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Old drugs: confronting recent advancements and challenges

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Old drugs: confronting recent advancements and challenges

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Editorial: Old drugs: confronting recent advancements and challenges

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Editorial on the Research Topic
Old drugs: confronting recent advancements and challenges

Introduction

Drug repurposing involves leveraging existing drugs or drug candidates, originally developed for other conditions, to address new therapeutic purposes or medical conditions. This strategy often draws from observations of unintended or off-target effects, including those initially seen as adverse or age-specific, which may reveal their potential to alleviate chronic, degenerative, or age-related conditions. The core characteristics of drug repurposing may be summarized as follows:

1. *Well-Known Safety*: Repurposed drugs are typically already tested for safety in humans, significantly reducing early-stage development risks.
2. *Streamlined Development*: These drugs can bypass initial drug discovery phases, proceeding directly to preclinical or clinical trials.
3. *Cost and Risk Red*uction: By skipping early development stages, repurposing lowers both financial costs and associated risks.
4. *Time Efficiency*: The process offers a quicker path to market, addressing unmet medical needs efficiently.

More recently, the search for repurposing candidates combines traditional and modern tools, including but not limited to computational analysis and database searches (i.e., utilizing public databases such as medical journals, regulatory agency archives, and patient advocacy group reports); newer experimental approaches (i.e., testing drug interactions in novel contexts); artificial intelligence and bioinformatics tools (i.e., advanced algorithms explore potential drug-protein interactions, integrating

pharmacological data with genetics, pharmacogenomics, biological pathways, and diagnostic/prognostic insights).

For the above reasons, over the years, there has been an increasing demand for a more effective and cost-efficient drug discovery process. To do so and in addition to searching for. In review entirely new molecules to be included in therapies, drug repositioning is a dynamically developing area in the search for effective therapy for many diseases. This approach is actually particularly appealing since it saves time and resources and reduces the risk of failure or unexpected safety issues.

The aim of the Research Topic was to inspire you to present your research, but also to encourage the interested readers to review drugs whose scope of action has expanded over the years and, additionally, open up new points of entry in the treatment of various diseases. The Research Topic features six review articles, including one systemic review. Additionally, the Research Topic includes four original research papers.

In the original papers, commonly known drugs were analyzed, but contextualized in new applications. For example, Chen et al. searched for antiviral treatment for parainfluenza 3 (PIV3) illness. The authors explored an integrated drug repurposing method, including disease similarity and chemical similarity as multi-similarity analysis approaches, molecular docking and molecular dynamic simulation methods as structure-based screening approaches, and network proximity analysis, in which they quantified the network distance between the disease module of PIV3 and the drug targets to probe the potential anti-PIV3 drugs. Using the aforementioned method, they confirmed that oseltamivir is the best potential drug against PIV3. Therefore, they recommend considering oseltamivir for clinical application in children.

The next compound analyzed for a potential new indication was rosiglitazone, which is a thiazolidinedione used in the treatment of diabetes (Lehmann et al., 1995). Rosiglitazone has been reported to ameliorate high-fat diet-induced obesity in mice (Petrovic et al., 2010). As Li et al. showed, this antidiabetic drug promotes adipocyte browning by inhibiting autophagy.

More specifically, inhibition of autophagy by rosiglitazone increased p62 nuclear translocation and stabilized the PPARγ-RXRα heterodimer for the transcription of browning genes. These studies underlined the promising role of rosiglitazone in the treatment of obesity. Interestingly, a new challenge has been set for dexmedetomidine (DEX), namely, its participation in the protection against many degenerative conditions, including neurodegenerative diseases, by reducing oxidative stress. Indeed, DEX is a highly selective α2-adrenoceptors agonist, has a sedative, analgesic effect and anti-sympathetic properties (Keating, 2015). Lou et al. research has shown that DEX effectively reduces oxidative stress, avoids apoptosis and protects osteoblasts. The authors propose it for the prevention of bone defects, which could also be utilized though for other non-bone defects.

The last of the original research tested in the diabetic retinopathy (DR) the purine analogue 6-thioguanine (6TG), an old drug approved in the 60s to treat acute myeloid leukemia (AML) – Trotta et al. The authors concluded that 6TG revealed a marked anti-angiogenic activity in HUVECs exposed to high glucose and in mice with DR, and this activity was mediated by MC1R and MC5R retinal receptors.

In turn, review papers considered the possibility of repurposing effect of cardiovascular-metabolic drugs to prolong life (Barinda et al.). This systematic review mainly addressed to animal models (short-lived, obese or diabetic mice), where numerous cardiovascular, antidiabetic and lipid-lowering medications proved to successfully extend the lifespan. The authors also provide summary of ongoing clinical trial in repurposing cardiometabolic drug (metformin, omega-3 fatty acid, acarbose, and atorvastatin) for aging, expressing an expectation that the obtained findings will give the support for the utility of these medications in healthy subjects' cardiovascular or neurological aging. The pleiotropic effects exerted by statins and beta-blockers gave the rationale to evaluate their possible complementary use in solid tumours, including breast, colorectal, lung, and prostate cancers (Braga et al.). The authors provided an update of the impact of these therapies on cancer treatment and surveillance, discussing the underlying mechanisms, and exploring their effects on the heart, contributing to the growing field of cardiooncology. The review of findings from retrospective analyses, and randomized clinical trials within the cancer continuum allowed the authors to conclude that β-blockers seemed to lead to better clinical outcomes for breast cancer, whereas statins were positively associated with greater outcomes in breast, colorectal and prostate treatment. Further evaluations of drug dosage, time of treatment and benefits of different classes of β-blockers and statins are needed, however.

Fatemi et al. provided a comprehensive review of research efforts, and examples of drugs repurposing in different types of gastrointestinal cancers, such as colorectal, pancreatic, and liver cancer. Moreover, the authors have addressed several barriers for drug repurposing for cancer therapy, including the legal, regulatory, patent issues and financial factors. In the work by Glajzner et al., the authors presented current data on the potential of drug repositioning across various therapeutic classes for the treatment of infectious diseases caused by multidrug-resistant bacteria. This review also explored the therapeutic possibilities that arise when these drugs are combined with antibiotics. Preclinical studies demonstrated considerable promises for drugs from a range of therapeutic areas, including oncology, psychiatry, and pain management. The review has shown that drug repositioning candidates for infectious diseases can impact bacterial metabolism and cellular structure, as well as damage their genetic material. However, authors emphasized the need for further research; this should exclude the risk that potential antibacterial candidates would induce resistance -either to the drug itself or to the antibiotics used in conjunction. In a mini-review by Huang et al., the protective effects of different anaesthetic agents in intestinal ischemia-reperfusion injury were presented.

The reviewed strategies of drug repurposing involved also exploring new therapeutic applications for anti-cancer drugs. Choi et al. suggested that they can be regarded as candidates for repurposing of Alzheimer's disease (AD) treatments. In particular, the authors emphasized the role of epidermal growth factor receptor (EGFR) as an important molecular target for both cancer and AD. The EGFR pathway is a widely recognized oncogenic pathway for non-small cell lung cancer. In the current review the authors mainly focused on the possible linkage between EGFR upregulation and inhibition of cancer cell migration or promotion of AD pathology

(e.g., production and deposition of the β-amyloid peptide, neuroinflammation, or cognitive function).

We are convinced that this Research Topic may offer a Research Topic of original and review manuscripts aiming to identify several selected drug candidates for specific repositioning treatments. Repurposing existing drugs with a well-known pharmacological and toxicological profile is an attractive method for quickly discovering new therapeutic indications. The off-label use of drugs for different diseases requires much less capital and time, and can hasten progress in the development of new drugs. Conversely, pharmaceutical industry seems to be, historically less interested in investing in further research with already approved or marketed drugs, and this may be direct consequence of the lack of economic incentives, regulatory protection or decreased chance for a new patent. Then, the potential novel indications, posologies or formulations for old medications are mainly studied in the independent clinical trials being conducted by research institutes, academia or collaborative groups. It is then clear that additional financial support is needed to confirm the possible results in terms of efficacy and safety of various but small I and II phase clinical studies, in large but still too expensive, time-consuming, and labor-intensive randomized controlled trials. Therefore some authors advocate for additional public funding as well as increased harmonization and centralization of such clinical research activities (Verbaanderd et al., 2021). This latter aspect becomes especially important in the light of the fact that in many circumstances the promises for repositioning old drugs from a wide range of therapeutic areas (even outside the original therapeutic area for which specific compounds were approved for) have been demonstrated in preclinical studies only, and further clinical trials are required to confirm their potential for such a clinical adoption, which in certain cases, could even provide higher efficacy, safety and increased wellbeing that initially thought (Aronson, 2007; Rechberger et al., 2025).

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EGFR is a potential dual molecular target for cancer and Alzheimer's disease

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Many researchers are attempting to identify drugs that can be repurposed as effective therapies for Alzheimer's disease (AD). Several recent studies have highlighted epidermal growth factor receptor (EGFR) inhibitors approved for use as anti-cancer drugs as potential candidates for repurposing as AD therapeutics. In cancer, EGFR inhibitors target cell proliferation and angiogenesis, and studies in AD mouse models have shown that EGFR inhibitors can attenuate amyloid-beta (Aβ) pathology and improve cognitive function. In this review, we discuss the different functions of EGFR in cancer and AD and the potential of EGFR as a dual molecular target for AD diseases. In addition, we describe the effects of anti-cancer EGFR tyrosine kinase inhibitors (TKIs) on AD pathology and their prospects as therapeutic interventions for AD. By summarizing the physiological functions of EGFR in cancer and AD, this review emphasizes the significance of EGFR as an important molecular target for these diseases.

KEYWORDS

Alzheimer's disease, EGFR, EGFR inhibitor, cancer, Aβ, learning and memory

1 Introduction

Heredity and aging are common risk factors for cancer and Alzheimer's disease (AD), which are the leading causes of death worldwide (White et al., 2014; Guerreiro and Bras, 2015; Majd et al., 2019). Numerous anti-cancer therapies are available, but therapeutic drugs for AD are scarce. AD is the most common neurodegenerative disease characterized by amyloid and tau protein aggregation and cognitive decline (Ates et al., 2016). Amyloid and tau protein aggregates are not only pathophysiological biomarkers of AD, but also cause neuronal loss, synapse destruction, and neuroinflammation (Serrano-Pozo et al., 2011; Krstic and Knuesel, 2013). Other potential mechanisms of AD progression are oxidative stress and epigenetic dysfunction.

Several studies have found an inverse association between cancer and AD, but others have argued for a parallel relationship (Majd et al., 2019; Nudelman et al., 2019; Branigan et al., 2021; Zhang et al., 2022). Although the underlying mechanism of the relationship between cancer and AD has not been thoroughly investigated, the diseases share hallmarks and risk factors. Risk factors for both cancer and AD include aging, smoking, obesity, and type 2 diabetes (Cannata et al., 2010; Cataldo et al., 2010; Mayeux and Stern, 2012; White et al., 2014; Emmerzaal et al., 2015). Strikingly, cell-cycle entry, which is required for cancer pathogenesis, is high in patients with AD (Majd et al., 2019). At the cellular level, the

pathogenesis of AD and cancer both involve the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway, which regulates cell proliferation, metabolism, growth, and autophagy (Pei and Hugon, 2008; Morgan et al., 2009; Advani, 2010; Talbot et al., 2012; Fumarola et al., 2014; Porta et al., 2014). Abnormal growth suppressor evasion is also observed in both cancer and AD. Specifically, cell growth and division in cancer often occur through inactivating mutations of tumor suppressors such as retinoblastoma transcriptional corepressor 1 (RB1) and TP53 (Hanahan and Weinberg, 2011; Fischer et al., 2016; Robinson et al., 2017). In patients with AD, levels of p27, a critical negative cell cycle regulator, are significantly reduced (Ogawa et al., 2003; Munoz et al., 2008). The systemic dysregulation of the cell cycle in both cancer and AD supports a correlation between these two diseases. Angiogenesis, cell adhesion inhibition, and inflammation are also shared by cancer and AD (Nudelman et al., 2019). Therefore, verifying the commonalities between cancer and AD might contribute to the development of effective therapeutic strategies.

A genome-wide association study found a significant positive genetic correlation between cancer and AD, implying that the pathophysiology of cancer and AD share common genetic variants (Feng et al., 2017). Specifically, super-enhancer, a broad enhancer domain affecting cell type identification and function, exhibits a significant positive genetic correlation with cancer and AD, indicating a potential role of gene expression regulation in the common genetic etiologies of cancer and AD (Feng et al., 2017; Zhao et al., 2022). Several genes [e.g., epidermal growth factor receptor (EGFR) and amyloid precursor protein (APP)] are associated with both cancer and AD, and we and others have recently found that anti-cancer drugs can penetrate the blood-brain barrier (BBB) and modulate AD pathology (Ryu and McLarnon, 2008; Cramer et al., 2012). Specifically, inhibitors of EGFR and other tyrosine kinases, which are multitarget enzymes, may have practical value for treating cancer and AD (Mansour et al., 2021c). This review provides insights into the potential roles of EGFR and EGFR inhibitors in cancer and AD and related therapeutic strategies.

2 EGFR

EGFR is a cell surface growth factor receptor that regulates cell proliferation, differentiation, and survival (Yewale et al., 2013). EGFR was the first receptor tyrosine kinase (RTK) to be discovered and is a member of the ErbB family of RTKs (Wong and Guillaud, 2004; Roskoski, 2019). The EGFR gene contains 31 exons and encodes a 170-kDa transmembrane glycoprotein (Mitsudomi and Yatabe, 2010; Sabbah et al., 2020). EGFR is stimulated by ligands such as epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) (Purba et al., 2017). Upon binding to the extracellular domain of EGFR, these ligands induce conformational changes in the receptor that facilitate the formation of receptor dimers or oligomers (Schlessinger, 2002). EGFR dimerization triggers the activation of its intrinsic tyrosine kinase activity and subsequent autophosphorylation of several tyrosine residues in the EGFR C-terminal domain (Yu et al., 2002). These phosphorylated tyrosine residues serve as docking sites for various signaling molecules and initiate canonical EGFR signaling pathways (Lemmon and Schlessinger, 2010). Although numerous reviews have discussed EGFR as a target in cancer, inflammatory diseases, and monogenic diseases, the regulation of EGFR expression or signaling as a multi-disease target requires further investigation.

3 EGFR in various cancers

EGFR plays an essential physiological role in regulating the development of epithelial tissue and homeostasis and hence is also linked to tumorigenesis, including lung cancer, breast cancer, and glioblastoma (Sigismund et al., 2018). EGFR is a critical modulator and a target for developing novel therapeutic strategies in various cancers. EGFR signaling modulates cancer cell proliferation through several metabolic processes (Sigismund et al., 2018). For example, Srivatsa et al. (2017) found that EGFR-expressing myeloid cells are abundant in the colorectal tumor stroma, indicating that EGFR in tumor-associated myeloid cells may be a diagnostic biomarker for colorectal cancer (CRC). CRISPR/Cas9-mediated elimination of EGFR significantly inhibits tumor cell growth and activates the mitogen-activated protein kinase (MAPK) (p-ERK1/2) pathway (Liu et al., 2020). Moreover, Song et al. (2020) identified upregulation of EGFR and phosphorylated signal transducer and activator of transcription 3 (p-STAT3) in breast cancer tissues.

The EGFR pathway is a widely recognized oncogenic pathway for non-small cell lung cancer (NSCLC), which represents approximately 75% of lung cancers (Bethune et al., 2010; Hsu et al., 2019). The EGFR pathway regulates the Bax/B-cell lymphoma 2 (Bcl-2) cascade, which is associated with apoptosis in NSCLC (Alam et al., 2022). Interestingly, a study identified EGFR overexpression or mutations in intracellular EGFR in 43%–89% of NSCLC cases (Gupta et al., 2009). Exon 19 deletion and L858R point mutation are the most frequent EGFR mutations in NSCLC (Khaddour et al., 2021). Activating somatic mutations in exons 18–21 of EGFR in NSCLC can continuously activate the EGFR kinase domain regardless of ligand binding and result in sustained downstream signaling. Several studies have reported that EGFR expression is increased by 40%–89% in NSCLC (Lu et al., 2001; Lynch et al., 2004; Al Olayan et al., 2012). Shao et al. (2022) found that upregulated EGFR signaling induces increased levels of the membrane-bound complement regulatory proteins (mCRPs) CD55 and CD59, thereby promoting tumor immune evasion in lung cancer cells ($CD8^+T$). In addition, Ohsaki et al. (2000) observed shorter survival of NSCLC patients with EGFR overexpression. Selvaggi et al. (2004) found that EGFR expression is significantly increased in stage III of NSCLC, implying that EGFR levels are a potential prognostic factor. Merrick et al. (2006) observed a significantly positive relationship between EGFR expression and the progression of bronchial dysplasia, a precursor of lung carcinoma, indicating a role of EGFR upregulation in lung cancer development and progression. Yang et al. (2015) reported that EGFR mutation-mediated lung cancer is associated with downregulation of cluster of differentiation 82 (CD82), which promotes EGFR expression. Shien et al. (2012) discovered that EGFR silencing by siRNA significantly reduces the cell viability of EGFR-mutant cell lines (PC-9, HCC827, NCI-H820, and NCI-1975), further supporting EGFR as a promising therapeutic target in NSCLC.

This critical role of EGFR upregulation in the development, progression, and longevity of lung cancer has led to the development of drugs that control EGFR activity and expression.

4 EGFR in Alzheimer's disease (AD)

The general functions of EGFR in the central nervous system (CNS) include neural stem cell pool maintenance, astrocyte differentiation and maturation, oligodendrocyte maturation, and neurite outgrowth (Romano and Bucci, 2020). EGFR isoforms are expressed in neurons in the hippocampus, cerebellum, and cerebral cortex (Wong and Guillaud, 2004). Several recent studies have demonstrated that EGFR and its related signaling pathways that it mediates are crucial targets for modulating AD pathology. For instance, Wang et al. (2013) found that EGFR activation (p-EGFR/EGFR) is significantly increased in 8-month-old APP/PS1 mice (a model of AD) compared with wild-type mice. Excessive EGFR expression induces memory impairment in Aβ-overexpressing *Drosophila* (Prüßing et al., 2013). More importantly, dual overexpression of EGFR and $A\beta_{42}$ synergistically promotes memory loss, implying that EGFR is upregulated in AD (Chiang et al., 2010). Notably, EGFR upregulation was recently shown to induce $A\beta_{42}$ neurotoxicity and neuroinflammation and activate astrocytes (Ozbeyli et al., 2017; Chen et al., 2019b). AD patients exhibit neuritic plaques with EGFR expression in the cerebral cortex and hippocampus (Birecree et al., 1988). However, EGF treatment does not alter EGFR or Aβ levels in the brain and prevents cognitive dysfunction in E4FAD mice (Thomas et al., 2016). Taken together, the literature suggests that inhibition of EGFR modulates Aβ plaque accumulation, neuroinflammation, and cognitive function in mouse models of AD. Although there are conflicting reports regarding the role of EGFR in Aβ pathology, there is strong evidence that EGFR is a dual molecular target for cancer and AD (Figure 1A).

5 Therapeutic applications of EGFR inhibitors

Despite substantial investments of resources and time in identifying new drugs for AD, clinical trials have produced disappointing results. Thus, the effects of EGFR on Aβ, neuroinflammation, and cognitive function have spurred growing interest in the potential repurposing of EGFR inhibitors used as anti-cancer drugs for the treatment of AD (Mansour et al., 2021c). The molecular mechanisms of EGFR in cancer and AD are also being investigated to develop disease-modifying drugs. Chen et al. (2019b) found that oxygen-glucose deprivation (OGD) increases EGFR phosphorylation and triggers downstream protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) signaling pathways in primary cultured astrocytes and CTX-TNA2 cells. Characterization of EGFR signaling pathways and downstream cascades may reveal promising strategies for utilizing tyrosine kinase inhibitors (TKIs) as disease-modifying therapies in cancer and AD. In addition, EGFR TKIs have greater BBB penetration potential than most intravenous chemotherapies (Ahluwalia et al., 2018). A recent high-performance liquid chromatography (HPLC) analysis showed that ibrutinib can cross the BBB in WT mice (Lee et al., 2021a), and gefitinib, erlotinib, afatinib, varlitinib, lapatinib, and osimertinib are all known to cross the BBB (Lin et al., 2008; Babu et al., 2015; Mansour et al., 2021b; Colclough et al., 2021). Colclough et al. (2021) compared the BBB permeability of EGFR TKIs and found that osimertinib has the highest BBB penetration, with a Kpuu of 0.21, followed by Kpuu values of 0.084 for erlotinib, 0.0092 for gefitinib, and 0.0046 for afatinib. The low BBB permeability of erlotinib and gefitinib means that these drugs do not exhibit significant or persistent effects in the brain (Ahluwalia et al., 2018). Although the abilities of lapatinib, osimertinib, and CL-387,785 to penetrate the BBB have not been determined, lapatinib is expected to cross the BBB due to its low molecular weight and lipophilicity (Mansour et al., 2021b). However, studies of the use of BBB-penetrating EGFR inhibitors to treat AD remain scarce, and the mechanisms of BBB-permeable EGFR TKIs in AD remain to be elucidated. Several anti-cancer EGFR inhibitors that are candidates for AD therapy are described below, and the therapeutic effects, safety, and toxicity profiles of EGFR TKIs in cancer and AD are shown in Tables 1, 2; Figure 1B.

5.1 Gefitinib

The EGFR/HER2 inhibitor Gefitinib is a first-generation EGFR TKI approved for lung cancer treatment (Arrieta et al., 2020). Gefitinib inhibits the binding of EGFR and adenosine triphosphate (ATP), thereby blocking EGFR autophosphorylation and downstream signaling cascades that control cell growth and trigger apoptosis (Jiang et al., 2005). Compared with erlotinib, gefitinib appears to be more effective and safer for treating NSCLC (Zhang et al., 2018).

Several studies have shown that gefitinib can ameliorate AD pathology. Gefitinib can penetrate the brain and thus positively affects non-EGFR targets that participate in AD pathology in E4FAD (APOE4-expressing) AD transgenic (Tg) mice (Thomas et al., 2016). A computational analysis indicated that gefitinib and the hydrophobic pocket of β-site APP cleaving enzyme (BACE) have complementary shapes and binding interactions, suggesting that gefitinib suppresses $A\beta_{40/42}$ through BACE (Niu et al., 2014). In addition, gefitinib prevents memory loss in an $A\beta_{42}$-overexpressing *Drosophila* model and rescues memory impairment in APP/PS1 Tg mice, a model of AD, indicating that EGFR inhibition can potentially improve cognitive function (Wang et al., 2012). In AD-induced Swiss albino mice, gefitinib attenuates hippocampal-dependent memory impairment as assessed by the Morris water maze (MWM) test and reduces acetylcholinesterase (AChE) activity (Dhamodharan et al., 2022). The ability of gefitinib to reduce Aβ-mediated AChE levels and attenuate cognitive impairments supports its potential as an AD treatment, but whether gefitinib directly affects other AD-associated factors (e.g., tau pathology) and its molecular mechanisms of action on AD pathology remain to be clarified.

5.2 Erlotinib

The EGFR-TKI erlotinib is an FDA-approved drug used to treat patients with NSCLC with mutations in the ATP-binding pocket of EGFR (Cohen et al., 2005; Smith, 2005; Nishimura et al., 2015; Lee

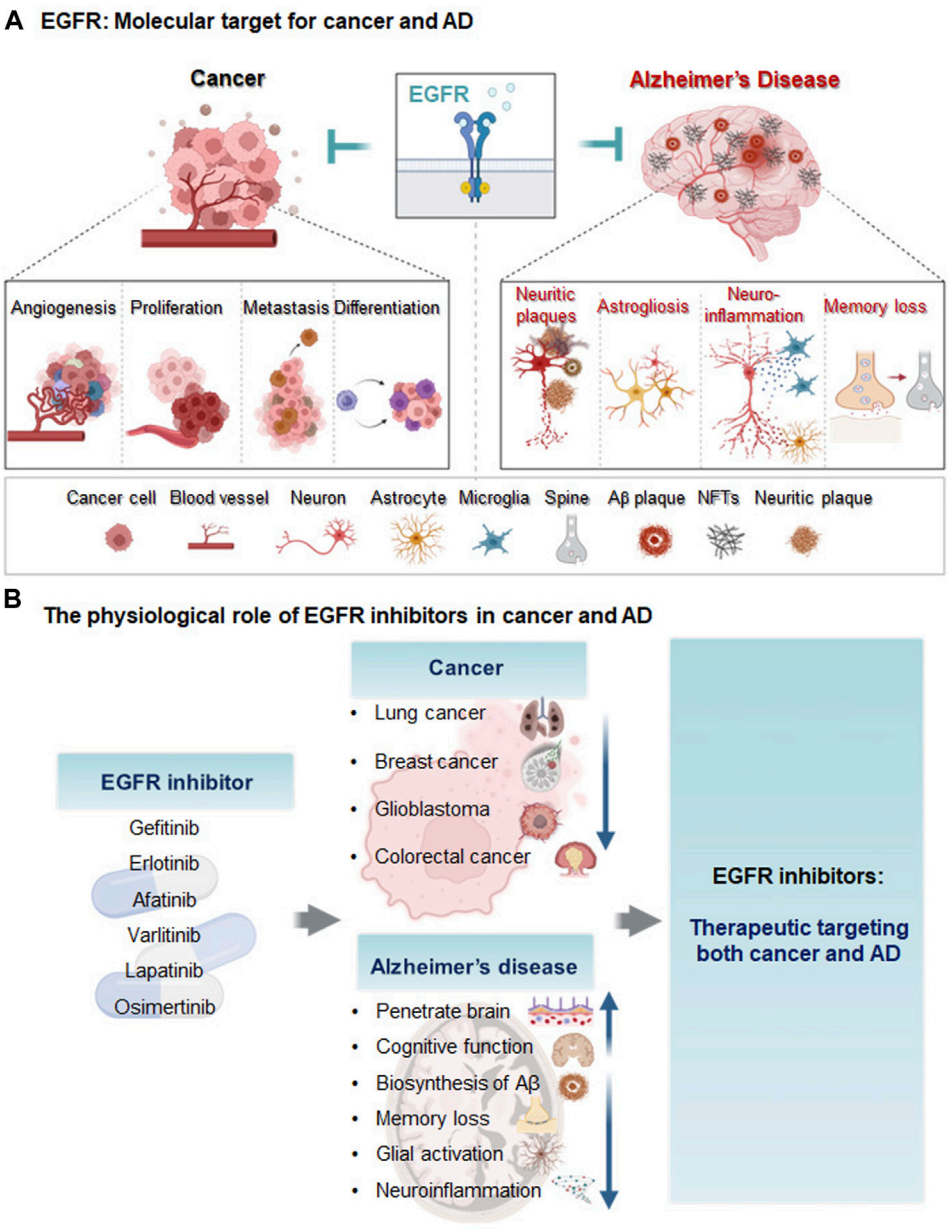

FIGURE 1
Diagram of EGFR as a molecular target and the effects of EGFR inhibitors on cancer and AD. **(A)** Epidermal growth factor receptor (EGFR) is a transmembrane protein receptor for the epidermal growth factor family that regulates cell growth and proliferation. In cancer, EGFR upregulation increases metastasis, angiogenesis, cancer cell proliferation, differentiation, and cancer viability. In animal models of AD and/or AD patients, EGFR levels are increased, leading to memory loss, astrogliosis, neuroinflammation and Aβ plaque formation. **(B)** The EGFR inhibitors gefitinib, afatinib, varlitinib, erlotinib, osimertinib, and lapatinib are anti-cancer drugs targeting EGFR. These EGFR inhibitors reduce AD pathology and improve cognitive function and thus may be potential therapeutic agents for cancer and AD.

et al., 2021b). Erlotinib suppresses the tumor growth of the human endometrial adenocarcinoma cell line HEC-1A, which expresses high levels of EGFR (Nishimura et al., 2015). Deep learning and machine learning algorithms predict that erlotinib is a BBB-permeable compound (Jang et al., 2022). Erlotinib blocks lipopolysaccharide (LPS)-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent cytokine production in C57BL/6J mice, implying that erlotinib modulates neuroinflammatory responses in the brain (De et al., 2015). However, due to their low BBB penetration, the effects of erlotinib and gefitinib on brain metastasis are neither significant nor persistent (Ahluwalia et al., 2018).

Importantly, erlotinib rescues memory deficits in APP/PS1 Tg mice as assessed by the MWM test, suggesting that erlotinib can modulate cognitive function (Wang et al., 2012). Although research has focused on the potential utility of erlotinib in treating AD, the effects of erlotinib on Aβ/tau pathology and its mechanisms of action in mouse models of AD require further study.

TABLE 1 The effects of EGFR inhibitors on cancer and AD.

EGFR Inhibitor	BBB Penetration	Effects in Cancer	Effects in AD	References
Gefitinib	0.0092 Brain Kpuu in WT rats	- First-generation EGFR TKI for lung cancer treatment	- Ameliorates Aβ-induced memory loss in APP/PS1 transgenic mice and a *Drosophila* AD model	Wang et al. (2012), Niu et al. (2014), Arrieta et al. (2020), Colclough et al. (2021), Dhamodharan et al. (2022)
			- Inhibits extracellular $A\beta_{40/42}$ levels and reduces β-secretase (BACE-1) activity in APP-overexpressing N2a cells	
			- Improves cognition function in Swiss albino mice	
Erlotinib	0.084 Brain Kpuu in WT rats	- Suppresses tumor growth in the human endometrial adenocarcinoma cell line HEC-1A	- Significantly increases the performance index of Drosophila with $A\beta_{42}$-induced memory loss	Wang et al. (2012), Nishimura et al. (2015), Colclough et al. (2021)
Afatinib	0.0046 Brain Kpuu in WT rats	- Second-generation EGFR-TKI with anti-inflammatory effects	- Prevents astrocyte activation	Chen et al. (2019a), Chen et al. (2019b), Vengoji et al. (2019), Colclough et al. (2021)
		- Inhibits the proliferation, migration, and invasion of hepatocellular carcinoma (HCC) cells		
		- Inhibits brain tumor formation by regulating EGFRvIII-cMet signaling when combined with temozolomide in glioblastoma cells	- Reduces proinflammatory cytokine levels and caspase-1 activation in CTXTNA2 cells	
Varlitinib	Crosses BBB	- FDA-approved EGFR/HER2 inhibitor	- Downregulates LPS-mediated neuroinflammatory responses and tau pathology in wild-type and tauoverexpressing PS19 mice	Babu et al. (2015), Liu et al. (2019), Dokduang et al. (2020), Kim et al. (2022)
		- Suppresses cell migration, invasion, and mammosphere formation in triple-negative breast cancer (TNBC) cells		
Lapatinib	Crosses BBB	- Dual TKI targeting EGFR and HER2	- Decreases $A\beta_{1-42}$ and p-tau levels and ameliorates cognitive impairment in D-galactose/ovariectomized rats	Oakman et al. (2010), Matsumoto et al. (2018), Cihan (2019), Mansour et al. (2021b)
		- Antitumor effects in HER2-positive breast cancer cells		
Osimertinib	0.21 Brain Kpuu in WT rats	- Clinical activity against EGFR-mutant glioblastoma and non-small cell lung cancer (NSCLC)	- No specific studies of osimertinib as an AD therapy	Makhlin et al. (2019), Colclough et al. (2021), Gen et al. (2022)
CL-387,785	Expected to cross BBB	- Inhibits EGFR mutants more effectively than first/secondgeneration EGFR TKIs	- Decreases C99 and AICD levels in cellular, zebrafish, and mouse models of AD	Greulich et al. (2005), Kobayashi et al. (2005), Wang et al. (2017)
			- Rescues cognitive impairment in APP/ PS1 mice	

5.3 Afatinib

Afatinib is a second-generation EGFR-TKI with anti-inflammatory effects and is widely used to treat NSCLC (Chen et al., 2019b; Moosavi and Polineni, 2023). Afatinib also inhibits the migration, proliferation, and invasion of hepatocellular carcinoma (HCC) cells, implying anti-tumorigenic effects (Chen et al., 2019a). Treatment with a combination of afatinib and temozolomide suppresses brain tumor formation by inhibiting crosstalk between EGFRvIII, a constitutively active EGFR mutant, and the RTK cross-activation of tyrosine kinase receptor (cMet) (Vengoji et al., 2019). Interestingly, afatinib (1 or 10 nM) inhibits OGD-induced EGFR phosphorylation, astrocyte activation, and reduced proinflammatory cytokine levels in CTX-TNA2 cells (Chen et al., 2019b). These results suggest that afatinib has anti-inflammatory effects on OGD-induced neuroinflammation. However, whether afatinib regulates AD pathology and cognitive function in mouse models of AD is unknown. Although the direct effects of afatinib on AD pathology have not been comprehensively investigated, the anti-cancer and anti-inflammatory effects of afatinib indicate promising potential for repurposing as an AD therapy.

5.4 Varlitinib

Studies have examined the effects of varlitinib, an FDA-approved EGFR/HER2 inhibitor, on various cancers, including gastric, pancreatic, colorectal, and breast cancers (Dokduang et al., 2020). Varlitinib can penetrate the BBB and suppresses cell migration, invasion, and mammosphere formation through ERK/AKT signaling in triple-negative breast cancer (TNBC) cells (Babu et al., 2015; Liu et al., 2019). In addition, EGFR/HER2 inhibition by varlitinib has therapeutic effects on cholangiocarcinoma (CCA) (Dokduang et al., 2020). Whereas other EGFR inhibitors have side effects, varlitinib does not have significant toxicity in CCA-inoculated mice (Dokduang et al., 2020). Importantly, we recently demonstrated that varlitinib downregulates LPS-mediated neuroinflammation and tau pathology through dual specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A), a tau kinase (Kim et al., 2022). In addition, we found that varlitinib significantly diminishes LPS-induced neuroinflammation in both BV2 microglial cells and primary astrocytes, suggesting that varlitinib has therapeutic effects on neuroinflammation and

TABLE 2 Safety, toxicity profiles, and target cancers of EGFR inhibitors.

EGFR Inhibitor	Target group	Target cancers	Safety profile	Toxicity profile	References
Gefitinib	- ATP binding sites of EGFR	- NSCLC	- Treated with optimal biological dosage (250 mg/day)	- Rash - Diarrhea - Xeroderma - Pruritus	Noble and Faulds (1999), Van Zandwijk (2003), Jiang et al. (2005), Arrieta et al. (2020)
Erlotinib	- ATP binding sites of EGFR	- NSCLC	- Tolerate total 1,600 mg weekly dosing for cancer patients	- Skin rash - Xeroderma - Pruritus - Paronychia	Cohen et al., 2005 Smith (2005), Kiyohara et al. (2013), Carter and Tadi (2020)
Afatinib	- EGFR, HER2/ErbB2 and ErbB4	- NSCLC	- Increased up to a maximum dosage of 50 mg/day	- Skin deformity - Diarrhea - Paronychia - Oral mucositis - Anorexia	Ingelheim (2016), Lai et al. (2017), Zhang et al. (2017), Tanaka et al. (2019)
Varlitinib	- EGFR and HER2/ErbB2	- Gastric cancer - Pancreatic cancer - Colorectal cancer - Breast cancer	- Maximum tolerated dosage of 300 mg began twice-daily (BID)	- No toxicity observed in CCA-inoculated mouse	Hotte et al. (2009), Dokduang et al. (2020)
Lapatinib	- EGFR and HER2/ErbB2	- Breast cancer	- Maximum tolerated dosage of 1,500 mg began twice-daily (BID)	- Diarrhea - Rash	Moy and Goss (2007), Oakman et al. (2010), Morikawa et al. (2019)
Osimertinib	- Mutant-selective EGFR (exon 19 deletion EGFR)	- NSCLC	- Optimal toxic limit of 259 ng/mL	- Skin rash - Paronychia - Acrodermatitis	Jiang and Zhou (2014), Makhlin et al. (2019), Agema et al. (2022)
CL-387,785	- EGFR and mutant EGFR	- NSCLC	- Studies regarding toxicity not reported	- Studies regarding toxicity not reported	Greulich et al. (2005), Kobayashi et al. (2005), Wang et al. (2017)

tau pathology (Kim et al., 2022). In contrast to its effects on cancer, the impact of varlitinib on AD pathology (including Aβ pathology and its mechanism of action) is poorly understood; thus, further studies are needed to evaluate the feasibility of using varlitinib for the treatment of AD.

5.5 Lapatinib

Like varlitinib, lapatinib is a dual TKI targeting both EGFR and HER2 and is currently used to treat cancer (Oakman et al., 2010). Lapatinib is a low-molecular-weight and lipophilic molecule and can penetrate the BBB (Mansour et al., 2021b). In HER2-positive breast cancer cells, a combination of lapatinib and capecitabine has synergistic anti-tumor effects (Matsumoto et al., 2018).

Lapatinib has been shown to ameliorate autoimmune encephalomyelitis, a functional disorder of the CNS (Akama-Garren et al., 2015). In addition, lapatinib has neuroprotective effects against neuronal ferroptosis, indicating the involvement of ferroptosis in AD pathologies (Jia et al., 2020; Chen et al., 2021). A study of the effects of lapatinib on cognitive function *in vivo* found that lapatinib rescues short/long-term recognition memory impairment by activating the PI3K/AKT/glycogen synthase kinase-3 beta (GSK-3β) pathway in D-galactose/ovariectomized (D-gal/OVX) rats (Mansour et al., 2021b). The same study demonstrated that lapatinib decreases $A\beta_{1\text{-}42}$ and p-tau levels and suppresses HER2 expression in D-gal/OVX rats (Mansour et al., 2021b). Thus, inhibition of HER2 by lapatinib promotes autophagy and reduces $A\beta_{1-42}$ and p-tau levels, consistent with the results of previous studies of the role of EGFR/HER2 in autophagy (Mansour et al., 2021a;

Mansour et al., 2022). Overall, lapatinib is a promising candidate anti-cancer drug for repositioning as an AD therapeutic. However, further studies of the effects of lapatinib on AD pathophysiology are needed.

5.6 Osimertinib

Osimertinib is an irreversible EGFR inhibitor and a third-generation TKI with high brain penetration (Makhlin et al., 2019). Osimertinib targets EGFR T790, which is resistant to most first- and second-generation EGFR TKIs (Janjigian et al., 2014; Jiang and Zhou, 2014; Barbuti et al., 2019). Osimertinib is known for its clinical activities in glioblastoma and NSCLC (Makhlin et al., 2019; Gen et al., 2022). *In vitro*, osimertinib has higher affinity for EGFR L858R/T790M than for wild-type EGFR (Kuijper et al., 2015; Greig, 2016). Interestingly, a recent report indicated that osimertinib is also highly effective against CNS metastasis (Liam, 2019). Furthermore, Ge et al. (2017) observed a higher probability and frequency of EGFR mutations in NSCLC patients with brain metastasis than in NSCLC patients without brain metastasis. In addition, the incidence of brain metastasis is higher in NSCLC patients with mutated EGFR than in NSCLC patients with wild-type EGFR, implying a correlation between EGFR mutation and brain metastasis (Ge et al., 2017). Whether the potential effectiveness of osimertinib in the CNS extends to AD pathology is unknown.

The relationships between specific EGFR mutations (exon 19 deletions and exon 21 L858R) and AD have not been examined. Jolly et al. (2022) investigated the associations of the locations of mutations in EGF-like repeats (EGFr) with vascular cognitive impairment (VCI) in patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), one of the most common forms of stroke and early-onset dementia, and found no significant relationship (Jolly et al., 2022). Although these results are not consistent with the mechanism of osimertinib, different results might be obtained for the relationships between EGFR mutations and ADs. Osimertinib may have utility as an EGFR inhibitor for treating AD pathology due to its high brain penetration, but the impact of osimertinib on AD has not been studied.

5.7 CL-387,785

CL-387,785 is an irreversible selective EGFR inhibitor designed to specifically inhibit EGFR autophosphorylation and tumor cell proliferation (Discafani et al., 1999). CL-387,785 inhibits not only wild-type EGFR but also EGFR T790M, which is resistant to EGFR TKIs such as erlotinib, gefitinib, and afatinib (Kobayashi et al., 2005; Yun et al., 2008; Mok et al., 2009; Rosell et al., 2012; Zhang et al., 2012; Chi et al., 2013; Sequist et al., 2013; Niu and Wu, 2014). Thus, CL-387,785 is expected to solve the cause of drug resistance. Although CL-387,785 is only approved for research purposes, several studies have indicated therapeutic effects of CL-387,785 on lung cancer. For instance, CL-387,785 inhibits colony formation by lung cancer cells expressing an EGFR missense or deletion mutant more effectively than gefitinib and erlotinib, suggesting that CL-387,785 may be a good therapeutic for lung cancer with exon 20 insertion mutations of EGFR (Greulich et al., 2005). In addition, CL-387,785 inhibits the proliferation and apoptosis of NSCLC H1975 cells expressing EGFR T790M, indicating that CL-387,785 can restrict the invasion and metastasis of NSCLC H1975 cells (Cai et al., 2023).

