamethasone; however, he was able to come off the ventilator and progressed to 10 L/min of oxygen within 10 days of receiving 6288 mg of BiS, consistent with a strong response (+2).

Patient 5 is a 38-year-old African-American female admitted in October 2021, with a PMH of hypothyroidism who remained on high dose oxygen (ie, 50 L/min) flow for the first 5 days of her admission despite receiving remdesivir, tocilizumab and dexamethasone. (Figure 5). She received a total of 3144 mg of

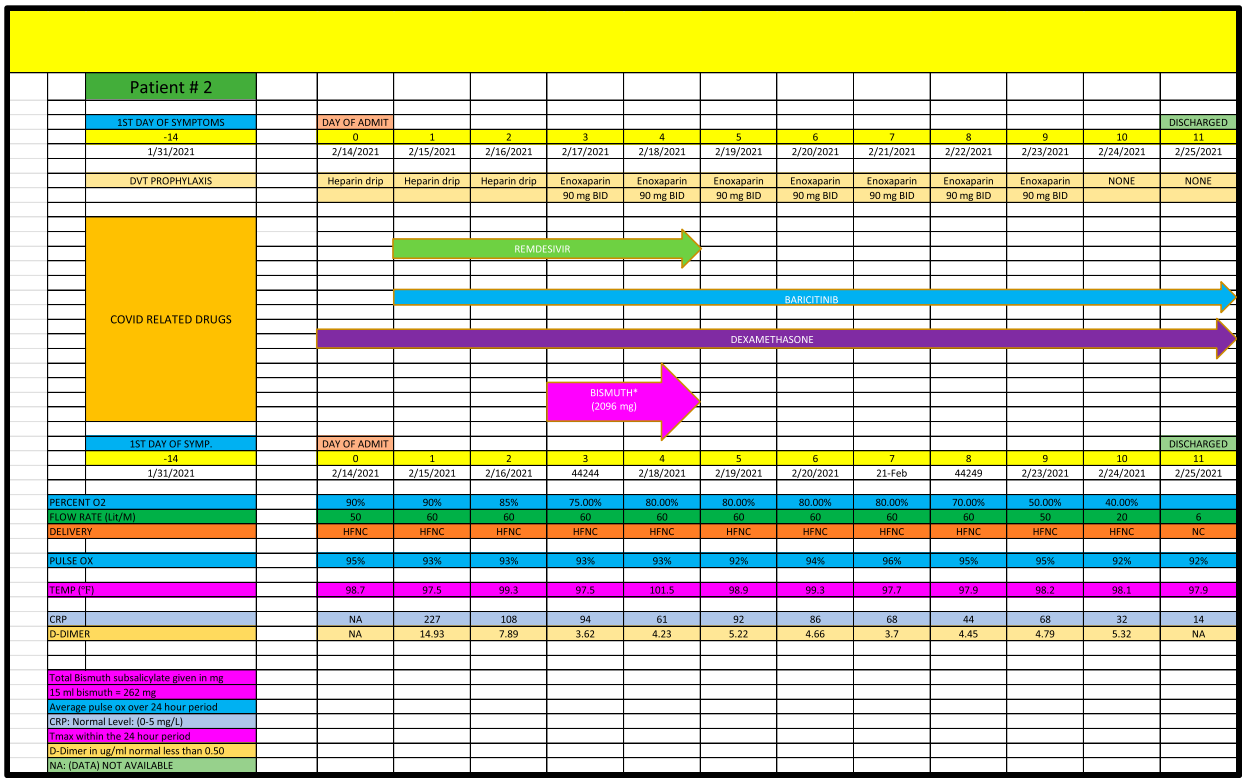


FIGURE 2
Medical Data for Patient #2.