With respect to potential effects on AD, CL-387,785 significantly reduces C99-CTF (c-terminal fragment) and APP intracellular domain (AICD) levels in C99-YFP–overexpressing HEK293 cells and C99 CTF-expressing zebrafish (Wang et al., 2017). More importantly, CL-387,785 rescues spatial learning and memory and reduces Aβ levels in APP/PS1 Tg mice (Wang et al., 2017). CL-387,785 also reduces the LC3-II/LC3-I ratio, which is crucial for activating autophagy, promoting the clearance of $A\beta_{40}$ and $A\beta_{42}$, and improving memory (Wang et al., 2017). Although the effects of CL-387,785 on AD pathology (i.e., tau) and its mechanism of action require further investigation, CL-387,785 can be considered a potential EGFR TKI for both cancer and AD.

Conclusion and future directions

Several recent studies have revealed associations of EGFR with cancer and AD; thus, regulating EGFR expression may be a strategy for treating both diseases. However, comprehensive studies of the roles of EGFR and EGFR inhibitors (TKIs) in cancer and AD are not available. In addition, although EGFR is a potential target for AD treatment, the effectiveness of major anti-cancer EGFR TKIs as AD therapeutics has received little attention. This review highlights the functional roles of EGFR and EGFR TKIs in cancer and AD. Specifically, EGFR upregulation induces various types of cancer and promotes Aβ pathology. EGFR inhibition has promising effects on both diseases, including inhibiting cancer cell migration and AD pathology (e.g., Aβ, neuroinflammation, and cognitive function). The literature and our findings suggest that anti-cancer drugs can be regarded as candidates for repurposing as AD treatments. However, the direct relationship between EGFR and AD, the effects of EGFR on tau pathology in mouse models of AD, and the mechanisms of action of EGFR in the brain are still unclear. Moreover, the effects of EGFR TKIs on AD pathology have not been well examined. Further studies are required to address these issues and may provide significant insights into cancer therapy and AD progression.

Author contributions

H-JC, YJJ, JK, and H-SH wrote the manuscript. JK and H-SH conceived the study. All authors contributed to the article and approved the submitted version.

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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.

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Current trends and future prospects of drug repositioning in gastrointestinal oncology

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Gastrointestinal (GI) cancers comprise a significant number of cancer cases worldwide and contribute to a high percentage of cancer-related deaths. To improve survival rates of GI cancer patients, it is important to find and implement more effective therapeutic strategies with better prognoses and fewer side effects. The development of new drugs can be a lengthy and expensive process, often involving clinical trials that may fail in the early stages. One strategy to address these challenges is drug repurposing (DR). Drug repurposing is a developmental strategy that involves using existing drugs approved for other diseases and leveraging their safety and pharmacological data to explore their potential use in treating different diseases. In this paper, we outline the existing therapeutic strategies and challenges associated with GI cancers and explore DR as a promising alternative approach. We have presented an extensive review of different DR methodologies, research efforts and examples of repurposed drugs within various GI cancer types, such as colorectal, pancreatic and liver cancers. Our aim is to provide a comprehensive overview of employing the DR approach in GI cancers to inform future research endeavors and clinical trials in this field.

KEYWORDS

gastrointestinal cancers, therapeutic strategies, drug repurposing, colorectal cacner, pancreatic cancer, liver cancer

1 Introduction

Gastrointestinal (GI) cancers, a group of malignancies occurring within the digestive system including colorectal cancer (CRC), esophageal cancer (EC), gastric cancer (GC), liver cancer (LC), and pancreatic cancer (PC) comprised a substantial proportion of global cancer cases. They were responsible for approximately 27.7% of cancer cases (5.5 million cases out of 18.1 million worldwide) and 35.8% of global cancer-related deaths in 2020 (Sung et al., 2021). GI cancers have different global prevalence and mortality rates. CRC is the most frequently occurring type, ranking third in prevalence and the second in cancer-related

TABLE 1 List of repurposed drugs proposed for targeting colorectal carcinoma.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Adapalene	Inhibition of proliferation, and induction of cell cycle arrest	Acne	Colorectal cancer	PTGS2	*In vitro* (LoVo and DLD1 cell lines), and *in vivo* (mouse xenograft model)	Shi et al. (2015)
Aflibercept	Inhibition of angiogenesis	Neovascular (wet) age-related macular degeneration, macular edema following retinal vein occlusion, diabetic macular edema, and diabetic retinopathy	Metastatic colorectal cancer	VEGFA	Clinical trial: Phase 3 (NCT04392479)	Li et al. (2018a)
Amantadine	Inhibition of cell proliferation, and induction of apoptosis	Parkinson	Colorectal cancer	Endoretroviruses (HERV-WE1, HERV-FRD1, HERV-31, and HERV-V1)	*In vitro* (HCT8 cell line)	Díaz-Carballo et al. (2015)
Artesunate	Induction of apoptosis and cytotoxicity	Antimalarial	Colorectal cancer	Downregulation of β-catenin	Clinical Trial: Phase 2 (NCT02633098)	Kumar et al. (2019)
Aspirin	Inhibition of tumor proliferation	Analgesia	Colorectal cancer, Gastrointestinal, esophageal cancer, etc.	COX-1/2, ANXA1-NF–κB axis, CDX2, COMMD1–RelA axis	Clinical trial: Phase 3 (NCT02301286)	Frouws et al. (2017)
Azithromycin	Inhibition of autophagy	Antibiotic	Colorectal cancer	Inhibition of autophagy by upregulating p62 and LC-3B	Clinical trial: Phase 4 (NCT04454151)	Qiao et al. (2018)
Berberine	Inhibition of invasion and metastasis	A chemical found in some plants and is typically used to treat bacterial diarrhea	Gastric, colorectal, lung cancer, etc.	Ephrin-B2, MMP-2/ MMP-9, EMT, miR-101, VEGF	Clinical trial: Phase 3 (NCT02226185)	Yu et al. (2014)
Captopril	Inhibition of cell proliferation and metastasis	Hypertension	Colorectal cancer	Angiotensin converting enzyme (ACE)	*In vivo* (mouse model)	Neo et al. (2007)
Celecoxib	Inhibition of proliferation through apoptosis	Pain and inflammation	Familial adenomatous polyps	COX-2	Clinical trial: Phase 3 (NCT00005094)	Grösch et al. (2001)
Chloroquine and related-derivatives	Inhibition of tumor growth and induction of apoptosis	Malaria, rheumatoid arthritis	Colorectal cancer	Autophagy, PPT1, TLR9/NFκB	*In vitro* (HCT116, HT29, and CT26) and *in vivo* (mouse xenograft model)	Anselmino et al. (2023)
Clarithromycin	Inhibition of cell growth, autophagy and angiogenesis	Antibiotic	Colorectal cancer	Inhibition of autophagy by targeting hERG1, PI3K	*In vitro* (HCT116 and LS174T, HEK293, and HT29 cell lines), and *in vivo* (mouse xenograft model)	Petroni et al. (2020)
Dalteparin	Inhibition of angiogenesis	Anticoagulant	Colorectal cancer	VEGFA	Clinical trial: Phase 2 (NCT00323011)	Agnelli et al. (2022)
Dapagliflozin	Reduction of cell adhesion and proliferation	Antihyperglycemic	Colorectal cancer	ADAM10, DDR1, cellular interaction with Collagen types I and IV Increased Erk phosphorylation	*In vitro* (HCT116 cell line)	Okada et al. (2020)
Diclofenac	Inhibition of cell proliferation via MYC-dependent and -independent mechanisms	Pain of osteoarthritis	Colorectal cancer	Bcl-2, COX-1, COX-2, MCP-1, MIP-1α and VEGF	*In vitro* (sw480, and Caco-2 cell lines) and *in vivo* (rat model)	Kaur and Sanyal, (2011)
Disulfiram	Reprogramming energy metabolism	Alcohol dependence	Colorectal cancer	NF-κB, NPL4	*In vitro* (H630, DLD-1 and RKO cell lines)	Wang et al. (2003)

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TABLE 1 (*Continued*) List of repurposed drugs proposed for targeting colorectal carcinoma.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Doxycycline	Induction of apoptosis and inhibition of proliferation and invasive potential	Antibiotic	Colorectal cancer	Inhibition of matrix metalloproteinases Activation of caspase-3, -8, and -9 Release of cytochrome c and Bax translocation	*In vitro* (LS174T, and HT29 cell lines)	Onoda et al. (2004)
Ebselen	Inhibition of tumor growth	Multifunctional compound	Colorectal cancer	ATG4B, autophagy and tumor suppression	*In vitro* (HCT116, and RKO cell lines), and *in vivo* (mouse xenograft model)	Xie et al. (2022)
Efavirenz	Induction of apoptosis	Anti-retroviral (anti-HIV drug)	Colorectal cancer	Activation of the phosphorylation of p53	*In vitro* (HCT-15 cell line)	Hecht et al. (2013)
Fluoxetine	Inhibition of colitis-associated tumorigenesis, dysplasia and angiogenesis	Antidepressant	Colorectal cancer	Inhibition of NF-κB activation and IKK phosphorylation Cell-cycle arrest at G0/ G1 Enhanced p27 expression Reduced VEGF expression	*In vitro* (HT29 cell line), *in vivo* (mouse model)	Kannen et al. (2012)
Gemifloxacin	Inhibition of cell migration and invasion	Antibiotic	Colorectal cancer	Inhibition of NF-κB activation Inhibition of TNF-α, IL-6, IL-8, and VEGF	*In vitro* (SW620, and LoVo cell lines)	Kan et al. (2013)
Indinavir	Inhibition of tumor growth	Anti-retroviral (anti-HIV drug)	Colorectal cancer	Proteasome-independent block of angiogenesis and matrix metalloproteinases	*In vitro* (SW480 cell line), *in vivo* (mouse xenograft model)	Toschi et al. (2011)
Indomethacin	Induction of G1 arrest and apoptosis	Rheumatic disease	Colorectalcancer	Shc-ERK axis, PKCζ-p38-DRP1 axis, Wnt/β-catenin	Clinical trial: Phase 4 (NCT00473980)	Lin et al. (2019), Mazumder et al. (2019), Bahmad et al. (2022)
Irbesartan	Inhibition of metastasis	Hypertension	Colorectal cancer	Angiotensin receptor	*In vivo* (mouse xenograft model)	Neo et al. (2007)
Ivermectin	Inhibition of proliferation and induction of apoptosis	Antihelmintic drug	Colorectal cancer	WNT-TCF signaling	*In vitro* (SW480 and SW1116 cell lines)	Zhou et al. (2021)
Linagliptin	Inhibition of metastasis	Type-2 diabetes	Colorectal cancer	Rb/Bcl-2/p53	*In vitro* (HCT 116 cell line), and *in vivo* (mouse xenograft model)	Li et al. (2020b)
Lovastatin	Inhibition of cancer progression and metastasis	Antilipidemic	Colorectal cancer	Inhibition of MACC1	*In vitro* (SW480 cell line) and *in vivo* (mouse model)	Xiao et al. (2022)
Mebendazole	Inhibition of metastasis	Antihelmintic drug	Colorectal cancer	MYC and COX2 pathways	Clinical trial: Phase 3 (NCT03925662)	Nygren and Larsson (2014), Hegazy et al. (2022)
Mefloquine	Induction of apoptosis and growth arrest	Antimalarial	Colorectal cancer	Inhibition of NF-κB activation	*In vitro* (HT-29, HCT116, RKO, SW620 and Lovo cell lines), and *in vivo* (mouse xenograft model)	Xu et al. (2018)
Metformin	Reprogramming energy metabolism	Obese type 2 diabetes	Colorectal cancer	AMPK, PI3K-mTOR pathways, BACH1	Clinical trial: Phase 3 (NCT05921942)	Higurashi and Nakajima (2018)

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TABLE 1 (*Continued*) List of repurposed drugs proposed for targeting colorectal carcinoma.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Midostaurin	Inhibition of cell growth and cell cycle arresting	A protein kinase inhibitor that has been developed for the treatment of acute myeloid leukemia, myelodysplastic syndrome and advanced systemic mastocytosis	Rectal cancer	cGAS, STING, IRF3, IFNAR1, Trex-1, c-Kit, and Flt3	Clinical trial: Phase 1 (NCT01282502)	Lai et al. (2022)
Nebivolol	Inhibition of tumor growth	Hypertension and other indications	Colorectal cancer	Inhibition of mitochondrial respiration by decreasing the activity of Complex I of the respiratory chain	*In vitro* (HCT116 cell line), and *in vivo* (mouse xenograft model)	Nuevo-Tapioles et al. (2020)
Niclosamide	Inhibition of invasion and metastasis	Antihelminthic drug	Colorectal cancer	Wnt/β-catenin, STAT3, NF-κB	Clinical trial: Phase 2 (NCT02519582)	Wang et al. (2019)
Nitazoxanide	Induction of G1 arrest, Modulation of angiogenesis and metabolism	Anti-Parasite	Colorectal cancer	mTOR	Clinical trial: Phase 3 (NCT06049901)	Senkowski et al. (2015), Ripani et al. (2020)
Oxiconazole	Inhibition of tumor growth	Antifungal agent	Colorectal cancer	Inhibiting autophagy through downregulation of peroxiredoxin-2 (PRDX2)	*In vitro* (HCT116, SW480, RKO, DLD-1, SW620, LoVo cell lines), and *in vivo* (mouse xenograft model)	Shi et al. (2022a)
Parecoxib	Prevention of inflammation and tumor-promotion	Pain	Colorectal cancer	COX2, PTGS2	*In vitro* (HCT116 and HT29 cell lines), and *in vivo* (mouse xenograft model)	Xiong et al. (2015)
Perhexiline	Induction of apoptosis, and reduction of cell viability	Anti-anginal	Colorectal cancer	-	*In vitro* (SW480, SW620, HCT116, HT29 and COLO205 cell lines), and *in vivo* (Patient-derived organoids)	Dhakal et al. (2022)
Propranolol	Induction of apoptosis	Hypertension	Colorectal cancer	Activating autologous $CD8^+$ T cells and decreasing the expression of p-AKT/ p-ERK/p-MEK, inhibiting the expression of p-ERK.	Clinical trial: Phase 3 (NCT00888797)	Liao et al. (2020)
Raltegravir	Inhibition of invasion	Anti-retroviral (anti-HIV drug)	Colorectal cancer	Blockage of fascin-1	*In vitro* (HCT-116 and DLD-1), and *in vivo* (mouse xenograft model)	Alburquerque-González et al. (2021)
Rapamycin or Sirolimus	Inhibition of cell proliferation, invasion, and angiogenesis, and induction of apoptosis	Immunosuppressant, anti-restenosis agent, Prevention of kidney transplant rejection	Rectum, and colorectalcancers	mTOR and associated signaling networks, CHOP-dependent DR5 induction on 4E-BP1 dephosphorylation Suppressed FBXW7 loss-driven EMT	Clinical trial: Phase 2 (NCT00409994) and (NCT03439462)	Gulhati et al. (2009), Mussin et al. (2017)
Ritonavir	Induction of apoptosis and inhibition of angiogenesis	Anti-retroviral (anti-HIV drug)	Colorectal cancer	Inhibition proteolytic degradation and accumulation of p21 Decreased	*In vitro* (DLD-1 cell line)	Mühl et al. (2004)

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TABLE 1 (*Continued*) List of repurposed drugs proposed for targeting colorectal carcinoma.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
				production of TNF-α, IL-6, IL-8, and VEGF Increased expression of heme oxygenase-1		
Rofecoxib (Withdrawn) (Phase 3)	Inhibition of mMetastasis	Osteoarthritis, rheumatoid arthritis, juvenile rheumatoid arthritis, acute pain conditions, migraine, and dysmenorrhea	Colorectal cancer	COX-2	Clinical trial: Phase 3 (NCT00031863)	Midgley et al. (2010)
Simvastatin	Inhibition of metastasis	Dyslipidemia	Colorectal cancer	KRAS	Clinical trial: Phase 3 (NCT01238094)	Lee et al. (2011)
Spiperone	Induction of apoptosis	Schizophrenia	Colorectal cancer	Activating phospholipase C, disrupting intracellular calcium balance, inducing irreversible endoplasmic reticulum stress, causing lipid metabolism changes, damaging the Golgi apparatus	*In vitro* (HCT116, SW620, HCT8, and MDA-MB-231 cell lines)	Antona et al. (2022)
Sulindac	Prevention of inflammation and tumor-promotion, and induction of apoptosis	Pain, swelling, and joint stiffness from arthritis	Colorectal cancer	PTGS2, Cyclin G2	Clinical trial: Phase 2 (NCT01856322)	Wang et al. (2022)
Thalidomide	Inhibition of angiogenesis	Sedative, antiemetic	Colorectal cancer	Various proangiogenic factors, VEGF receptor, NF-κB	Clinical trial: Phase 3 (NCT02748772)	Sakamoto and Maeda (2010), Zhang and Luo (2018)
Tolfenamic acid	Inhibition of cell proliferation, metastasis, and induction of apoptosis	Migraine	Colorectal cancer	Cyclin D, cyclin E, Cdk2, E2F-1, c-Myc, Mmp7, S100a9, Nppb and Aldh1a3, PTGS, VEGF, survivin, XIPA	*In vitro* (HCT116 and LoVo cell lines)	Jeong et al. (2013)
Valproate	Reduction of cell proliferation and cytotoxicity enhancement	Antipsychotic	Colorectal cancer	Histone hyperacetylation Relief of HDAC-mediated transcriptional repression	Clinical trial: Phase 2 (NCT05694936)	Jeong et al. (2013), Patel and Patel (2018)
Zidovudine	Induction of apoptosis, and cell cycle arrest	Anti-retroviral (anti-HIV drug)	Colorectal cancer	Increased expression of the p53-Puma/Bax/ Noxa pathways Activation of the p53-p21 pathway	Clinical trial: Phase 2 (NCT03144804)	Falcone et al. (1997)

deaths. GC, LC and EC rank as the fifth, sixth and eighth most commonly diagnosed cancers, with mortality rates ranking fourth, third and sixth, respectively. PC which has a poor prognosis with low curability rates compared to the other GI cancers, ranks 12th in frequency and the seventh in cancer death (Ferlay et al., 2021).

GI cancers exhibit diverse incidence patterns across regions, with PC and CRC being more prevalent in Europe and Northern America, and GC, LC, and EC in Asia. Interestingly, the countries with high human development indicators (HDIs) report a higher incidence of PC cases (Li et al., 2021a; Huang et al., 2021). In addition, the incidence of GI cancers is influenced by several factors such as lifestyle habits and dietary choices as well as age, with elderly males having a higher risk of incidence and mortality compared to females. This emphasizes the complex interplay of environmental and biological factors in the development of GI cancers (Sung et al., 2021).

The existing standard treatment options for GI cancers comprise surgery, chemotherapy, radiation therapy, targeted therapy, and immunotherapy (Nakayama et al., 2013; Anwanwan et al., 2020; Biller and Schrag, 2021; Joshi and Badgwell, 2021). Although combination regimens offer higher response rates and improved survival rates than single-agent therapy, it is crucial to consider the toxicity profile of these regimens closely (Chakraborty and Rahman, 2012; Miller et al., 2016; Jin and Mills, 2020). Reducing GI cancer mortality involves

TABLE 2 List of repurposed drugs proposed for targeting pancreatic cancer.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
AM580	Increasing tumor sensitivity to chemotherapy	Acute promyelocytic leukemia	Pancreatic ductal adenocarcinoma	Upregulation of Meflin expression	*In vitro* (Pancreatic Stellate Cells)	Iida et al. (2022)
Bazedoxifene	Inhibition of cell viability and migration	Selective estrogen receptor modulator	Pancreatic ductal adenocarcinoma	Inhibition of STAT3 activation mediated by interleukin 6 (IL-6) and 11 (IL-11)	*In vitro* (AsPC-1, PANC-1, HPAF-II, BxPC-3, HPAC, Capan-1 cell lines), and *in vivo* (mouse xenograft model)	Wu et al. (2016)
Carglumic acid	Induction of apoptosis	Hyperammonemia	Pancreatic ductal adenocarcinoma	COX-1/2, ANXA1-NF–κB axis, CDX2, COMMD1–RelA axis	*In vitro* (Capan1, AsPc1/luc, and PanO2/luc cell lines), and *in vivo* (mouse model)	Chen et al. (2015a)
(Hydroxy)-Chloroquine	Inhibition of proliferation	Malaria Systemic Lupus Erythematosus Rheumatoid arthritis	Pancreatic Cancer	Inhibition of autophagy in PSCs through reduced IL-6 expression and ECM protein production, Reduction of metastatic PC cells, ERK/MAPK inhibitors, Inhibition of CXCL12/ CXCR4 signaling, reduced phosphorylation of ERK and STAT3, downregulation of Hedgehog signaling	Clinical trial: Phase 1 (NCT01777477)	Samaras et al. (2017)
Doxycycline	Induction of apoptosis, and cell cycle arrest	Antibiotic	Pancreatic ductal adenocarcinoma	Impairment of mitochondrial biogenesis and oxidative phosphorylation, downregulation of PAR1/FAK/PI3K/AKT signaling	Clinical trial: Phase 2 (NCT02775695)	Liu et al. (2020c)
Digoxin	Induction of apoptosis	Atrial fibrillation, atrial flutter, and heart failure	Pancreatic Cancer	Nrf2 inhibitor	Clinical trial: Phase 2 (NCT04141995)	Zhou et al. (2019b)
Disulfiram	Induction of autophagy-dependent apoptosis, and ER stress	Drugs used in addictive disorders, Chronic alcoholism	Pancreatic ductal adenocarcinoma	Activation of the IRE1a-XBP1 pathway upregulation of p27 Inhibition of the NF-kB signaling pathway and downregulate stemness-related genes (HER2, c-myc and SOX9), Promotion of aponecrosis death pathways in K-Ras mutant PC cells, activation of the ER stress/IRE1α-XBP1 pathway	Clinical trial: Phase 2 (NCT03714555)	Zhang et al. (2019)
Efavirenz	Inhibition of cell proliferation, and induction of apoptosis	HIV infection	Pancreatic Cancer	ROS production and mitochondrial membrane depolarization, phosphorylation of both ERK1/2 and p38 MAPK stress pathways	Clinical trial: Phase 2 (NCT00964171)	Hecht et al. (2018)

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TABLE 2 (*Continued*) List of repurposed drugs proposed for targeting pancreatic cancer.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Emetine, Ouabain	Induction of cancer cell death	Emetine; Anti-protozoal, Ouabain; The cardiac glycoside	Pancreatic ductal adenocarcinoma	Interfering in hypoxia response	*In vitro* (ASPC-1, and PANC-1 cell lines), *in vivo* (patient-derived organoid)	Hirt et al. (2022)
Fulvestrant	This study focused on bioinformatic approaches	Fulvestrant; Metastatic breast cancer, Midostaurin; AML	Pancreatic ductal adenocarcinoma	Fulvestrant; target *ESR1,* Midostaurin; target *PRKA*	Clinical trial: Phase 1 (NCT04247126)	Mugiyanto et al. (2022)
Gemcitabine	Inhibition of DNA synthesis and induction of apoptosis	Antiviral	Bladder cancer; Pancreatic ductal adenocarcinoma; Non-small cell lung cancer; Ovarian cancer; Breast cancer	–	Clinical trial: Phase 4 (NCT02812992), FDA approved	Rebelo et al. (2021)
Haloperidol	Inhibition of proliferation by promoting ER stress, and induction of apoptosis	Psychosis	Pancreatic Cancer	*DUSP6*	*In vitro* (MIA PaCa-2, and PANC-1 cell lines)	Kim et al. (2012), Heer et al. (2022)
Ibrutinib	Induction of apoptosis	Antineoplastic agents (protein kinase inhibitors)	Pancreatic ductal adenocarcinoma	Mast cell-dependent antifibrotic effect	Clinical trial: Phase 3 (NCT02436668)	Massó-Vallés et al. (2015), Gunderson et al. (2016), Overman et al. (2020)
Itraconazole	Induction of apoptosis, and Inhibition of cell proliferation	Antifungal	Pancreatic ductal adenocarcinoma	Inhibition of TGF-β/ SMAD2/3 signaling ROS production and mitochondrial membrane depolarization, Bak-1 activation, TGF-β/ SMAD2/3 signaling suppression	*In vitro* (CFPAC-1, MiaPaCa-2, Panc-1, and BxPC-3 cell lines), and *in vivo* (mouse xenograft model)	Chen et al. (2018), Jiang et al. (2019)
Losartan	Inhibition of cell proliferation	Angiotensin II receptor antagonist	Pancreatic ductal adenocarcinoma	Inhibition of collagen I synthesis, Blockade of AT1R leading to inhibition of VEGF synthesis, increasing $CD8^+$ T cells, decreasing IL-1β, TANs and Tregs, inhibiting aberrant TGF-β activity	Clinical trial: Phase 2 (NCT03563248)	Diop-Frimpong et al. (2011)
Metformin	Inhibition of proliferation	Antidiabetic	Pancreatic ductal adenocarcinoma	Inhibition of mTOR, STAT3 and TGF-β1/ Smad2/3 signaling suppression of insulin/ IGF-I receptor activation and downstream signaling mediators IRS-1 and Akt Activation of AMPK	Clinical trial: Phase 2 (NCT01210911)	Karnevi et al. (2013), Nair et al. (2014), Kordes et al. (2015)
Nitroxoline	Induction of cell cycle arrest and apoptosis	Antiviral (Nelfinavir), Antibiotic (Nitroxoline)	Pancreatic ductal adenocarcinoma	ROS production, DNA damage response, mitochondrial depolarization and deregulation of cytosolic iron homeostasis	*In vitro* (AsPC-1, BxPC-3, and Capan-2 cell lines)	Veschi et al. (2018), Veschi et al. (2020)
Olanzapine	Inhibition of tumor proliferation	Antipsychotic	Pancreatic ductal adenocarcinoma	Inhibition of surviving in CSCs	*In vitro* (PANC-1, and PSN-1 cell lines)	Sanomachi et al. (2017)

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TABLE 2 (*Continued*) List of repurposed drugs proposed for targeting pancreatic cancer.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Parbendazole	Induction of apoptosis, and cell cycle arrest	Anthelmintic	Pancreatic ductal adenocarcinoma	Apoptosis induction, DNA damage, cell cycle arrest and alterations of tubulin distribution	*In vitro* (AsPC-1, and Capan-2 cell lines)	Florio et al. (2019)
Pentoxifylline	Inhibition of metastasis	Vasodilator	Pancreatic ductal adenocarcinoma	Reduction in collagen I and downregulation of alpha-smooth muscle actin and connective tissue growth factor Inhibition of chitinase 3-like-1	*In vitro* (BxPC3, and PANC-1 cell lines)	Xavier et al. (2021)
Pimavanserin	Induction of apoptosis	Parkinson disease psychosis	Pancreatic Cancer	Abrogation of Akt/ Gli1 signaling cascade leading to the downregulation of Oct-4, SOX2 and NANOG cancer stem cell markers	*In vitro* (AsPC1, BxPC3, MIAPaCa2, and PANC1 cell lines), and *in vivo* (mouse xenograft model)	Ramachandran and Srivastava (2020)
Pimozide	Induction of ER stress, cell cycle arrest, apoptosis and activation of the UPR	Antipsychotic	Pancreatic ductal adenocarcinoma	Inhibition of DRD2	*In vitro* (BxPC-3, Panc-1, MiaPaCa-2, Capan-1, and CFPAC-1 cell lines), and *in vivo* (mouse xenograft model)	Jandaghi et al. (2016)
Pirfenidone	Inhibition of proliferation and promotion of cell cycle arrest	Antifibrotic	Pancreatic ductal adenocarcinoma	Suppression of desmoplasia through regulation of PSCs, Suppression of PDGF-A, HGF, periostin, collagen type I and fibronectin, Cell cycle arrest and upregulation of p21 of PDAC cells, Inhibition of CHI3L1 and FN1, Downregulation of collagen I and TGF-β, Inhibition of fibronectin	*In vitro* (SW1990 cell line), and *in vivo* (mouse xenograft model)	Ji et al. (2017), Gao et al. (2019)
Propranolol	Induction of apoptosis	Hypertension	Pancreatic Cancer	Inhibiting the expression of NF-kB, AP-1 and CREB, as well as the expression of MMP-9, MMP-2 and VEGF target genes, decreasing Fz1, Wnt-1 and vimentin expression, downregulation of α7nAChR, ERK1/2 and p-CREB	Clinical trial: Phase 2 (NCT03838029)	Al-Wadei et al. (2009), Zhang et al. (2009), Zhang et al. (2010), Li and Xu (2019)
Pyrvinium	Inhibition of tumor cells in nutrient-depleted condition by targeting mitochondria	Anthelmintic	Pancreatic ductal adenocarcinoma	Inhibition of mitochondrial function, the WNT pathway, and cancer stem cell renewal	Clinical trial: Phase 1 (NCT05055323)	Schultz et al. (2021); Ponzini et al. (2023)
Ritonavir	Induction of apoptosis and cell cycle arrest	Antiviral	Pancreatic ductal adenocarcinoma	Induction of apoptosis and cell cycle arrest, through Inhibition of E2F-1 and AKT pathway, suppression of Akt and Rb phosphorylation	*In vitro* (BxPC-3, MIA PaCa-2, and PANC-1 cell lines)	Batchu et al. (2014)

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TABLE 2 (*Continued*) List of repurposed drugs proposed for targeting pancreatic cancer.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Somatostatin	Inhibition of angiogenesis and cell migration	Neuroendocrine inhibitor	Pancreatic Cancer	Cytotoxic, somatostatin receptors (SSTR) targeted therapy	*In vitro* (Capan-1, Capan-2, CAV, MIA PaCa-2, and Panc-1 cell lines), and *in vivo* (pancreatic tumor xenografts)	Li et al. (2005)
Simvastatin	Inhibition of metastasis	Dyslipidemia	Pancreatic ductal adenocarcinoma	KRAS	Clinical trial: Phase 2 (NCT00944463)	Liu et al. (2020b)
Trifluoperazine	Induction of apoptosis and necroptosis	Antipsychotic	Pancreatic ductal adenocarcinoma	Impairment of mitochondrial and ER homeostasis, induction of apoptosis and necroptosis and activation of the UPR	*In vitro* (MiaPaCa-2), and *in vivo* (patient-derived xenograft)	Huang et al. (2019)
Verteporfin	Induction of apoptosis	Antineovascularization agent (Verteporfin), Sensitizers in photodynamic therapy (protoporphyrin IX)	Pancreatic ductal adenocarcinoma	Activation of apoptosis via TAp73 activation, Inhibition of thioredoxin reductase, Inhibition of Hippo/ YAP signaling pathway	Clinical trial: Phase 2 (NCT03033225)	Huggett et al. (2014); Hanada et al. (2021)
Vorinostat	Inhibition of proliferation	HDAC inhibitors	Pancreatic Cancer	*FBP1*	Clinical trial: Phase 2 (NCT00831493)	Emamzaden et al. (2022)

identifying and implementing more efficient therapy strategies that lead to superior prognosis and/or fewer side effects. Although the discovery and development of novel drugs are crucial for halting and reversing disease effects, they demand substantial funding, broad experimentation, and subsequent investigations into efficacy, pharmacokinetics, and toxicity. Moreover, only a small percentage of these drugs, approximately 5%, undergo clinical trials which may be approved for clinical use after successful results from the three phases of clinical trials (Gupta et al., 2013). Thus, developing new drugs is a costly and time-consuming procedure that requires extensive resources and expertise.

Drug repurposing (DR), also known as drug repositioning, is an alternative and promising strategy for cancer treatment. It involves exploring the potential therapeutic applications of already approved drugs or withdrawn/outdated agents in the clinic (Tables 1–4; Supplementary Table S2; Figure 1). DR offers a substantial asset in drug development, uses the prior knowledge of the pharmacodynamics, pharmacokinetics and toxicity of already approved drugs which have undergone extensive animal and human studies (Gupta et al., 2013; Bertolini et al., 2015). Accordingly, repurposed compounds can be authorized for use in cancer treatment and other therapeutic applications rapidly, relying on the established safety profiles and efficacy data. Moreover, the regulatory approval process for repurposed drugs is often faster and less expensive, making DR an attractive alternative strategy for drug development (Chong and Sullivan, 2007). In this review, we aim to provide a critical overview of the current therapies for GI cancers, as well as examples of repositioned components, and the most promising candidate for drugs repurposing in GI cancers.

2 Current gastrointestinal cancers therapies, challenges, and limitations

The treatment approaches for GI cancer patients are diverse and contingent upon factors such as the patient's performance status, medical comorbidities, cancer type, stage, potential side effects and overall health (Joshi and Badgwell, 2021; Catalano et al., 2022; Ducreux et al., 2023). Treatment strategies encompass adjuvant chemotherapy, adjuvant chemoradiotherapy, preoperative chemoradiotherapy, endoscopic/colonoscopy resection, surgical resection, and perioperative chemotherapy. A comprehensive therapy regimen involves a combination of suitable treatment options to effectively address GI cancers (Sugarbaker, 2005).

Targeted therapy serves as a viable treatment option within various therapeutic regimens for GI cancers. Notably, for GC, HER2-targeted therapy (trastuzumab) and anti-angiogenesis therapy (ramucirumab) are major targeted therapies employed (Joshi and Badgwell, 2021). Ramucirumab is also considered as a targeted therapy alternative for patients with EC who have not responded well to initial treatment approaches (Fuchs et al., 2014). Erlotinib, an epidermal growth factor receptor (EGFR) blocking agent, has obtained Food and Drug Administration (FDA) approval for advanced PC patients (Moore et al., 2007). In the treatment of CRC, the commonly employed anti-angiogenesis agent, bevacizumab, in combination with chemotherapy can prolong the survival rate of advanced CRC patients (Xiong et al., 2021). Unresectable or metastatic hepatocellular carcinoma (HCC) patients commonly undergo a combination of anti-angiogenesis targeted therapy and immunotherapy as the predominant treatment strategies for HCC, while systemic chemotherapy proves ineffective in such cases (Greten et al., 2008).

TABLE 3 List of repurposed drugs proposed for targeting hepatocellular carcinoma.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Amiodarone	Inhibition of proliferation and induction of apoptosis, and autophagy	Antiarrhythmic	Hepatocellular Carcinoma	*mTOR*	*In vitro* (Hep 3B, HepG2 and Hu-H7 cell lines), and *in vivo* (HBx-transgenic mice)	Favoulet et al. (2001); Lan et al. (2014)
Atovaquone	Induction of apoptosis	Pneumonia	Hepatocellular Carcinoma	DNA double-stranded breaks	*In vitro* (HepG2, Hep3B, and Huh7 cell lines), and *in vivo* (mouse xenograft model)	Gao et al. (2018)
Bortezomib	Inhibition of proliferation	26S proteasome inhibitor	Hepatocellular Carcinoma	*FBP1*	Clinical trial: Phase 2 (NCT00077441)	Jin et al. (2017)
Canagliflozin	Inhibition of Cell proliferation, differentiation, stress response, and induction of apoptosis	Oral hypoglycemic	Hepatocellular Carcinoma	*ERK*, *p38*, *AKT*	*In vitro* (Huh7, HepG2, and HLE cell lines), and *in vivo* (patient-derived xenograft)	Kaji et al. (2018)
Dexamethasone	Inhibition of proliferation	Synthesized glucocorticoid	Hepatocellular Carcinoma	*FBP1*	Clinical trial: Phase 3 (NCT05711823)	Zhao et al. (2021)
Fenofibrate	Metabolic reprogramming	Antihypercholesterolemia	Hepatocellular Carcinoma	*PPARa*, *AKT*, *CTMP*	*In vitro* (Hep3B, Li7, Huh7, and HepG2 cell lines), and *in vivo* (mouse xenograft model)	Yamasaki et al. (2011), Chen et al. (2023)
Genistein	Inhibition of glycolysis and induction of mitochondrial apoptosis	Inhibits *HK2*	Hepatocellular Carcinoma	Downregulates *HIF-1α*, therefore inactivating *GLUT1* and *HK2*, enhances the antitumor effect of sorafenib in sorafenib-resistant HCC cells	*In vitro* (CC-LM3, SMMC-7721, Hep3B, Bel-7402, and Huh-7 cell lines), and *in vivo* (mouse xenograft model)	Li et al. (2017b)
Guanabenz acetate	Induction of apoptosis	Antihypertensive	Hepatocellular Carcinoma	DNA damage-inducible, *p34*, eukaryotic initiation factor 2α	*In vitro* (NU398, SNU423, SNU 449, SNU475, Huh7 cell lines), and *in vivo* (patient derived xenograft)	Kang et al. (2019)
Ketoconazole	Inhibition of tumor growth, invasion, and metastasis	Antifungal	Hepatocellular Carcinoma	*PTGS2*	*In vitro* (cell line-derived xenograft), and *in vivo* (patient-derived xenograft)	Chen et al. (2019a); Chen et al. (2019b)
LBH589	Inhibition of proliferation	HDAC inhibitors	Hepatocellular Carcinoma	*FBP1*	Clinical trial: Phase 1 (NCT00823290)	Yang et al. (2017)
Linagliptin	Immune destruction	Oral hypoglycemic	Hepatocellular Carcinoma	*ADORA3*	*In vitro* (HepG2, and Huh7 cell lines)	Ayoub et al. (2018)
Metformin	Inhibition of proliferation	Oral hypoglycemic	Hepatocellular Carcinoma	*KLF6/p21*, *AMPK*	Clinical trial: Phase 3 (NCT02319200)	Vacante et al. (2019)
Niclosamide ethanolamine	Inhibition of proliferation and angiogenesis, induction of apoptosis	Anthelmintic	Hepatocellular Carcinoma	*STAT3*	*In vitro* (HepG2, Huh7, Hep3B, Hep40, PLC/PRF/5, SNU-398, SNU-449, SNU-182, SNU-475 and SNU-423 cell lines), and *in vivo* (patient-derived xenograft)	Chen et al. (2017a)
Obeticholic acid	Inhibition of proliferation and angiogenesis and metastasis, induction of apoptosis	Primary biliary cholangitis	Hepatocellular Carcinoma	IL-6/STAT3 pathway	*In vitro* (HepG2, Huh7, and SNU-449 cell lines), and *in vivo* (orthotopic liver tumor model)	Attia et al. (2017), Gou et al. (2022)
Simvastatin	Controlling of tumor growth via metabolism reprogramming	Antihypercholesterolemia	Hepatocellular Carcinoma	*AMPK*, *STAT3*	Clinical trial: Phase 2 (NCT02968810)	Wang et al. (2017), Dehnavi et al. (2021)

(Continued on following page)

TABLE 3 (*Continued*) List of repurposed drugs proposed for targeting hepatocellular carcinoma.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	References
Tranylcypromine	Inhibition of proliferation	LSD1 inhibitor	Hepatocellular Carcinoma	*FBP1*	Asian cohort study: population-based nested case-control study	Chen et al. (2017c)
Valproate	Prevention of proliferation via Reactive Oxygen Species (ROS)-mediated cytotoxicity	Antiepileptic	Hepatocellular Carcinoma	*HDAC*	*In vitro* (HepG2 cell line)	Rithanya and Ezhilarasan (2021)
Vorinostat	Blocking growth promoting signal transduction pathways and inhibition of proliferation	Histone deacetylase inhibitor	Hepatocellular Carcinoma	*ERK/NF-κB signaling*	Clinical trial: Phase 1 (NCT01075113)	Gordon et al. (2019)

TABLE 4 List of repurposed drugs proposed for targeting Gastric cancer.

Drug name	Mechanism of action	Original indication	Proposed indication	Reported targets/ pathways	Status	Ref
Sulfasalazine	Inhibition of proliferation and metastasis and induction of ferroptosis	Rheumatoid arthritis	Gastric cancer	_	Clinical trial (EPOC1205)	Shitara et al. (2017)
Trastuzumab	Inhibition of cell proliferation	Oncology drug, HER2 positive breast cancer	HER2 positive Gastric cancer	HER2	Approved (NCT01260194)	Rose and Bekaii-Saab (2011)
6-Thioguanine	Induction of cell death	Antimetabolite, guanine analog, acute and chronic myelogenous leukemias	Gastric cancer	Ferroptosis inducer	*In vitro* and *in vivo* (xenograft mouse model)	Zhang et al. (2022a)

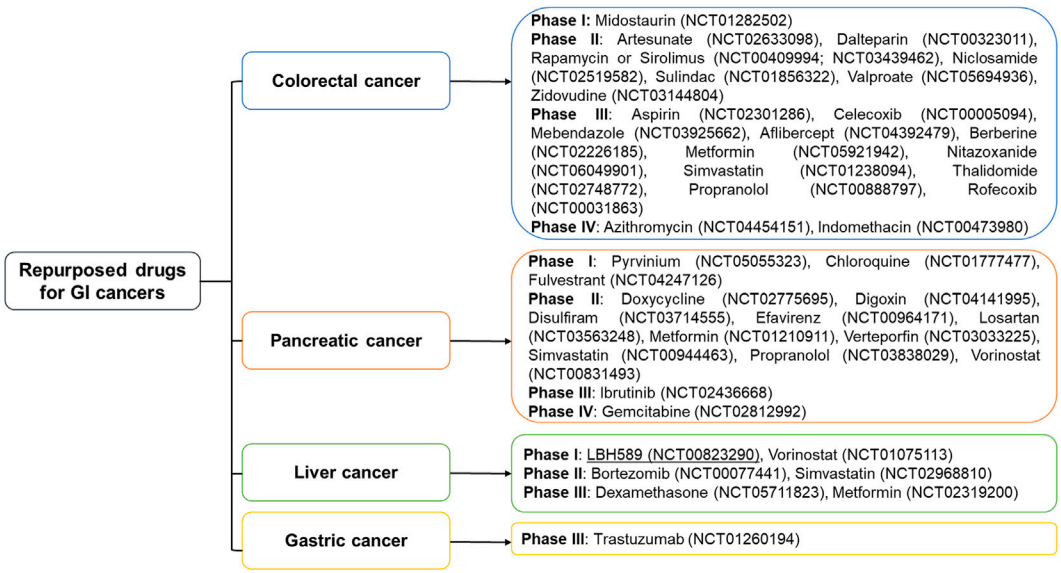

FIGURE 1
Repurposed drugs currently being evaluated against gastrointestinal cancers in various clinical phases. Metformin, simvastatin, and propranolol are entering phases 2 and 3 to treat more than 2 gastrointestinal cancers. Two drugs indomethacin and azithromycin for colorectal cancer, and gemcitabine for pancreatic cancer have reached phase IV.

Immunotherapy, aimed at restoring immune system function, represents another line of therapy for patients with advanced GI cancer. An alternative treatment for metastatic CRC, HER2-positive EC, and advanced GC patients who exhibit Programmed Cell Death Ligand 1 (PD-L1) or microsatellite instability-high (MSI-H) biomarkers and are resistant to chemotherapy is pembrolizumab, an anti-PD-1 antibody, which functions by targeting the PD-1 receptor on tumor cells and preventing their evasion from the immune system. In addition, dostarlimab is another alternative option for the treatment of PCs with MSI-H or deficient mismatch repair (dMMR). Nivolumab either alone or in combination with ipilimumab is employed to treat adults with MSI-H or dMMR who have metastatic and drug-resistant CRC or EC (Zhang X. et al., 2022; Teixeira Farinha et al., 2022). It is worth noting that dostarlimab and nivolumab act upon the PD-1 receptor, whereas ipilimumab specifically targets CTLA-4. For additional information on this subject, refer to the Supplementary Material, Supplementary Table S1.

Despite the range of therapy options available for GI cancers, the high mortality rate highlights the limited efficiency of current treatments for these malignancies (Sung et al., 2021). Several challenges impede the improvement of existing therapeutic strategies, including chemotherapy resistance, tumor heterogeneity, late diagnosis, and limited efficiency of certain treatments, all contributing to treatment failure in GI cancer patients (Au et al., 2017; Parikh et al., 2019; Raziq et al., 2020). The development of drug resistance involves a complex multi-step process influenced by a variety of contributing factors. Numerous studies have shown that intensified DNA repair, apoptosis or autophagy disorders, epithelial-mesenchymal transition, inactivation of drug-metabolizing enzymes, and changes in expression or activity of membrane transporters are potential factors that promote chemotherapy resistance (Zheng, 2017). Furthermore, In the context of GI cancers, the unique characteristics of certain gastrointestinal tumors pose significant challenges to their effective treatment. For instance, the pancreas is anatomically situated in a hard-to-reach location, leading to difficulties in early diagnosis and the absence of effective screening techniques presents a significant challenge in detecting PC during its initial stages (McGuigan et al., 2018). Despite endeavors to implement personalized treatments, PC has not exhibited convincing outcomes comparable to those seen in other cancer types (Chantrill et al., 2015). The efficiency of radiotherapy in EC is limited due to the prevention of TAZ (Transcriptional Activator with PDZ-Binding Motif) ubiquitination and degradation (He et al., 2021). Furthermore, epigenetics and non-coding RNAs play a critical role in EC-related multidrug resistance and affect the effectiveness of therapies. In fact, EC's susceptibility to recurrence, metastasis, and drug resistance development after first-line treatment, highlights the urgent requirement for optimizing the medicine regimen (Liu et al., 2021; Wei et al., 2021).

3 Drug repurposing

DR, is a strategic approach aimed at investigating alternative therapeutic applications of existing approved medications, beyond their originally intended uses. This method offers significant advantages in improving treatment outcomes, primarily by circumventing several essential stages of drug development. This results in reduced expenditure, shorter clinical trial durations, and mitigated risks associated with clinical trial failures due to adverse reactions (Ashburn and Thor, 2004; Chong and Sullivan, 2007; Zamami et al., 2017).

Repurposing FDA-approved medications is an efficient and inexpensive method to address oncology needs, including cancer treatment and reducing problems from existing anticancer treatments or radiation therapy (Ciociola et al., 2014). The drug-repurposing strategy has various advantages, including a shorter development period (usually 3–5 years), reduced costs (under $10 million), and higher success rates than original drug research (Morgan et al., 2011). Furthermore, DR can offer numerous benefits in overcoming therapeutic challenges by targeting various components both inside and outside of cancer cells (Würth et al., 2016). The concomitant use of multiple drugs that can target different tumor subtypes simultaneously can be a remarkable plan to overcome tumor heterogeneity (Li and Jones, 2012). Moreover, DR reveals the anticancer potential of non-oncology drugs with fewer side effects than traditional chemotherapy, making it a valuable option for cancer treatment (Crawford, 2014; Ferioli et al., 2018). Repositioned drugs can be used in combination with regular chemotherapy, and some demonstrate selectivity in causing cytotoxicity to cancer cells while sparing non-cancerous cells (Foglietta et al., 2021).

Drug repositioning involves a multi-step process. Initially, drug selection can start with *in silico* methods like molecular docking, pathway matching, and genome-wide association studies to create a ranked list of compounds. The next step is secondary analysis, which includes experimental techniques to refine and prioritize these compounds. Tertiary analysis aims to validate these compounds using cell cultures and animal models. Finally, the chosen drugs are advanced to clinical trials, and successful ones are repurposed for new uses (Shameer et al., 2015; Kulkarni et al., 2023). The key stages of DR are elucidated in (Figure 2).

4 Approaches used for drug repurposing in cancer therapy

To evaluate the repurposing of an existing drug as a potentially effective anti-cancer agent, a mechanistic assessment of the drug's action in preclinical models is crucial and requires systematic approaches (El-Hachem et al., 2017; Nagaraj et al., 2018). These approaches can be classified into two categories, computational and experimental, which use available data and biochemical experiments, respectively, to explore the potential of repurposing existing drugs as novel cancer treatments (Luo et al., 2017; Fernández-Torras et al., 2019). Indeed, successful DR is contingent upon the integrated and synergistic use of both approaches (Mottini et al., 2021; Palve et al., 2021).

4.1 Computational approaches

Data-driven computational approaches involve the systematic analysis of data from different sources such as gene expression,

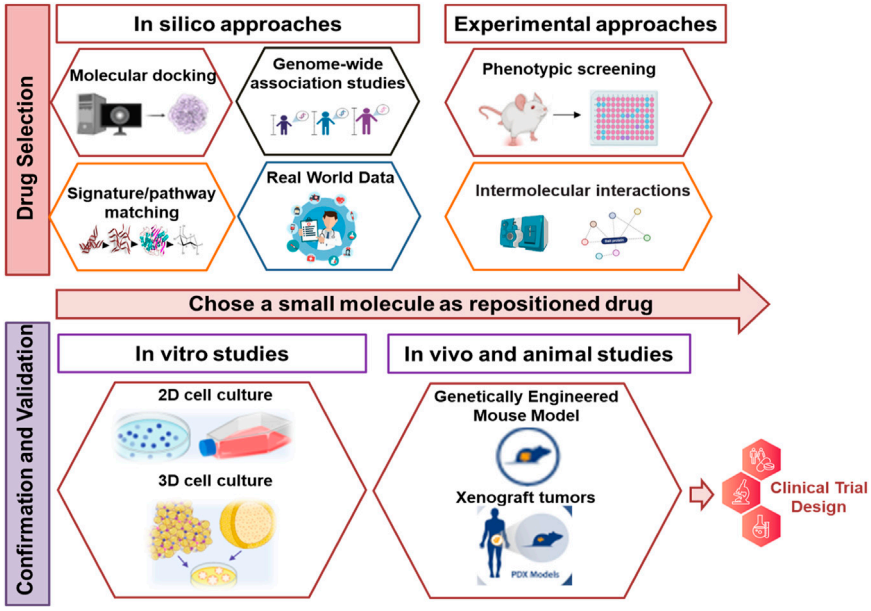

FIGURE 2
The key stages of drug repurposing. The process of DR begins with the use of computational or experimental techniques to select candidate drugs. Subsequently, these selected drugs are subjected to additional validation through potential evidence and additional experimental methods. Compounds that successfully pass through this rigorous evaluation then advance to clinical trials to obtain the FDA approval. Upon approval, these drugs are introduced to the market and their labeling is updated to reflect their new applications.

chemical structure, genotype or proteomic data, or electronic health records (EHRs), which lead to the establishment of hypotheses on DR (Hurle et al., 2013). Carla Mottini et al. (2021) thoroughly reviewed computer-aided DR strategies for cancer therapy, offering examples of this approach in cancer studies within their article (Mottini et al., 2021). Future research should improve advanced computational tools, like machine learning and artificial intelligence, to improve therapeutic efficacy and safety prediction (Prasad and Kumar, 2021). Furthermore, computational methods can be used to address current anticancer medications or radiation therapy issues in addition to cancer treatment (Dalwadi et al., 2023). The most commonly used computational approaches are discussed below.

4.1.1 Molecular docking

Molecular docking, a structure-based computational strategy, predicts complementarity of binding sites between the ligand (drug) and the receptor (target) (Hurle et al., 2013; Knapp, 2018; Mottini et al., 2021; Palve et al., 2021). If prior knowledge is available about a receptor target involved in cancer, multiple drugs could be investigated against that specific target, or drug libraries can be screened for a collection of target receptors to identify new interactions suitable for repurposing (Honarparvar et al., 2014; Nero et al., 2014). Dakshanamurthy and others performed molecular fit computations on a list of 3,671 FDA-approved drugs against 2,335 crystal structures of human proteins. They experimentally validated that mebendazole, an anti-parasitic agent, shows significant potential to bind to the vascular endothelial growth factor receptor 2 (VEGFR2) and effectively block angiogenesis (Dakshanamurthy et al., 2012). Furthermore, it has been predicted that levosimendan, a heart failure drug, could serve as a potential inhibitor of several kinases including RIO Kinase 1 (RIOK1), through ligand-binding site comparison and protein-ligand docking. Levosimendan shows anti-cancer activity against various cancers by directly inhibiting RIOK1 and RNA processing enzymes (Lim et al., 2019). Similarly, Arulanandam CD et al. (2021) suggested itraconazole as a better inhibitor of platelet-derived growth factor receptor alpha (PDGFRA) compared to other antifungal drugs for treating gastrointestinal stromal tumors (GISTs) (Arulanandam et al., 2021).

4.1.2 Genome-wide association studies

Genome-Wide Association Studies (GWAS) aim to identify variants associated with common genetic disorders, thereby providing new insights into the biology of the disease. The novel associations between genes and cancer through GWAS and Phenome-Wide Association Studies (PheWAS) enable the identification of new targets for existing drugs leading to the repositioning of drugs (Zhang et al., 2015; Sud et al., 2017; Kim et al., 2018). Grover et al. (2015) employed a bioinformatics strategy to match gene targets for coronary artery disease (CAD) with drug information collected from three different drug–target databases (DrugBank, Therapeutic Target Database, and PharmGKB) to identify potential therapeutics for repositioning toward treatment of CAD (Grover et al., 2015). Another valuable resource for medication indications, called Medication Indication Resource (MEDI), can validate the plausibility of inferring novel drug indications with clinical potential (Wei et al., 2013; Bejan et al., 2015). Furthermore, a genome-wide positioning systems network algorithm uncovered the antitumor activity of ouabain, an approved drug for cardiac arrhythmia and heart failure, in lung adenocarcinoma cells (Cheng et al., 2019).

GWAS results may pose challenges when applied to DR due to several reasons. Firstly, GWAS signals in gene-rich loci, where linkage disequilibrium commonly occur, can complicate the identification of the associated genes and their specific variants. Secondly, the direction of the gene variant's effect may not be immediately apparent, necessitating functional studies to determine whether they act as activators or suppressors for disease control (Sanseau et al., 2012). Moreover, GWAS results may not provide detailed pathogenic information on genetic diseases (Wang and Zhang, 2013). It is important to acknowledge that understanding of the human genome is continually evolving, and new genes may continue to be discovered (Willyard, 2018). Therefore, careful consideration and further research are necessary when leveraging GWAS data for DR endeavors.

4.1.3 Signature or pathway matching

The signature matching method represents an innovative and promising approach within the realm of DR, as it involves comparing the distinct characteristics of a drug with those of other drugs, disease or clinical phenotype (Hieronymus et al., 2006; Keiser et al., 2009). The drug signatures can be derived from three different sources of data including omics data, chemical structures and adverse event profiles (Pushpakom et al., 2019). As a specific example, a study employed an optimal approach including two novel benchmarking standards, namely, area under the curve (AUC)-based standard and Kolmogorov-Smirnov (KS) statistic-based standard for signature-based DR and reported homoharringtonine (HHT) as a potential agent in the treatment and prevention of LC (Yang et al., 2022). Furthermore, various omics studies (transcriptomic, proteomic, or metabolomics) in the field of cancer not only provide large-scale data for supporting DR through applying advanced bioinformatics, but also expand our knowledge of hallmarks of cancer at the molecular level (Fernandez-Banet et al., 2016; Chen B. et al., 2020). Chemical structures serve as another type of signature matching used in DR; where the comparison of the chemical features of each drug with others helps identify possible chemical similarities that could signify shared biological activity (Pushpakom et al., 2019). A recent research employed this method along with other conventional DR methods to create a multilayer network algorithm. This algorithm was used to rank drugs that possibly can be repurposed for various types of cancer, including GI cancers and to explore new therapeutic possibilities for existing drugs (Cheng et al., 2021).

The unique adverse event profile of a drug can serve as a proxy for its related phenotypic effects, and drugs with similar side effects may act on the same target protein or pathway (Dudley et al., 2011; Cheng et al., 2021). In addition, if a drug's phenotypic response resembles that of a disease, it may indicate that the drug and disease share pathways and physiological mechanisms (Pushpakom et al., 2019). Pathway and network-based approaches have been broadly used to identify drugs or drug targets with the potential for repurposing (Smith et al., 2012). Moreover, despite some of the targets identified by GWAS or other analytical networks appearing potent for drug targets, many of these genes may not be ideal in practical drug targeting applications. In such circumstances, a pathway-based approach can provide insights into genes upstream or downstream of the GWAS-associated target and could be considered as potential repurposing opportunities (Greene and Voight, 2016). In a recent study, the network-wide association study (NetWAS) technique has been employed, combining GWAS-identified genetic variant information with tissue-specific functional networks to identify disease-relevant genes more accurately than GWAS alone. Using NetWAS on the concept of hypertension and incorporating drug–target data from the DrugBank, Greene et al. (2015) observed an expansion in the number of the top target genes for the anti-hypertensive drugs compared to the GWAS (Greene et al., 2015). Additionally, pathway analysis of gene expression data obtained from a wide range of studies of human viral respiratory diseases identified 67 signaling pathways which may play important roles in respiratory viral infections (Smith et al., 2012). In summary, DR candidates can be identified through construction of drug or disease networks using gene expression patterns, protein interactions, disease pathology or GWAS data along with the signature matching studies to complement the network analysis approach (Iorio et al., 2010, 2013).

4.1.4 Real World Data

Real World Data (RWD) includes information from electronic health records (EHRs) of patients, characterized by large and complex datasets (Booth et al., 2019; Eichler et al., 2019). EHRs store patient and population health information in digital format, providing a wealth of data on patient outcomes (Miriovsky et al., 2012; Luhn et al., 2019). While diagnostic and pathophysiological data including experimental and drug prescribing data are structured, a significant portion of EHR data, such as clinical descriptions of patient symptoms and signs as well as imaging data remains unstructured (Khozin et al., 2017; Booth et al., 2019). Both structured and unstructured data from patients can serve as valuable sources to identify consistent signals for drug repurposing (Hurle et al., 2013; Low et al., 2017).

The retrospective clinical trial analysis is a commonly used computational approach based on RWD, with data commonly extracted from EHRs (Gray et al., 2019; Zheng et al., 2020). Sildenafil is an interesting example of retrospective clinical analysis which led to the repurposing of a candidate molecule (Ashburn and Thor, 2004). A classical example of repurposing a noncancer drug for cancer therapy through retrospective clinical analysis is metformin, which has shown to reduce cancer mortality in a dose-dependent manner (Sadeghi et al., 2012; Chaiteerakij et al., 2016). Furthermore, Xu et al. (2015) conducted a clinical cohort study using EHRs from Vanderbilt University Medical Center and Mayo Clinic, confirming the favorable effects of metformin in cancer survival including breast, colorectal, lung and prostate cancers in independent populations (Xu et al., 2015). Other successful examples of repurposing opportunities through retrospective clinical and/or pharmacological analyses include raloxifene in breast cancer, aspirin in CRC, propranolol in osteoporosis, and valproate in acute myeloid leukemia and glioblastoma (Cavalla and Singal, 2012; Happold et al., 2016; Lübbert et al., 2020).

4.2 Experimental approaches

4.2.1 Phenotypic screening

Phenotypic screening, a direct method of DR, involves identifying compounds based on their effects in model systems

without prior knowledge of candidate drug targets (Moffat et al., 2014, 2017; Kim et al., 2019). This approach typically uses a wide range of cell-based *in vitro* assays in a 96- or 384-well format (Sala et al., 2010). Iljin and others (2009) have performed high-throughput cell-based screening of a library including 4,910 drug-like small molecules against prostate cancer and non-malignant prostate epithelial cell lines using proliferation as the primary phenotypic criteria. They found an anticancer effect of disulfiram, a medication used to treat alcohol abuse, which was subsequently validated through genome-wide-gene expression studies (Iljin et al., 2009). Moreover, whole animal screening assays can be employed for DR which offer insights into efficient anticancer drugs, as well as pharmacokinetic and organ-toxicity potential results (Yadav et al., 2016; Baxendale et al., 2017). Cousin et al. (2014) evaluated 39 FDA-approved medications using a zebrafish model for tobacco dependence treatment and identified compounds like apomorphine and topiramate that modulate the behavioral effects of nicotine and ethanol in this model (Cousin et al., 2014). Similarly, over 26,000 small molecules were evaluated for their efficacy against leukemia using a genetically engineered T-cell reporting zebrafish model and discovered the remarkable activity of lenaldekar, against various hematologic neoplasms (Clements and Traver, 2012; Ridges et al., 2012).

4.2.2 Intermolecular interactions

Proteomic techniques including affinity chromatography and mass spectrometry reveal protein-protein interactions based on the intermolecular force. These techniques have been used to identify binding partners for various drugs, thus facilitating DR due to an experimentally based pharmacological analysis (Brehmer et al., 2005; Shantikumar et al., 2015). In this approach, drug treatments are administered to cells or animals, followed by a deep proteome analysis to identify protein changes (Savitski et al., 2018). An early successful example of this technique involves the validation of more than 20 cellular targets for the epidermal growth factor receptor kinase inhibitor gefitinib using mass spectrometry (Brehmer et al., 2005).

The Cellular Thermo Stability Assay (CETSA) has been introduced as a method based on altered protein thermal stabilization/destabilization in response to ligand binding which can predict drug targets when combined with thermal proteome profiling (Martinez Molina et al., 2013; Li J. et al., 2020; Mateus et al., 2020; Perrin et al., 2020). Additionally, chemical genetic approaches such as kinase drug discovery also rely on intermolecular forces and can provide insights into the relationship between binding and drug efficacy (Sloane et al., 2010; Cong et al., 2012; Wong et al., 2016). These findings can be interpreted quickly into new clinical applications or to improve drug resistance outcomes which are near-inevitable phenotypic responses to protein kinase inhibitors for the cancer treatment (Carter et al., 2005; Bago et al., 2016). Karaman et al. (2008) used a competition binding assay *in vitro* to evaluate 38 protein kinase inhibitors against a panel of 317 pathologically significant human protein kinases. Their analysis identified 3,175 binding interactions, revealing that some drugs such as sorafenib and dasatinib showed higher affinity to other kinase targets than their known target (Karaman et al., 2008). The development of chemical-genetic approaches, particularly non-kinase targets of small molecules planned to inhibit kinases is becoming increasingly recognized (Munoz, 2017) resulting in new repurposing opportunities for cancer treatment, such as the use of anthelmintic drug niclosamide to treat Zika virus infection (Xu et al., 2016) and the potential to treat drug-resistant pathogens (Sun et al., 2016).

4.3 Experimental approaches to validate repurposed drugs

In order to understand the mechanism of action of repurposed drugs for cancer treatment, it is often essential to evaluate and validate their effects within a comprehensive system that considers safety, dosage and toxicity before advancing to clinical trials. This evaluation can be accomplished using various models, which are divided into two main categories: *in vivo* and *in vitro* models. These models should be reproducible, cost-effective, and quickly constructed (Würth et al., 2016).

4.3.1 *In vitro* studies

In *in vitro* tumor models, cancer cell lines are the predominant choice, followed by primary cells, although these models may also incorporate immune cells, stem cells, and stromal cells alongside cancer cells (Martinez-Pacheco and O'Driscoll, 2021). When using these cell types for detecting repositioning activity, *in vitro* assays offer several advantages including the ability to examine multiple substances with distinct mechanisms of action across a wide concentration-effect range, depending on the throughput of the experiment. Furthermore, these assays provide direct knowledge about potential new disease settings and allow testing of different drugs with novel mechanisms of action (Wilkinson and Pritchard, 2015).

In vitro screening approaches have been used for medication and DR in the various GI cancer types such as CRC, PC, GC and LC. A deep understanding of cancer progression and treatment has prompted the development of accurate *in vitro* tumor models that better represent the physiological features of the tumor microenvironment. Consequently, recent *in vitro* tumor models have become increasingly complex, extending beyond monitoring primary cell behaviors such as proliferation, invasion and cytotoxicity. These advanced *in vitro* models recapitulate important metastasis steps including angiogenesis, extracellular matrix remodeling and tumor cell metabolism reprogramming and dormancy (Wu and Swartz, 2014).

4.3.1.1 Two-dimensional and three-dimensional cell cultures

Cell culture plates and Transwell-based models are widely employed to assess viability, apoptosis, intra/extravasation and matrix remodeling of cancer cells. Two-dimensional (2D) models have been extensively used for drug screening and drug repurposing (Hulkower and Herber, 2011; Yu et al., 2020; Tomi-Andrino et al., 2022). In recent years, three-dimensional (3D) cell culture methods have become popular as they replicate *in vivo* microenvironmental providing data with greater predictive value for clinical outcomes. These authentic 3D cell culture models using human cells can overcome the limitations of mice models, which in addition to their high cost and ethical implications, are not always capable of

accurately mimicking human illnesses or capturing medication side effects such as liver damage (Sivaraman et al., 2005). Moreover, by simulating the cell culture environment, 3D cell cultures can encourage specific cell activity, allowing drug discovery to target cell behavior with greater precision, such as enhancing cellular motility, promoting epithelial cell proliferation and differentiation, inducing cell dormancy, supporting of stem cell-like characteristics or mimicking desired microenvironment, like metastatic niches (Valastyan and Weinberg, 2011). According to their importance and applications in cancer research or DR studies, 3D cell culture models can be categorized into two major groups: Spheroids and Organoids.

4.3.1.2 Spheroids

Spheroids are cell aggressions that grow in suspension or are embedded in a three-dimensional matrix using three-dimensional culture methods. Cancer cell spheroids represent avascular tumor nodules, also known as micro-metastases and are widely used for drug screening despite being more expensive and time-consuming than 2D cell cultures. Spheroids also recapitulate interaction between cells and matrix in the tumor microenvironment and their size-dependent structure includes a necrotic central nucleus, resembling tumors with poor angiogenesis. Moreover, tumor spheroids offer valuable insights into how tumors respond to candidate drugs and combination therapies which reduces the need for animal testing and providing a more realistic representation of the tumor microenvironment (El Harane et al., 2023).

4.3.1.3 Organoids

Organoids are novel *ex vivo* tumor models composed of self-organized three-dimensional multicellular tissue cultures derived from stem cells, primary tissue specimens or cancer cell lines. These models are capable of mimicking the *in vivo* organ (Simian and Bissell, 2017). Patient-derived cancer organoids provide a promising opportunity to predict drug efficiency and treatment response in GI cancers. Furthermore, incorporating 3D cell culture technology with primary patient-derived cancer cells, molecular characterization data, or the establishment of GI organoid banks representing molecular tumor subtypes could lead to preclinical assessment of personalized drug targets to improve treatment outcomes by reducing side effects in cancer therapy (Perrone and Zilbauer, 2021).

The first CRC organoid biobank was established in 2015 for drug screening, enabling the study of gene-drug interactions to recognize potential treatment response biomarkers and understand the molecular basis of drug response (van de Wetering et al., 2015). Notably, numerous studies have reported a significant overlap between esophageal adenocarcinoma organoids and tumor response to standard chemotherapy (Donohoe and Reynolds, 2017; Li et al., 2018) and a similar approach yielded comparable outcomes using GC organoids (Verduin et al., 2021). These results highlight the benefits and advantages of 3D *ex vivo* models in DR and repositioned drug efficiency assessments.

4.3.2 *In vivo* studies of drug repurposing

Animal models offer a powerful tool to simulate physiological conditions and complicated interactions, as well as the responses of reactions of different cell types and tissues to chemicals (Ruggeri et al., 2014). During the drug development process, *in vivo* testing plays a crucial role as it allows the study of interactions between the drug and both target and non-target cells.

Based on the premise of evolutionarily conserved pathogenetic mechanisms, animal models such as zebrafish, mice, fruit flies, worms and yeast have been used to give a comprehensive understanding of the biological mechanisms underlying the effect of drug administration. Xenograft models and genetically engineered mouse cancer models were among the *in vivo* models used in drug repurposing (Freires et al., 2017).

4.3.2.1 Xenograft tumors

The human tumor xenograft is a widely used *in vivo* model where human cancer cells are transferred into immunocompromised (nude) mice through either ectopic or orthotopic implantation. Although the cell line-derived xenograft (CDX) model is considered as the gold-standard model for cancer research and investigation of anti-tumor therapies, patient-derived xenograft (PDX) tumors can also be used for this purpose (Tentler et al., 2012).

Xenograft tumors are highly used as animal models in GI cancer and DR research (Onaciu et al., 2020). *In situ* GI cancer can be induced by locally injecting of cell lines or implantation of tumor cells, while the metastatic models are established by injection of the tumor cells through the tail vein or into the specific organ (Morton and Houghton, 2007; Tentler et al., 2012). For decades, athymic nude mice and mouse xenograft models using human tumor cell lines have been used to study tumor progression factors. In the DR approaches, the use of xenograft tumors can be very helpful in assessing the accuracy of *in vitro* study results and the effectiveness of drugs on cancer cells in safe doses under *in vivo* situations. Doxycycline, a tetracycline-class antibiotic commonly used to treat bacterial and parasitic infections, has demonstrated to reduce tumor growth by ~80% in pancreatic tumor xenografts (Son et al., 2009; Liu H. et al., 2020).

4.3.2.2 Genetically engineered mouse models

The Genetically Engineered Mouse Model (GEMM) is another animal model for studying human cancer and conducting preclinical study of repurposed drugs to target special gene-derived GI tumors. Genetic technologies have been recently applied by an increasing number of studies to introduce oncogenes into mouse embryonic or somatic cells through tissue-specific promoters targeting the GI tract and inducing GI cancers (Kersten et al., 2017). Transgenic, gene knock-in, and gene knock-out techniques are used to modify the genetic sequence of these mice in order to transfer, mutate, delete or overexpress one or more genes associated with transformation or malignancy. For instance, transgenic mice overexpressing *KRAS* mutant genes can mimic pancreatic tumorigenesis. Indeed, while general single-gene modified models may not fully represent the entire process of GI tumorigenesis, it has been discovered that physiological levels of *KRAS* G12D induce ductal lesions that serve as putative precursors to invasive PC (Westphalen and Olive, 2012). Additional genetic modifications, such as *P53* mutation, can promote tumorigenesis and metastasis (Walrath et al., 2010). Another gene that can be engineered to produce GI cancer GEMM is carbonic anhydrase, present in the basolateral membranes of gastrointestinal epithelial cells and Its overexpression has been reported in many carcinomas including GC. Mice with null mutations of the *Car9* gene develop gastric hyperplasia in glandular

epithelium after 1 month (Gut et al., 2002). Additionally, ApcMin/Pten−/− mice are developed to study CRC (Stastna et al., 2019).

GEMM animals can be used to study the impact of a specific gene on tumorigenesis, or to investigate whether a *de novo*/repositioned drug acts through predicted pathways or genes to control the disease (Richmond and Su, 2008). Kobayashi et al. (2017) used mogp-TAg transgenic mice for DR to target the mevalonate pathway as a key tumorigenesis pathway in ovarian cancer (Kobayashi et al., 2017). Furthermore, the effect of metformin cancer initiation and progression suppression was studied using transgenic KPC mice (Chen K. et al., 2017). This animal model was also employed to evaluate the efficiency of repurposed histone deacetylase (HDAC) and mammalian target of rapamycin (mTOR) inhibitors on PC treatment (Biermann et al., 2022). Transgenic animals can be used to evaluate safe, first-in-human (FIH) doses during preclinical studies in drug development. For example, human-CYP3A4-expressing transgenic (Cyp3aXAV) mouse serve as practical model to evaluate the safe dosage and efficiency of CYP3A4-metabolized small-molecule drugs (Damoiseaux et al., 2022). The application of GEMM animal models can prove advantageous in specialized DR studies and allows researchers to focus on specific molecular pathways.

4.3.2.3 Chemically induced gastrointestinal tumors

Chemical agents can be used to induce GI cancers in mice, potentially leading to mutations in relevant human cancer genes. The most well-established chemically induced GC model is produced by the administration of N-Methyl-N-nitrosourea (MNU) (Tomita et al., 2011; Ji et al., 2020; Rabben et al., 2021b). MNU is an N-nitroso compound mostly generated by anaerobic gut bacteria in the presence of nitrates and nitrites (Sobko et al., 2005; Zhuang et al., 2017). Furthermore, 1,2-dimethylhydrazine (DMH) and its metabolite, azoxymethane (AOM), are the most commonly used chemical compounds and carcinogens to induce CRC in mice. It has also shown that intraperitoneal injection of azaserine in rats induces metastatic pancreatic acinar cell carcinoma, although 10% of animals develop tumors in other organs (Rosenberg et al., 2009). Another method to produce a chemically induced PC model is through topical application of benzopyrene which induces adenocarcinoma (Kong et al., 2020). This group of animal models is widely employed in DR studies for GI cancers. Kannen et al. (2012) used C57BL/6 mice exposed to the carcinogen N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) as a colonic carcinogen mouse model to evaluate the antiproliferative effect of fluoxetine, an antidepressant medicine, on colon cancer (Kannen et al., 2012). In a similar study, the inhibitory effects of Liuwei Dihuang Pill (LDP) were investigated on MNU-induced gastric tumorigenesis in diabetic mice (Zhuang et al., 2017).

5 Repurposed drugs for gastrointestinal cancers treatment

5.1 Colorectal cancer

Researchers in the field of Gl cancer treatment are exploring innovative therapeutic strategies through drug repurposing, with an extensive focus on leveraging existing medications for the management of CRC. Among the studies of significance, Antona et al. (2022) have recently showed that spiperone, an approved drug for schizophrenia treatment, triggers apoptosis in CRC cells by activating phospholipase C, disrupting intracellular calcium balance, inducing irreversible endoplasmic reticulum stress, causing lipid metabolism changes, and damaging the Golgi apparatus (Antona et al., 2022). Furthermore, ten small molecules/drugs were identified via bioinformatic techniques for treating CRC, specifically targeting the upregulated Tissue Inhibitor of Matrix Metalloproteinases-1 (*TIMP1*) gene; these included established agents like formaldehyde and paclitaxel, as well as promising new drug candidates (Leng et al., 2022). Another study discovered that ebselen effectively inhibits Autophagy related protease 4B (*ATG4B*) through oxidative modification. This was based on the FDA-approved drug library, using Fluorescence Resonance Energy Transfer (FRET)-based high-throughput screening and gel-based analysis. The study showcased the potential of ebselen as an anti-CRC agent by influencing autophagy and tumor suppression (Xie et al., 2022). Mao et al. (2023) developed an efficient method for identifying repurposed drugs for CRC using organoid-based screening and computational drug prediction. Out of 335 tested drugs, 34 showed anti-CRC effects, with distinct transcriptome patterns including differentiation induction, growth inhibition, metabolism inhibition, immune response promotion, and cell cycle inhibition. Validation in patient-derived organoid-based xenograft (PDOX) systems demonstrated the anticancer effectiveness of drugs like fedratinib, trametinib, and bortezomib. (Mao et al., 2023). It has been revealed that the antifungal agent oxiconazole induces anti-tumor effects in CRC cells by inhibiting autophagy through downregulation of peroxiredoxin-2 (*PRDX2*), leading to growth suppression, and suggests its potential therapeutic use in combination with oxaliplatin for CRC treatment (Shi J. et al., 2022). Moreover, Dhakal et al. (2022) investigated the cytotoxic effects of the anti-anginal drug perhexiline and its enantiomers on CRC cells and demonstrated their ability to induce apoptosis and reduce cell viability in both monolayers and spheroids, as well as patient-derived organoids (Dhakal et al., 2022). Liñares-Blanco et al. (2020) proposed abemaciclib, an inhibitor of the CDK4/6 protein, as a promising option for the treatment of colon cancer (Liñares-Blanco et al., 2020). Furthermore, mebendazole, an antihelminthic medication used to treat gut worm infections, exhibited a cytotoxic effect on the RKO and HCT-116 colon cancer cell lines. Mebendazole was evaluated against a panel of kinases to determine the mechanism of its cytotoxic effect, which indicated significant inhibitory action against Abl and BRAF proteins. Additionally, in a case study of a patient with resistant metastatic colon cancer, twice-daily therapy with the normal antihelminthic dose of mebendazole led to a substantial reduction in metastasis (Nygren et al., 2013). A comprehensive investigation introduced several promising repurposing drugs (crizotinib, arsenic trioxide, vorinostat, dasatinib, estramustine, and tamibarotene) for CRC by prioritizing candidate genes obtained from the GWAS data (Zhang et al., 2015). Zhao P et al. (2022) highlighted the integration of metabolomics and transcriptomics as a powerful approach to gain insights into the antitumor mechanism of tadalafil, a phosphodiesterase type 5 (PDE5) inhibitor, in patients with CRC

(Zhao et al., 2022). The anti-cancer effect of niclosamide was confirmed in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice implanted with human CRC xenografts from patients with metastatic CRC and remarkable results were obtained with a non-lethal dose of 200 mg/kg (Osada et al., 2011). Tioconazole, originally used to treat vaginal yeast infections, was reported to enhance chemotherapy efficacy in colorectal tumor xenografts (Liu et al., 2018). Table 1 provides a comprehensive summary of various repurposed drugs in CRC.

5.2 Pancreatic cancer

In the field of PC treatment research, recent developments in DR have yielded promising strategies and novel candidates for therapeutic intervention. Pham et al. (2022) have recently introduced a deep learning framework that employs various genome-wide chemical-induced gene expression datasets to predict gene rankings in expression profiles induced by *de novo* chemicals, based on their chemical structures. They used this model for DR to identify potential treatments for PC from all existing drugs in DrugBank, and proposed candidates including dipyridamole, AZD-8055, linagliptin, and preladenant, which were subsequently validated *in vitro* (Pham et al., 2022). A recent study established a biobank of over 30 genetically distinct human pancreatic ductal adenocarcinoma (PDAC) organoid lines, demonstrating their correlation with the molecular and phenotypic heterogeneity observed in primary PDAC tissue and *in vivo* drug responses. Using a fully automated screening platform, this study conducted a DR analysis covering 1,172 FDA-approved drugs. Among the *in vivo* validated hits were several drugs currently approved for non-cancer indications, including emetine and ouabain. These drugs were found to specifically target PDAC organoids by disrupting their response to hypoxia. Notably, a dose of 0.56 mg/kg/d of ouabain significantly reduced PDAC xenograft growth in mice (Hirt et al., 2022). Mugiyanto et al. (2022) used genomic data from the cBio Cancer Genomics Portal to identify PC-associated and drug target genes. Through functional annotations, they prioritized 318 PC risk genes, of which 216 were druggable according to DrugBank. The Connectivity Map (CMap) Touchstone analysis revealed 13 potential PC drugs, including midostaurin and fulvestrant, which target *PRKA* and *ESR1* respectively, as promising candidates for PC treatment (Mugiyanto et al., 2022). Another study investigated whether aspirin (ASA) and oseltamivir phosphate (OP) treatment could enhance PC cell sensitivity to gemcitabine-induced cytotoxicity and hinder chemoresistance development. The combination of ASA and OP with gemcitabine significantly disrupted PC cell viability, clonogenicity, ECM protein expression, migration, and induced apoptosis in MiaPaCa-2 and PANC-1 cells (Qorri et al., 2022). Several studies have recently identified Meflin, a glycosylphosphatidylinositol-anchored membrane molecule, as a functional maker of cancer-restraining CAFs (rCAFs) in PDAC (Kobayashi et al., 2019, 2021; Mizutani et al., 2019; Takahashi et al., 2021). Lida et al. conducted a screening of nuclear receptor ligands and identified Am580, a synthetic retinoid and RARα-selective agonist, as a compound that upregulates Meflin expression in both human pancreatic stellate cells (PSCs) and mouse mesenchymal stem cells (MSCs). Furthermore, Increasing Meflin enhances tumor sensitivity to chemotherapy in a PDAC xenograft model (Iida et al., 2022). The phase I and II clinical trials are conducting to investigate the efficacy of a combination of AM80 and gemcitabine and nab-paclitaxel in patients with advanced PC (Mizutani et al., 2022). Molecular docking technique confirmed that ZINC000001612996, ZINC000052955754, ZINC000003978005, and ZINC000006716957 could potentially act as small molecule drugs and co-ligands for *TRPC3* and *TRPC7*, both of which are part of the Transient Receptor Potential Channels (TRPs)-related gene signature in PDAC (Shi W. et al., 2022). Chen et al. (2020) reported dose-dependent negative effects of albendazole, an anthelmintic drug, on proliferation, migration and viability of the human PC cell lines SW1990 and PANC-1. The cytotoxicity of albendazole was further confirmed *in vivo* using a nude mouse xenograft model, showing a significant reduction in tumor growth (Chen H. et al., 2020). Table 2 presents details on various repurposed drugs for PC.

5.3 Liver cancer

Recently, several innovative transcriptomics-based DR methods were employed to uncover novel therapeutic candidates and combinations for the treatment of hepatocellular carcinoma (HCC or LC). Regan-Fendt K et al. (2020) showed that fostamatinib and dasatinib could be effective for sorafenib-resistant HCC (Regan-Fendt et al., 2020). Additionally, an mRNA expression profile-based DR method revealed that *TOP2A* has consistently unfavorable association with HCC patient survival and successfully repositioned withaferin-a (WFA) and mitoxantrone (MTX) through molecular docking studies as potential inhibitors of HepG2 cell proliferation for HCC treatment (Yuan et al., 2022). Tan et al. (2023) have screened a compound library containing 419 FDA-approved drugs and discovered that desloratadine, an antiallergic drug, inhibits proliferation in HCC cell lines as well as CDX, patient-derived organoid (PDO) and PDX. The study also identified N-myristoyl transferase 1 (NMT1) as its target, linking high *NMT1* and *VILIP3* expression to advanced HCC stages and poor survival (Tan et al., 2023). Furthermore, the combined effects of sorafenib, raloxifene, and loratadine on LC cells were assessed, finding that these two- or three-drug combinations significantly reduced metabolic activity, increased apoptosis, and decreased colony formation compared to single-drug treatments, suggesting the potential of the triple combination as a promising approach for LC treatment (Villarruel-Melquiades et al., 2023). We have summarized several repurposed drugs for HCC in Table 3.

5.4 Gastric cancer

Zhang et al. (2022) identified 6-Thioguanine (6-TG) as a potential therapeutic agent for GC by inducing ferroptosis through inactivation of system xc^-, inhibition of glutathione (GSH) production, downregulation of GPX4, and elevation of lipid reactive oxygen species (ROS) levels in MGC-803 and AGS

cell lines, with *in vivo* data supporting its anti-tumor activity (Zhang et al., 2022a). Furthermore, it has been shown that HC-056456, a CatSper channel blocker, as a novel ferroptosis-inducing compound inhibits GC cell growth by reducing GSH through the p53/SLC7A11 pathway, leading to increased Fe2+ and lipid peroxides *in vitro* and *in vivo* (Zhang et al., 2022b). In a comprehensive bioinformatic analysis of highly differentially expressed genes in GC, Hossain et al. (2023) found that *CDH2*, *COL4A1*, and *COL5A* are associated with the survival of GC patients. They also identified docetaxel, lanreotide, venetoclax, temsirolimus, and nilotinib as the top six candidate drugs, targeting aforementioned proteins, for the treatment of GC patients (Hossain et al., 2023). Nitazoxanide also yielded favorable outcomes, as it exhibited activity across GC cell lines tested (Ribeiro et al., 2023). In addition, Rabben et al. (2021a) computationally predicted the repositioning of ivermectin for the treatment of GC based on gene expression profiles of both human and mouse models of GC. They further validated their *in silico* prediction used human GC cell lines MKN74 and KATO-III *in vitro*. Transgenic insulin–gastrin (INS-GAS) mice were employed for experimental validation of ivermectin in GC treatment (Rabben et al., 2021a). Furthermore, *in vivo* and *in vitro* anti-tumor and growth suppression effects of ivermectin were demonstrated on GC, showing that ivermectin suppressed MKN1 cells growth through yes-associated protein 1 (YAP1) downregulation (Nambara et al., 2017). To identify drugs capable of inhibiting the DNA-binding activity of the *helicobacter pylori* transcription factor HP104, a combination of computational and *in vitro* methods led to the discovery of three promising drugs, including temoporfin, trientine, and tetraethylenepentamine, for potential antibacterial applications (Antoniciello et al., 2022). Table 4 provides a summary of available repurposed drugs in GC.