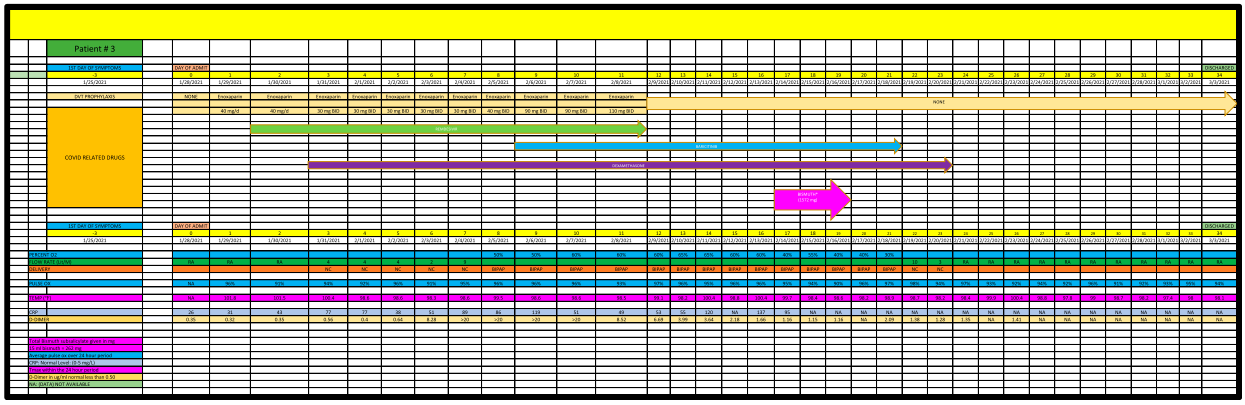


FIGURE 3
Medical Data for Patient #3.

BiS and progressed to an oxygen requirement of only 1 L/min within 5 days of her last dose of BiS, consistent with a strong (2+) response.

Patient 6 is a 57-year-old man admitted in September 2021, with a PMH of coronary heart disease and hypertension (Figure 6) who remained on high dose oxygen (ie, 50 L/min) flow for the first 5 days of his admission despite receiving remdesivir, tocilizumab and dexamethasone. He received 3144 mg of BiS and eventually progressed down to 1.5 L/Min of oxygen but received a score of 0 (minimal change)

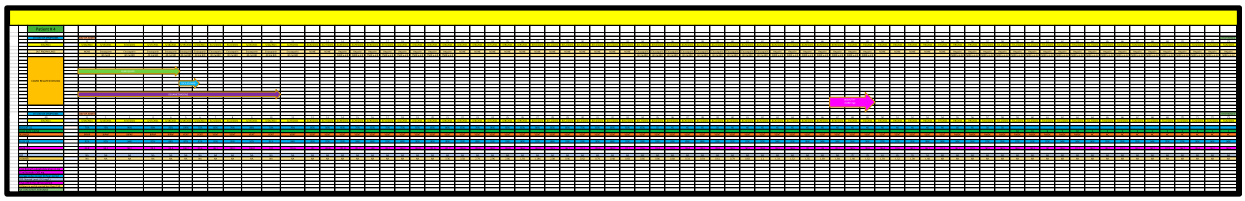


FIGURE 4
Medical Data for Patient #4.