5.5 Other examples of drug repurposing in gastrointestinal cancers

Surveillance for individuals at risk of GI cancers is essential for early diagnosis and prognosis improvement, and in choosing long-term chemoprotective drugs, approved molecules with well-known long-term effects are preferred. Aspirin (acetylsalicylic acid), introduced at the end of the 19th century, has been proposed for various diseases, such as cardiovascular diseases, strokes (Brighton et al., 2012; Dimitriadis et al., 2022; Gdovinova et al., 2022) and the chronic treatment of Fabry Disease (Monticelli et al., 2022). Specifically, aspirin has been shown to prevent CRC (Baron et al., 2003; Benamouzig et al., 2012; Guirguis-Blake et al., 2022) and PC (Streicher et al., 2014). Other non-steroid anti-inflammatory molecules like celecoxib (Arber et al., 2006; Bertagnolli et al., 2006) and sulindac (Long et al., 2020) have also been suggested for CRC.

Recent studies have highlighted the promising potential of repurposing non-oncology drugs for future cancer therapy. Examples include anticoagulant agents (warfarin (Rebelo et al., 2021) and dalteparin (Agnelli et al., 2022)), anti-fungi (itraconazole (Shen et al., 2021)), antidiabetic drugs (metformin (Cunha Júnior et al., 2021) and linagliptin (Li Y. et al., 2020)), antiparasitic (ivermectin (Nambara et al., 2017)), anthelminthic (parbendazole (Son et al., 2020)), antibiotics (nitroxoline (Mitrović and Kos, 2019), doxycycline (Ghasemi and Ghasemi, 2022), azithromycin (Qiao et al., 2018) and tigecycline (ElHefnawi et al., 2022)).

Brefeldin A, originally used as a macrolide antibiotic, has shown significant induction of autophagy in CRC cells both *in vitro* and *in vivo* (Bei et al., 2022). It functions by provoking endoplasmic reticulum stress (ER-stress) and upregulating Bip which decreases Akt phosphorylation through increased Bip/Akt interaction leading to autophagy induction in CRC cells (Zhou L. et al., 2019). In addition, antifungal drug ketoconazole has been reported to induce PINK1/Parkin-mediated mitophagy and accelerate apoptosis in HCC cells via COX-2 downregulation (Chen Y. et al., 2019).

Genistein, originally prescribed for reducing symptoms of menopause, osteoporosis, and obesity, has shown promising effects in cancer therapy. Genistein has been reported to inhibit proliferation, induce apoptosis and cell cycle arrest in G2 by inhibiting the Wnt/β-catenin signaling pathway in CRC cells (Oliveira et al., 2022). It also promotes apoptosis in HT29 CRC cells by modulating the caspase-3 and p38 MAPK signaling pathways (Shafiee et al., 2016). Furthermore, genistein inhibits glycolysis and induces mitochondrial apoptosis through downregulating of HIF-1α which leads to GLUT1 and HK2 inactivation and apoptosis induction in drug-resistant HCC cells (Li J. et al., 2017). Genistein modulates telomerase activity and reduces tumorigenesis by *hTERT* downregulation as well as modulation of *Gli1* gene expression to weaken cancer stem-like properties in GC cells (Jian-Hui et al., 2016). Genistein, which shows promise as an anticancer drug candidate, is currently in phase II of clinical trials (Cao et al., 2022; Chu et al., 2023).

Metformin is another successful repositioned drug for GI cancers which is currently in phase II of the clinical trial. Metformin reduces cell survival and tumorigenesis by lowering serum insulin levels and downregulation of IGF-1 (Sarfstein et al., 2013). Additionally, it induces G1-arrest via AMPK activation and cyclin D1 downregulation (Wang Y. et al., 2018), inhibits proliferation through mTOR signaling pathway regulation regardless of AMPK dependency (Demaré et al., 2021). It has been shown that metformin inhibits the progression of GC through the inhabitation of HIF1α/PKM2 signaling (Chen G. et al., 2015). Furthermore, it inactivates RAS/ERK and AKT/mTOR signaling pathways and reduces proliferation in KRAS-derived tumors. Metformin has been reported to selectively inhibit *KRAS*-driven metastatic CRC by silencing MATE1 (Xie et al., 2020).

Several Studies have demonstrated that the combination of repurposed drugs with cytotoxic drugs, radiotherapy or even the combination of multiple repositioned drugs can exhibit synergistic antitumor effects. A recent meta-analysis by Heer et al. (2022) revealed that aspirin when co-administered with sulindac and difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase used to treat facial hirsutism, showed significantly more effective results in protecting against CRC adenomas (Heer et al., 2022). The combination of bortezomib and chloroquine has been shown to suppress proliferation and induce apoptosis in human liver tumors, whether orthotopically or subcutaneously xenografted in mice (Hui et al., 2012). In clinical research, combining nelfinavir with a short course of hypofractionated radiotherapy (SCHRT) showed increased sensitivity of CRC tumors to radiotherapy (Meyn et al., 2016). In addition, FOLFIRINOX, a chemotherapy regimen comprising leucovorin

calcium (folinic acid), fluorouracil, irinotecan hydrochloride and oxaliplatin, is used for the treatment of advanced PC. In a phase II clinical trial, FOLFIRINOX is combined with losartan as neoadjuvant therapy, followed by chemoradiotherapy, for locally advanced PC (Murphy et al., 2019). Another successful example of DR is a combination of metformin, digoxin and somatostatin, which has shown significant suppression of PC cell proliferation in clinically relevant animal models. Currently, this combination is being evaluated in a clinical trial (Liu S.-H. et al., 2020). Tables 1–4 provide comprehensive information on various drugs that have been successfully repurposed to treat GI cancers and approved for clinical use by the FDA. Furthermore, we have prepared a Supplementary Table S2, which presents a list of repurposed unapproved or withdrawn drugs/natural components targeting GI-related cancers.

6 Challenges and future perspectives

Drug repurposing for cancer therapy is widely used to discover new indications for existing compounds; However, only a few repurposed drugs have been formally subjected to the clinical treatment guidelines. Despite advantages such as anticancer pharmacokinetic parameters, acceptable safety and tolerability in humans, there is still a risk of late-phase clinical trial failure due to competition with new drug development. The legal and regulatory barriers such as patents issues and prescription charges must also be addressed (Vickers, 2017; Breckenridge and Jacob, 2019; Pushpakom et al., 2019).

The financial factors significantly influence the clinical development and approval of repurposing drugs, as private sector organizations prioritize higher returns due to intellectual property rights (Vickers, 2017). Most registered clinical trials listed in the Repurposing Drugs in Oncology (ReDo) project are sponsored by Universities or Hospitals (67%), research institutes or non-profit organizations (28%), and only a small percentage by pharmaceutical companies (Pantziarka et al., 2018). Patent considerations for off-patent drugs can pose significant barriers to DR, requiring credible and statistically strong evidence for a new indication and legislative efforts to address this issue (Pushpakom et al., 2019).

Physicians prescribe drugs based on scientific evidence from clinical trials, and both generic and repurposed drugs should be used when suitable. Nevertheless, the pharmaceutical industry can exert substantial investments on drug promotion, physician marketing and consumer advertising. A clear example is thalidomide, originally used as a sedative or antiemetic, which has been repurposed for multiple myeloma. Despite phase III clinical trials showing no survival advantage for the combination of melphalan-prednisone-lenalidomide over melphalan-prednisone-thalidomide (Stewart et al., 2015; Zweegman et al., 2016) lenalidomide became the standard treatment approach, even with a higher estimated cost than thalidomide. Thus, underfunding for clinical trials in oncology and prescribing rejection bias remain significant challenges for cancer drug repurposing.

Another challenge lies in the genetic diversity of individuals and the complex nature of disease (Pritchard et al., 2017). While there are many common pathways involved in cancer development, differences in pathways and genes exist among subgroups and individuals. This genetic diversity results in varying side effects and treatment responses to routine drugs and therapies.

Moreover, current GI cancer therapies may be ineffective for specific patients or cancer types due to inadequate drug targeting or inefficient drug interactions (Apicella et al., 2017). In rectal cancer, personalized DR based on gene expression signatures and reverse drug-induced gene expression profiles has shown promising results. For instance, Carvalho et al. (2021) identified potential topoisomerase II inhibitors like doxorubicin, teniposide, idarubicin, mitoxantrone and epirubicin for CRC therapy, leading to a significant reversal of rectal cancer gene expression signatures (Carvalho et al., 2021). Given the significant differences in drug efficacy among individuals due to gene profiles and tumor heterogeneity, it becomes crucial to focus on DR based on tumor/subject molecular profiles to reduce inefficiencies in cancer treatment (Li and Jones, 2012).

While drug repurposing offers various benefits compared to the conventional *de novo* approach, it may not always lead to success due to lack of efficacy or toxicity issues. Bevacizumab, initially developed to treat CRC, was determined to be a strong candidate to treat other kinds of cancer such as colon, rectal, brain, lung, and kidney through drug repositioning. However, it failed in phase III trials despite positive results (Kim and Oh, 2018). These failed drug candidates in clinical trials still represent an affluent resource for repositioning, as they are well studied pharmacokinetically and clinically. Personalized genomics studies focusing on patient and disease heterogeneities may reveal that many of these failures were tested in inappropriate subject groups, making them practical options for future personalized medicine approaches, particularly for subjects with limited treatment options.

7 Conclusion

Drug repurposing is increasingly considered by both academia and the pharmaceutical industry as a cost and time-saving alternative to *de novo* drug development. Repurposing non-oncology drugs in cancer therapy provides a promising therapeutic opportunity, especially for patients with rare cancers, advanced diseases, or chemo-resistant tumors. In the present review, we have explored the potential of DR approaches, with a particular focus on their application in GI cancers. Repurposed drugs can target known pathways and key molecular targets in cancer biology due to their established functional mechanisms. DR has received attention owing to its potential to enhance treatment effectiveness and ability to overwhelm resistance to standard chemotherapy as well as improve therapy outcomes in tumors with limited response to conventional treatments. Additionally, when repurposed drugs are used in combination with routine oncology drugs, they offer a unique opportunity to target multiple pathways and molecular targets in cancer cells, going beyond the scope of traditional chemotherapy drugs and modulating diverse cancer-relevant pathways. However, it should be noted that the interaction of repurposed drugs with standard cancer drugs may pose challenges during the clinical trials.

The repositioning of drugs to treat GI cancers presents an attractive option given the increasing number of new cases, annual deaths, and the challenges in treating certain tumors. This review outlines various DR approaches that can be used to improve the efficiency of existing GI therapies. However, further clinical studies are needed to determine their potential for clinical adoption.

Author contributions

NF: Conceptualization, Visualization, Writing–original draft. MK: Conceptualization, Visualization, Writing–original draft. HB: Conceptualization, Visualization, Writing–original draft. MZ: Conceptualization, Supervision, Writing–review and editing. VC: Conceptualization, Visualization, Writing–original draft. AR: Conceptualization, Supervision, Writing–original draft. EN-M: Conceptualization, Writing–original draft, Visualization. MT: Conceptualization, Supervision, Writing–review and editing.

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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

Supplementary material

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Attenuation of intestinal ischemia-reperfusion-injury by anesthetics: a potentially protective effect of anesthetic management in experimental studies

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Intestinal ischemia-reperfusion injury (IRI) is a potentially severe clinical syndrome after major surgical procedures. In addition to causing intestinal mucosa injury, intestinal IRI further damages distant organs, causing the severity of the condition in patients. So far, effective therapy for intestinal IRI is still absent, and the survival rate of the patients is low. Previous experimental studies have shown that some anesthetics can alleviate intestinal IRI and protect organs while exerting their pharmacological effects, indicating that reasonable perioperative anesthesia management may provide potential benefits for patients to avoid intestinal IRI. These meaningful findings drive scholars to investigate the mechanism of anesthetics in treating intestinal IRI in-depth to discuss the possible new clinical uses. In the present mini-review, we will introduce the protective effects of different anesthetics in intestinal IRI to help us enrich our knowledge in this area.

KEYWORDS

ischemia-reperfusion injury, anesthetic management, gas anesthetics, intravenous anesthetics, analgesics

Introduction

Intestinal ischemia-reperfusion injury (IRI) is a potentially severe clinical syndrome of several major surgical procedures, including cardio-pulmonary bypass surgery, liver transplantation, bowel resection and transplantation, abdominal aortic aneurysm surgery, and strangulated hernias (Yasuhara, 2005; Abboud et al., 2008; Nickkholgh et al., 2013). Intestinal IRI is a serial cascade of pathophysiologic changes of mucosal barrier failure, bacteria translocation, and inflammation caused by the disruption of blood and oxygen supply and the subsequent reperfusion of the intestine after arterial obstruction, venous thrombosis, and diffuse vasospasm (Shen et al., 2014). The intestine is more sensitive to IRI, which damages the integrity of the intestinal mucosal barrier and may lead

to bacterial translocation through the intestinal wall, peritonitis, and subsequent systemic inflammatory response syndrome (SIRS) (Collard and Gelman, 2001; Cheng et al., 2013). Intestinal IRI can also cause damage to distant organs, especially acute lung injury (ALI), and may even lead to multiple organ dysfunction syndrome (MODS), endangering the patient's life (Li et al., 2020; Liao et al., 2022). Therefore, how to attenuate intestinal IRI after major surgical procedures encourages scholars to focus on exploring effective and safe prevention and treatment methods. Although several therapeutic strategies for intestinal IRI have been reported so far, including energy therapy, anti-free radical therapy, anti-leukocyte adhesion therapy, glucocorticoids, and mesenchymal stem cells (MSCs) therapy, and even some miRNAs are considered new targets for the research and development of innovative drugs, there is no definite ideal treatment available for it (Akbari, 2020; Liao et al., 2022; Shi et al., 2022).

In recent years, many studies have demonstrated that some anesthetics attenuate operative stress during the perioperative period and have organ protective effects while exerting their pharmacological effects, particularly against IRI in various organs, attracting extensive attention and reflection (Erturk, 2014; Álvarez et al., 2014; Motayagheni et al., 2017). In addition to ischemic preconditioning (IPC) and remote ischemic preconditioning (RIPC), reasonable anesthetic management in the perioperative period may be another strategy to supply some benefits for patients to avoid intestinal IRI. Some scholars have attempted to select specific anesthetics from gas anesthetics, intravenous anesthetics, analgesics, and sedatives to attenuate intestinal IRI and further explore the underlying mechanism. In particular, the research results of sevoflurane, propofol, dexmedetomidine, and remifentanil have provided experimental evidence of attenuation of intestinal IRI, suggesting that they may represent a prioritized selection of anesthetic management. In the present mini-review, we will introduce the protective effects of different anesthetics in intestinal IRI to help us enrich our knowledge in this area.

Sevoflurane

Sevoflurane is a widely used inhalation anesthetic in clinical practice, which has the advantage of inducing rapid and rapid resuscitation compared with other inhalation anesthetics (Duffy and Matta, 2000; Liang et al., 2021). Sevoflurane is potentially neurotoxic and may cause weak effects on cognitive function after a short or single exposure and cognitive dysfunction after prolonged or repeated exposure. It raises our concern about the potential neurotoxic effect of sevoflurane, particularly in brain development in childhood (Sun et al., 2022). However, existing findings demonstrated that sevoflurane has the activity of anti-oxidative stress, anti-inflammation, and neuroprotective effects and also can attenuate IRI, for instance, cerebral IRI injury (Liang et al., 2021; Lyu and Li, 2023; Xing et al., 2024). Hei and his research team first explore the protection effect of sevoflurane preconditioning on small intestinal IRI in Sprague-Dawley (SD) rats. In their study, the SD rats were first exposed to 2.3% sevoflurane for 1 h per day for 3 days. Subsequently, they clamped the rats' superior mesenteric artery (SMA) for 75 min and then released the clamp to maintain reperfusion for 2 h. They found no difference in the survival rates between the rats treated with sevoflurane preconditioning or oxygen alone. However, a degree of intestine injury and Chiu's scores of the rats treated with sevoflurane decreased obviously. Sevoflurane preconditioning also downregulated the myeloperoxidase (MPO) activities, ICAM-1 protein expression, and IL-6 concentrations, indicating sevoflurane possibly through inhibiting neutrophil sequestration and systemic inflammation to attenuate small intestinal IRI. They suggested that sevoflurane preconditioning may provide some benefits in alleviating postoperative intestinal ischemia and mortality (Gan et al., 2013). They also confirmed that sevoflurane preconditioning protects SD rats from intestinal ischemia-reperfusion-induced ALI via inhibiting NADPH oxidase and the synergistic action between oxidative stress and mast cell activation in a subsequent study, which may have a positive meaning for the clinical treatment of IIR-mediated ALI (Luo et al., 2015). Liu et al. have successively studied and reported on the effects of clinically relevant concentrations of sevoflurane in attenuating intestinal IRI. They first exposed the intestinal IRI rat models to 0.25, 0.5, and 1.0 minimum alveolar concentration (MAC) sevoflurane before, during, or after intestinal ischemia-reperfusion, respectively. They observed that 0.5 MAC sevoflurane can reduce epithelial apoptosis for protecting the intestinal mucosa without severe respiratory inhibition during ischemia-reperfusion. However, the protective effect can be partially reversed by phosphatidylinositol 3 kinases (PI3K) inhibitor LY294002, suggesting that sevoflurane inhibits intestinal mucosal epithelial apoptosis via the activation of the PI3K/Akt pathway (Liu et al., 2015a). In the subsequent research, they observed the changes in protein kinase C (PKC) and mitochondrial ATP-sensitive potassium channel (mKATP) in intestinal IRI rats treated with sevoflurane preconditioning, suggesting that the protecting effects of sevoflurane preconditioning on intestinal IRI was also dependent on the activation of PKC and mKATP (Liu et al., 2015b). Later, they found that sevoflurane preconditioning reversed the high expression of NF-κB P65 protein, proinflammatory cytokine tumor necrosis factor-α (TNF-a), and interleukin-6 (IL-6) and upregulated PPARγ protein in the intestinal mucosa of intestinal IRI rats, indicating that sevoflurane inhibits the intestinal inflammatory reaction via activation of the PPARγ/NF-κB pathway (Liu et al., 2020). Notably, they compared the efficacy of sevoflurane preconditioning and IPC attenuating intestinal IRI while studying the potential mechanism (Luo et al., 2015a). The results of the histopathological test and Chiu's scores showed both sevoflurane preconditioning and IPC attenuated intestinal injury in rats, indicating that sevoflurane preconditioning can provide similar anti-intestinal IRI effects as IPC. These findings provide valuable experimental evidence of clinically relevant concentrations of sevoflurane in attenuating intestinal IRI, which may have great translational potential in patients at risk of intestinal IRI.

Propofol

Propofol, as a commonly used short-acting intravenous anesthetic, has some advantages in the anesthetic management of the perioperative period, including fast onset of anesthesia, short

recovery time, repeated administration, and antiemetic effect. Hence, propofol has been widely administered for the induction and maintenance of anesthesia so far (Lundström et al., 2010; Walsh, 2022). Previous studies have shown that propofol also has anti-inflammatory, antioxidant, and immunomodulatory properties, contributing to its neuroprotective effects but even affecting the cancer prognosis (Marik, 2005; Kotani et al., 2008; Gao et al., 2020). Given the unique pharmacological effects of propofol, many scholars have investigated the effect of propofol on intestinal IRI. Liu et al. selected three propofol regimens to investigate the effect on intestinal mucosal injury after intestinal IRI. Wistar rats were treated with propofol at a sedative dose before, during, or after the intestinal IRI, respectively. The histological measurement of intestinal mucosal injury and Chiu's scores showed that three different propofol treatment regimens significantly alleviated intestinal mucosal injury after intestinal IRI, especially propofol preconditioning displayed the best protective effect. Propofol preconditioning can inhibit the levels of lipid peroxidation product malondialdehyde (MDA) via attenuating nitric oxide (NO) and endothelin-1 (ET-1) production and stimulate an over-production of endogenous superoxide dismutase (SOD) activity in the intestinal mucosa, indicating that propofol attenuates intestinal IRI may be attributable to its antioxidant properties. They suggested that propofol preconditioning at a sedative dose provides a profound protective effect in intestinal IRI rats, and it is worth exploring the clinical translational potential of propofol in patients at risk for intestinal IRI following major cardiac surgery or for critical care (Liu et al., 2007). In addition to inhibiting MDA in intestinal IRI rats, Kaplan et al. (2007) found that propofol can also inhibit the production of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-a) and interleukin-6 (IL-6). They suggested that the anti-inflammatory and antioxidant properties may contribute to the protection of propofol in intestinal ischemia/reperfusion-induced liver injury. Vasileiou et al. (2012) believed those properties also seem to be the crucial mediating mechanisms of propofol for efficiently preventing intestinal ischemia/reperfusion-induced lung injury. Li et al. (2021a) confirmed that the anti-inflammatory of propofol comes from downregulating the p38 MAPK/NF-κB signaling pathway to inhibit the production of inflammatory cytokines in intestinal IRI rats. Wu et al. (2020) observed propofol decreased the number of cell apoptosis in the intestinal tissue of intestinal IRI rats besides anti-inflammatory and antioxidant. Liu et al. (2008) attributed this anti-apoptotic effect of propofol preconditioning to its antioxidant property modulating the ceramide pathway. Some studies proved mucosal mast cell (IMMC) activation is critical in intestinal IRI by secreting many mediators to induce intestinal epithelial injury and integrity disruption. Propofol preconditioning can suppress IMMC activation, and it can explain why propofol can attenuate Intestinal IRI, restore intestinal epithelial cell integrity, and prevent intestinal IRI-induced lung injury in rodents and even pigs from other perspectives (Zhao et al., 2014; Gan et al., 2015; Bian et al., 2021; Li et al., 2022a). The above findings showed propofol may provide a meaningful anesthetic management regimen for preventing intestinal IRI and organ injury following major surgery and is worthy of a further clinical study to examine the clinical significance.

Dexmedetomidine

Dexmedetomidine (DEX) is a highly selective α2-adrenoceptor agonist with unique sedative and analgesic properties and widespread use in the perioperative period (Cai et al., 2022). Previous studies have demonstrated that DEX also has anti-inflammatory and anti-apoptotic properties, allowing it to provide multiple organ-protective effects in animal models of IRI (Cai et al., 2014; Zhao et al., 2022). Zhang et al. compared the effects of different doses of DEX given 1 h before intestinal ischemia or 1 h after the beginning of reperfusion on the intestinal injury of rats. They found that DEX at 2.5 μg/kg/h has no beneficial effects before or after ischemia, while DEX at 10 μg/kg/h led to severe hemodynamic suppression. Only when DEX at 5 μg/kg/h was infusion before ischemia can decrease intestinal injury and rat mortality by inhibiting the inflammatory response and intestinal mucosal epithelial apoptosis via α2 adrenoreceptor activation. Notably, the dose of 5 μg/kg/h DEX used in rats is equal to approximately 0.8 μg/kg/h in humans, which is a safe dose to apply in cardiovascular surgery and the ICU. However, DEX lacks a protective effect after ischemia. They speculated that this ineffectiveness may due to the slow onset of dexmedetomidine, which reaches its effect approximately 15 min after intravenous administration (Zhang et al., 2012). Some scholars also suggested this ineffectiveness may be due to DEX not inhibiting JAK/STAT signaling after ischemia, and the JAK/STAT signaling regulates the signal transduction for various cytokines and growth factors in inflammation processes and plays a pivotal role in intestinal IRI (Zhang et al., 2020). Another study also demonstrated the intestinal protection of DEX preconditioning in intestinal IRI rats. The authors suggested that DEX has good free radical scavenging and antioxidant properties, anti-apoptotic effects, and anti-inflammatory effects during the progress of intestinal IRI (Zhang et al., 2015). Shen et al. (2013) further evaluated the protective effect of DEX's anti-inflammatory on intestinal IRI-induced lung injury, and they suggested the inhibitory effect of DEX on cytokine production and the immune response in lung tissue via modulating the TLR4/MyD88 pathway may provide valuable and effective protection to intestinal IRI rats. Chen et al. (2020a) suggested that the cannabinoid receptor CB2-mediated PI3K/Akt pathway is also involved in the function of DEX against lung injury in intestinal IRI rats. Interestingly, Li et al. (2022b) suggested that the anti-inflammatory effect of DEX contributes to attenuating early cognitive dysfunction induced by intestinal IRI mice, indicating that DEX may also provide some benefits in reducing the incidence of cognitive dysfunction. Some new protective mechanisms of DEX in intestinal IRI in animal models have been reported in recent years. DEX can attenuate intestinal I/R injury by decreasing ferroptosis and pyroptosis, enhancing mitophagy, promoting the mitochondrial localization of TERT, and microbiota-related mechanisms (Liu et al., 2021; Zhang et al., 2021; Dong et al., 2022; Hu et al., 2022; Hou et al., 2023). These findings provide new experimental evidence supporting the protective effect of DEX against intestinal IRI and a unique insight into the clinical use of DEX, which has positive significance for DEX administration in the perioperative period.

Remifentanil

Remifentanil is a short-acting opioid with high analgetic potency, widely used in intra-operative or postoperative analgesia. It also takes part in general anesthesia induction and maintenance as a component of total intravenous anesthesia (Grape et al., 2019; Sridharan and Sivaramakrishnan, 2019; Ren et al., 2022). Remifentanil can attenuate the IRI of organs through multiple mechanisms, such as anti-inflammatory, antioxidant, and anti-apoptotic signaling pathways, that have attracted the attention of scholars (Yi et al., 2023). Some scholars have confirmed the protective effects of remifentanil on intestinal IRI in their studies. Cho et al. (2013) injected 1 μg/kg of remifentanil into C57BL/6J mice before clamping the SMA for 30 min and then 60 min of reperfusion. The tissue injury and lipid peroxidation of jejunum and ileum and systemic IL-6 were analyzed by histology, malondialdehyde (MDA), and ELISA, respectively. They found that remifentanil preconditioning can attenuate the intestinal IRI and inhibit lipid peroxidation and systemic inflammatory response, indicating that pre-treatment of remifentanil may bring potential benefits in the clinical prevention of intestinal IRI. Shen et al. (2016) used the rat models and the rat intestinal epithelial IEC-6 cells to evaluate the protective effects of remifentanil preconditioning. They found that remifentanil preconditioning attenuated intestinal injury in intestinal IRI rats and IEC-6 cell apoptosis after being subjected to oxygen and glucose deprivation (OGD), but naltrindole (a δ-OR selective antagonist) and CTOP (a μ-OR selective antagonist) can markedly attenuate these changes. They suggested that δ- and μ-opioid receptors may play a critical role in the protection against intestinal IRI of remifentanil preconditioning. Then, they proposed a new perspective to explain the mechanism of remifentanil preconditioning in protection against intestinal IRI in a recent study. They found that remifentanil preconditioning attenuated intestinal IRI by reducing oxidative and ER stress, and the PDIA3 gene played an essential role in this protection process, but p38MAPK inhibitor (SB203580) can suppress the PDIA3 expression and abolish the intestinal protection of remifentanil, indicating that remifentanil activates p38MAPK to PDIA3 expression for inhibiting intestinal IRI-induced oxidative and ER stress (Shen et al., 2022). In addition to the anti-inflammatory and antioxidant activity, Sayan-Ozacmak et al. (2015) suggested that remifentanil preconditioning can also improve intestinal contractility in intestinal IRI rats, resulting in restoring dysfunction of intestinal motility induced by the IRI. Therefore, the protection mechanism of remifentanil in intestinal IRI may be multifactorial, and the exact mechanism still needs further elucidation.

Other anesthetics

Ketamine is often used for anesthesia, analgesia, and sedation, but its applications beyond anesthesia are involved in the treatment of addiction, depressive episodes, asthma, and even inhibit cancer growth (Ivan Ezquerra-Romano et al., 2018; Nowacka and Borczyk, 2019; Mihaljević et al., 2020; Ritter et al., 2020; Adegbola et al., 2023). In addition, ketamine can also protect various tissues from IRI (Xiao et al., 2012; Li et al., 2018). Previous studies found that pre-treatment of ketamine can reduce inflammatory cell infiltration and intestinal injury in intestinal IRI rats (Cámara et al., 2008; Guzmán-De la Garza et al., 2010a; Guzmán-De la Garza et al., 2010b). Ketamine also has some anticoagulant and platelet anti-aggregation properties and can improve the intestinal transit delay, contributing to the protective effects of ketamine. Guzmán-De la Garza et al. (2010b) suggested that an intact enteric nervous system seems to need in the protective action of ketamine in intestinal IRI rats. Parecoxib sodium is an injectable COX-2-specific inhibitor and usually used for postoperative analgesia (Cheer and Goa, 2023). Li et al. reported that parecoxib sodium also exerts protective effects in intestinal IRI rats. They found that parecoxib sodium preconditioning attenuated intestinal injury and increased the rat survival rate by inhibiting inflammation, oxidative stress, and apoptosis (Li and Zheng, 2021). Moreover, Clarysse et al. (2023) observed that isoflurane has anti-inflammatory effects and can reduce intestinal epithelial damage and permeability in intestinal IRI rats, and combined with oxygen-supplementation will provide additional benefits in the attenuation of intestinal IRI, indicating that anesthetic management will bring substantial positive influence in a rodent model of intestinal IRI.

Summary and outlook

Intestinal IRI causes severe intestinal mucosa histopathological injury and further damage to distant organs, causing the severity of the condition in patients. Unfortunately, effective therapy for intestinal IRI is still absent, leading to a high rate of mortality. According to statistics, about 26% of patients unable to live for more than a year (Wang et al., 2021). Exploring the protection against intestinal IRI of anesthetic management is very important for patients who will suffer major surgical procedures. The research on specific anesthetics introduced above indicates that pre-treatment of these agents confers protection against intestinal IRI and distant organ injury by their anti-inflammatory, anti-oxidant, and anti-apoptotic properties. Preconditioning with specific anesthetics may be advantageous in patients with intestinal IRI, providing a referable opinion for anesthetic management in the perioperative period. However, some limits of current studies need attention and to be discussed.

First, the existing exciting research results are mainly from preclinical studies, but heterogeneity and methodological quality of these research are still unavoidable limitations. Animal experiments serve as a basis for exploring the efficacy and potential mechanisms of the anesthetics in treating intestinal IRI (Hou et al., 2023). The animals commonly used to build animal models of intestinal IRI include rodents, pigs, cats, and dogs (Gonzalez et al., 2015). Previous studies on intestinal IRI mainly employed rodent models (see Table 1). Compared to large animals, rodents have the advantage of low cost, rapid reproduction rate, number of available and well-established models. Wang et al. (2021) summarized the establishment methods of the intestinal IRI rat model from strains, gender, age, weight, anesthesia, surgical details, ischemia and reperfusion time, and perioperative care in their published review. They realized that building a uniform standard for the intestinal IRI rat model would provide a reliable basis for the horizontal comparison of mechanism research. However, there are difficulties in surgery and a high mortality rate in the procedure of

TABLE 1 Literature examples of anesthetic application in Intestinal IRI animal models.

Anesthetics	Dose	Animal species	IRI modeling method	References
Sevoflurane	2.3%	SD rat	75 min SMA occlusion followed by 120 min reperfusion	Gan et al. (2013), Luo et al. (2015)
Sevoflurane	0.5, 1.0, and 2.0%	SD rat	60 min SMA occlusion followed by 120 min reperfusion	Liu et al. (2015a), Liu et al. (2015b), Liu et al. (2020)
Propofol	50 mg/kg	Wistar rats	60 min SMA occlusion followed by 180 min reperfusion	Liu et al. (2007), Liu et al. (2008)
Propofol	10 mg/kg	Wistar rats	30 min SMA occlusion followed by 120 min reperfusion	Kaplan et al. (2007)
Propofol	60 mg/kg	Wistar rats	45 min SMA occlusion followed by 240 min reperfusion	Vasileiou et al. (2012)
Propofol	50 mg/kg	SD rats	45 min SMA occlusion followed by 90 min reperfusion	Li et al. (2021a)
Propofol	60 mg/kg	SD rats	60 min SMA occlusion followed by 120 min reperfusion	Wu et al. (2020)
Propofol	50 mg/kg	SD rats	75 min SMA occlusion followed by 120 min reperfusion	Zhao et al. (2014), Gan et al. (2015)
Propofol	10 mg/kg/h	miniature pigs	120 min SMA occlusion followed by 240 min reperfusion	Bian et al. (2021)
Dexmedetomidine	2.5, 5, and 10 μg/kg/h	SD rats	60 min SMA occlusion followed by 120 min reperfusion	Zhang et al. (2012)
Dexmedetomidine	10, 20, and 50 μg/kg	Wistar rats	60 min SMA occlusion followed by 120 min reperfusion	Zhang et al. (2020)
Dexmedetomidine	50 μg	Wistar rats	60 min SMA occlusion followed by reperfusion	Zhang et al. (2015)
Dexmedetomidine	2.5 and 5.0 μg/kg/h	SD rats	60 min SMA occlusion followed by 120 min reperfusion	Shen et al. (2013), Liu et al. (2021)
Dexmedetomidine	5.0 μg/kg/h	SD rats	60 min SMA occlusion followed by 120 min reperfusion	Chen et al. (2020a)
Dexmedetomidine	50 μg/kg	C57BL/6J mice	45 min SMA occlusion followed by 24 h reperfusion	Li et al. (2022a)
Dexmedetomidine	10 and 100 μg/kg	Wistar rats	60 min SMA occlusion followed by 60 min reperfusion	Hu et al. (2022)
Dexmedetomidine	400 μg/kg	C57BL/6 mice	60 min SMA occlusion followed by 180 min reperfusion	Dong et al. (2022)
Remifentanil	1.0 μg/kg	C57BL/6 mice	30 min SMA occlusion followed by 60 min reperfusion	Cho et al. (2013)
Remifentanil	0.1, 0.2, 0.6, and 1.0 μg/kg	SD rats	60 min SMA occlusion followed by 120 min reperfusion	Shen et al. (2016)
Remifentanil	1.0 μg/kg	C57BL/6 mice	45 min SMA occlusion followed by 240 min reperfusion	Shen et al. (2022)
Remifentanil	2.0 μg/kg/min	Wistar rats	30 min SMA occlusion followed by 180 min reperfusion	Sayan-Ozacmak et al. (2015)
Ketamine	100 mg/kg	Wistar rats	45 min SMA occlusion followed by 60 min or 24 h reperfusion	Cámara et al. (2008)
Ketamine	6.25, 12.5, 50, and 100 mg/kg	Wistar rats	30 min SMA occlusion followed by 60 min reperfusion	Guzmán-De la Garza et al. (2010a)
Ketamine	50 mg/kg	Wistar rats	30 min SMA occlusion followed by 60 min reperfusion	Guzmán-De la Garza et al. (2010b)

(Continued on following page)

TABLE 1 (*Continued*) Literature examples of anesthetic application in Intestinal IRI animal models.

Anesthetics	Dose	Animal species	IRI modeling method	References
Parecoxib sodium	10 and 20 mg/kg	SD rats	60 min SMA occlusion followed by 120 min reperfusion	Li and Zheng (2021)
Isoflurane	induction with 5% and maintenance with 1.5%–1.75% at 1 L/min	SD rats	60 min SMA occlusion followed by 60 min reperfusion	Clarysse et al. (2023)

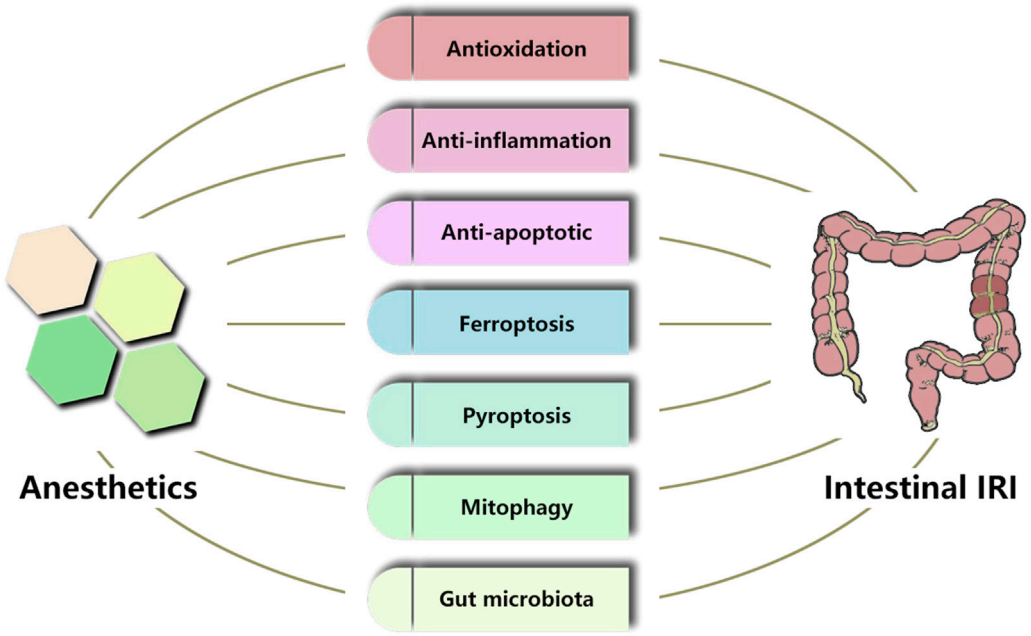

FIGURE 1
The possible mechanisms of protection effects of anesthetics in intestinal IRI.

building IRI rodent models. In addition, the pathological and physiological changes of the rodent models' intestinal tract differ to some extent from those in humans. Some scholars employed big animals to build the IRI animal models because it may be easier to develop safe preclinical protocols directly transferable to humans using those animals. For example, pigs are considered the ideal model for human intestinal IRI research because of their approximate morphology and function of human intestines. The bigger size of pigs is the ease of surgical manipulation. Scholars can temporarily occlude the smaller vessels within the mesentery without compromising the integrity and then reperfusion to cause intestinal IRI in pigs. However, the experimental costs, feeding management, and the number of models also limit the conduct of related experiments on intestinal IRI pig models. In addition to the animal models, the appropriate model-making method is critical to experimental design. Gonzalez et al. (2015) classified the intestinal IRI modeling methods as complete vascular occlusion (SMA ligation and SMA embolization), low-flow ischemia, and segmental mesenteric vascular occlusion. They also compared the advantages and disadvantages of these methods in their published review. They suggested that the mesenteric vascular occlusion model of intestinal ischemia may be the prioritized modeling method because it can be readily performed in large animals (e.g., pigs) and rodents. Given current intestinal IRI animal experiments still lack optimal choices in animal species and modeling methods, the methodologic of the preclinical studies about anesthetics still needs to be further well-designed before clinical implication.

Second, the dosage of anesthetics in most intestinal IRI animal experiments is clinically relevant concentration, but there are growing concerns about the safety of anesthetic use. It is well-known that the neurotoxicity of anesthetics may inhibit the development of children's nervous systems, weaken memory and learning functions, cause postoperative delirium, and even induce long-term cognitive dysfunction (Jungwirth et al., 2009; Kang et al., 2017; Johnson et al., 2019; Johnson et al., 2019; McCann and Soriano, 2019). Research has confirmed that long-term exposure to sevoflurane in young animals could lead to a 50-fold increase in the rate of neuroapoptosis (Sun et al., 2022). Some scholars suggested that anesthetic-induced neuroapoptosis is the leading cause of neurotoxicity, and exposure to anesthetics can cause neuroapoptosis through several different molecular mechanisms. For example, sevoflurane induces neuroapoptosis through the brain-derived neurotrophic factor (BDNF)-modulated apoptotic cascade, mitochondria-mediated apoptosis, death receptor signaling, intracellular ROS, and intracellular calcium homeostasis (Sun

et al., 2022). Other evidence has emerged that anesthetics can also induce neuroinflammation, impair hippocampal synaptic plasticity, and cause neurodegenerative changes, indicating the possible detrimental effects of anesthetics on both the young developing and the elderly aging brain (Wan et al., 2021; Yang et al., 2021; Rump and Adamzik, 2022). Still, some experimental evidence of potential neuroprotective effects has been reported, making it somewhat equivocal. Therefore, further preclinical research is needed to enrich our understanding of anesthetics. In addition, it usually combines several anesthetics rather than a single anesthetic in perioperative anesthesia management, especially major surgeries and time-consuming surgeries. For instance, dexmedetomidine combined with propofol may decrease the demand doses and side effects of sedation (Elbakry and Ibrahim, 2017; Mason et al., 2021). We suggest that researchers pay attention to the adverse risks of anesthesia regimens when exploring the potential beneficial effects of synergistic use of anesthetics on intestinal IRI treatment. Developing appropriate anesthesia plans based on the patient's condition is essential for achieving safe medication.

Third, we have learned that pre-treatment of specific anesthetics may provide outstanding protection effects for intestinal IRI and ALI from the introduced research in this review. Most studies confirmed that antioxidation, anti-inflammation, and anti-apoptotic are possible mechanisms of these protection effects. In recent years, scholars have also found that anesthetics regulate ferroptosis, pyroptosis, mitophagy, and gut microbiota to protect the intestinal mucosa (Figure 1). However, the exact mechanism of intestinal IRI is still unclear (Zhang et al., 2023a). Intestinal IRI causes injury to multiple extraintestinal organs and is a multi-step process involving intestinal flora disturbance, bacterial translocation, endotoxin release, and multiple signaling pathways are involved (Chen et al., 2021a; Li et al., 2021b; Deng et al., 2023). Some scholars are also committed to revealing the pathological mechanisms of intestinal IRI from new perspectives. Non-coding RNAs, extracellular vesicles (EVs), intestinal microbiota derivatives, complement activation, and neutrophil extracellular traps play essential roles in the pathogenesis of intestinal IRI (Chen et al., 2020b; Chen et al., 2021b; Wu et al., 2021; Zhang et al., 2023a; Zhang et al., 2023b). These findings will also further drive scholars to explore the mechanism of anesthetics to protect against intestinal IRI and help refine our knowledge in this aspect.

In a word, our review shows the beneficial effects of anesthetics on intestinal IRI, indicating that reasonable anesthetic management in the perioperative period may be an important strategy for avoiding intestinal IRI in major surgical procedures. Although current related research is still focused on preclinical studies, we should encourage further research efforts in this direction.

Author contributions

ZH: Writing–original draft. YB: Writing–original draft. YiC: Writing–original draft. YeC: Writing–original draft. YJ: Writing–original draft, Writing–review and editing. JZ: Writing–review and editing.

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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.

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Ocular pharmacological and biochemical profiles of 6-thioguanine: a drug repurposing study

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Introduction: The purine analog 6-thioguanine (6TG), an old drug approved in the 60s to treat acute myeloid leukemia (AML), was tested in the diabetic retinopathy (DR) experimental *in vivo* setting along with a molecular modeling approach.

Methods: A computational analysis was performed to investigate the interaction of 6TG with MC1R and MC5R. This was confirmed in human umbilical vein endothelial cells (HUVECs) exposed to high glucose (25 mM) for 24 h. Cell viability in HUVECs exposed to high glucose and treated with 6TG (0.05–0.5–5 μM) was performed. To assess tube formation, HUVECs were treated for 24 h with 6TG 5 μM and AGRP (0.5–1–5 μM) or PG20N (0.5–1–5–10 μM), which are MC1R and MC5R antagonists, respectively. For the *in vivo* DR setting, diabetes was induced in C57BL/6J mice through a single streptozotocin (STZ) injection. After 2, 6, and 10 weeks, diabetic and control mice received 6TG intravitreally (0.5–1–2.5 mg/kg) alone or in combination with AGRP or PG20N. Fluorescein angiography (FA) was performed after 4 and 14 weeks after the onset of diabetes. After 14 weeks, mice were euthanized, and immunohistochemical analysis was performed to assess retinal levels of CD34, a marker of endothelial progenitor cell formation during neo-angiogenesis.

Results: The computational analysis evidenced a more stable binding of 6TG binding at MC5R than MC1R. This was confirmed by the tube formation assay in HUVECs exposed to high glucose. Indeed, the anti-angiogenic activity of 6TG was eradicated by a higher dose of the MC5R antagonist PG20N (10 μM) compared to the MC1R antagonist AGRP (5 μM). The retinal anti-angiogenic effect of 6TG was evident also in diabetic mice, showing a reduction in retinal vascular alterations by FA analysis. This effect was not observed in diabetic mice

receiving 6TG in combination with AGRP or PG20N. Accordingly, retinal CD34 staining was reduced in diabetic mice treated with 6TG. Conversely, it was not decreased in diabetic mice receiving 6TG combined with AGRP or PG20N.

Conclusion: 6TG evidenced a marked anti-angiogenic activity in HUVECs exposed to high glucose and in mice with DR. This seems to be mediated by MC1R and MC5R retinal receptors.

KEYWORDS

6-thioguanine, diabetic retinopathy, melanocortin receptors, angiogenesis, drug discovery

1 Introduction

Diabetic retinopathy (DR), one of the most severe diabetic complications, is the leading cause of blindness among working people in industrialized countries, with a significant socio-economic impact (Wild et al., 2004; Brownlee, 2005; Fletcher et al., 2005). The early and less severe DR form, known as non-proliferative DR (NPDR), is associated with long-term diabetes with inadequate glycemic control (Bloomgarden, 2009; Araszkiewicz and Zozulinska-Ziolkiewicz, 2016). NPDR is characterized by microaneurysms, microhemorrhages, retinal vascular abnormalities, exudates, and retinal occlusion (Park, 2016; Altmann and Schmidt, 2018). The progression to proliferative DR (PDR), the highly disabling pathology with the risk of pre-retinal hemorrhages and secondary retinal detachments (Dao-Yi et al., 2001), is triggered by the formation of the retinal ischemic area and the consequent stimulation of the vascular endothelial growth factor (VEGF) actions (Kollias and Ulbig, 2010). These lead to retinal neo-angiogenesis and increased vascular permeability (Arrigo et al., 2022).

Currently, anti-VEGF therapy is the pharmacological gold standard for DR; however, its management remains still challenging, with the 40% of inadequately treated NPDR cases evolving to PDR within 12 months (Hendrick et al., 2015; Wang and Lo, 2018). Therefore, new anti-angiogenic molecules targeting retinal vessel remodeling and angiogenesis could be considered novel pharmacological tools to prevent the DR progression (Lieth et al., 2000; Barber, 2003; Barber et al., 2011; Fehér et al., 2018). With regard to this finding, the purine analog 6-thioguanine (6TG), an old drug approved in the 60s to treat acute myeloid leukemia (AML), has shown a remarkable anti-angiogenic activity in AML experimental and clinical settings, by modulating endothelial cell motility, sprout formation, collagen gel invasion, and morphogenesis (Presta et al., 2002). Thus, 6TG could be repurposed as a drug to deal with pathologies characterized by pathological angiogenesis, such as DR.

We hereby identified, through a virtual screening approach, 6TG as a putative melanocortin receptor type 1 and type 5 (MC1R and MC5R) ligand, using the same *in silico* repurposing campaign, which was previously carried out by our group (Gesualdo et al., 2021). In this regard, since it has already been demonstrated that retinal MC1R and MC5R ligands exert an anti-angiogenic effect, beneficial for DR resolution (Maisto et al., 2017; Rossi et al., 2021), we have analyzed the binding of 6TG to MC1R and MC5R, through a structure-based computational approach. To confirm the computational findings, we investigated the interaction between 6TG and melanocortin receptors by using selective MC1R and MC5R antagonists in human umbilical vein endothelial cells (HUVECs) exposed to high glucose and in a mouse model of early DR.

2 Materials and methods

2.1 Molecular modeling, virtual screening, MM-GBSA calculations, and molecular dynamics simulations

Computational studies have been carried out within the Schrödinger Maestro environment, specifically using the modules of the drug discovery bundle. For methodology regarding homology modeling, molecular dynamics simulation, molecular docking, and virtual screening of approved FDA compounds, please refer to our previous paper authored by Gesualdo et al., 2021. Human MC1 and MC5 receptor (hMC1R and hMC5R) structures were modeled using the FASTA files from accession numbers Q01726.2 and NP_005904.1 as primary sequences for hMC1R and hMC5R, respectively. Homology models were built using the X-ray structure of human melanocortin receptor 4 (PDB:6W25) as a common template. Models were optimized through the all-atom molecular dynamics (MD) simulation of membrane protein systems in explicit water. MD trajectories were clustered, and clusters were chosen on the basis of affinity for known selective MC1R and MC5R compounds (Gesualdo et al., 2021). The molecular docking of 6TG was carried out with the extreme precision option of Glide within the Schrödinger Maestro environment. The complexes 6TG/hMC1R and 6TG/hMC5R were subjected to MM-GBSA calculations, and residues within 15 Å from the ligands were set free to move during the minimization protocol, applying the VSGB 2.0 implicit solvation model and implicit membrane model.

Therefore, 6TG/hMC1R and 6TG/hMC5R complexes were inserted in a 30 $Å^3$ 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) lipid membrane system according to the output from the OMP database (https://opm.phar.umich.edu/). The TIP3P water model was selected, each system was neutralized, and NaCl was added to a final 150 mM concentration. The system ionization state was set for a pH = 7.4. Following the membrane protein equilibration protocol, 50 ns NPγT ensemble production runs were carried out. The simulations were analyzed, within the Schrödinger Maestro environment, and ligand–receptor interactions were described in terms of protein–ligand contact frequency, protein and ligand root mean deviation (RMSD), and protein–ligand root mean square fluctuation.

2.2 Compounds

6TG and streptozotocin (STZ) were purchased, respectively, from R&D system (Milan, Italy, 4061) and Santa Cruz Biotechnology (Heidelberg, Germany, sc-200719). The MC1R antagonist AGRP and the MC5R antagonist PG20N were synthetized as previously described (Carotenuto et al., 2015; Merlino et al., 2018; 2019).

2.3 Cell viability and tube formation assay

The effects of 6TG were assessed *in vitro* on HUVECs, purchased from Lonza (Milan, Italy), grown in a basal medium (cod. EGM2, Lonza), and enriched with SingleQuots™ (Palinski et al., 2021).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out to determine cell viability, starting from 1×10^4 HUVECs/well, seeded in 96-well plates (Li et al., 2017). HUVECs were then cultured for 14 h under normal (5 mmol/L) or high (25 mmol/L) glucose conditions (respectively, NG or HG groups) (Xu et al., 2020) and exposed for 24 h to 6TG dissolved in EGM-2 media (6TG groups) at different concentrations (0.05–0.5–5 μM). All the treatments were carried out in quadruplicate. At the end of the treatments, MTT (1:10, Elabscience) was added to the medium, and the plates were incubated at 37°C for 4 h. Then, the medium was removed, and dimethyl sulfoxide (DMSO, 150 μL/well) was added to solubilize the formazan crystals. Optical density (OD) at 570 nm was determined using a microplate reader (TECAN 2000 Infinity). HUVEC viability was expressed as a percentage (%) of the control group (NG).

For the tube formation assay, 6×10^4 HUVECs were grown in μ-slide IbiDI culture plates with reduced Matrigel (GIBCO Lonza) (Palinski et al., 2021). NG or HG cells were exposed at 6TG 5 μM alone or in combination with MCR antagonists, dissolved in a phosphate saline buffer (PBS). Specifically, HUVECs were exposed to the following combinations: 6TG (5 μM) + AGRP (0.5–1–5 μM) and 6TG (5 μM) + PG20N (0.5–1–5–10 μM). All the treatments were carried out in quadruplicate. After capturing images using the ZEISS confocal microscope, the branch number for each field ($n = 3$) was quantified using ZEN Microscopy software (ZEISS, Germany).

2.4 Animals

For the induction of the *in vivo* DR model, a single dose of STZ (65 mg/kg) (Gesualdo et al., 2021) was used to induce diabetes in 6-week-old C57BL/6J male mice (23.4 ± 2.1 g) (Envigo, Italy). All the animal experimental procedures, in line with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, were approved by the Italian Ministry of Health (number 522/2019-PR, 19 July 2019) and by the Institutional Ethical Committee of the "Vasile Goldis" Western University of Arad (number 135, 1 March 2019). Mice had free access to standard chow and mineral water in single standard cages and were exposed to controlled temperature, humidity, and light/dark (12 h/12 h) cycle.

After an overnight fast, mice received a single intraperitoneal (i.p.) injection of sodium citrate (SCT) buffer (pH 4.5) as non-diabetic controls (CTR group) or STZ (65 mg/kg) freshly dissolved in 50 mM SCT (STZ group). Blood glucose levels were measured after 4 h fasting, using a one-touch glucometer (Accu-Chek Active, Roche Diagnostics, United States). Only STZ mice with blood glucose levels beyond 2.5 g/L on two consecutive weeks were considered diabetic (Gesualdo et al., 2021) and were included in the study to test the effects of intravitreal injections of 6TG. Because this study has been designed as a proof-of-concept, the intravitreal administration of 6TG was used to reach reproducible drug levels in the retina.

Four mice per group were randomized as follows: I—non-diabetic mice, used as controls (CTR group); II—non-diabetic mice receiving 6TG (2.5 mg/kg) intravitreally (CTR + 6TG); III—diabetic mice receiving PBS (pH 7.4) intravitreally (STZ group); IV–V–VI—diabetic mice receiving intravitreal injections of 6TG (0.5–1–2.5 mg/kg) (STZ + 6TG); VII–VIII—diabetic mice receiving intravitreal injections of the MC1R antagonist AGRP and 6TG (1–2.5 mg/kg) (STZ + 6TG + AGRP group); IX–X—diabetic mice receiving intravitreal injections of the MC5R antagonist PG20N and 6TG (1–2.5 mg/kg) (STZ + 6TG + PG20N).

Specifically, STZ and STZ + 6TG mice received intravitreal injections (5 μL) of PBS or 6TG (0.5–1–2.5 mg/kg) (Oancea et al., 2017) after 2 weeks from STZ injection (T0), and then every 4 weeks (at 4 and 8 weeks, respectively, indicated as T2 and T3) (Figure 1) (Gesualdo et al., 2021). 6TG was dissolved in DMSO and then diluted in PBS to a final concentration of 0.1% DMSO (Tsai et al., 2009; Seo and Suh, 2017). STZ + 6TG + ANTA MCR groups received intravitreal injections (5 μL) of the MC1R antagonist AGRP (14.3 μM in sterile PBS) or the MC5R antagonist PG20N (130 nM in sterile PBS) (Rossi et al., 2021) at T0, followed by intravitreal injections of 6TG (1–2.5 mg/kg) after 24 h. The same procedures were repeated every 4 weeks, at T2 and T3 (Figure 1).

2.5 Intravitreal injections and fluorescein angiography

For intravitreal injections, mice were anesthetized with pentobarbital (45 mg/kg in saline) and received tetracaine (1%) for local anesthesia into the right eye, along with tropicamide (5%) for pupils' dilatation. PBS, 6TG, AGRP, and PG20N preparations (5 μL) were injected into the mice vitreous with a sterile syringe fitted with a 30-gauge needle (Micro-fine; Becton Dickinson AG, Meylan, France), after performing anterior chamber paracentesis (5 μL) to avoid an intraocular pressure increase (Biswas et al., 2007).

For fluorescein angiography (FA), mice received an i.p. injection of 10% fluorescein (1 mL/kg, AK-Fluor; Akorn, Inc.). Retinal vasculature was evaluated with a Topcon TRC-50DX apparatus (Topcon, Tokyo, Japan) in the same animal after 4 weeks from STZ (T1) and then after 12 weeks from T0 (T4)

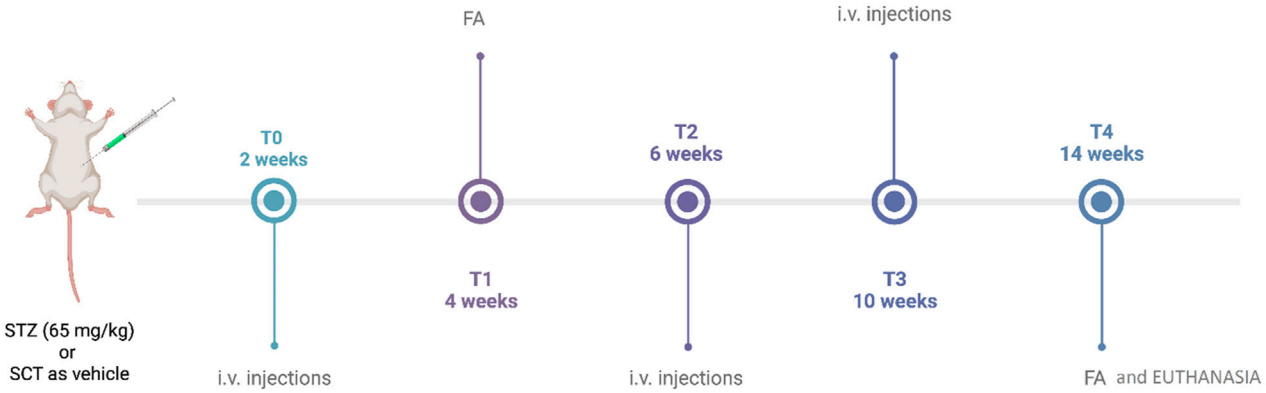

FIGURE 1
Timeline of the experimental design. STZ, streptozotocin; SCT, sodium citrate; FA, fluorescein angiography; i.v., intravitreal. This figure was created with BioRender.com.

(Figure 1), the first time point reported to be associated with marked vessel alterations in the same DR mouse model (Butler and Sullivan, 2015; Gesualdo et al., 2021; Rossi et al., 2021). Vessel abnormalities (VAs), reported as mean observed at T1 and T4 by two different ophthalmologists unaware of the treatment, were scored at T4 as 0 = absence of VA; 1 = presence of vessel thinning; 2 = presence of vessel thinning and tortuosity; 3 = presence of thinning, tortuosity, and/or crushing; 4 = presence of vessel thinning and tortuosity, venous beading, and rosary-like vessels (Gesualdo et al., 2021).

After FA at T4, mice were euthanized to remove the eyes for immunohistochemical analysis. These were placed in cooled PBS, fixed in 10% neutral buffered formalin, and paraffin-embedded.

2.6 Immunohistochemistry

After deparaffinization, 5-μm ocular sections were incubated overnight at 4°C with the CD34 primary antibody (1:100; sc-74499 Santa Cruz Biotechnology, United States), which was used as a marker of endothelial progenitor cells (EPCs) during neo-angiogenesis (Di Filippo et al., 2014). After being washed with PBS, the sections were incubated with the biotin-conjugated anti-mouse IgG secondary antibody and avidin–biotin peroxidase complex (DBA, Milan, Italy). Then, six microscopic fields for each retina (n = 4 per group) were visualized at ×200 magnification and analyzed by an expert pathologist (intra-observer variability 5%) unaware of the experimental protocol. CD34 positive particles per area were expressed as % of the positive stained area/total area.

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism v.8 (GraphPad Software, La Jolla, CA, United States). Differences were considered statistically significant for p values < 0.05 by one-way ANOVA, followed by Tukey's multiple comparisons test.

3 Results

3.1 Virtual screening, molecular docking, and MM-GBSA calculations

Virtual screening carried out by Gesualdo et al. (2021) provided evidence for 6TG favorable binding for the hMC1 receptor ($\Delta G_{binding}$ = −23 kcal/mol), but virtual screening on hMC5R did not evidence any binding for 6TG. We indeed carried out extreme precision molecular docking of 6TG to predict the binding at hMC1 and hMC5; therefore, complexes have been optimized through the MM-GBSA calculation. We found that for the 6TG/hMC5R complex, the binding was characterized by a lower $\Delta G_{binding}$ −68.05 kcal/mol, compared to the $\Delta G_{binding}$ predicted for the optimized 6TG/hMC1R complex, −40.29 kcal/mol (Figure 2). The difference of approximately 20 kcal/mol for the 6TG/hMC1 receptor complex, as previously reported by Gesualdo et al. (2021), is related to the difference in MM-GBSA rescoring carried out within the virtual screening process, which does not consider the implicit model for the membrane.

3.2 Molecular dynamics simulations

To explain $\Delta G_{binding}$ differences between 6TG/hMC1 and 6TG/hMC5 complexes, we carried out 50 ns of molecular dynamics simulation of these two complexes in an explicit water–membrane model. We found that besides a greater number of contacts of 6TG at hMC1R, compared to the 6TG/hMC5 complex, 6TG/hMC1 was characterized by increased protein RMSF (particularly at the first extracellular loop, ECL1) and increased ligand RMSD, in comparison to the 6TG/hMC5 complex (Figures 2, 3).

Additionally, the number of contacts of 6TG at hMC1 had a lower frequency during 50 ns simulation, compared to the frequency of contacts of 6TG at the hMC5 receptor (Figure 3). Overall, the computational approaches evidenced that 6TG binding at the hMC5 receptor is more stable than binding at the hMC1 receptor.

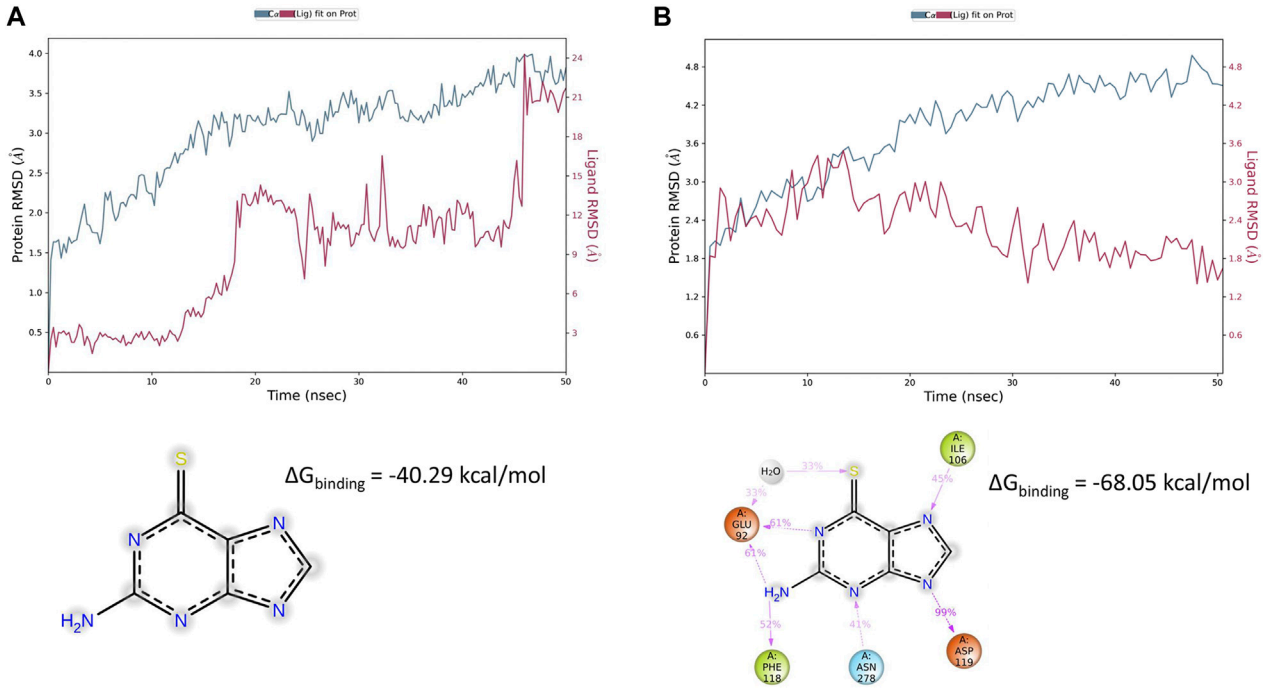

FIGURE 2
Thioguanine (6TG) interaction with hMC1 and hMC5 receptors during 50 ns simulation. **(A)** hMC1R root mean square fluctuation (blue line) during molecular dynamics simulation of the 6TG/hMC1 complex embedded in an explicit membrane model. 6TG RMSD is represented with a red line. The frequency of thioguanine contacts with hMC1R was below 35% during 50 ns simulation, and 6TG was preferentially exposed to the solvent, outside of the hMC1R-binding pocket. **(B)** hMC5R root mean square fluctuation (blue line) during molecular dynamics simulation of the 6TG/hMC5 complex embedded in an explicit membrane model. 6TG RMSD is represented with a red line. The frequency of thioguanine contacts with hMC5R was above 30% during the 50 ns simulation. Tioguanine was found to bind with a high frequency to Glu92 (also through a water bridge), Ile 106, Asp 119, Phe 118, and Asn 278.

3.3 Cell viability in HUVECs

6TG (0.05–0.5–5 μM) did not show any toxic effects in HUVECs exposed to normal glucose (5 mmol/L, NG) and has not reduced cell viability (Supplementary Figure S1S). Similarly, cell viability of HUVECs exposed to high glucose (25 mmol/L, HG; 94% ± 5%) did not show any reduction when treated with 6TG 0.05 μM (89% ± 1%, $p > 0.05$ *vs.* HG) and 0.5 μM (89% ± 10%, $p > 0.05$ *vs.* HG), while 6TG 5 μM significantly increased HUVEC viability (120% ± 10%, $p < 0.05$ *vs.* HG) (Figure 4A).

3.4 Angiogenesis assessment in HUVECs

The number of branches in HUVECs exposed to HG (30 ± 3) was significantly reduced by 6TG 5 μM (12 ± 2, $p < 0.05$ *vs.* HG). The anti-angiogenic effect of 6TG 5 μM on HUVECs exposed to HG was eradicated by the MC3R antagonist AGRP 5 μM (55 ± 13, $p < 0.05$ *vs.* HG + 6TG 5) and the MC5R antagonist PG20N 10 μM (28 ± 6, $p < 0.05$ *vs.* HG + 6TG 5). 6TG 5 μM co-treatment with AGRP 0.5–1 μM (respectively, 2 ± 1 and 9 ± 3, both $p > 0.05$ *vs.* HG + 6TG 5) or PG20N 0.5–1–5 μM (respectively, 2 ± 1, 7 ± 2 and 4 ± 2, all $p > 0.05$ *vs.* HG + 6TG 5) had no effects on HUVECs exposed to HG (Figures 4B, C).

3.5 Fluorescein angiography assessment

FA evaluations in non-diabetic mice receiving intravitreal injections of 6TG 2.5 mg/kg (CTR + 6TG group) evidenced a retinal VAs score (0.3 ± 0.2) like non-diabetic mice (CTR group; 0.2 ± 0.1), with a normal vessel caliber and course. At 14 weeks (T4) after STZ injection, retinal VAs were evident in both diabetic mice (STZ group), showing irregular vessel caliber and thinning with marked vessel tortuosity (2.9 ± 0.3, $p < 0.05$ *vs.* CTR), and diabetic mice receiving intravitreal injections of 6TG at the dose of 0.5 mg/kg (STZ + 6TG 0.5 group) showed the presence of microaneurysms and arteriovenous nicking (2.7 ± 0.3, $p > 0.05$ *vs.* STZ). Conversely, higher 6TG doses (1 and 2.5 mg/kg) intravitreally injected in diabetic mice (STZ + 6TG 1 and STZ + 6TG 2.5 groups) significantly reduced the retinal VAs score. In particular, the 1 mg/kg STZ + 6TG group showed diffused vessel tortuosity (2.0 ± 0.2, $p < 0.05$ *vs.* STZ) and 2.5 mg/kg STZ + 6TG evidenced marked vessel thinning and rare microaneurysms (1.7 ± 0.3, $p < 0.05$ *vs.* STZ). Both 6TG doses 1 and 2.5 mg/kg were not effective in reducing the VAs score at T0 when intravitreally administered in combination with the MC1R antagonist AGRP 14.3 μM (STZ + 6TG + AGRP group) or the MC5R antagonist PG20N 130 nM (STZ + 6TG + PG20N group). Indeed, STZ + 6TG 1 + AGRP mice evidenced an irregular vessel caliber with stacking of red blood cells and blood column stasis (3.0 ± 0.2, $p < 0.05$ *vs.* STZ + 6TG 1), as well as the STZ + 6TG 2.5 +

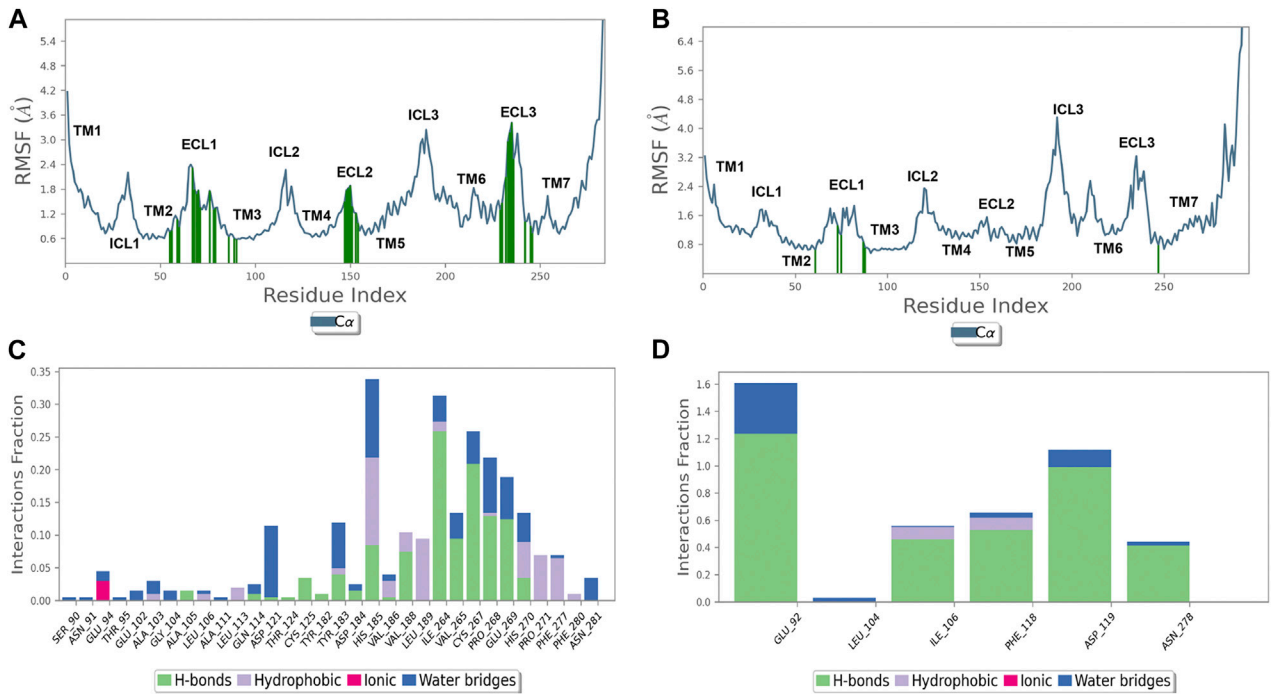

FIGURE 3
Thioguanine (6TG) binds with the hMC5 receptor with few residues but with a high frequency. **(A)** hMC1R root mean square fluctuation (RMSF, blue line) and 6TG contacts (green lines). **(B)** hMC5R root mean square fluctuation (RMSF, blue line) and 6TG contacts (green lines). **(C)** Frequency (interaction fraction) of contacts of 6TG with hMC1R during 50 ns of simulation. Tioguanine binds to hMC1R through several contacts but with a frequency below 35% (interaction fraction <0.35). **(D)** Frequency (interaction fraction) of contacts of 6TG with hMC1R during 50 ns of simulation. Tioguanine binds to hMC5R with few contacts but with a high frequency, also above 150% (interaction fraction >1.5). Interaction fraction above 1 stands for multiple forms of interaction with a given amino acid (H-bond, water bridge, hydrophobic, and coulombic).

AGRP group, showing an irregular vessel caliber and vessel thinning (3.0 ± 0.2, $p < 0.05$ *vs.* STZ + 6TG 2.5). Similarly, in STZ + 6TG 1 +PG20N and STZ + 6TG 2.5 + PG20N groups, a hyperfluorescent area along the vessel course as microaneurysm (2.8 ± 0.1, $p < 0.05$ *vs.* STZ + 6TG 1) and an irregular vessel caliber and thinning were present, respectively (2.6 ± 0.2, $p < 0.05$ *vs.* STZ + 6TG 2.5) (Figures 5A, B).

3.6 Retinal CD34 staining in STZ mice

CTR and CTR + 6TG groups exhibited weak CD34-positive retinal staining (respectively, 23% ± 4% and 22% ± 3%) as a marker of neo-angiogenesis (Di Filippo et al., 2014), predominantly in the outer plexiform layer (OPL) and in the inner nuclear layer (INL). This was significantly increased in the OPL and INL of STZ mice (53 ± 5, $p < 0.05$ *vs.* HG) and STZ mice receiving 6TG 0.5 mg/kg intravitreally (STZ+6TG 0.5; 50 ± 9, $p > 0.05$). Conversely, intravitreal injections of 6TG 1 mg/kg and 2.5 mg/kg were able to significantly reduce CD34 retinal labeling in both OPL and INL of STZ mice (respectively, 33% ± 4% and 31% ± 6%, both $p < 0.05$ *vs.* STZ). Interestingly, the combination with AGRP 14.3 μM reverted the 6TG (1–2.5 mg/kg) effects, by showing high CD34 retinal staining (respectively, 48% ± 12% and 56% ± 6%, both $p < 0.05$ *vs.* STZ+6TG at the same dose), as well as the combination of 6TG (1–2.5 mg/kg) with PG20N 130 nM (respectively, 49% ± 7% and 50% ± 7%, both $p < 0.05$ *vs.* STZ + 6TG at the same dose) (Figures 6A, B).

4 Discussion

Retinal neovascularization, a PDR hallmark, is a multiphasic process that starts with basal membrane degradation by activated retinal endothelial cells (Kusuhara et al., 2018). Then, these cells migrate and proliferate, leading to sprout formation in the stromal space. The formation of vascular loops is then followed by the capillary tube development and new basal membrane deposition. Each phase of this process represents a potential target for the inhibitory action of angiostatic molecules, potentially able to prevent DR complications and improve DR prognosis (Presta et al., 2002; Barber et al., 2011). Retinal endothelial cell activation is mainly triggered by VEGF-A, an endothelial cell-specific mitogen growth factor (Simons et al., 2016; Gui et al., 2020). Along with other angiogenic factors such as fibroblast growth factor (FGF), placental growth factor (PlGF), platelet-derived growth factor (PDGF), and angiopoietin-1/2 (Ang-1/2), VEGF-A is overproduced in retinal endothelial cells, following hyperglycemia, inflammation, hypoxia, advanced glycation end products (AGEs), and oxidative stress (Gui et al., 2020). In addition to VEGF-A, the expression of VEGF receptors 1 and 2 (VEGFR1 and VEGFR2) is induced by AGEs, stimulating, respectively, endothelial cell sprouting and vascular

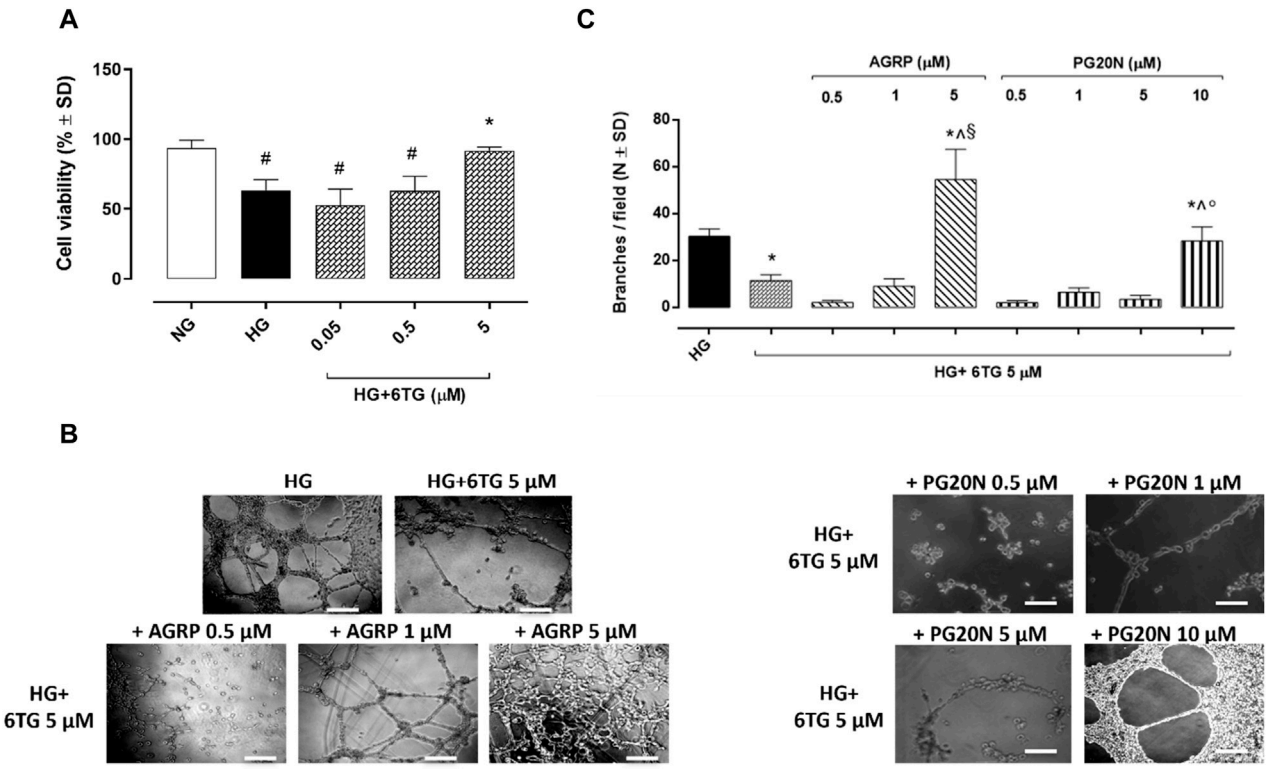

FIGURE 4
Effects of 6TG on HUVEC viability and angiogenesis under high glucose conditions. **(A)** MTT assay assessing HUVEC viability. Cells were cultured in normal glucose (5 mmol/L) (NG), high glucose (25 mmol/L) (HG), and treated with 6TG 0.05–0.5–5 μM (HG+6TG). Cell viability was reported as a percentage (%) of NG ± SD ($n = 4$). $^{\#}p < 0.05$ *vs.* NG; $^{*}p < 0.05$ *vs.* HG. **(B)** Representative images of the Matrigel assay for **(C)** the number of branches/field (as a marker of angiogenesis) formed by HUVEC (N = 4) cultured in high glucose (25 mmol/L) (HG) and treated with 6TG 5 μM (HG+6TG) alone and combined with the MCR1 antagonist AGRP (0.5–1–5 μM) (HG+6TG + AGRP) or with the MCR5 antagonist PG20N (0.5–1–5–10 μM) (HG+6TG + PG20N). $^{*}p < 0.05$ *vs.* HG; $^{\wedge}p < 0.05$ *vs.* HG+6TG 5; $^{\S}p < 0.05$ *vs.* HG+6TG + AGRP (0.5–1); $^{\circ}p < 0.05$ *vs.* HG+6TG + PG20N (0.5–1–5). Scale bar: 100 μm.

permeability (Wu et al., 2014; Kanda et al., 2017; Gesualdo et al., 2021). Diabetic macular edema (DME) is strongly associated with DR severity. The current gold standard for DME treatment is using intravitreal injections of anti-VEGF agents or steroids (Tomita et al., 2021). In particular, the current therapeutic approaches, mainly targeting VEGF-A, involve using monoclonal antibodies such as ranibizumab, brolucizumab, faricimab, and bevacizumab (off-label), as well as fusion proteins such as aflibercept (Zhao and Singh, 2018).