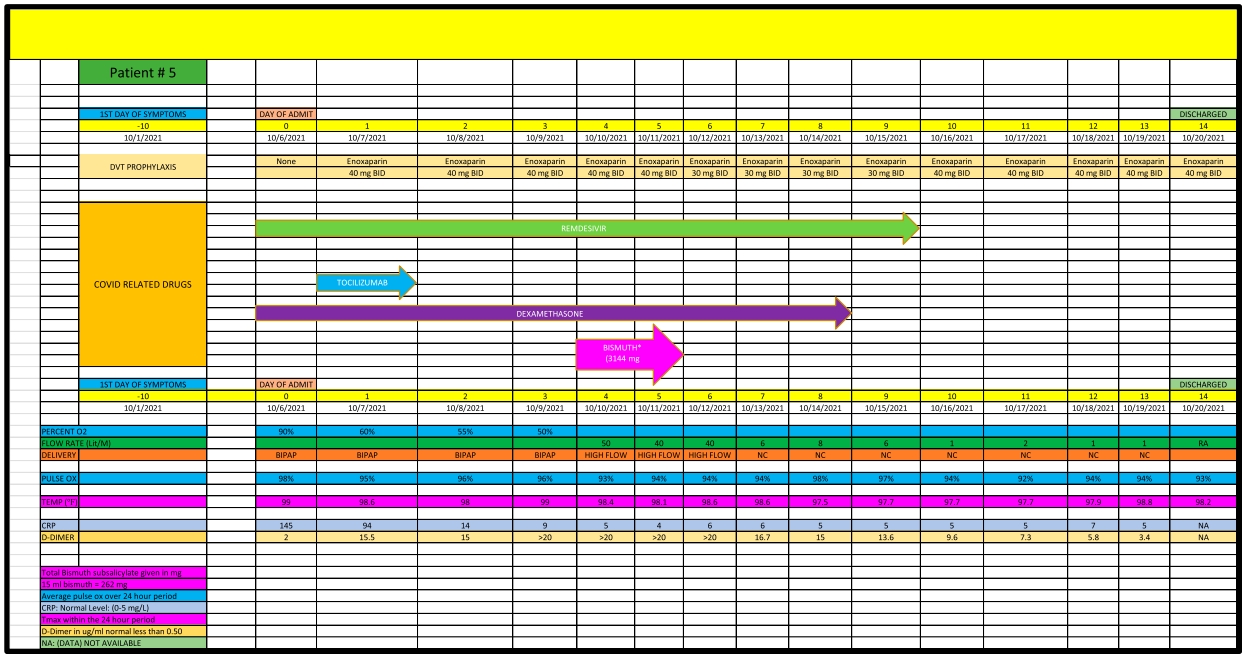


FIGURE 5
Medical Data for Patient #5.

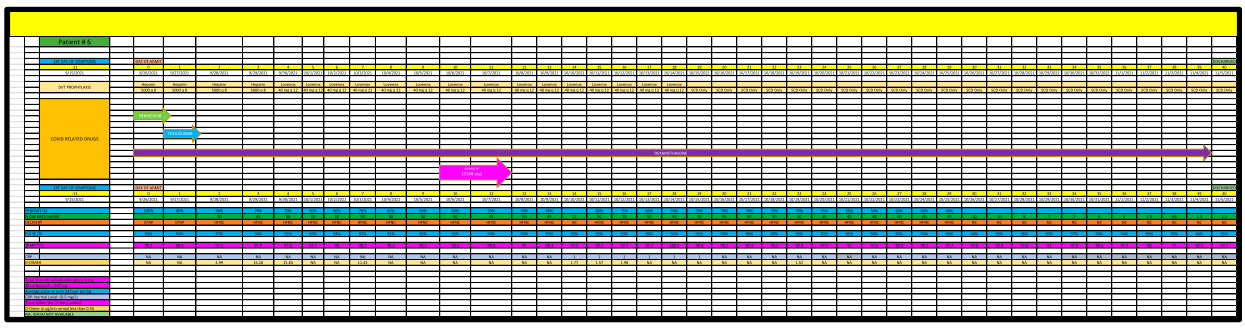


FIGURE 6
Medical Data for Patient #6.