Recently, we have shown that the reduction in retinal VEGF-A, VEGFR1, VEGFR2, and blood retinal barrier alterations could be obtained in a DR mouse model by the selective activation of melanocortin receptors 1 and 5 (MC1R and MC5R) (Gesualdo et al., 2021; Rossi et al., 2021). MC1R and MC5R agonists also led to a restoration of antioxidant enzymes in primary retinal cells exposed to high glucose, with the consequent reduction in pro-inflammatory markers (Maisto et al., 2017). Interestingly, by a drug repurposing study, we suggested that, in addition to their selective agonists, MC1R and MC5R could have a good affinity also for some Food and Drug Administration (FDA)-approved compounds (Gesualdo et al., 2021). In particular, the sphingosine 1-phosphate receptor agonist fingolimod, approved for relapsing–remitting multiple sclerosis (RR-MS) therapy (Cohen et al., 2010; Kappos et al., 2010), emerged as a potential MCR1 agonist by a structure-based computational approach (Gesualdo et al., 2021). This was confirmed also in a DR mouse model by the selective MCR1 blockade. Although the molecular dynamic and structural simulations were less straightforward for MCR5, the *in vivo* experiments blocking this receptor suggested the interaction between MC5R and fingolimod (Gesualdo et al., 2021). By the same virtual screening approach as evidenced by Gesualdo et al. (2021), 6TG (labeled as L01) has shown a predicted binding free energy −23 kcal/mol for hMC1R. In the same virtual screening campaign, 6TG has not been identified as a ligand for hMC5R. Therefore, we carried out an extreme precision docking protocol of 6TG to predict again the 6TG pose for hMC1 receptor and *de novo* for hMC5R. These two complexes have been rescored and optimized with MM-GBSA calculations and simulated in an explicit water–POPC) membrane environment. MM-GBSA rescoring for 6TG/hMC1R evidenced a more favorable binding free energy of approximately 20 kcal/mol, when compared to the predicted value reported in Gesualdo et al. (2021), and this difference could be related to the parameters regarding the implicit membrane model, which were added in the hereby presented study. MM-GBSA rescoring evidenced a higher affinity of 6TG for hMC5R, when compared to the value predicted for hMC1R. These data have been confirmed by molecular dynamics simulation of the two complexes. 6TG binds with low and stable RMSD to the hMC5R receptor, and ligand–protein contacts have a greater interaction fraction, when

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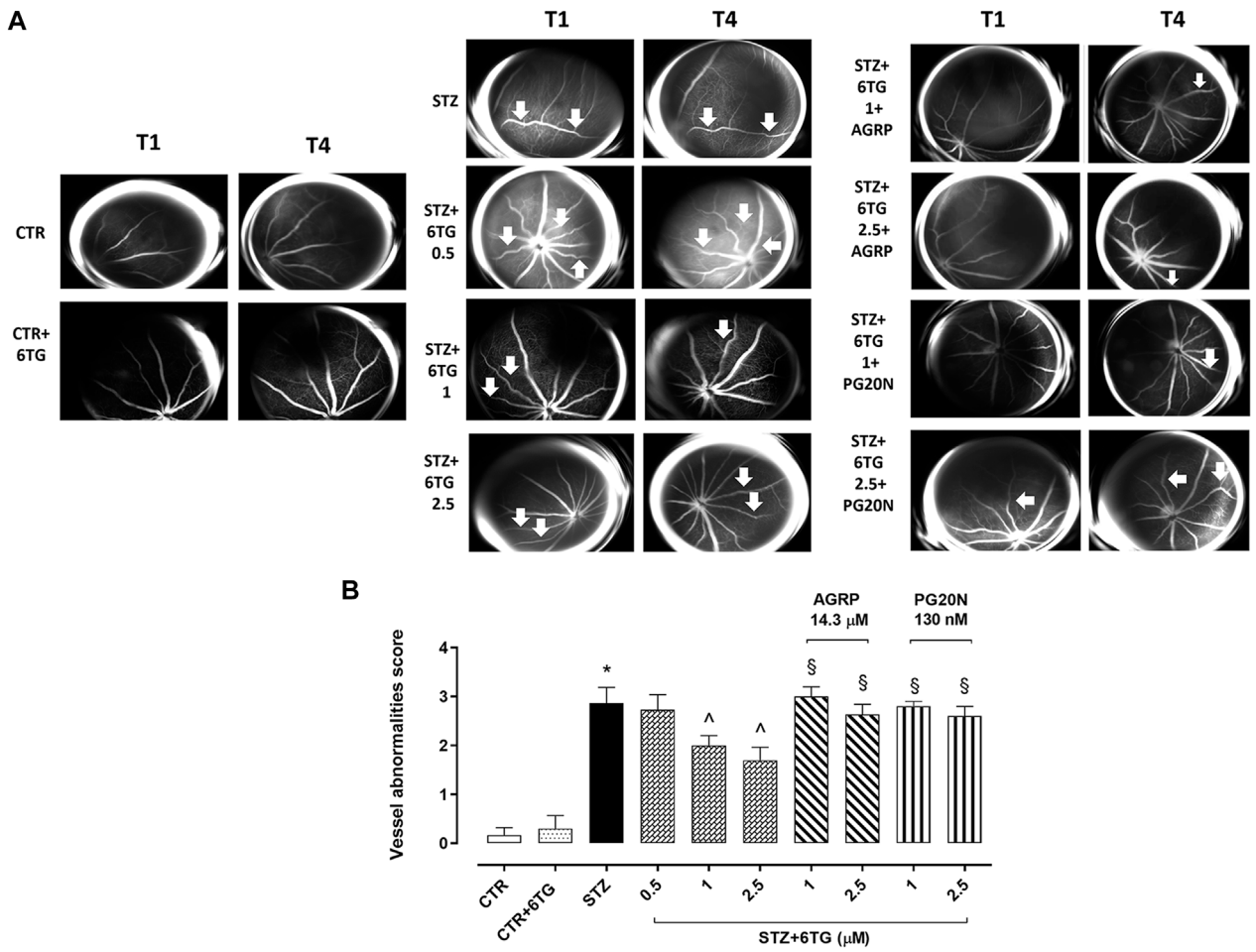

FIGURE 5
Effects of 6TG on retinal vascular alterations in diabetic mice. **(A)** Representative FA analyses showing the mouse retina (n = 4 per group) after 4 weeks of STZ (T1) and after 12 weeks from T0 (T4). CTR: normal vascularization; CTR+6TG normal vessel caliber and course; STZ: irregular vessel caliber and thinning, with marked vessel tortuosity (arrow); STZ+6TG 0.5: microaneurysm and arteriovenous nicking (arrow); STZ+6TG 1: diffused vessel tortuosity (arrow); STZ+6TG 2.5: marked vessel thinning, rare microaneurysms (arrow); STZ+6TG 1 + AGRP: irregular vessel caliber with stacking of red blood cells and blood column stasis (arrow); STZ + 6TG 2.5 + AGRP: irregular vessel caliber and vessel thinning (arrow); STZ + 6TG 1 + PG20N: hyperfluorescent area along the vessel course as microaneurysm (arrow); STZ + 6TG 2.5 + PG20N: irregular vessel caliber and thinning (arrow). **(B)** Vessel abnormalities at T4 graded from 0 to 4, based on the presence of vessel thinning, tortuosity, venous beading, and rosary-like vessels. CTR: non-diabetic control mice; CTR+6TG: non-diabetic mice receiving 6-TG (2.5 mg/kg) intravitreally; STZ: diabetic mice receiving PBS (pH 7.4) intravitreally; STZ+6TG: diabetic mice receiving intravitreal injections of 6-TG (0.5–1–2.5 mg/kg); STZ + 6TG + AGRP: mice receiving intravitreal injections of the MCR1 antagonist AGRP (14.3 μM) and 6-TG (1–2.5 mg/kg); STZ+6TG + PG20N: diabetic mice receiving intravitreal injections of the MCR5 antagonist PG20N (130 nM) and 6-TG (1–2.5 mg/kg). *$p < 0.05$ *vs.* CTR; ^$p < 0.05$ *vs.* STZ; §$p < 0.05$ *vs.* STZ+6TG (same dose).

compared to the 6TG-hMC1R complex. These findings correlated with the higher concentration of the hMC5R antagonist used to revert anti-angiogenic effects of 6TG in the *in vitro* experiments hereby presented.

Interestingly, 6TG (2-amino 6-mercaptopurine), along with 6-metilmercaptourine (6MP) riboside, has shown anti-angiogenic properties (Presta et al., 1999; 2002). These are both pro-drugs commonly used in the management of cancer, post-transplant immunosuppression, and autoimmune diseases (Petit et al., 2008). After intestinal and hepatic metabolism, 6MP and 6TG are transformed into thioguanine nucleotides, by replacing the endogenous purine guanine during DNA synthesis, causing DNA strand breaks and modulating gene expression (Vora et al., 2019). In this contest, purine analogs can interfere with molecular mechanisms of intracellular signaling and growth factors, including VEGF (Keshet and Ben-Sasson, 1999). In particular, 6TG was able to inhibit neo-angiogenesis in endothelial GM 7373 cells, in the chick embryo chorioallantoic membrane, and in the rabbit cornea (Presta et al., 1999). Accordingly, 6TG has been found effective as a potential anti-angiogenic molecule since it reduced endothelial cell proliferation induced by VEGF or fibroblast growth factor-2 (FGF2), thus inhibiting endothelial cell sprouting (Presta et al., 2002). *In vivo*, 6TG prevented neovascularization stimulated by VEGF, FGF2, or human leukemia cells (LIK) in the chick embryo chorioallantoic membrane (Presta et al., 2002). Based on the above findings, we tested 6TG effects in the retinal angiogenesis process to assess the prevention or delay of the onset and/or progression of DR using the mouse model. Before the *in vivo* study, we evaluated the safety profile of

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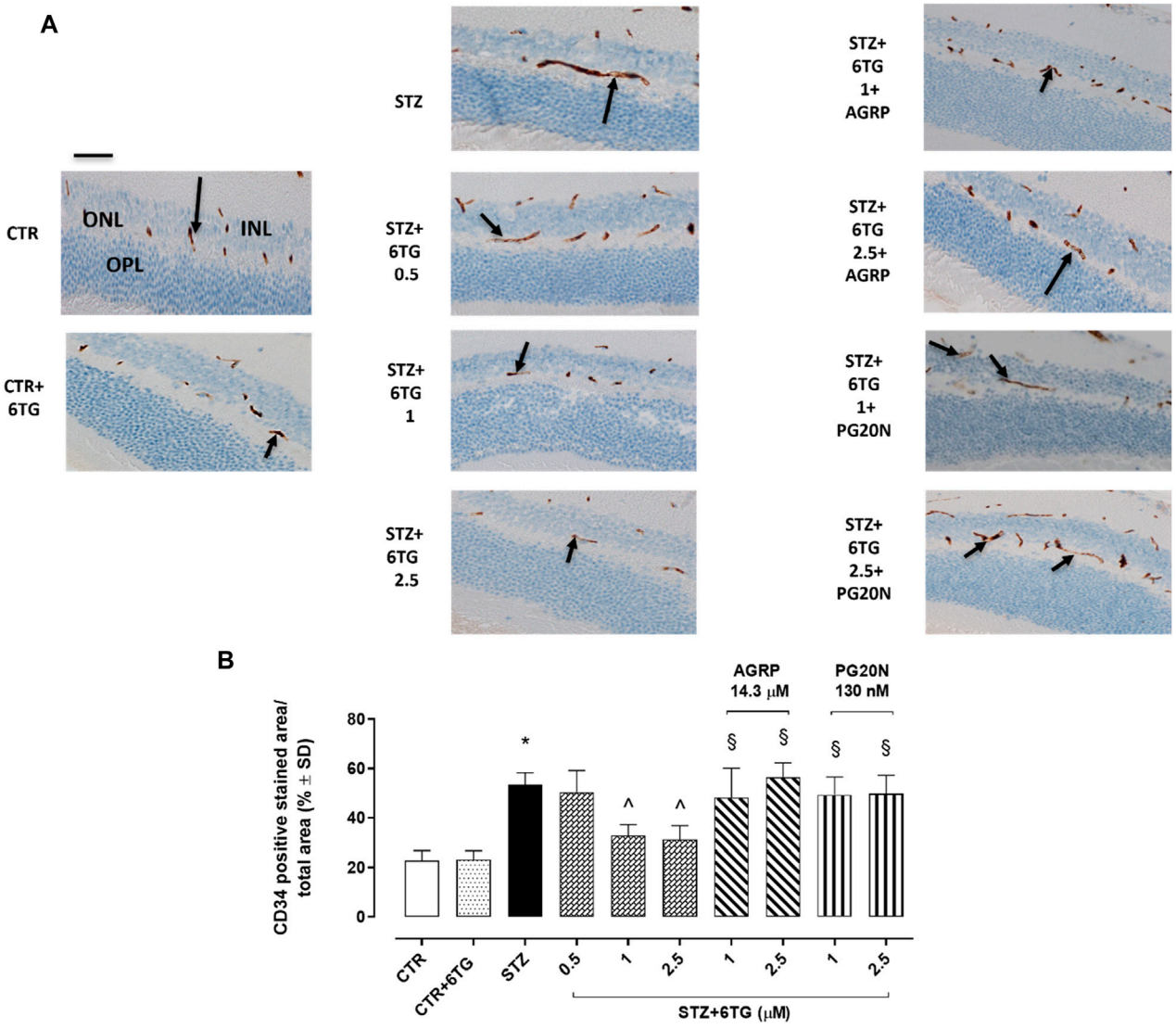

FIGURE 6
Effects of 6TG on CD34 retinal neo-angiogenesis in diabetic mice. **(A)** Representative retinal IHC images showing C34 labeling (black arrow), as a marker of neo-angiogenesis after 12 weeks from T0 (T4) in CTR, CTR + 6TG, STZ, STZ + 6TG, STZ + 6TG + AGRP, and STZ + 6TG + PG20N groups. Scale bar: 20 μm. **(B)** Percentage (%) of the CD34-positive stained area/total area analyzed ($n = 4$). *$p < 0.05$ *vs.* CTR; ^$p < 0.05$ *vs.* STZ; §$p < 0.05$ *vs.* STZ+6TG (same dose). CTR: non-diabetic control mice; CTR+6TG: non-diabetic mice receiving 6-TG (2.5 mg/kg) intravitreally; STZ: diabetic mice receiving PBS (pH 7.4) intravitreally; STZ+6TG: diabetic mice receiving intravitreal injections of 6-TG (0.5–1–2.5 mg/kg); STZ+6TG + AGRP: mice receiving intravitreal injections of the MCR1 antagonist AGRP (14.3 μM) and 6-TG (1.–2.5 mg/kg); STZ+6TG + PG20N: diabetic mice receiving intravitreal injections of the MCR5 antagonist PG20N (130 nM) and 6-TG (1.–2.5 mg/kg); INL: inner nuclear layer; IPL: inner plexiform layer; ONL: outer nuclear layer; OPL: outer plexiform layer.

HUVECs. The compound had no detrimental effects on HUVEC viability under normal or high glucose conditions at all the doses tested. This is in line with the previous evidence reporting 6TG as able to promote apoptosis only in cancer cells (Laera et al., 2019; Li et al., 2020). In particular, 5 μM 6TG significantly increased the cell viability of HUVECs exposed to high glucose and also reduced their vasculogenic activity, confirming its anti-angiogenic effects previously reported in endothelial cells or the chick embryo chorioallantoic membrane, as well as in AML patients showing a reduced bone marrow microvessel density when treated with 6TG (Padró et al., 2000; Presta et al., 2002; Dean, 2012). It is worth noting that AML is frequently associated with severe retinal alterations characterized by retinal microvascular involvement, such as choroidal thickness and abnormalities in retinal circulation, known as leukemic retinopathy (Yang et al., 2023). The anti-angiogenic effects of 6TG (1 and 2.5 mg/kg) were also shown in a DR mouse model by FA analysis, evidencing a reduction in retinal vascular alterations present in diabetic mice, such as irregular vessel caliber, vessel tortuosity and thinning, microaneurysm, or arteriovenous nicking. A further confirmation was obtained by immunohistochemical analysis, showing a remarkable reduction in retinal CD34 staining, a marker of pathological retinal neovascularization, in diabetic mice treated with 6TG (Kollias and Ulbig, 2010; Kady et al., 2017). Interestingly, the selective blocking of MC1R and MC5R reversed the effects of 6TG both *in vitro* and *in vivo*

settings. In particular, a higher dose of MC5R antagonist was necessary to eradicate the anti-angiogenic effects of 6TG on HUVECs exposed to high glucose, compared to the MC1R antagonist. This corroborates the results of our simulations, showing that 6TG seems to have a higher affinity for MC5R. The MC1R and MC5R selective antagonist suppressed the protective effects of 6TG on vessel abnormalities, as evidenced by FA analysis. Furthermore, CD34 staining was evident in the retina of diabetic mice receiving 6TG co-administered with MC1R and MC5R antagonists. We already demonstrated that MC1 and MC5 receptor agonists inhibit angiogenesis, decreasing the VEGF-A release, while MC1 and MC5 receptor antagonists increased VEGF-A retinal levels (Gesualdo et al., 2021). Tioguanine has been found to decrease VEGF protein levels through ERK pathway inhibition in malignant glioma cells (U87 cells) (Mukhopadhyay et al., 1998). These findings were also confirmed in breast cancer cells (MCF-7) treated with 10 μM 6TG (Gallwitz et al., 2002). In our study, 6TG elicited anti-angiogenic effects *in vitro* and *in vivo* through MC1 and MC5 activation; however, further studies are needed to investigate the modulation on VEGF-A levels in the retina treated with 6TG. In conclusion, the present findings suggest a new repurposing of 6TG to handle DR. Future research should focus also on topical 6TG delivery using innovative and stable ocular formulation.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by the Italian Ministry of Health (number 522/2019-PR, 19 July 2019) and by the Institutional Ethical Committee of the "Vasile Goldis" Western University of Arad (number 135, 1 March 2019). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MT: writing–original draft, methodology, investigation, formal analysis, and conceptualization. CG: writing–original draft, methodology, investigation, formal analysis, and conceptualization. CL: writing–original draft, methodology, and investigation. MR: writing–original draft, methodology, and investigation. FF: writing–original draft, methodology, and investigation. IP: writing–original draft, methodology, and investigation. EM: writing–original draft, methodology, and investigation. PG: writing–original draft, methodology, and investigation. FP: writing–original draft, methodology, and investigation. AH: data curation, writing–original draft, methodology, and investigation. FS: writing–original draft. MD'A: writing–review and editing, supervision, project administration, and conceptualization. CB: writing–review and editing. FL: writing–review and editing and formal analysis. FD: methodology, investigation, and writing–original draft. SR: writing–review and editing, supervision, project administration, funding acquisition, conceptualization, and writing–original draft. CBMP: writing–original draft, editing and investigation.

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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.

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Protective effect of dexmedetomidine against delayed bone healing caused by morphine via PI3K/Akt mediated Nrf2 antioxidant defense system

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Background: As a class of analgesics, opioids are frequently used to treat both acute and chronic moderate to severe pain. Patients frequently receive opioid painkillers after orthopedic accidents or surgeries. Evidence suggests that opioid drug users have a 55.1% higher risk of fracture and poor bone repair than non-users of opioid drugs. The key pathogenic alterations in the incidence and progression of poor bone repair are over apoptosis and aging of osteoblasts due to the stress caused by oxidation. Dexmedetomidine (Dex) has been proven to protect against a variety of degenerative illnesses by reducing oxidative stress. However, nothing is known about how it affects bone repair.

Methods: PI3K/Akt/Nrf2 pathway was detected by immunofluorescence and Western blot. SOD, CAT, JC-1, dihydroethidium and mitosox were used in the Oxidative Stress. Micro-CT, H&E and Masson's staining, immunohistochemically were performed to evaluate the therapeutic effects of DEX on calvarial defects in the morphine-induced rat model.

Results: We found that morphine-induced an imbalance in the metabolism and catabolism of primary rat Osteoblasts. However, these conditions could be inhibited by DEX treatment. In the meantime, DEX induced the expression of Nrf2-regulated antioxidant enzymes such as NQO1, HO-1, GCLm, GCLc, and TrxR1. DEX-mediated Nrf2 activation is linked to the PI3K/Akt signaling system. Furthermore, it has been established that intravenous DEX enhanced the growth of bone healing in a model of a surgically produced rat cranial lesion.

Conclusion: This is the first description of the unique DEX mechanism acting as a Nrf2 activator against morphine-mediated oxidative harm, raising the possibility that the substance may be used to prevent bone defects.

KEYWORDS

morphine, oxidative stress, dexmedetomidine, calvarial defect, PI3K/Akt/Nrf2 pathway

1 Introduction

One of the best opioid analgesics for treating severe acute and chronic pain is morphine (Sverrisdóttir et al., 2015). The analgesic withdrawal symptoms of morphine, which are primarily mediated by μ-opioid receptors, are mediated by numerous types of signaling mechanisms (Zhuang et al., 2022). However, a number of unwanted side effects, including headache, gastrointestinal issues, cough suppression, or respiratory depression, come along with these beneficial antinociceptive benefits (Tiseo et al., 1995). Evidence suggests that compared to non-users of opioids, opioid users had a 55.1% higher risk of fracture and poor bone repair (Saunders et al., 2010; Coluzzi et al., 2020). Numerous studies imply that oxidative stress may contribute to the onset of these unfavorable occurrences (Cai et al., 2016). Reactive oxygen species (ROS), which are created when oxygen is partially reduced, are produced and degraded in an unbalanced manner during oxidative stress events (Filomeni et al., 2015). According to several studies, morphine increases the process of creation of ROS and decreases the activity of several antioxidant-producing enzymes (Reymond et al., 2022; Taghavi et al., 2023).

The cellular antioxidant defense system is heavily dependent on the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), an omnipresent regulator of the antioxidant consequence (Kobayashi et al., 2016). Numerous research have been carried on to uncover Nrf2-downstream target genes, which involve antioxidant phase II detoxifying enzymes, despite the Nrf2-dependent antioxidant reaction being an intricate in addition well-structured cellular mechanism (Zhang et al., 2013). Numerous signaling mechanisms, including nuclear localization and nuclear rejection indications, control the nuclear accumulation of Nrf2 (Wang et al., 2022). The phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been identified as a key upstream controller of Nrf2 nuclear localization and controls a broad range of processes inside cells, which include proliferation, advancement, differentiation, and movement (Yu and Xiao, 2021). Phosphoinositide-dependent protein kinase-1 phosphorylates and triggers Akt once PI3K has been triggered by a variety of stimuli, which then causes the stimulation of Nrf2 and the induction of Nrf2-mediated production of antioxidant/phase II detoxification enzymes (Su et al., 2012).

DEX is a highly selective α_2-adrenoceptors agonist (AR), it has sedative, analgesic,opioid-sparing, sympatholytic effects (Keating, 2015). DEX also has anti-apoptotic effects and inhibits the production of inflammatory mediators in patients with craniocerebral injury. These actions effectively increase vascular stability, lessen cerebral edema brought on by craniocerebral injury, and improve perioperative brain function in ischemic attack (IA) patients (Benggon et al., 2012; Burlacu et al., 2022). Numerous studies have documented how DEX reduces oxidative stress to protect against a variety of degenerative illnesses (Wang et al., 2020; Shi et al., 2021). The therapeutic effects of DEX on bone abnormalities as a condition brought on by oxidative stress, however, have not been shown. The main objective of this research investigation was to figure out how DEX influences the bone-healing process.

2 Materials and methods

2.1 Antibodies and reagents

Abcam (Cambridge, MA, United States) provided the COL1A1, RUNX2, OCN, PI3K, p-PI3K, and GAPDH; and ProteinTech (Wuhan, China) provided the primary antibodies against Nrf2, NQO1, GCLc, and Gclm. The antibodies were purchased from Cell Signaling Technologies (Danvers, MA, United States) and were directed against P-Akt, Akt, TrxR1, Keap1, and HO-1. Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), and penicillin/streptomycin were purchased from Gibco BRL (Thermo Fisher Scientific, Waltham, MA, United States). The criteria for tissue and cell culture were followed by all other substances, which were all of analytical quality.

2.2 Isolation and primary culture of osteoblasts

One-day-old Sprague Dawley rats' calvarial bones were used to separate primary osteoblasts, which were then grown in full DMEM complemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin under 5% CO2 at 37°C. (Jonason and O'Keefe, 2014). The cells were transmitted when they amounted to 80%–90% intersection, and the medium was replaced every other day. Cells underwent the following therapy, in brief: 1) Morphine group: cells were cultured in full DMEM media for 2 h after being exposed to 100 μM morphine for 48 h 2) Morphine + DEX group: cells were exposed to 100 μM DEX for 48 h, then co-cultured with 0.1 and 1 μM DEX for 2 hours. 3) Control group: Osteoblasts that had not been given any treatment were cultivated for a comparable period.

2.3 Animal model

At the Shanghai Animal Center of the Chinese Academy of Sciences, 46 male Sprague-Dawley rats were acquired. They were given unlimited access to food and drink while being kept in an SPF environment with a 12-h light/12-h dark cycle. The Wenzhou Medical University Animal Research Committee approved all tests using rats, and the surgical procedures followed the guidelines established by the Ethics Committee for Animal Research (Animal Ethics Number:WYDW 2020-0564). After acclimation of the rats for 1 week, they were divided into three groups.: control, morphine, and DEX + morphine. The rats were subsequently rendered unconscious by an intraperitoneal dose of 50 mg/kg pentobarbital sodium. The dissection was carried down to the calvarium after a 1.5 cm incision was performed in the scalp. A 5-mm-diameter trephine was used to make a critical-size calvarial defect after the periosteum was removed, with saline water being given for cooling. For 12 weeks following craniotomy, rats in the DEX + morphine group received DEX injections at 7 mg/kg using a 5 L microinjector (flat tip diameter, 0.3 mm; needle length, 2.5 cm). A single dosage of 100 mg/kg of morphine hydrochloride was slowly injected into the rats in the morphine group.

2.4 Western blot assay

With the use of the RIPA lysis solution containing 1 mM PMSF (phenylmethanesulfonyl fluoride), the total protein in the

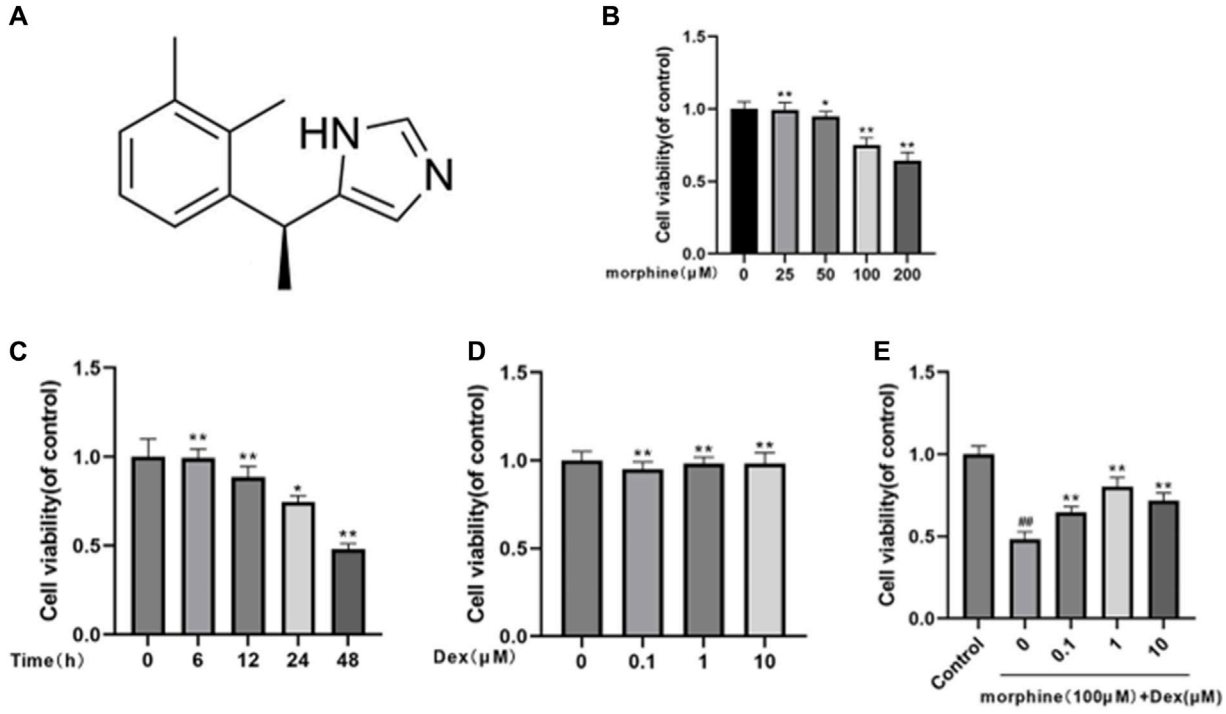

FIGURE 1
Effects of morphine and/or DEX administration on the viability of rat osteoblastic cells. **(A)** The dexmedetomidine chemical structure. **(B)** The percentage of viable cells treated for 24 h with various dosages of morphine. **(C)** Morphine treatment percentage is given to live cells throughout a time course of 0, 6, 12, 24, and 48 h at a 100 μM concentration. **(D)** How various DEX dosages affect cell viability. **(E)** The impact of morphine on cell survival when combined with various DEX concentrations. Data were evaluated as mean + SD. *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, compared to the morphine stimulation group, $n = 3$.

osteoblasts was extracted. The BCA protein assay kit (Beyotime) was used to calculate the protein concentration. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, the protein (40 ng) was separated, and then it was transferred to a polyvinylidene difluoride (PVDF) membrane. Following a 2-h blocking step in which the membrane was incubated with 5% non-fat milk, the membrane was then incubated overnight at 4°C with the primary antibodies COL1A1, NRF2, RUNX2, OCN, PI3K, p-PI3K, GAPDH, NQO1, GCLc, Gclm, HO-1, P-Akt, Akt, TrxR1, and Keap1. The bands were then identified using the matching secondary antibodies and an electrochemiluminescence reagent (Invitrogen) for 2 hours at room temperature. This was done after three TBST washes. The intensity of blots was then calculated using Image Lab 3.0 software (Bio-Rad).

2.5 Immunofluorescence

Six-well plates with glass coverslips were used to seed osteoblasts, which were then washed with PBS, fixed in 4% paraformaldehyde, and permeated with 0.1% Triton X-100 for 15 min. The osteoblasts were treated overnight at 4°C with the primary antibody against Nrf2 (1:200) following blocking with 5% bovine serum albumin for 30 min. The cells were washed the next day and labeled for 1 h at room temperature using Alexa Fluor 488 or Alexa Fluor 594. The slides were examined with a confocal scanning microscope (Nikon, Japan), and ImageJ software 2.1 (Bethesda, MD, United States) was used to evaluate the fluorescence intensity.

2.6 Osteogenic differentiation of Primary Calvarial Osteoblasts

Primary Calvarial Osteoblasts' Osteogenic Differentiation. A 24-well plate containing the cells was planted with 5 104 cells per well. Osteoblasts were grown in DMEM with 20 mM ascorbic acid and 10 mM -glycerophosphate after receiving the recommended therapy. Every other day, the media was switched out. After 7 days of differentiation, the alkaline phosphatase (ALP) activity was assessed using the ALP Staining Kit from the Beyotime Institute of Biotechnology in Jiangsu, China. The cells were grown in osteogenic conditions for 21 days to induce mineralization and the development of bone nodules. They were then fixed and stained with Alizarin Red S (ARS) by Solarbio Science and Technology, Beijing, China.

2.7 Quantification of SOD and CAT activities

The appropriately treated cells were lysed on ice for 30 min after being washed twice with PBS. According to the manufacturer's instructions, commercial assay kits (Jiancheng Biotechnology, Nanjing, China) were used to measure the activity of SOD and CAT in the lysates.

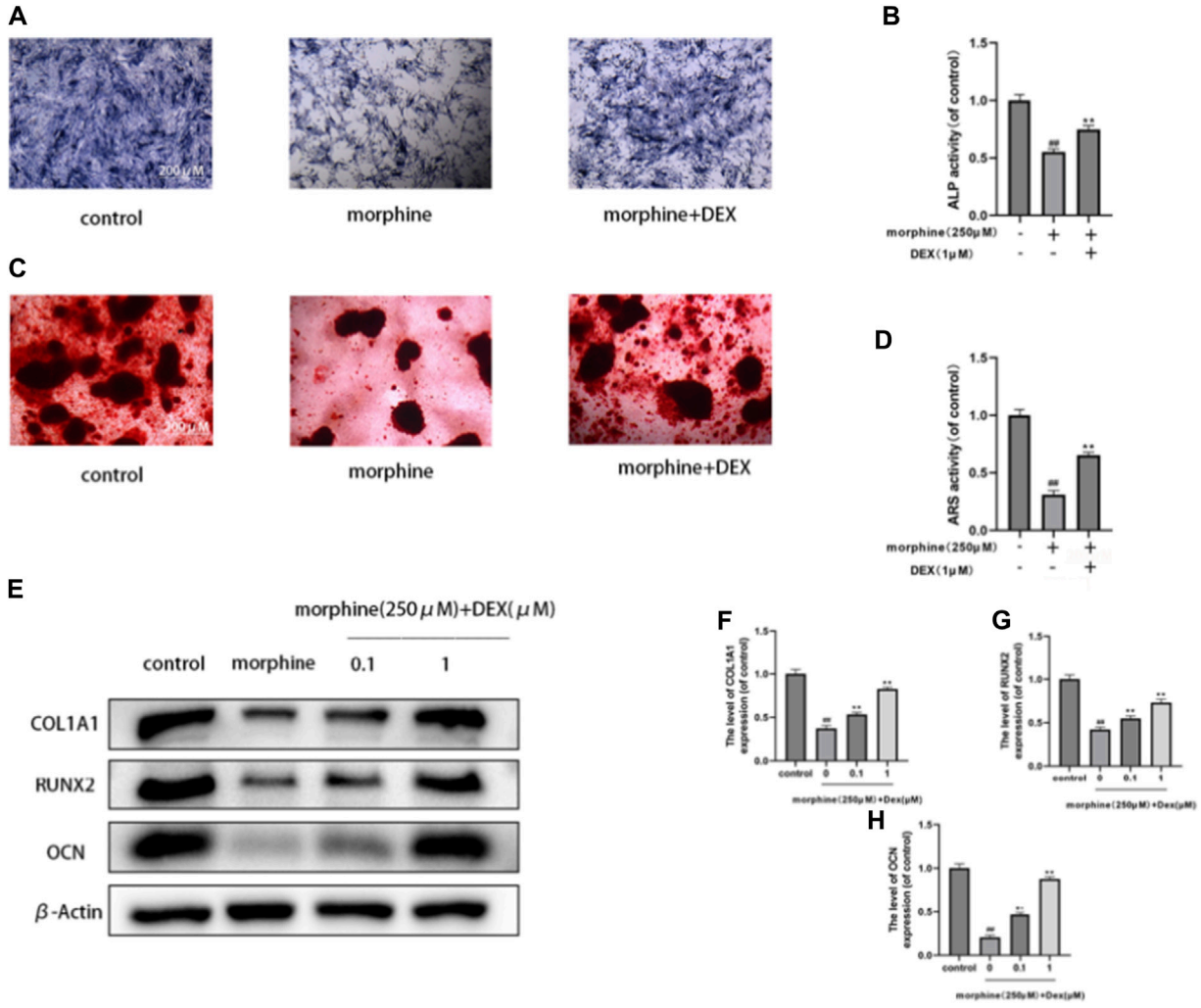

FIGURE 2
DEX reestablished the mineralization and differentiation of morphine-treated osteobiasts. **(A–D)** Differentially treated osteoblasts ALP stained on the 7th day and ARS stained on the 21st day after osteogenic induction. DEX restored differentiation and mineralization. **(E–H)** OCN, RUNX2, and COL1A1 expression levels in the variously treated osteoblasts. Data were evaluated as mean +SD. *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, compared to the morphine stimulation group, $n = 3$.

2.8 Intracellular ROS assay and mitochondrial function assays

Reactive oxygen species within cells were measured using a dihydroethidium (DHE) probe (Yeasen, Shanghai, China). According to the manufacturer's instructions (Beyotime, Shanghai, China), the amounts of superoxide ions and mitochondrial membrane potential (MMP) in the properly treated osteoblasts were measured by, respectively, staining with JC-1 and MitoSox. A fluorescent microscope (Olympus Life Science; Tokyo, Japan) and confocal scanning microscopy (Nikon; Japan) were used to examine stained cells.

2.9 Micro-CT analysis

Utilizing a cabinet conebeam micro-CT system and related software (CT 50, Scanco Medical; Brüttisellen, Switzerland), a microstructural investigation of the calvarial defect was carried out. The photos were taken at a voltage of 70 kV, an amperage of 200 A, and a spatial resolution of 14.8 mm all around. The trabecular compartment 2 mm below the greatest point of the growth plate to the distal 100 CT slices was included in the volume of interest (VOI), which was used to create three-dimensional reconstructed images. The ratio of bone volume to tissue volume (BV/TV), the mean trabecular number (Tb. N, 1/mm), the mean trabecular thickness (Tb. Th, mm), the mean connective density (Conn.D, 1/mm3) and the mean trabecular separation (Tb. Sp, mm) were among the quantitative bone characteristics evaluated inside the VOI.

2.10 Histopathologic analysis

After removing the dependent tissues, the calvarial defect tissues were collected and preserved with 4% formaldehyde for 48 h. The

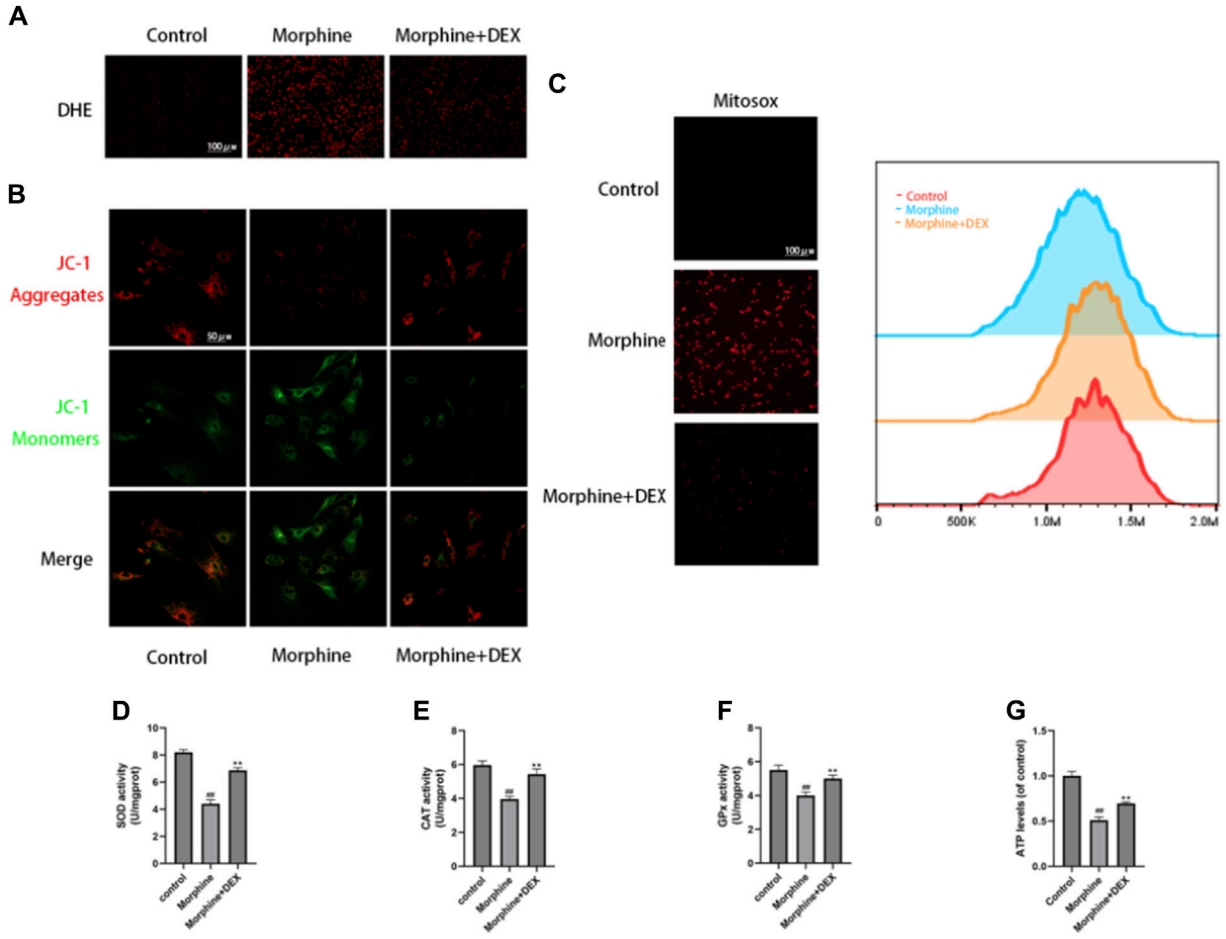

FIGURE 3
DEX Reduced Oxidative Stress in Osteoblasts Caused by Morphine. **(A)** Images of the DHE stains tnat are representative. **(B)** Confocal fluorescence microscopy was used to identify JC-1 aggregates (red) and monomers (green) in osteoblasts. **(C)** MitoSox (red) was used for mtROS staining. MitoSox (red) staining representative photos are displayed as the relative mean fluorescence intensity as determined by flow cytometry. **(D–F)** The activities of GPx, CAT, and SOD in cells treated with morphine with or without DEX. **(G)** The osteoblasts received various treatments and their ATP levels. Data were evaluated as mean +SD. *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, compared to the morphine stimulation group, $n = 3$.

samples were then decalcified for a month at 4 °C using a 10% ethylenediamine-tetraacetic acid (EDTA) solution. 70%–100% ethanol gradient dehydration, xylene clearing, and paraffin embedding. According to the manufacturer's directions, longitudinal 4 m-thick serial slices were stained with hematoxylin-eosin and Mason trichrome. For IHC, 6-m-thick sections were treated with the anti-VEGF, CD31 primary antibody before being subjected to the manufacturer's recommended horseradish peroxidase detection procedure (Vector Laboratories; Burlingame, CA, United States).

2.11 Statistical analysis

The findings have been laid out as mean ± SD. The statistical application SPSS 18.0 was used to examine the data. ANOVA or t-tests were used to evaluate whether there were differences between the groups. At P 0.05, statistical differences within and/or between groups were taken into consideration. Three separate runs of each experiment showed that they could all be reliably repeated.