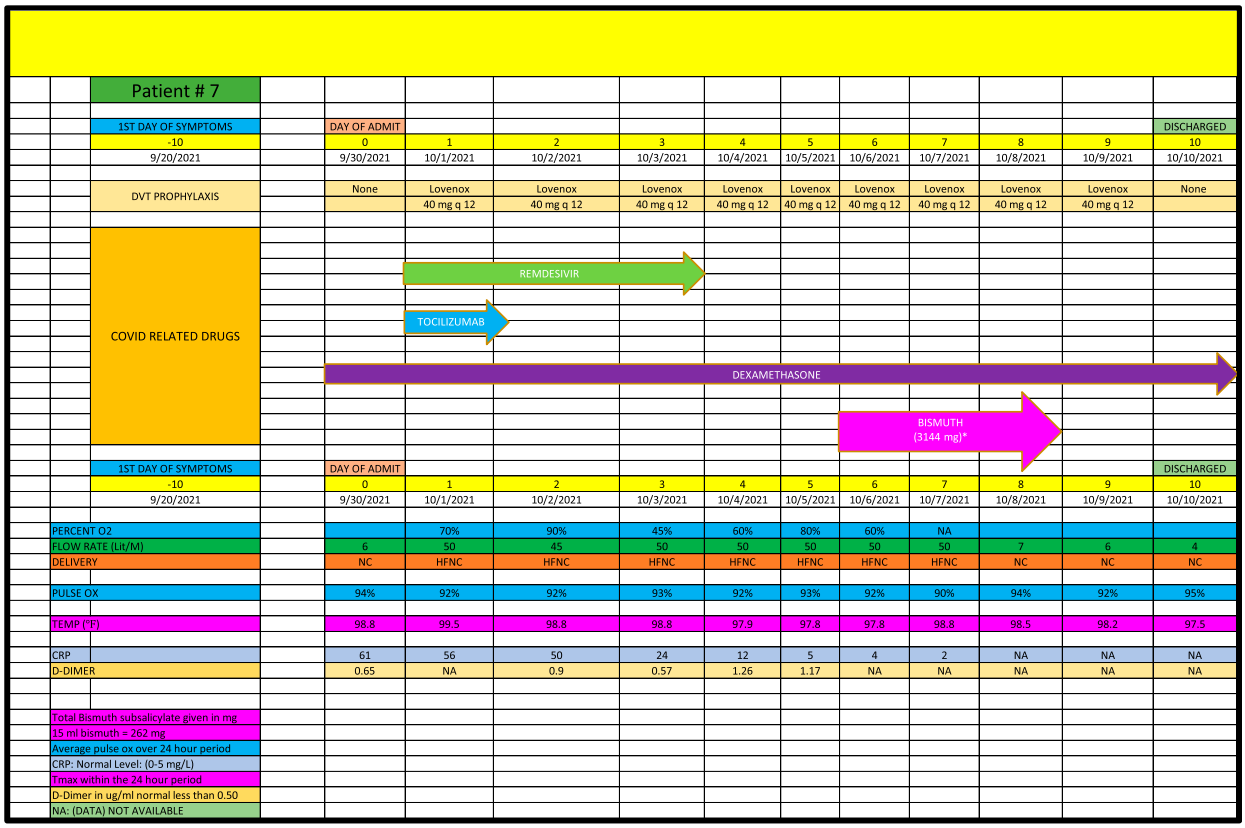


FIGURE 7
Medical Data for Patient #7.

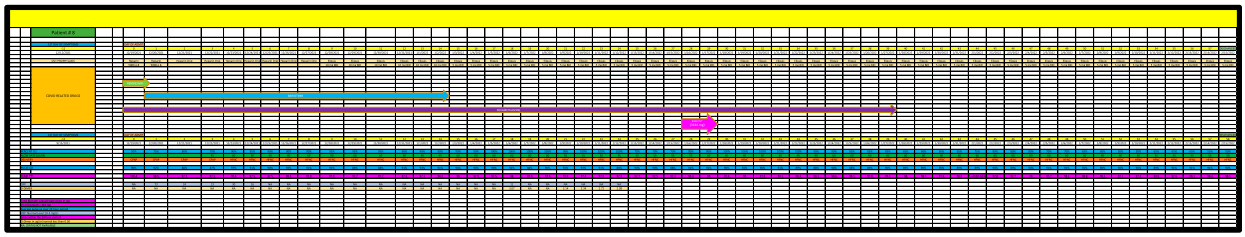


FIGURE 8
Medical Data for Patient #8.

since he remained on high flow oxygen for more than 7 days after receiving his last dose of BiS.

Patient 7 is a 50-year-old man admitted in September 2021, with a PMH of asthma (Figure 7) who remained on high dose oxygen (ie, 50 L/min) for the first 8 days of his admission despite receiving remdesivir, tocilizumab and dexamethasone. He progressed down to 4 L/min of oxygen within 2 days of receiving a total of 3144 mg of BiS consistent with a strong (+2) response.

Patient 8 is a 61-year-old admitted in December 2021, with PMH of COPD who developed acute respiratory failure due to Covid pneumonia and received remdesivir, baricitinib and dexamethasone but remained on high flow (ie, 60 L/min of oxygen) until day 28 of his admission at which time BiS was started; he received a total of 3144 mg of BiS (Figure 8). He eventually reduced his oxygen requirements to 50 L/min on day 58 and received a score of 0 since his response was well after the last day of BiS administration.

TABLE 2 Patients who improved after bismuth therapy.

PATIENT	AVERAGE O2 FLOW 5-10 DAYS PRIOR TO BISMUTH	02 FLOW WITHIN STUDY PARAMETER TIME AFTER BISMUTH
PATIENT 1	50	8
PATIENT 3	50	3
PATIENT 4	40	10
PATIENT 5	50	1
PATIENT 7	50	4

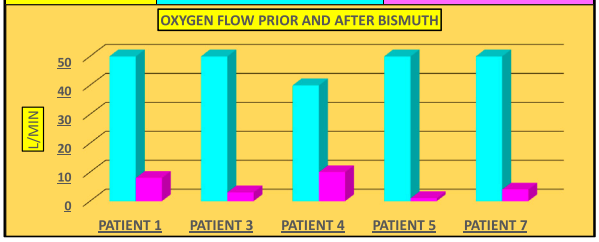
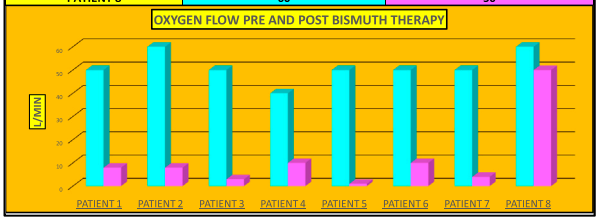


TABLE 3 Patients who improved after bismuth therapy (excluding parameters).