3 Results

3.1 Effects of morphine and/or DEX treatment on rats' osteoblasts viability

Figure 1A depicts DEX's chemical make-up. The CCK8 test was used to investigate the effectiveness of morphine and/or DEX in preserving osteoblast viability. To determine the proper dose and duration of the stimulus, osteoblasts were first given morphine in a dose-dependent manner (0, 25, 50, 100, 200 μM) for 24 h and in a time-dependent manner (0, 6, 12, 24, 48 h) at 100 μM (Figures 1B, C). At a dosage of 100 μM morphine for 48 h, it was found that cell viability was significantly decreased (survival rate <50%). For the following investigations, 100 μM morphine with a 48 h treatment duration was used. The effects of DEX on the cell, whether or not it was combined with morphine, were then also

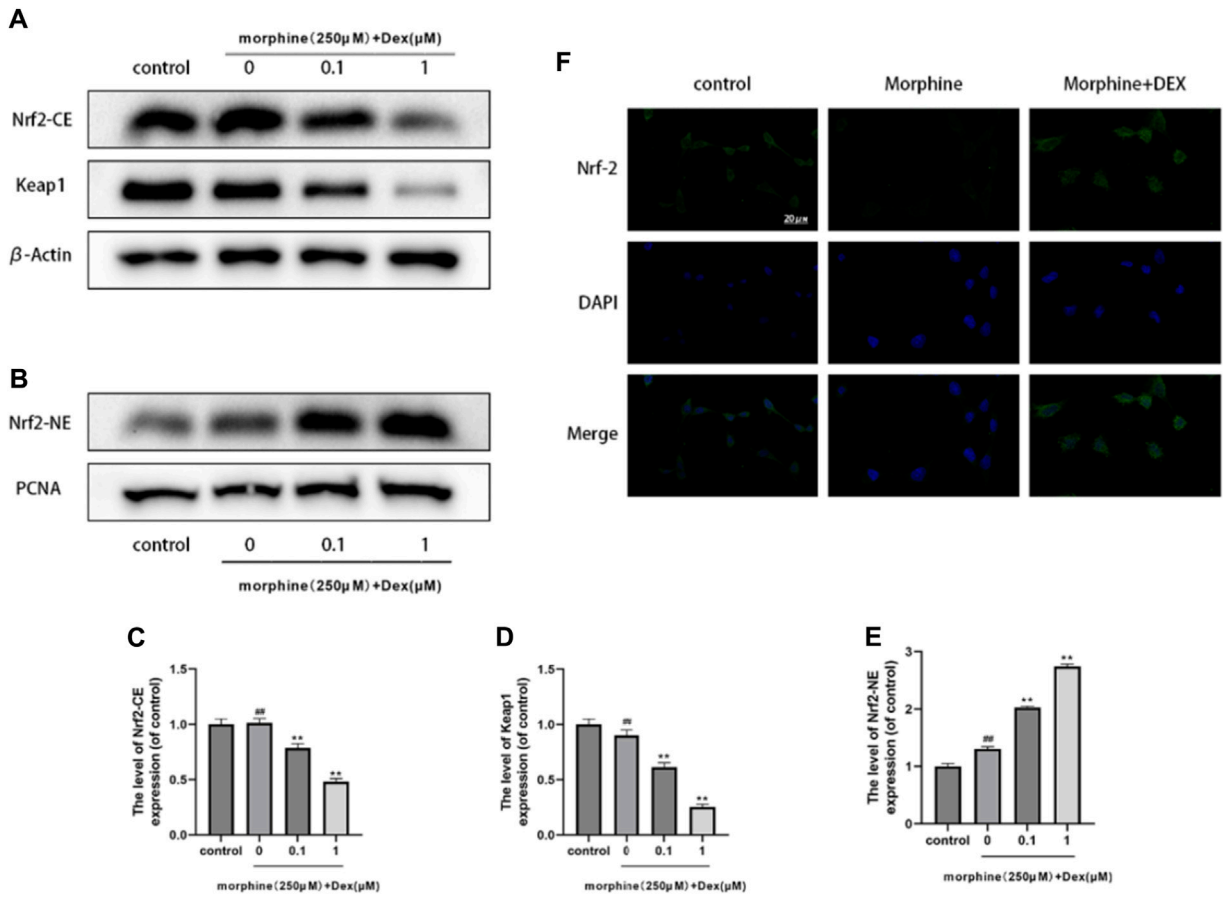

FIGURE 4
Effects of DEX on Nrf2 nuclear localization **(A–E)**. Immunoblot demonstrating Nrf2-CE, Nrf2-NE, and Keap1 levels in the variously treated osteoblasts. **(F)** Typical fluorescence pictures displaying Nrf2 localization in the cells that underwent different treatments. Data were evaluated as mean +SD. *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, compared to the morphine stimulation group, $n = 3$.

investigated. CCK-8 tests were used to determine how DEX affected cell viability. The findings showed that, in comparison to exposure to 0 μM DEX, there was no significant difference in cell viability when Osteoblasts were subjected to 0.1–10 μM DEX (Figure 1D). The CCK-8 results indicated that the safeguarding effect of DEX towards morphine-induced cytotoxicity was best at the concentration of 1 μM after the cells had been treated with 100 μM morphine and various doses of DEX (Figure 1E). The results mentioned above imply that DEX corrected the reduction in cell viability caused by morphine.

3.2 DEX reestablished the mineralization and differentiation of morphine-treated osteoblasts

Osteoblasts' capacity for bone formation is compromised by oxidative stress and mitochondrial malfunction. Next, we evaluated how DEX affected the morphine-treated cells' early differentiation and mineralization. Morphine significantly lowered calcium nodule development by the 21st day (Figures 2A–D) and significantly inhibited osteogenic differentiation, as seen by the ALP activity following a 7-day culture. After 7 days of osteogenic induction, cotreatment with DEX increased the levels of the osteogenic transcription factors OCN, COL1A1, and RUNX2, which also restored ALP activity and the degree of mineralization in the morphine-treated osteoblasts (Figures 2E–H). When combined, DEX shields osteoblasts from morphine's inhibitory effects.

3.3 DEX neutralized morphine-induced oxidative stress in osteoblasts

The primary pathogenic changes in delayed bone repair are increased apoptosis and senescence of osteoblasts caused by oxidative stress. We thus assessed the effects of DEX on the generation of ROS, dysfunction of the mitochondria, and the functioning of antioxidant enzymes in the morphine-treated osteoblasts. Using the DHE fluorescence to analyze intracellular ROS, it was shown that morphine administration enhanced ROS buildup, which was countered by DEX pretreatment (Figure 3A). JC-1 and MitoSox probes were used, respectively, to identify the MMP and mitochondrial ROS. In comparison to the control cells, the morphine-treated cells exhibited considerably decreased MMP and higher amounts of superoxide anion, both of which were nearly restored to normal levels by DEX administration (Figures 3B, C). Along with restoring ATP levels, morphine-treated osteoblasts

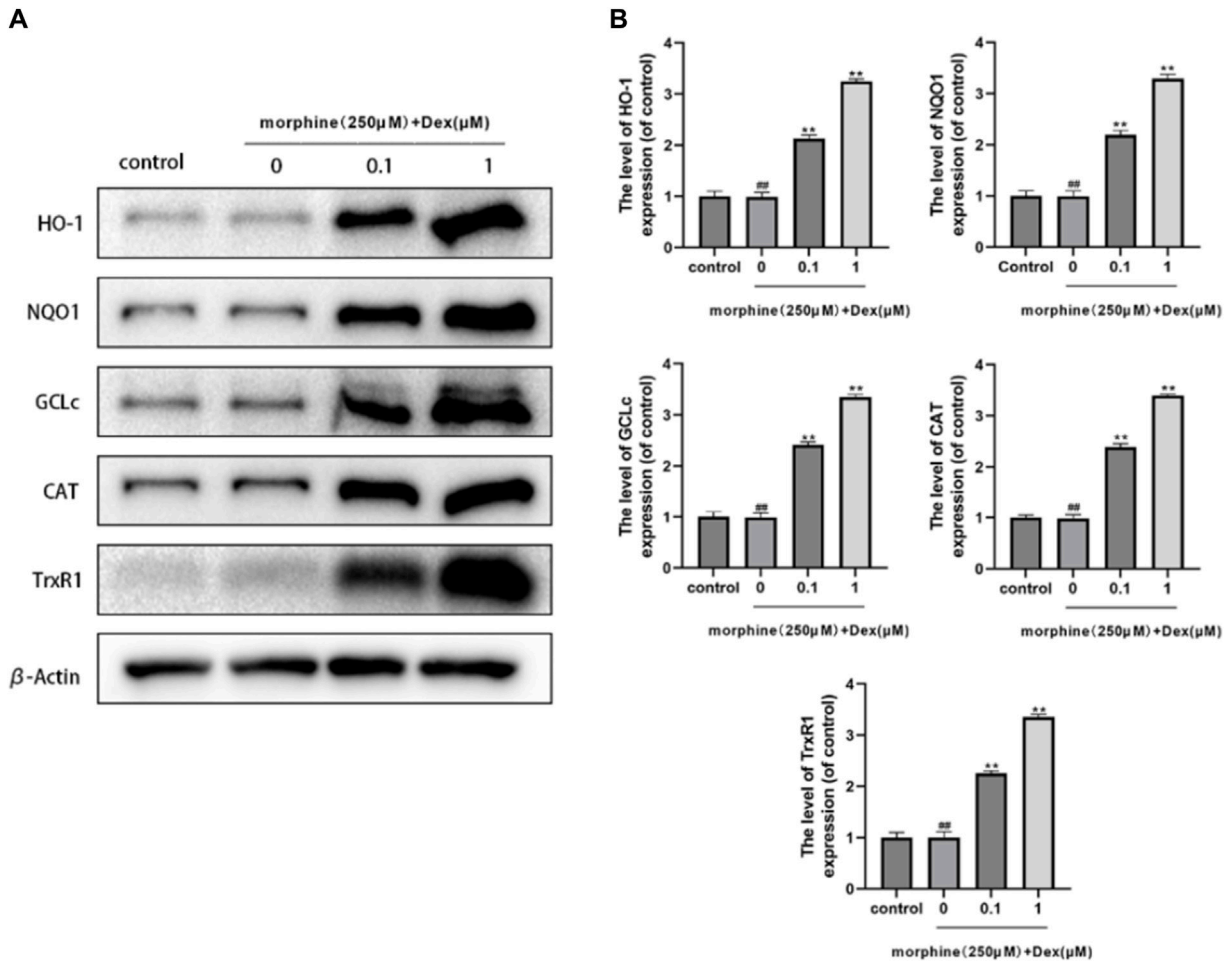

FIGURE 5
Effects of DEX on antioxidant/phase-I| detoxification enzymes. **(A,B)** The protein expression of HO-1, NQO1, GCLc, CAT, and TrxR1 is shown in the osteoblasts treated as described above. Data were evaluated as mean +SD. *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, compared to the morphine stimulation group, $n = 3$.

cocultured with DEX also restored the activities of CAT, GPx, and SOD (Figures 3D–G).

3.4 Effects of DEX on Nrf2 nuclear localization

The expression of Intracellular Nrf2 and Keapl was examined to establish DEX as the powerful Nrf2 activator and to demonstrate Nrf2 nuclear accumulation by DEX. Keapl typically locks Nrf2 away in the cytoplasm, however when Nrf2 is let out into the nucleus, antioxidant/phase II detoxification enzymes can be activated. The level of intracellular Nrf2 expression did not change after morphine treatment, as shown in Figure 4A (p 0.05). However, DEX significantly decreased cytoplasmic Keapl expression at all doses (Figure 4A). The nuclear translocation of Nrf2 was examined to identify the strong Nrf2 promoter as a need for stimulation of the endogenous protective antioxidant system. As seen in Figures 4B–E, DEX caused a more than threefold increase in Nrf2 nuclear accumulation whereas morphine had no impact on Nrf2 translocation. The findings of the immunofluorescence labeling showed that DEX co-administration considerably increased the expression of Nrf2 in comparison to the morphine-treated cells (Figure 4F). These findings indicate that Nrf2 activation is linked to DEX's protective effect against morphine-induced oxidative damage.

3.5 DEX's effects on phase-II detoxification enzymes and antioxidants

The production of antioxidant/phase II detoxifying enzymes, Nrf2's downstream target, provided evidence of the transcriptional action of Nrf2. DEX had an impact on the expression of Nrf2-driven antioxidant/phase II detoxification enzymes such as GCLc, CAT, TrxR1, HO-1, and NQO1. When compared to the morphine-treated group, the expression of HO-1, NQO1, GCLc, CAT, and TrxR1 was dramatically increased more than two times, demonstrating that DEX is a powerful trigger of Nrf2-driven antioxidant responses (Figure 5).

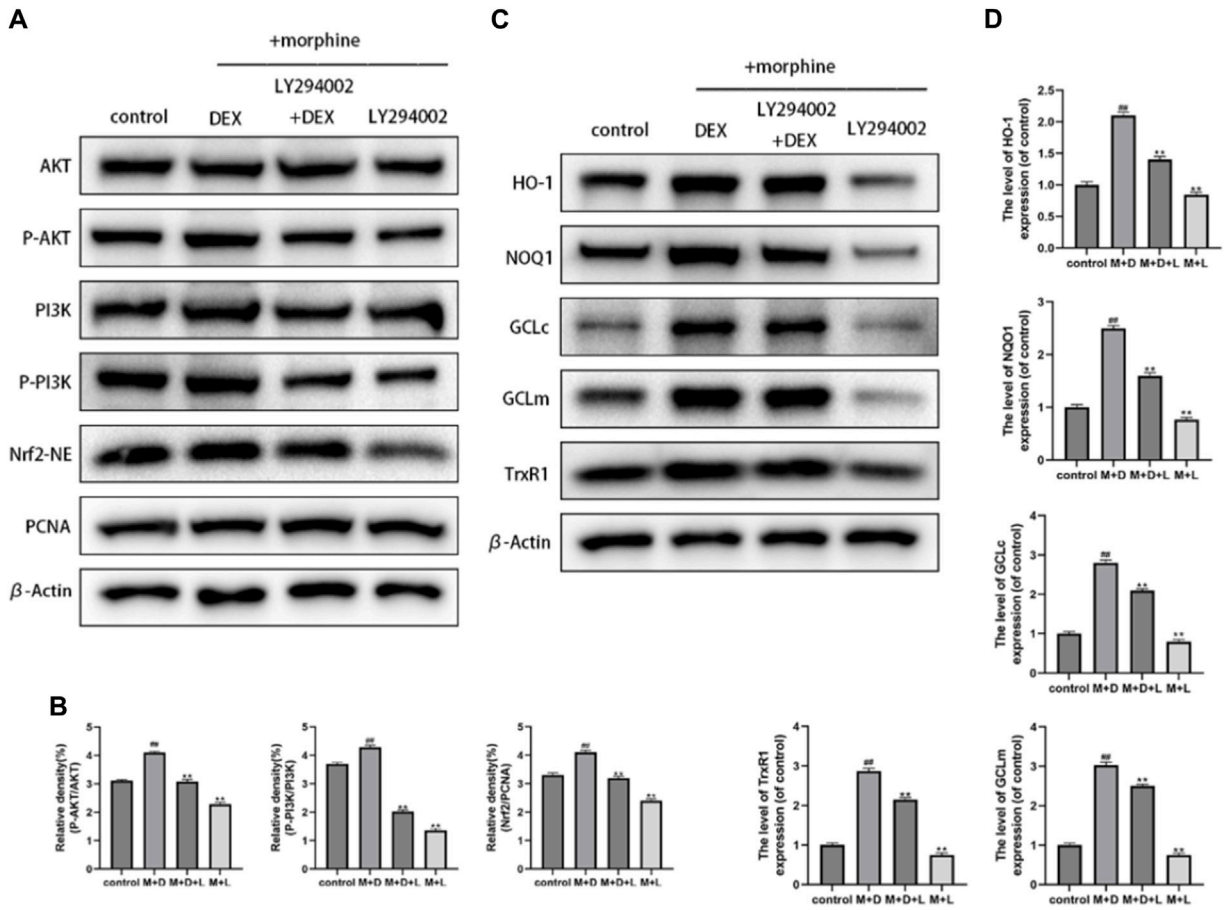

FIGURE 6
Effects of DEX on the PI3K/Akt/Nrf2 signaling pathway and downstream antioxidant enzymes **(A,B)** Immunoblot demonstrating the degree of PI3K and AKT phosphorylation. The osteoblasts that had received different treatments for Nrf2-NE levels using Western blotting. **(C,D)** The protein expression of GCLc, NQO1, GCLm, HO-1, and TrxR in the osteoblasts after the aforementioned treatment is displayed. Data were evaluated as mean +SD. *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, compared to the morphine stimulation group, $n = 3$.

3.6 Impacts of DEX on the downstream antioxidant enzymes and the PI3K/akt/Nrf2 signaling pathway

Recent research suggests that the Nrf2 function involves the PI3K/Akt signaling pathway. The activation of downstream targets like Akt and Nrf2 was suppressed using a particular PI3K inhibitor (LY294002) to investigate the mechanism by which DEX upregulates Nrf2 nuclear localization. LY294002 at 10 M significantly reduced the expression of phosphorylated Akt, phosphorylated PI3K, and nuclear Nrf2, as seen in Figures 6A, B. Furthermore, DEX and LY294002 together significantly reduced the increased Akt, PI3K phosphorylation, and Nrf2 nuclear expression caused by DEX at 1 M, showing that DEX-mediated Nrf2 activation was closely related to the PI3K/Akt signaling pathway. The corresponding changes in the gene expression of antioxidant enzymes by the PI3K blocker were discovered to comprehend the protective effectiveness of DEX against morphine-induced oxidative damage through the PI3K/Akt/Nrf2 pathway. The results demonstrated that co-treatment with DEX and LY294002 significantly reduced the expression levels of GCLc, GCLm, NQO1, and TrxR, and HO-1compared to DEX alone (Figures 6C, D).

3.7 DEX mitigates morphine-induced delayed bone healing *in vivo*

The essential size calvarial defect in the rats model was created by surgery to examine the preventive impact of DEX against delayed bone healing progression *in vivo*. Micro-CT scans of the morphine group showed minimal new bone growth compared with the control group. In mice treated with DEX, this behavior was, however, less severe (Figures 7A, B). Sections stained with H&E and Masson's revealed that the morphine group had negligible new bone development. Contrarily, a lot of bone growth was seen in the DEX group (Figures 7C, D). Immunohistochemical examination of VEGF and CD31 was carried out to better understand osteogenesis and angiogenesis (Figure 7E). Representative IHC staining in the morphine group did not show any significant positive staining for VEGF or CD31. The DEX group had more pronounced VEGF and CD31 positive brown staining. These findings were also validated by quantitative analysis (Figure 7F). These results showed that DEX therapy given in combination might enhance neovascularization and bone growth in rats.

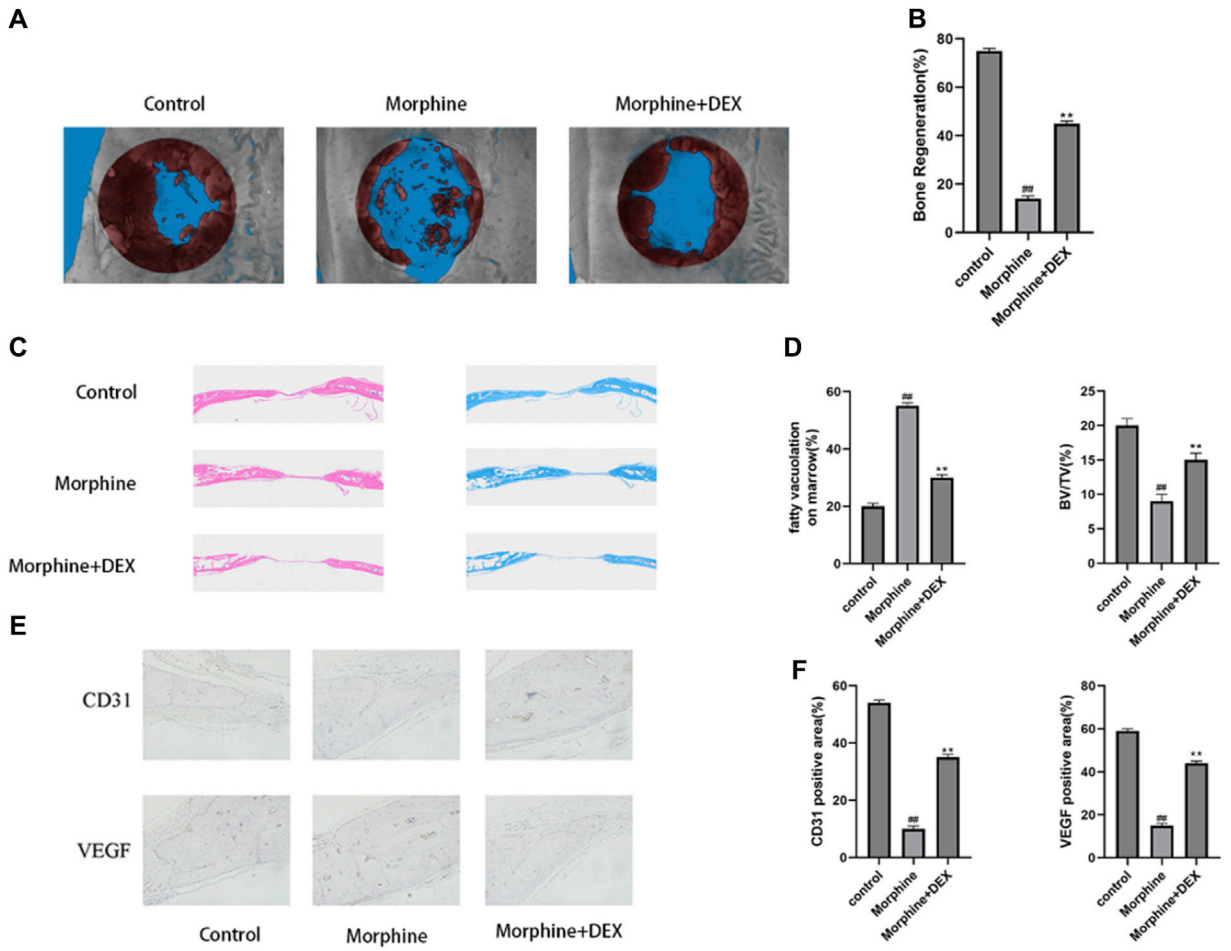

FIGURE 7
DEX slows down the delayed bone repair caused by morphine *in vivo*. **(A,B)** 12 weeks following surgery, a micro-CT examination of bone repair in calvarial defects was performed. **(C)** H&E staining and Masson's staining of calvarial bone. **(D)** Quantitative analysis of fatty vacuolation on marrow in HE and BV/TV in Masson staining. **(E,F)** Calvarial tissue was immunohistochemically stained for VEGF and CD31, and the IHC results were quantified. Data were evaluated as mean +SD. *$p < 0.05$, **$p < 0.01$ compared to the control group; #$p < 0.05$, ##$p < 0.01$, compared to the morphine stimulation group, $n = 3$.

4 Discussion

The common class of analgesic drugs known as opioids are used to treat both acute and chronic pain that is mild to severe (Corder et al., 2018). Evidence indicates that opioid users have a 55.1% higher risk of fractures. The exact mechanism is thought to be connected to opioid central nervous system side effects, which lower bone density. The secondary cause may be opioid endocrine effects (release of inhibitory hormones like prolactin and growth hormone, hypogonadism), or a direct impact on bone cells (King et al., 2007; Oladeji et al., 2021). Our goal was to research the effects of morphine exposure on Osteoblasts due to the disagreement around morphine's link with oxidative stress in the present literature and the paucity of information on osteoblasts. The nuclear factor-2 erythroid-related factor (Nrf2), the pathway's main transcription factor, is responsible for controlling the primary protective mechanisms against oxidative and electrophilic stresses (Ma, 2013). Indeed, it plays a vital role in many physiological functions, including inflammation and mitochondrial function, and it maintains redox homeostasis through endogenous antioxidant mechanisms (Chen, 2022). Numerous genes, including peroxiredoxins, and glutathione peroxidases, thioredoxin reductase 1 (TXNRD1), have been discovered as Nrf2 targets. Currently, more than 250 genes are known to be Nrf2 targets (Gao et al., 2020).

Dex is an agonist of the α-2 receptor that has analgesic, sedative, and anti-sympathetic properties (Lee, 2019). Dex preconditioning is protective against the IRI of important organs such as the brain, liver, heart, kidney, and lung in both *in vitro* and *in vivo* tests (Sun et al., 2019; Tang et al., 2020). Only a small number of research, nevertheless, have looked at how Dex postconditioning affects osteoblasts. Our research proved that Dex postconditioning effectively decreased oxidative stress, avoided apoptosis, and safeguarded osteoblasts. To promote apoptosis in osteoblasts during the *in vitro* experiment, we employed morphine as a ROS source in this work. DEX administration was shown to dramatically lower the levels of cyto and C-caspase3. Additionally, in the investigation at hand,

we found that Dex therapy enhanced the expression of the proteins OCN, RUNX2, and COL1A1 that are associated with osteogenesis. This improvement was accompanied by an increase in the osteogenic phenotype of ALP and ARS.

As a crucial transcription factor, Nrf2 is associated with stimulating the expression of downstream genes for a variety of antioxidant enzymes, including SOD, CAT, GPx, and HO-1 (Wang et al., 2021). In the critical size calvarial defect model systems, a variety of Nrf2-activating substances have demonstrated positive benefits, such as lowering inflammatory indicators, oxidative stress, and apoptosis and enhancing synaptic and mitochondrial function (Zhang et al., 2022). Enzymes involved in antioxidant/phase II detoxification contribute to protection by regulating the intracellular redox state (Li et al., 2008). The initial stepwise breakdown of heme is catalyzed by HO-1, producing powerful antioxidants such as free iron, carbon monoxide, and biliverdin (Gao et al., 2022). NQO1 detoxifies reactive quinones to its less harmful hydroquinones, protecting cells from oxidative stress (Ross and Siegel, 2021). TrxR is a homodimeric flavin enzyme that reduces oxidized thioredoxins through NADPH. It contains selenocysteine (Xu et al., 2022). In this work, an efficient antioxidant defense mechanism is provided by the overexpression of TrxR, NQO1, HO-1, and GCLs in response to DEX.

It has been proposed that the Nrf2 upstream regulator is the PI3K/Akt pathway (Liu et al., 2020). According to the results of the current investigation, Nrf2 nuclear accumulation and PI3K/Akt activation were trending in the same direction. Additionally, the DEX-induced production of Nrf2 and its downstream genes was effectively stopped by the PI3K inhibitor LY294002. These results show that DEX increases the nucleus localization of Nrf2, which is facilitated by activating the PI3K/Akt signaling pathway, and therefore stimulates the production of a group of antioxidant/phase II detoxification enzymes.

There is growing evidence that two major pathways can activate Nrf2 (Best et al., 2018). First, it specifically targets the Nrf2/Keapl complex by oxidizing the cysteine residues in Keapl, which modifies Keapl's conformation and causes the Nrf2-Keapl complex to dissociate (Zhao et al., 2018). The second mechanism involves the activation of upstream kinases such as mitogen-activated protein kinases (MAPKs), PI3K/Akt, AMP-activated protein kinase (AMPK), and protein kinase C (PKC). This causes Nrf2 to be phosphorylated and translocated into the nucleus, which encourages the expression of the antioxidant enzyme (Zhuang et al., 2019). According to the findings of the Western blot investigation, DEX activated Nrf2 and consequent downstream antioxidant enzymes through a twofold mechanism that involved the disruption of the Nrf2-Keapl complex and the PI3K/Akt axis. This allowed Nrf2 to be released and go into the nucleus, where it initiates the transcription of antioxidant genes. Our results offer the first proof that DEX may counteract the oxidative stress caused by morphine by triggering the antioxidant defense mechanism that is driven by Nrf2 and that DEX upregulates and activates Nrf2 through the PI3K/Akt pathway.

The result of the present study should be interpreted with some limitations. 1) In the preparation of the animal model for this study, a intraperitoneal dose of 50 mg/kg pentobarbital sodium was used to make rats lose consciousness. Therefore, the combined effect of dexmedetomidine and morphine was obtained under the premise of using pentobarbital sodium, and the combined effect of the two drugs under other anesthesia methods needs to be further explored. 2) There are multiple pathways involved in antioxidant activity, and studies have shown that dexmedetomidine also has an effect on other pathways. This experiment only studied the Nrf2-pI3k pathway. 3) Dexmedetomidine and morphine are commonly used drugs in clinical anesthesia, and the combined use of the two drugs still needs clinical validation for their impact on bone healing.

5 Conclusion

The above results give empirical support for the hypothesis that DEX prevents morphine-induced oxidative injury by enhancing the Nrf2-mediated antioxidant system and PI3K/Akt pathway. According to molecular docking research, Dex displayed substantial interactions with PI3K, Akt, and Nrf2-keapl through hydrogen bonds and van der Waals forces. The first time, the proof was shown that DEX exerted safeguarding properties by activating the antioxidant defense mechanism regulated by PI3K/Akt/Nrf2 in morphine-induced oxidative stress.

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 authors. The original raw data can be found here: https://pan.baidu.com/s/1bL7jX0Ba7E00Ly6Kzaq7vQ, password: 2t54.

Ethics statement

The animal study was approved by The Wenzhou Medical University Animal Research Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL: Data curation, Formal Analysis, Methodology, Project administration, Writing–original draft. LZ: Data curation, Formal Analysis, Methodology, Writing–original draft. ZS: Data curation, Formal Analysis, Writing–original draft. JZ: Data curation, Formal Analysis, Writing–original draft. YL: Data curation, Formal Analysis, Writing–original draft. ZZ: Data curation, Formal Analysis, Writing–original draft. XC: Data curation, Formal Analysis, Writing–original draft. JP: Conceptualization, Project administration, Supervision, Writing–review and editing. XZ: Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing–review and editing.

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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

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The autophagic regulation of rosiglitazone-promoted adipocyte browning

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Objective: Browning of white adipocytes is considered an efficient approach to combat obesity. Rosiglitazone induces the thermogenetic program of white adipocytes, but the underlying mechanisms remain elusive.

Methods: Expression levels of browning and autophagy flux markers were detected by real-time PCR and immunoblotting. H&E and Oil Red O staining were performed to evaluate the lipid droplets area. Nuclear protein extraction and immunoprecipitation were used to detect the proteins interaction.

Results: In this study, we reported that rosiglitazone promoted adipocyte browning and inhibited autophagy. Rapamycin, an autophagy inducer, reversed adipocyte browning induced by rosiglitazone. Autophagy inhibition by rosiglitazone does not prevent mitochondrial clearance, which was considered to promote adipose whitening. Instead, autophagy inhibition increased p62 nuclear translocation and stabilized the PPARγ–RXRα heterodimer, which is an essential transcription factor for adipocyte browning. We found that rosiglitazone activated NRF2 in mature adipocytes. Inhibition of NRF2 by ML385 reversed autophagy inhibition and the pro-browning effect of rosiglitazone.

Conclusion: Our study linked autophagy inhibition with rosiglitazone-promoted browning of adipocytes and provided a mechanistic insight into the pharmacological effects of rosiglitazone.

KEYWORDS

rosiglitazone, PPARγ agonist, autophagy, mitophagy, adipocyte browning, obesity

1 Introduction

Obesity has become a worldwide epidemic in recent decades (Wang et al., 2021; Chen et al., 2023). Development of obesity is caused by hypertrophy and hyperplasia of white adipocytes, which are a key component in lipid-storing adipose. Distinct from white adipocytes, beige/brown adipocytes dissipate energy as heat and are considered beneficial in combating obesity (Gao et al., 2018; Ying and Simmons, 2020). Interestingly, white adipocytes can be transdifferentiated into beige adipocytes upon cold stimulation or sympathetic nerve activation, which is termed browning. Adipocyte browning can be observed in human adults (Finlin et al., 2018), highlighting its potential value in combating obesity (Ro et al., 2019).

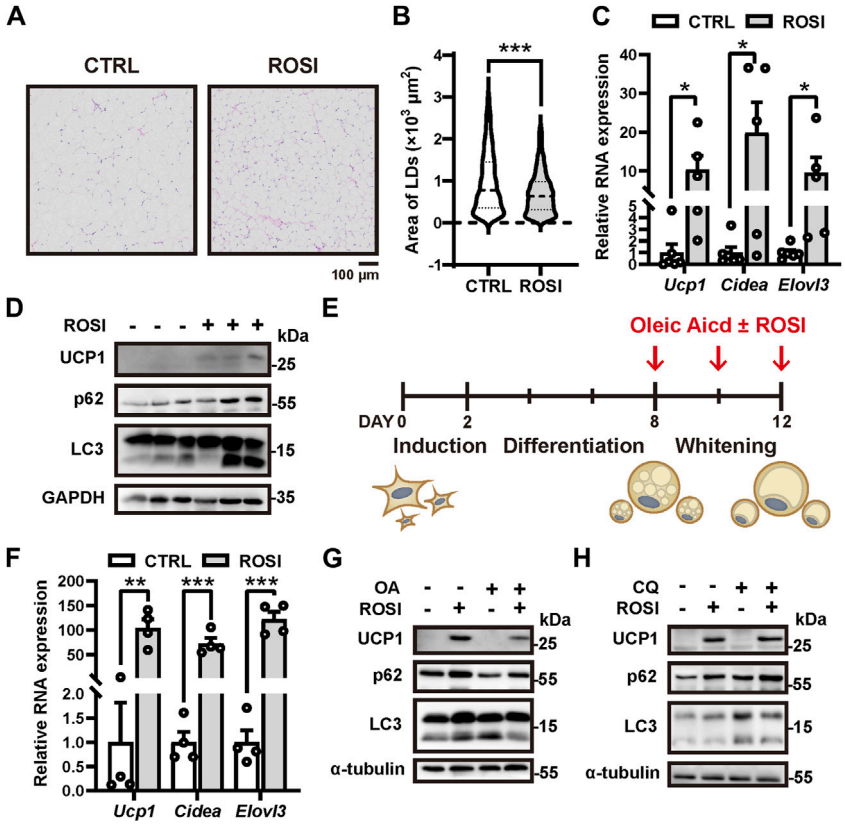

FIGURE 1
Rosiglitazone promotes white adipocyte browning while inhibiting autophagy in high-fat diet (HFD)-induced obesity mice and cultured adipocytes. **(A–D)** HFD-induced obesity mice were injected subcutaneously with 2.5 mg/kg solvent (CTRL) or rosiglitazone (ROSI) daily for 2 weeks. **(A)** Inguinal white adipose tissue (iWAT) depots were fixed and stained by H&E (n = 5–6 mice, per group). Scale bar, 100 μm. **(B)** Quantification of lipid droplet size in adipocytes from H&E staining shown in **(A)**. **(C)** Total RNA levels of *Ucp1*, *Cidea*, and *Elovl3* in iWAT were measured by qRT-PCR (n = 5–6 mice, per group). **(D)** UCP1, p62, and LC3 protein levels in iWAT were measured by Western blotting. GAPDH was blotted as a loading control. **(E)** Scheme for *in vitro* induction of adipocyte differentiation and whitening in **(F–H)**. When reaching confluence, stromal vascular fraction from iWAT was treated with the induction medium for 2 days and then switched to differentiation medium for 6 days. After differentiation, 30 μM oleic acid (OA) was treated for 4 days to induce mature adipocyte whitening. Any other reagents indicated in **(F–H)** were added together with OA. **(F)** Total RNA levels of *Ucp1*, *Cidea*, and *Elovl3* were measured by qRT-PCR in OA-treated cultured adipocytes from CTRL and ROSI (10 μM) groups. (n = 4 trials). **(G,H)** UCP1, p62, and LC3 protein levels were measured by Western blotting in matured adipocytes treated with OA, ROSI, and chloroquine (CQ, 10 μM) for 4 days. β-actin and α-tubulin were blotted as loading controls. Data in **(B,C, F)** are shown as mean ± SEM. Statistical analysis was performed by the Mann–Whitney test in **(B)** and t-test in **(C)** and **(F)**. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ *versus* CTRL.

Rosiglitazone is a thiazolidinedione used as an antidiabetic drug (Lehmann et al., 1995). Notably, treatment with rosiglitazone in inguinal white adipose tissue (iWAT) induces browning of adipocytes and alleviates high-fat diet-induced obesity in mice (Petrovic et al., 2010). It has been proposed that rosiglitazone activates peroxisome proliferator-activated receptor-γ (PPARγ), which enhances the transcription of genes essential for adipocyte browning (Digby et al., 1998). Nevertheless, the molecular and cellular alternations underlying the pro-browning effects of rosiglitazone have not been fully understood (Fayyad et al., 2019).

Autophagy is an evolutionary conserved process that degrades cellular molecules and organelles through the autophagosome–lysosome pathway (Reggiori and Klionsky, 2002; Wang and Klionsky, 2003; Levine and Klionsky, 2004). Accumulating evidence suggests that autophagy plays a crucial role in promoting brown-to-white adipocyte transition. White adipose tissue in adipocyte-specific *Atg7* knockout mice had increased browning features (Singh et al., 2009). In addition, adipocyte-specific deletion of *Atg5* or *Atg12* prevents loss of beige adipocytes in the whitening process after withdrawal of β3 adrenergic receptor agonist (Altshuler-Keylin et al., 2016). On the contrary, overactivation of BECN1, a pro-autophagy protein, by F121A mutation in primary adipocytes accelerates the loss of beige characteristics (Wu et al., 2023). Moreover, autophagy-induced mitochondrial clearance (mitophagy) also accelerates whitening of beige adipocytes (Taylor and Gottlieb, 2017; Lu et al., 2018). Rosiglitazone inhibits autophagy in hepatic cell line LX2 and spinal cord injury neurons, implying that rosiglitazone might play a role in autophagy inhibition (Li et al., 2017; Yum et al., 2023). Therefore, we aimed to explore whether and how rosiglitazone inhibits autophagy to induce adipocyte browning.

2 Materials and methods

2.1 Animal

Eight-week-old male C57BL/6 mice were fed on a high-fat diet (HFD) for 8 weeks. Then, two groups with five to six mice in each

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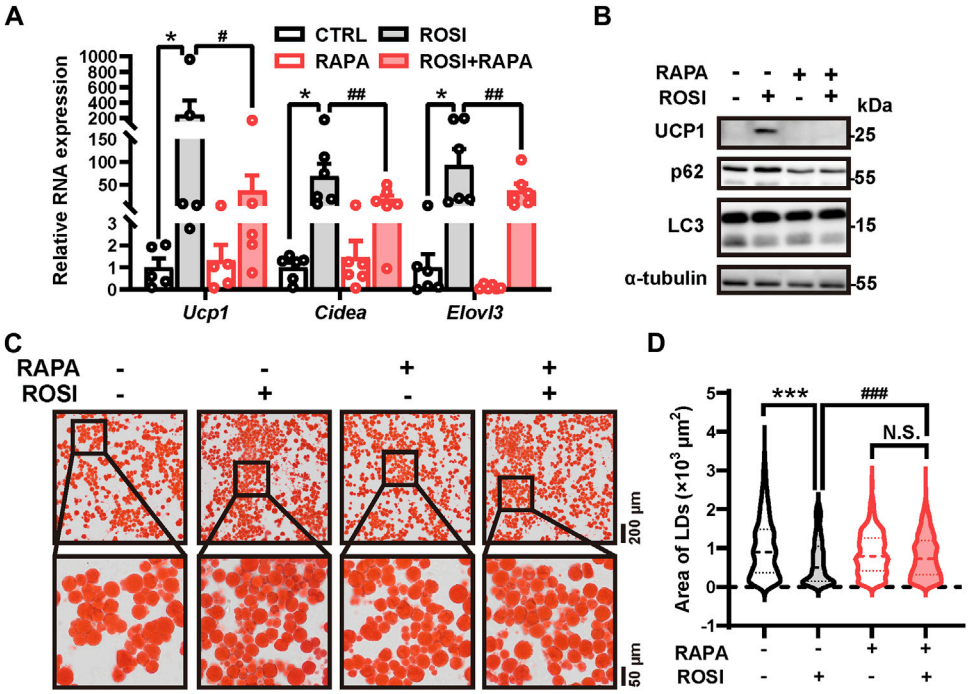

FIGURE 2
Rosiglitazone induces white adipocyte browning by inhibiting autophagy in oleic acid-treated adipocytes. **(A–D)** OA-induced whitened adipocytes were treated with rosiglitazone (ROSI, 10 μM) and rapamycin (RAPA, 5 nM) for 4 days. **(A)** Total RNA levels of *Ucp1*, *Cidea*, and *Elovl3* were measured by qRT-PCR (n = 5–6 trials). **(B)** UCP1, p62, and LC3 protein levels were measured by Western blotting. α-Tubulin was blotted as a loading control. **(C)** Lipid droplets in adipocytes were marked by Oil Red O staining (n = 3 trials). The square box shows the zoomed-in view of the indicated image. Upper scale bar, 200 μm; lower scale bar, 50 μm. **(D)** Quantification of lipid droplets' size from Oil Red O staining shown in **(C)**. (n = 600–1,000 lipid droplets per group) Data in **(A)** and **(D)** are shown as mean ± SEM. Statistical analysis was performed by one-way ANOVA and nonparametric test with Dunn's multiple comparisons. *p < 0.05, ***p < 0.001 *versus* CTRL. #p < 0.05, ##p < 0.01, and ###p < 0.001 *versus* ROSI.

group were treated with the solvent (containing 0.5% DMSO, 40% PEG 400, 50% Tween-80, and 54.5% saline) or rosiglitazone (2.5 mg/kg) for 2 weeks subcutaneously on both sides of inguinal white adipose tissue under isoflurane anesthesia. The mice were kept on a HFD during drug administration. At the end of rosiglitazone treatment, the mice were euthanized by CO_2 asphyxiation, and iWATs were collected for analysis.