PATIENT	AVERAGE O2 FLOW 5-10 DAYS PRIOR TO BISMUTH	02 FLOW AFTER BISMUTH (EXCLUDING PARAMETERS)
PATIENT 1	50	8
PATIENT 2	60	8
PATIENT 3	50	3
PATIENT 4	40	10
PATIENT 5	50	1
PATIENT 6	50	10
PATIENT 7	50	4
PATIENT 8	60	50



Results

Overall, we noted that five out of eight Covid pneumonia patients (ie, 62.5%) had a strong response to BiS treatment as per criteria (+2) (Table 2). In addition, seven of eight patients who received BiS got dramatically better (ie, 87.5%) (Table 3) although two of the cases got better outside the time period criteria noted in Table 1. No side effects of BiS treatment were observed.

Discussion

We acknowledge that a short series of case reports rarely provides definitive information of drug efficacy, mechanism of action or pharmacokinetic/pharmacodynamic properties. Nevertheless, consistent and unexpected clinical outcomes are noteworthy and raise questions worthy of answers. Herein, we describe the outcomes of eight diverse cases of Covid-19 pneumonia where patients were treated with common Covid drugs including corticosteroids, remdesivir, baricitinib and tocilizumab. A common denominator in these patients was the short-term use of BiS for gastric complaints/symptoms. Even when the prognosis was guarded, some of the patients showed a relatively rapid rebound and clinically improved lung function.

Bismuth's apparent efficacy may be partly due to its' inhibitory effect on viral enzymes. Bismuth salts inhibit two key enzymes (ie, NTPase and RNA helicase) which are necessary for viral replication (Shu *et al.*, 2020). The mechanism of action of bismuth in the post-acute phase is unclear, however, the dramatic response of most of the cases, often weeks after contracting Covid-19, raises the question of whether the Covid-19 virus is still alive in lung cells even after a patient has developed antibodies. There appears to be support for this: (Zhao *et al.*, 2020): noted that 100% of hospitalized patients developed antibodies to Covid by Day 15, while viral RNA detectability from upper respiratory samples remained positive in many patients weeks after a positive antibody response. They noted that critically ill patients may take a long time to clear Covid from their respiratory tracts: "It should be noted that the risings of antibodies were not always accompanied by RNA clearance, particularly in the 3 critical patients. This finding suggested that antibodies may not be sufficient to clear the virus" (Zhao *et al.*, 2020). Support for this phenomenon exists with other viruses (eg, herpes) which are known to live in the body for years despite positive antibody responses (eg, Ebstein Barr and Varicella viruses). We do not know if Covid-19 virus could still be living in lung cells long after the formation of antibodies, but the dramatic response in our cases who often responded even weeks after contracting Covid pneumonia, raises this possibility.

If further studies were to corroborate our case-reports, it might benefit people around the world who often have no access to expensive medications such as remdesivir or tocilizumab, monoclonal antibodies or the vaccine; for example, as of September 2021 only 1% of people from low-income countries had received even one dose of the vaccine [https://www.kff.org/coronavirus-covid-19/press-release/disparities-in-global-vaccination-progress-are-large-and-growing-with-low-income-countries-and-those-in-africa-lagging-behind/ (Accessed 6 September 2021)]. We also note that all of our patients received less than 9 doses of BiS: whether bismuth medications would have a more pronounced effect if given over longer periods of time is not known. In addition, all of our patients received BiS after several days/weeks of being hospitalized and requiring high-dose oxygen flows. Whether BiS could have even greater efficacy had it been given earlier in our patient's stay or even prophylactically are questions that merit further study. In addition, very little BiS is absorbed from the gastrointestinal tract when given orally. Patients who were given oral doses of ranitidine bismuth citrate 800 mg twice daily only absorbed 0.5% of

the dose with a peak plasma concentration did not exceed 19 ng/ml (Koch *et al.*, 1996). If higher absorption rates could be achieved, as noted in a recent study when BiS was combined with N-acetyl cysteine (Wang *et al.*, 2021) it potentially could increase BiS's efficacy.