All the animal experiments were approved by and conducted in accordance with the ethical guidelines of the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2 Cell culture

Two-week-old male C57BL/6 mice were euthanized by CO_2 asphyxiation, and iWATs were isolated on ice and then digested with collagenase type I for an hour in a 37°C water bath. Cells were filtered through a 70-μm filter and centrifuged for 5 min at 500 g. The cell pellet was resuspended and cultured in DMEM/F12 medium containing 10% FBS. On reaching confluence, the culture medium was changed with the induction medium (DMEM/F12 containing 10% FBS, 5 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 125 μM indomethacin) for 2 days. Then, it was changed into the differentiation medium (DMEM/F12 containing 10% FBS and 5 μg/mL insulin) for 6 days. For experiments pertaining to differentiating of adipocytes, 10 μM rosiglitazone was added in the differentiation medium for the indicated group. For experiments pertaining to mature adipocytes, the differentiation medium was changed with DMEM/F12 containing 10% FBS and 30 μM oleic acid for 4 days. Rosiglitazone (10 μM), 5 nM rapamycin, 10 μM chloroquine, or 10 μM ML385 were added in the media for 4 days in the indicated groups.

2.3 Histological analysis

iWATs from four mice of each group were isolated and fixed in 4% paraformaldehyde for 24 h and then embedded in paraffin. Tissue sections (6 μm) were stained with hematoxylin and eosin (H&E). Cells of the cultured adipocytes were grown on the microscope cover glasses. After the indicated treatment, cells were fixed in 4% paraformaldehyde for 10 min and stained with Oil Red O for 10 min. The experiments were repeated independently in at least triplicate. Both H&E staining of the iWAT sections and Oil Red O staining of the cultured adipocytes were visualized under 20-fold magnification by a Virtual Slide System (OLYMPUS, VS120).

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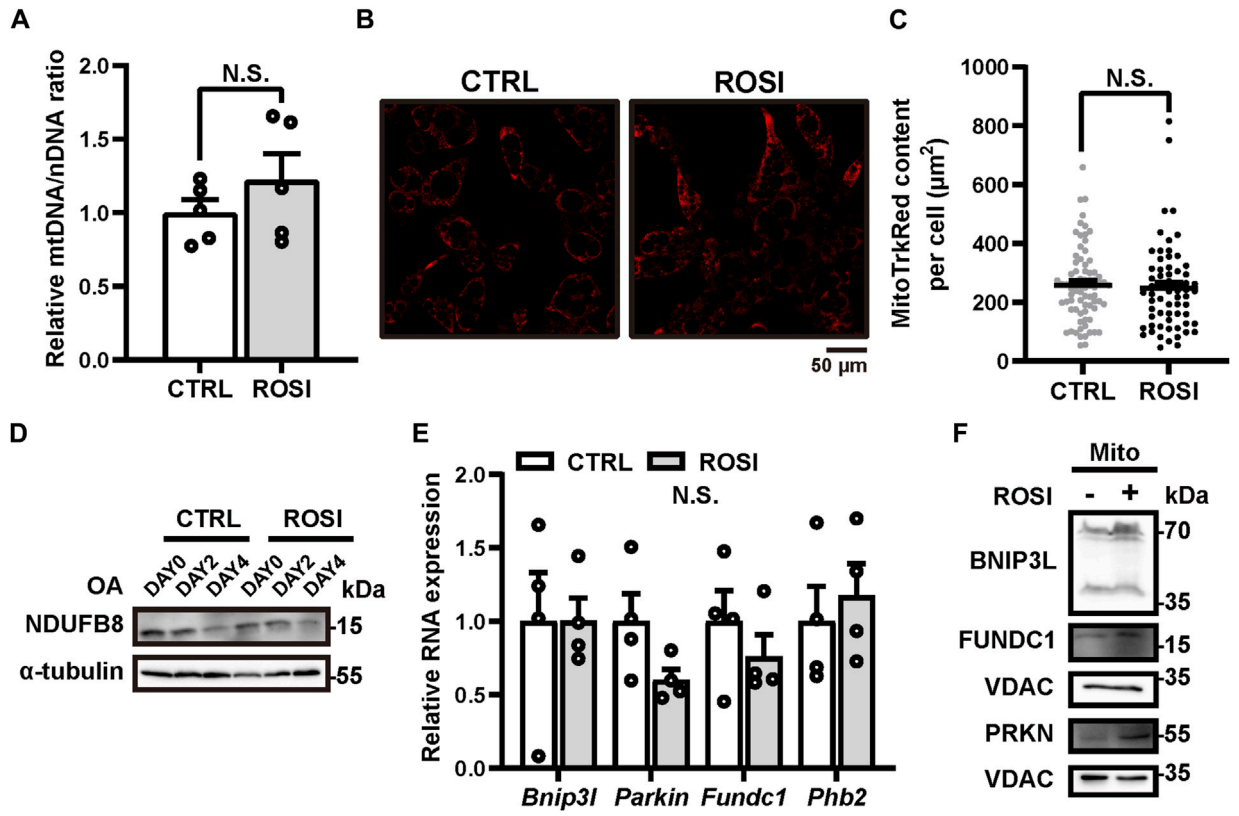

FIGURE 3
Rosiglitazone could not inhibit mitophagy in oleic acid-treated whitened adipocytes. **(A–F)** OA-induced whitened adipocytes were treated with rosiglitazone (ROSI, 10 μM) for 4 days. **(A)** Relative expressions of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) were measured by qRT-PCR (n = 5 trials). **(B)** Representative live image of MitoTracker Red staining in control or ROSI-treated adipocytes. Scale bar, 50 μm. **(C)** Quantification of MitoTracker Red fluorescence area showed in **(B)**. (n = 60–80 cells per group) **(D)** NDUFB8 protein levels were measured by Western blotting. α-Tubulin was blotted as a loading control. **(E)** Total RNA levels of *Bnip3l*, *Parkin*, *Fundc1*, and *Phb2* were measured by qRT-PCR (n = 4 trials). **(F)** BNIP3L, PRKN, and FUNDC1 protein levels in isolated mitochondria were measured by Western blotting. VDAC was blotted as a loading control. Data in **(A,C, E)** were shown as mean ± SEM. Statistical analysis by t-test shown in **(A)** and **(E)** and the Mann–Whitney test in **(C)**.

The area of lipid droplets was analyzed using ImageJ with at least 500 lipid droplets in randomly selected fields.

2.4 Cytoplasmic and nuclear protein extraction

Cytoplasmic and nuclear proteins from primary cultured adipocytes were extracted following the instructions of the Cytosolic and Cytoplasmic Protein Extraction Kit (Abbkine, KTP3001). Cells were washed with cold PBS, harvested with a spatula, and then centrifuged at 500 g for 5 min at 4°C. Two hundred microliters of working CESA (1:100 with protease inhibitor, 1:500 with DTT) was added and vortexed vigorously for 15 s to completely resuspend the cell pellets. After keeping it on ice for 15 min, 10 uL of pre-cooled CESB was added, vortexed for 10–15 s, and left on ice for 2 min. After centrifuging at 16,000 g for 5 min at 4°C, the supernatant extracted is the cytoplasmic protein. After resuspending the precipitate with pre-cooled working NES (1:100 for protease inhibitor and 1: 500 for DTT), it is allowed to stand on ice for 30 min and vortexed for 15 s every 10 min. Then, it is centrifuged at 16,000 g for 5 min at 4°C. The supernatant extracted is a nuclear protein, which is then subjected to immunoblotting.

2.5 Immunoblotting and immunoprecipitation

iWATs and primary cultured adipocytes were homogenized using RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS; pH 7.4). The Nuclear and Cytoplasmic Protein Extract Kit (Abbkine, KTP3001) and Mitochondria Protein Extract Kit (Sangon Biotech, C500051) were used for isolation of nuclear and mitochondria protein, respectively. The protein extracts were separated by SDS-PAGE and transferred onto nitrocellulose membrane (PALL, 66485). After blocking with 5% skim milk powder (BioFroxx, 3250GR500) for 1 h, the membranes were incubated with the following primary antibodies overnight at 4°C: BNIP3L (Cell Signaling Technology, 12396S; 1:1000), GAPDH (ABclonal, A19056; 1:3000), FUNDC1 (ABclonal, A16318; 1:

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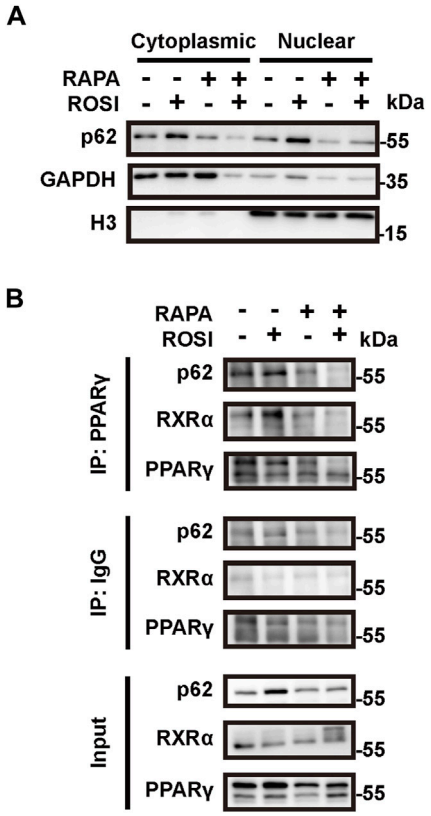

FIGURE 4
Rosiglitazone promotes nuclear translocation of p62 and stabilizes the PPARγ-RXRα heterodimer through autophagy inhibition. **(A,B)** OA-induced whitened adipocytes were treated with rosiglitazone (ROSI, 10 μM) and rapamycin (RAPA, 5 nM) for 4 days. **(A)** p62 protein levels in cytoplasmic and nuclear proteins of cultured adipocytes were measured by Western blotting. GAPDH was blotted as a loading control of the cytoplasmic protein, and H3 was blotted as a loading control of the nuclear protein. **(B)** Protein–protein interactions of p62, PPARγ, and RXRα in the nucleus were confirmed by immunoprecipitation.

1000), Histone H3 (ABclonal, A17562; 1:1000), LC3B (ABclonal, A19665; 1:1000), NRF2 (ABclonal, A0674; 1:1000), PPARγ (Proteintech, 16643-1-AP; 1:1000), Parkin (ABclonal, A0968; 1:1000), RXRα (ABclonal, A19105; 1:1000), SQSTM1/p62 (ABclonal, A19700; 1:1000), total OXPHOS (Abcam, ab110413; 1:1000), UCP1 (ABclonal, A21979; 1:1000), α-tubulin (ABclonal, A6830; 1:1000), and β-actin (ABclonal, AC026; 1:50000). After incubation with the secondary antibodies [HRP Goat Anti-Rabbit IgG (ABclonal, AS014; 1:5000) and HRP Goat Anti-Mouse IgG (ABclonal, AS003; 1:5000)] for 1 h, the blots were detected by the ECL Enhanced Kit (ABclonal, RM00021).

For immunoprecipitation experiments, primary cultured adipocytes were lysed using weak RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, and 0.25% sodium deoxycholate; pH 7.4). The indicated primary antibodies [PPARγ (Proteintech, 16643-1-AP; 5 μg/mL) or Rabbit IgG (Beyotime, A7016; 5 μg/mL)] and then protein extracts were bound with Protein A/G Magnetic Beads (MCE, HY-K0202) for 2 h at 4°C. The immunoprecipitates were eluted by 1 × loading buffer (ABclonal, RM00001) boiled for 5 min at 95°C, and subjected to immunoblotting.

2.6 Real-time PCR

Total RNA from iWATs and cultured adipocytes was extracted using TRIzol reagent (Vazyme, R411-01) and an RNA Extraction Kit (Accurate Biology, AG21017), respectively. After quantification of RNA concentration by NanoDrop One (Thermo Scientific), the RNA was reverse-transcribed into cDNA using the Evo M-MLV RT Mix Kit (Accurate Biology, AG11728). Relative RNA expression was analyzed by LightCycler 480 Instrument II (Roche) using SYBR Green Premix (Accurate Biology, AG11701). The reaction parameters were set at 95°C for 30 s, 95°C for 5 s, 60°C for 30 s (45 cycles), 95°C for 15 s, 60°C for 15 s, 95°C continuous for melt curves, and held at 40°C. The primers used for the experiments are listed in Supplementary Table S1.

2.7 Confocal imaging

For imaging of live adipocytes, cells were grown on confocal dishes (NEST). Before photographing, cells were treated with 100 nM MitoTracker Red (Invitrogen, M7512) for 30 min and Hoechst 33258 (Abcam, ab228550; 1:500) for 10 min. Cells were observed by using a confocal microscope (Leica, TCS SP8). At least five randomly selected fields were analyzed for each group by ImageJ.

2.8 Statistical analysis

Statistics were analyzed using GraphPad Prism 9.3. All the data were shown in mean ± SEM. A two-tailed Student's t-test or one-way ANOVA was used for single or multiple comparisons. The nonparametric test was used for non-normally distributed data. A value of $p < 0.05$ was considered significantly different.

3 Results

3.1 Rosiglitazone inhibits autophagy while promoting adipocyte browning

Rosiglitazone (ROSI) promotes white adipocyte browning, but the molecular mechanism is not fully understood (Nedergaard and Cannon, 2014). High-fat diet (HFD)-treated mice were administrated rosiglitazone (2.5 mg/kg, *s.c.*, daily) for 2 weeks. We observed a significant reduction in the size of adipocyte lipid droplets (LD) in the inguinal white adipose tissue (iWAT), indicating white-to-beige adipocyte transition (Figures 1A, B). Moreover, transcription of browning-related genes *Ucp1*, *Cidea*, and *Elovl3* and the protein level of UCP1 were significantly enhanced in iWAT with ROSI treatment (Figures 1C, D). These results confirmed that ROSI induced adipose browning. To explore the involvement of autophagy activity in the pro-browning effects of ROSI, we detected the autophagy-related proteins in iWAT. It showed that both p62 and LC3-II were accumulated with ROSI treatment, suggesting an autophagic flux blockage by ROSI in iWAT (Figure 1D). Moreover, primary cultured mouse adipocytes were incubated with oleic acid (OA) for 4 days to induce whitening, and ROSI was treated to adipocytes during D8 to D12 (Figure 1E). Consistent with the findings in mice iWAT, ROSI significantly upregulated the transcription of *Ucp1*, *Cidea*, and *Elovl3* and enhanced the protein level

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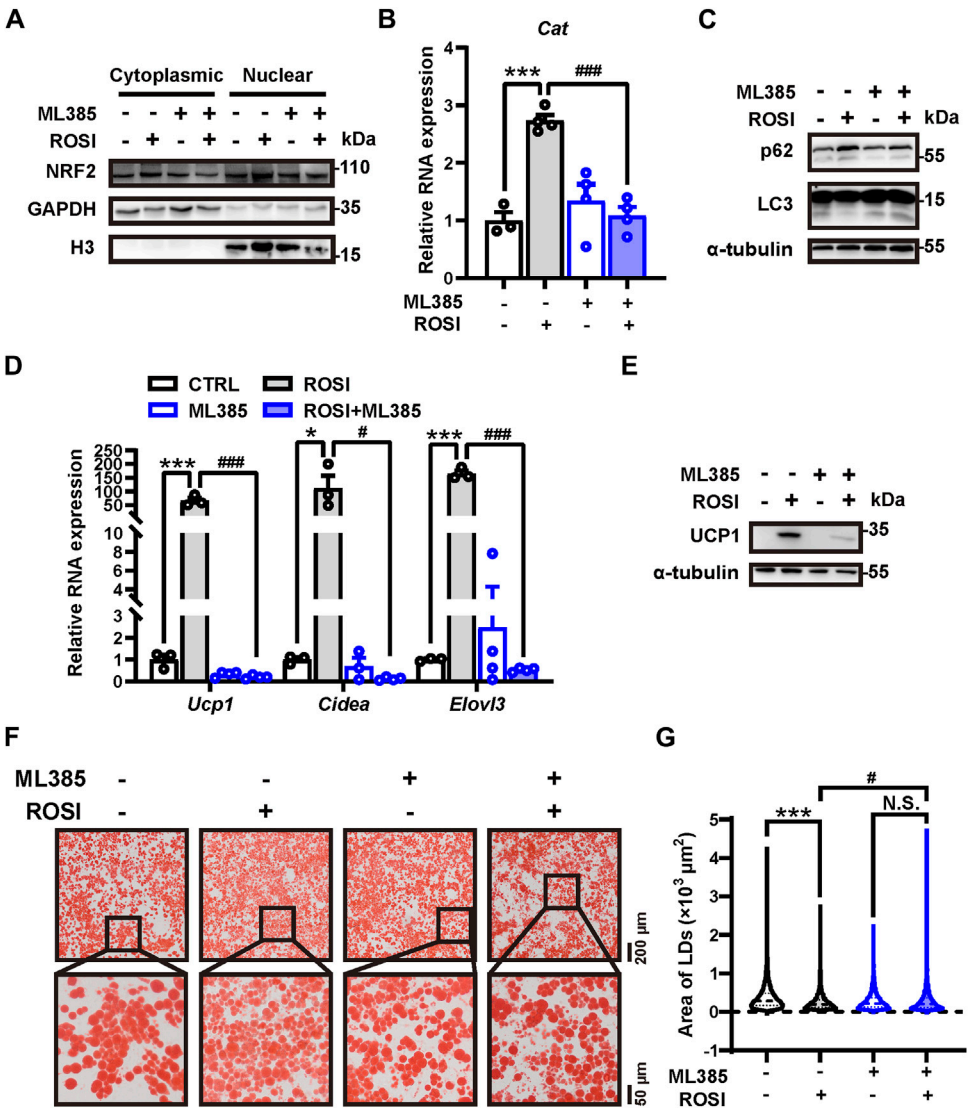

FIGURE 5
Rosiglitazone inhibits autophagy and promotes adipocyte browning through NRF2 activation **(A–G)** OA-induced whitened adipocytes were treated with rosiglitazone (ROSI, 10 μM) and ML385 (10 μM) for 4 days. **(A)** NRF2 protein levels in cytoplasmic and nuclear proteins of cultured adipocytes were measured by Western blotting. GAPDH was blotted as a loading control of cytoplasmic proteins, and H3 was blotted as a loading control of nuclear proteins. **(B)** Total RNA levels of *Cat* were measured by qRT-PCR (n = 3–4 trials). **(C)** p62 and LC3 protein levels were measured by Western blotting. α-Tubulin was blotted as a loading control. **(D)** Total RNA levels of *Ucp1*, *Cidea*, and *Elovl3* were measured by qRT-PCR (n = 3–4 trials). **(E)** UCP1 protein levels were measured by Western blotting. α-Tubulin was blotted as a loading control. **(F)** Lipid droplets in adipocytes were marked by Oil Red O staining (n = 3 trials). The square box shows the zoomed-in view of the indicated image. Upper scale bar, 200 μm; lower scale bar, 50 μm. **(G)** Quantification of lipid droplets' size from Oil Red O staining shown in **(F)**. (n = 1,800–3,000 lipid droplets per group) Data in **(B,D, G)** are shown as mean ± SEM. Statistical analysis was performed by one-way ANOVA and nonparametric test with Tukey's multiple comparisons in **(B)** and **(D)** and Dunn's multiple comparisons in **(G)**. *p < 0.05; ***p < 0.001 *versus* CTRL. #p < 0.05; ###p < 0.001 *versus* ROSI.

of UCP1 (Figures 1F, G). Furthermore, OA treatment alone decreased p62 and increased LC3-II, suggesting autophagy activation along with whitening, and these alternations were reversed by ROSI treatment (Figure 1G). To confirm the autophagic flux blockage by ROSI, cultured white adipocytes were simultaneously incubated with ROSI and lysosome inhibitor chloroquine (CQ). As expected, CQ alone induced dramatic accumulation of LC3-II and p62, which was observed to a less extent in the presence of ROSI, confirming the blockage of autophagy flux by ROSI in cultured adipocytes (Figure 1H). Taken together, these results suggested that rosiglitazone inhibits autophagy while promoting adipocyte browning.

3.2 Rosiglitazone induces adipocyte browning through autophagy inhibition

To identify the contribution of autophagy inhibition in ROSI-induced browning of adipocytes, an autophagy activator rapamycin was employed. Rapamycin significantly abolished the effects of ROSI on browning-related gene expression, UCP1 protein level, and autophagy inhibition (Figures 2A, B). Additionally, as shown in Oil Red O staining, ROSI reduced the area of lipid droplets, indicating the transition to beige-like multilocular adipocytes, and pro-browning transition was also reversed by rapamycin

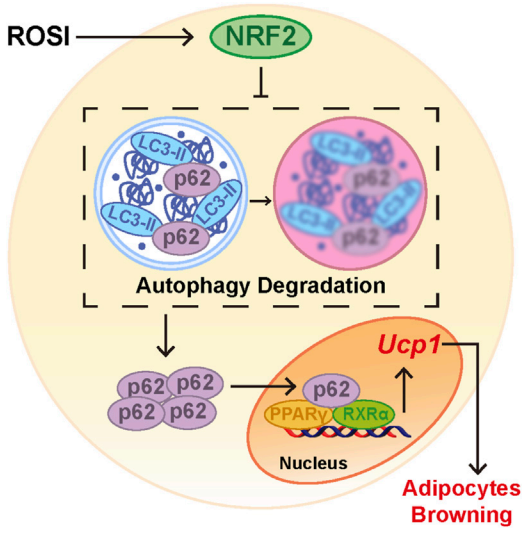

FIGURE 6
Rosiglitazone promoted adipocyte browning by NRF2-induced autophagy inhibition. When adipocytes were treated with rosiglitazone, nuclear translocation of NRF2 was activated. Activation of NRF2 inhibited autophagy and led to accumulation of p62 in the cytoplasm. Cumulative p62 translocated into the nucleus and bound with the PPARγ-RXRα heterodimer as a coactivator. Activated PPARγ increased the RNA expression of browning-related genes such as *Ucp1* and, thus, initiated adipocyte browning.

(Figures 2C, D). These findings indicated that autophagy inhibition was required for the effects of ROSI in promoting adipocyte browning.

As autophagy inhibition may lead to deficits in adipogenesis, a process where adipocytes develop from precursor cells (Baerga et al., 2009; Zhang et al., 2009), we wonder whether ROSI inhibits autophagy and impairs adipogenesis. To this end, we applied ROSI to premature adipocytes for 8 days during their differentiation (Supplementary Figure S1A). It showed that more lipid droplets, organelles of mature adipocytes, were stained by Oil Red O in ROSI-treated adipocytes (Supplementary Figure S1B, C). In addition, ROSI did not suppress the expression of the late-stage adipogenesis marker FABP4 and even accelerated the appearance of UCP1 in differentiating adipocytes, indicating ROSI did not impair adipogenesis in differentiating adipocytes (Supplementary Figure S1D). Notably, neither ROSI alone nor in combination with rapamycin suppressed the transcription of browning markers, further suggesting that ROSI-inhibited autophagy has a limited impact on adipogenesis (Supplementary Figure S1E).

3.3 Rosiglitazone has no effect on mitophagy in differentiated adipocytes

Mitochondria clearance via autophagy (mitophagy) promotes beige-to-white transition of adipocytes, and mitophagy inhibition maintains the brown-like phenotype of adipose (Altshuler-Keylin et al., 2016; Lu et al., 2018; Ro et al., 2019). We, therefore, hypothesized that ROSI inhibited mitophagy and, thus, promoted adipocyte browning. We measured the mitochondrial DNA (mtDNA) level to reflect the mitochondrial content in OA-treated adipocytes. Unexpectedly, ROSI did not alter mtDNA level (Figure 3A), and it did not change the quantity of MitoTracker Red-labeled mitochondria (Figures 3B, C), suggesting no mitophagy inhibition by ROSI in the adipocytes. Along with OA-induced adipocyte whitening, the protein level of mitochondria marker NDUFB8 gradually decreased, suggesting mitophagy activation. This trend of mitochondrial reduction was not affected by ROSI (Figure 3D). These results suggested that ROSI cannot reinforce mitophagy during OA-induced whitening of adipocytes. We next evaluated the RNA and protein levels of several mitophagy-related genes, *i.e.*, *Bnip3L*, *Parkin*, *Fundc1*, and *Phb2* (Schweers et al., 2007; Geisler et al., 2010; Liu et al., 2012; Wei et al., 2017). It showed that ROSI neither downregulated the transcription nor the expression of these genes (Figures 3E, F), further suggesting that ROSI did not promote mitophagy during adipocyte whitening. Collectively, our findings indicated that mitophagy was not required for ROSI-induced adipocyte browning.

3.4 Rosiglitazone-mediated autophagy inhibition stabilizes the PPARγ-RXRα heterodimer

We next explored through which mechanism ROSI-inhibited autophagy contributed to adipocyte browning. As an autophagy adapter protein, p62 is reported to fine-tune the transcriptional activity of the PPARγ-RXRα heterodimer and, thus, leads to expression of thermogenetic genes in brown adipocytes (Huang et al., 2021). To determine whether p62 was involved in ROSI-induced adipocyte browning, we isolated the nuclear protein of OA-treated adipocytes. We found p62 accumulation in the nucleus with ROSI treatment, which was reversed by rapamycin (Figure 4A). These data suggested that ROSI inhibited autophagy and led to p62 translocation to the nucleus. Moreover, the interactions between p62, PPARγ, and RXRα in the nucleus were also enhanced by ROSI and partly abolished by rapamycin (Figure 4B). These observations suggested that inhibition of autophagy by ROSI activated PPARγ-RXRα via promoting p62 nuclear translocation in adipocytes.

3.5 Activation of NRF2 by rosiglitazone inhibits autophagy and promotes adipocyte browning

We next explored how ROSI inhibited autophagy in adipocytes. Nuclear factor erythroid 2-related factor 2 (NRF2) is a transcriptional factor that transcripts autophagy-related genes, including p62 (Liu et al., 2022). We found that ML385, an NRF2 inhibitor, prevented ROSI-induced p62 nuclear translocation (Figure 5A). The transcription of *Cat* mRNA, a target gene of NRF2, was upregulated by ROSI (Figure 5B), suggesting that ROSI activated NRF2. ML385 restored the inhibited autophagy flux in adipocytes with ROSI treatment (Figure 5C). Moreover, ROSI-induced browning characteristics including increase in RNA and protein levels of browning-related genes and the multilocular phenotype of adipocytes were also diminished by ML385 (Figures 5D–G). These data collectively indicated that ROSI inhibited autophagy through NRF2 activation and, thus, promoted adipocyte browning (Figure 6).

4 Discussion

As a PPARγ agonist, ROSI was reported to increase the expression of browning-related genes (Digby et al., 1998; Petrovic et al., 2010; Ohno et al., 2012), but the cellular and molecular mechanisms of how ROSI promotes adipocyte browning has not been fully elucidated. Autophagy has a vital role in beige-to-white adipocyte transition. Blockage of autophagy by deletion of autophagy-related genes or pharmacological methods induces adipocyte browning (Singh et al., 2009; Altshuler-Keylin et al., 2016). There are compounds that enhance adipocyte browning that were also reported to suppress autophagy, further highlighting the role of autophagy inhibition in promoting adipocyte browning (Leu et al., 2018; Duan et al., 2020). As ROSI leads to autophagy inhibition in hepatic cell line LX2 and spinal cord injury neurons (Li et al., 2017; Yum et al., 2023), we hypothesized whether ROSI promoted adipocyte browning through inhibition of autophagy. Our results showed that ROSI inhibited autophagy flux in high-fat diet-induced obese mice and oleic acid-induced whitened primary cultured adipocytes. Moreover, autophagy activation by rapamycin reversed the thermogenesis pattern and multilocular phenotype of white adipocytes under ROSI treatment, suggesting that autophagy inhibition is required in ROSI-induced adipocyte browning.

In addition to browning of adipocytes, autophagy inhibition could also lead to a deficiency in adipogenesis, the process through which adipocytes develop. *Atg5* or *Atg7*-deleted mouse embryonic fibroblasts are arrested at the early stage of adipogenesis (Baerga et al., 2009; Zhang et al., 2009). Autophagy is required to stabilize PPARγ2 by suppressing its proteasomal degradation and, thus, induce adipogenesis (Zhang et al., 2013). Interestingly, we found that ROSI inhibited autophagy without arresting adipogenesis. It has been documented that ROSI stimulates adipogenesis of mice and human preadipocytes (Lehmann et al., 1995; Yamauchi et al., 2001; Hutley et al., 2003). Another PPARγ agonist troglitazone could induce adipogenesis in *Ulk1* or *Atg5*-knockdown 3T3-L1 cells (Ro et al., 2013). Therefore, it seems that PPARγ activation by ROSI might have overwhelming effects on adipogenesis, regardless of whether autophagy is inhibited. This assumption should be verified by further investigation. Taken together, we exclude the possibility that ROSI-induced small multilocular phenotype of white adipocytes was due to autophagy inhibition-mediated adipogenesis deficiency.

Mitophagy has been reported to promote the beige-to-white adipocyte transition. Adipocyte-specific deletion of *Atg5* or *Atg12* rescued the mitochondria clearance process after β3 receptor agonist withdrawal and maintained beige adipocyte characteristics (Altshuler-Keylin et al., 2016). Similarly, mitophagy receptor *Parkin*-deficient mice showed reduced mitochondrial degradation and retained the beige adipocyte phenotype (Lu et al., 2018). These studies indicated that mitophagy inhibition facilitates adipocyte browning. However, our results suggested that ROSI has no impact on mitophagy. This result implied that mitophagy activity is dispensable for the pro-browning effects of ROSI. Consistently, ectopic PRKN expression failed to fully abolish ROSI-induced UCP1 in differentiated 3T3-L1 adipocytes (Taylor and Gottlieb, 2017). Nevertheless, knockdown of *Bnip3*, a mitophagy receptor, canceled ROSI-induced UCP1 expression in differentiated 3T3-L1 adipocytes (Choi et al., 2016). In addition to the non-mitophagy roles of PRKN and BNIP3, these controversial results may be attributable to the different measurements in revealing adipocyte transition. For example, the reduction of UCP1 in adipocytes, which indicates whitening, may result from its degradation within the mitochondria, where it is primarily located.

Canonical transactivation by PPARγ requires binding with RXRα as a heterodimer (Tontonoz et al., 1994), which interacts with coactivators upon agonist binding (Hernandez-Quiles et al., 2021). Autophagy adapter p62 serves as a coactivator of the PPARγ-RXRα heterodimer (Huang et al., 2021). Our results showed that ROSI-mediated autophagy inhibition triggered nuclear translocation of p62 and enhanced its binding with PPARγ and RXRα, suggesting that inhibiting autophagy can enhance the browning of adipocytes by increasing the expression and interaction of PPARγ coactivators. Similarly, PRDM16 was also stabilized by ROSI through inhibition of the ubiquitin–proteasome pathway, thus inducing a white-to-brown adipocyte transition (Ohno et al., 2012). In combination with the present study, these findings suggest that ROSI promotes adipocyte browning through fine-tuning of the interaction between PPARγ and its coactivators.

NRF2 was reported to play a vital role in adipocyte browning. Knockdown of *Nrf2* significantly reversed β3 receptor agonist CL316,243-induced adipose browning (Chang et al., 2021; Bauzá-Thorbrügge et al., 2023). Moreover, NRF2 activator tert-butylhydroquinone (tBHQ) and antioxidant N-acetylcysteine (NAC) induce adipocyte browning (Chang et al., 2021; Bauzá-Thorbrügge et al., 2023). However, the cellular and molecular mechanisms of how NRF2 activates adipocyte browning have not been fully understood. Our study suggested that autophagy inhibition is required for NRF2-induced browning, at least in ROSI-treated adipocytes. These findings are in line with the finding that ROSI inhibited autophagy and activated NRF2 in status epilepticus rats (Peng et al., 2021). However, another study indicated that autophagy impairment by *Atg3* knockout led to NRF2 activation in the adipose tissue (Cai et al., 2018), implying a positive feedback regulation of NRF2 by autophagy inhibition. p62 may serve as a hub in integrating autophagy and NRF2 signaling in adipocyte browning. As a substrate of autophagy, p62 accumulates in autophagy-impaired cells and competitively binds to Keap1, thus stabilizing NRF2 (Komatsu et al., 2010). NRF2 translocated in the nucleus could also increase transcription of p62 (Frias et al., 2020; Zhang et al., 2021). NRF2 or autophagy-related gene knockout mice may increase the reliability of the autophagy inhibitory effect of ROSI in future studies. Additionally, the transcriptional factor MiT/TFE was reported to enhance autophagy flux and promote browning of adipocytes (Altshuler-Keylin et al., 2016). It remains elusive whether MiT/TFE underscores the pro-browning effects of ROSI.

ROSI was used as an antidiabetic drug with the effects of promoting adipocyte browning and alleviating high-fat diet-induced obesity (Lehmann et al., 1995; Petrovic et al., 2010). It was reported that subcutaneous ROSI administration reduced iWAT size and promoted adipose browning in a diet-induced obesity mouse model. In combination with the present study, these investigations highlighted a promising role of ROSI in obesity treatment.

5 Conclusion

Our study revealed that ROSI promoted adipocyte browning through inhibition of autophagy. Autophagy inhibition by ROSI increased p62 nuclear translocation and stabilized the PPARγ-RXRα heterodimer for transcription of browning genes. We proposed that NRF2 activation was involved in autophagy inhibition by ROSI in adipocytes. The present study provided a new insight into the pharmacological mechanisms underlying ROSI-induced adipocyte browning.

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

The animal study was approved by the Zhejiang University Animal Experimentation Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YeL: writing–original draft and writing–review and editing. WZ: writing–original draft and writing–review and editing. XL: writing–review and editing. ZL: writing–review and editing. YnL: writing–review and editing. JW: writing–review and editing. XZ: writing–original draft and writing–review and editing.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1412520/full#supplementary-material

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Improving the treatment of bacterial infections caused by multidrug-resistant bacteria through drug repositioning

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Drug repurposing (repositioning) is a dynamically-developing area in the search for effective therapy of infectious diseases. Repositioning existing drugs with a well-known pharmacological and toxicological profile is an attractive method for quickly discovering new therapeutic indications. The off-label use of drugs for infectious diseases requires much less capital and time, and can hasten progress in the development of new antimicrobial drugs, including antibiotics. The use of drug repositioning in searching for new therapeutic options has brought promising results for many viral infectious diseases, such as Ebola, ZIKA, Dengue, and HCV. This review describes the most favorable results for repositioned drugs for the treatment of bacterial infections. It comprises publications from various databases including PubMed and Web of Science published from 2015 to 2023. The following search keywords/strings were used: drug repositioning and/or repurposing and/or antibacterial activity and/or infectious diseases. Treatment options for infections caused by multidrug-resistant bacteria were taken into account, including methicillin-resistant staphylococci, multidrug-resistant *Mycobacterium tuberculosis*, or carbapenem-resistant bacteria from the *Enterobacteriaceae* family. It analyses the safety profiles of the included drugs and their synergistic combinations with antibiotics and discusses the potential of antibacterial drugs with antiparasitic, anticancer, antipsychotic effects, and those used in metabolic diseases. Drug repositioning may be an effective response to public health threats related to the spread of multidrug-resistant bacterial strains and the growing antibiotic resistance of microorganisms.

KEYWORDS

drug repositioning, bacterial infections, antimicrobial drug resistance, carbapenem-resistant enterobacteriaceae, methicillin resistance, tuberculosis

1 Introduction

For several years, an increase in antibiotic resistance of bacterial strains has been observed in various types of infections, both hospital and community-acquired, and in various age groups (Wattal and Goel, 2020). One of the most important problems in the treatment of infections is the occurrence of multidrug-resistant bacteria, where resistance affects one (multidrug-resistance, MDR), several (extensively drug-resistant, XDR) or all (pandrug-resistance, PDR) groups of the used antibacterial drugs (Magiorakos et al., 2012). The highest priority pathogens include both Gram-negative and Gram-positive bacteria

(Fritsche, 2021). They constitute the ESKAPE group of alert pathogens (E—*Enterococcus faecium*, S—*Staphylococcus aureus*, K—*Klebsiella pneumoniae*, A—*Acinetobacter baumannii*, P—*Pseudomonas aeruginosa*, E—*Enterobacter* spp.), which constitute the greatest threat in the case of nosocomial infections (Loyola-Cruz et al., 2023). The key pathogens, in addition to *K. pneumoniae*, also include other genera from the *Enterobacteriaceae* family—*Escherichia coli*, *Serratia* sp., and *Proteus* sp. (WHO, 2017; Asokan et al., 2019). In recent years, the problem of drug resistance has also affected bacteria of the *Mycobacterium* genus. Tuberculosis has also become a major global health problem. Cases of extensively drug-resistant tuberculosis (XDR-TB) have been reported in over 100 countries around the world (Kanvatirth et al., 2019; Kaya et al., 2023).

The problem of antibiotic resistance results, among others, from their unjustified application, use in inappropriate clinical situations, and in inappropriate doses for the treatment of infections. In addition to natural antibiotic resistance, as a result of the improper use of antibiotics, bacteria have developed various mechanisms as a result of mutations in chromosomal genes or genes constituting mobile genetic elements and as a result of horizontal gene transfer. This is related to the phenomenon of selective pressure among microorganisms (Bassetti et al., 2022; Hryniewicz and Struzycka, 2023). However, the development of bacterial resistance to antibiotics due to genome changes may occur not only due to inappropriate drug applications but also independently of them. Multidrug-resistant bacteria are estimated to cause 25,000 deaths per year in Europe (Morehead and Scarbrough, 2018).

In recent years, the number of new antibiotics approved for use in medicine has been insufficient, given the growing problem of drug resistance. A 2021 WHO analysis found that 217 antibacterial drugs were in preclinical development. However, according to the WHO report from 2022, in 2017–2021 only 12 new antibiotics were registered and approved for use (WHO, 2022). The answer to the problem of too few new, effective antibiotics may be drug repositioning. This process is based on the use of old drugs belonging to different therapeutic classes in a new medical indication (Foletto et al., 2021a). It provides a solution to the high costs and slow process of discovering new drugs (Hua et al., 2022). Repositioning allows bypassing many stages on the way to the registration of a new drug due to the known pharmacokinetic and toxicity profiles of the drug. In the case of repositioned drugs, the preclinical phase is only related to demonstrating their effectiveness in a new indication in a cellular or animal model (Farha and Brown, 2019; Kanvatirth et al., 2019).

Various techniques are used to discover a new application for a drug. One of them includes molecular modeling techniques (including, among others, molecular docking, molecular dynamic simulations and quantitative structure activity relationships), which allows predicting biological activity and virtual screening of molecules (Kumar et al., 2022). Important tools in the process of drug repositioning are databases that contain pathways connecting genes and proteins responsible for biological processes that may be influenced by the interactions of drugs with their therapeutic targets (Pan et al., 2014; Gonzalez-Cavazos et al., 2023).

Drugs that have been successfully repositioned include: bupropion, originally used to treat depression and now also used in the treatment of addictions (tobacco smoking); sildenafil, which was originally registered for treatment of pulmonary arterial hypertension and is now also indicated for erectile dysfunction; or minoxidil registered as a drug for hypertension, currently used to treat androgenic alopecia (Ashburn and Thor, 2004; Farha and Brown, 2019). Currently, research on the repositioning of drugs for the treatment of viral diseases is developing rapidly. In recent years, this has also been demonstrated by the pandemic caused by SARS-CoV-2 (Hua et al., 2022; Vaz et al., 2023). Research into drugs repositioned for the treatment of ZIKA virus infections include substances such as mycophenolic acid used as an immunomodulator and memantine used to treat Alzheimer's disease. Repositioning clomiphene and toremifene, used in the treatment of breast cancer and infertility, for the treatment of Ebola virus infection is also being considered (Sirohi and Kuhn, 2017; Mani et al., 2019).

Due to the spread of multidrug-resistant strains, the possibility of changing the purpose of classes of non-antibiotic drugs in the treatment of severe bacterial infections is also emphasized (Foletto et al., 2021a; Caldara and Marmiroli, 2021). This review focuses on the potential repurposing of several drugs and their use in the treatment of bacterial infections. Articles published between 2015 and 2023 were searched, including *in vitro* and *in vivo* studies of drug candidates for repositioning. Keywords included: drug repositioning and/or repurposing and/or antimicrobial activity and/or infectious diseases. The applied search strategy allowed for the selection of nearly 1,700 publications. The focus was on work taking into account the spectrum of activity of the tested potential drug candidates in the case of bacterial infections. Works on viral and fungal infections were not included. Drug structures, probable mechanisms of action, and bacterial species used in the research were taken into account. Attention was also paid to the role of synergism in the case of combining tested drugs and antimicrobial substances, which would allow for dose reduction and would reduce the selection pressure among bacteria. These applied criteria allowed the search for 113 scientific articles that were used to write this manuscript. The Clinical Trials database was also searched (studies reported since 2015), where the keywords were the names of active substances included in the manuscript.

2 Potential candidates for repositioning in the treatment of bacterial infections

Research on drug repositioning in antimicrobial therapy is currently developing rapidly. The applied article search strategy allowed us to filter over 1,700 scientific articles. Figure 1 presents the strategy and summary of the literature search results. The multitude of articles published in recent years indicates a growing interest in the topic of drug repositioning in infectious diseases. According to many of them, which concern repositioning not only in bacterial diseases, but also research is ongoing in fungal and viral diseases, which is also related to the recent COVID-19 pandemic.

More than 70 out of 1,600 non-antimicrobial drugs with antimicrobial activity were selected in *in vitro* tests in one of the screening studies aimed at discovering new antibacterial drugs. They were active against at least one pathogen from the ESKAPE group