Bismuth is available in many forms such as bismuth subsalicylate, bismuth citrate, bismuth subcitrate and bismuth subgallate, many of which are widely available around the world and often over-the counter. While bismuth may alter the absorption of other medications there are few risks of taking a short course although theoretically BiS could increase the risk of bleeding slightly due to the salicylate effect. However, taking high doses of bismuth for short or extended periods of time may cause severe renal or neurological problems (Cengiz *et al.*, 2005; Borbinha *et al.*, 2019).

Conclusion

During the care of hospitalized Covid-19 patients we administered bismuth subsalicylate to those patients having diarrhea or other gastric issues. Upon retrospective review, we noticed that these patients became less dependent on supplemental oxygen. Using arbitrary yet conservative criteria, we reviewed these cases and herein report the results. All patients showed at least some improvement in their oxygen needs and several showed marked improvement. Noteworthy, none had any side effects from the bismuth, and the improvement came after the patients had already been treated with common Covid drugs. Based on these observations and literature relating to bismuth and Covid we recommend a detailed clinical evaluation of bismuth's potential role as an adjunct treatment for Covid 19.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Each patient gave informed consent to use his or her medical data for publication; no names or other non-medical information were revealed.

Author contributions

CK gathered initial information on case reports; both CK and KN reviewed all clinical data. CK, KN, and WS all contributed to writing, analyzing and reviewing this paper.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Beigel, J. H., Tomashek, K. M., Dodd, L. E., Mehta, A. K., Zingman, B. S., Kalil, A. C., et al. (2020). Remdesivir for the treatment of covid-19 - final report. *N. Engl. J. Med.* 383, 1813–1826. doi:10.1056/NEJMoa2007764

Beran, A., Srour, O., Malhas, S. E., Mhanna, M., Ayesh, H., Sajdeya, O., et al. (2022). High-flow nasal cannula oxygen versus non-invasive ventilation in subjects with COVID-19: A systematic review and meta-analysis of comparative studies. *Respir. Care. Respcare.*, 09987. doi:10.4187/respcare.09987

Borbinha, C., Serrazina, F., Salavisa, M., and Viana-Baptista, M. (2019). Bismuth encephalopathy- a rare complication of long-standing use of bismuth subsalicylate. *BMC Neurol.* 19, 212. doi:10.1186/s12883-019-1437-9

Cengiz, N., Uslu, Y., Gök, F., and Anarat, A. (2005). Acute renal failure after overdose of colloidal bismuth subcitrate. *Pediatr. Nephrol.* 20, 1355–1358. doi:10.1007/s00467-005-1993-7

Horby, P., Lim, W. S., Emberson, J. R., Mafham, M., Bell, J. L., Linsell, L., et al. (2021). Dexamethasone in hospitalized patients with covid-19. *N. Engl. J. Med. Overseas. Ed.* 384, 693–704. doi:10.1056/NEJMoa2021436

Koch, K. M., Kerr, B. M., Gooding, A. E., and Davis, I. M. (1996). Pharmacokinetics of bismuth and ranitidine following multiple doses of ranitidine bismuth citrate. *Br. J. Clin. Pharmacol.* 42, 207–211. doi:10.1046/j.1365-2125.1996.39310.x

Koo, H. L., Ajami, N., Atmar, R. L., and DuPont, H. L. (2010). Noroviruses: The leading cause of gastroenteritis worldwide. *Discov. Med.* 10 (50), 61–70. PMID: 20670600; PMCID: PMC3150746.

Pasricha, P. J. (2006). "Treatment of disorders of bowel motility and water flux; antiemetics: Agents used in biliary and pancreatic disease" in *Goodman & Gilmans The Pharmacological Basis of Therapeutics*. 11th ed. (New York, NY: McGraw-Hill), 983–1008.

Shoukri, A. M. (2021). High flow nasal cannula oxygen and non-invasive mechanical ventilation in management of COVID-19 patients with acute respiratory failure: A retrospective observational study. *Egypt. J. Bronchol.* 15, 17. doi:10.1186/s43168-021-00063-0

Shu, T., Huang, M., Wu, D., Ren, Y., Zhang, X., Han, Y., et al. (2020). SARS-Coronavirus-2 Nsp13 possesses NTPase and RNA helicase activities that can Be inhibited by bismuth salts. *Virol. Sin.* 35 (3), 321–329. doi:10.1007/s12250-020-00242-1

Waldum, H. D., Nordbø, S. A., and Bevanger, L. S. (1998). A bismuth salt inhibits herpes simplex virus. *Microb. Ecol. Health Dis.* 10, 13–15. doi:10.1080/089106098435395

Wang, R., Chan, J. F., Wang, S., Li, H., Zhao, J., Ip, T., et al. (2021). Orally administered bismuth drug together with *N*-acetyl cysteine as a broad-spectrum anti-coronavirus cocktail therapy. *Chem. Sci.* 13 (8), 2238–2248. doi:10.1039/d1sc04515f

Ward, R. L., Sander, D. S., and Knowlton, D. R. (1985). *In vitro* activities of bismuth salts against rotaviruses and other enteric viruses. *Antimicrob. Agents Chemother.* 27 (3), 306–308. doi:10.1128/AAC.27.3.306

Wolf, D. C., Wolf, C. H., and Rubin, D. T. (2020). Temporal improvement of a COVID-19-positive crohn's disease patient treated with bismuth subsalicylate. *Am. J. Gastroenterol.* 115 (8), 1298. doi:10.14309/ajg.0000000000000725

Yacyshyn, M. B., Collins, J., Chua, M., Siegwald, A., Yacyshyn, S., Briones-Pryor, V., et al. (2022). Feasibility study of Bismuth Subsalicylate (BSS) as an addition to standard of care for COVID-19 therapy. *Curr. Ther. Res. Clin. Exp.* 96, 100667. doi:10.1016/j.curtheres.2022.100667

Yang, N., Tanner, J. A., Wang, Z., Huang, J. D., Zheng, B. J., Zhu, N., et al. (2007). Inhibition of SARS coronavirus helicase by bismuth complexes. *Chem. Commun.* (42), 4413. doi:10.1039/b709515e

Yuan, S., Wang, R., Chan, J. F., Jinxia, A., Cheng, T., Ka-Heng, K., et al. (2020). Metallodrug ranitidine bismuth citrate suppresses SARS-CoV-2 replication and relieves virus-associated pneumonia in Syrian hamsters. *Nat. Microbiol.* 5, 1439–1448. doi:10.1038/s41564-020-00802-x

Zhao, J., Yuan, Q., Wang, H., Liu, W., Liao, X., Su, Y., et al. (2020). Antibody responses to SARS-CoV-2 in patients with novel coronavirus disease 2019. *Clin. Infect. Dis.* 71, 2027–2034. doi:10.1093/cid/ciaa344

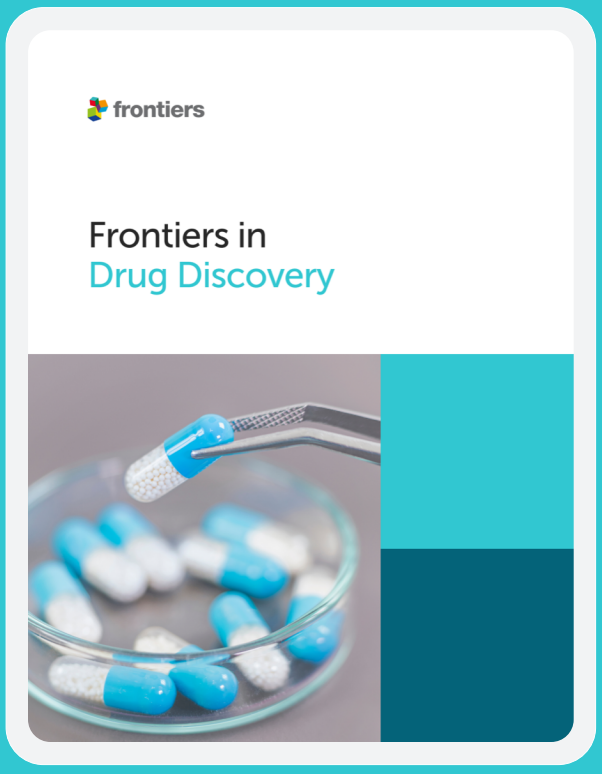
Frontiers in
Drug Discovery

Explores the discovery and development of new drugs and therapies

A multidisciplinary journal that focuses on drug discovery and the development lifecycle, from the identification of drug entities to the design and development of new candidate medications and therapies.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact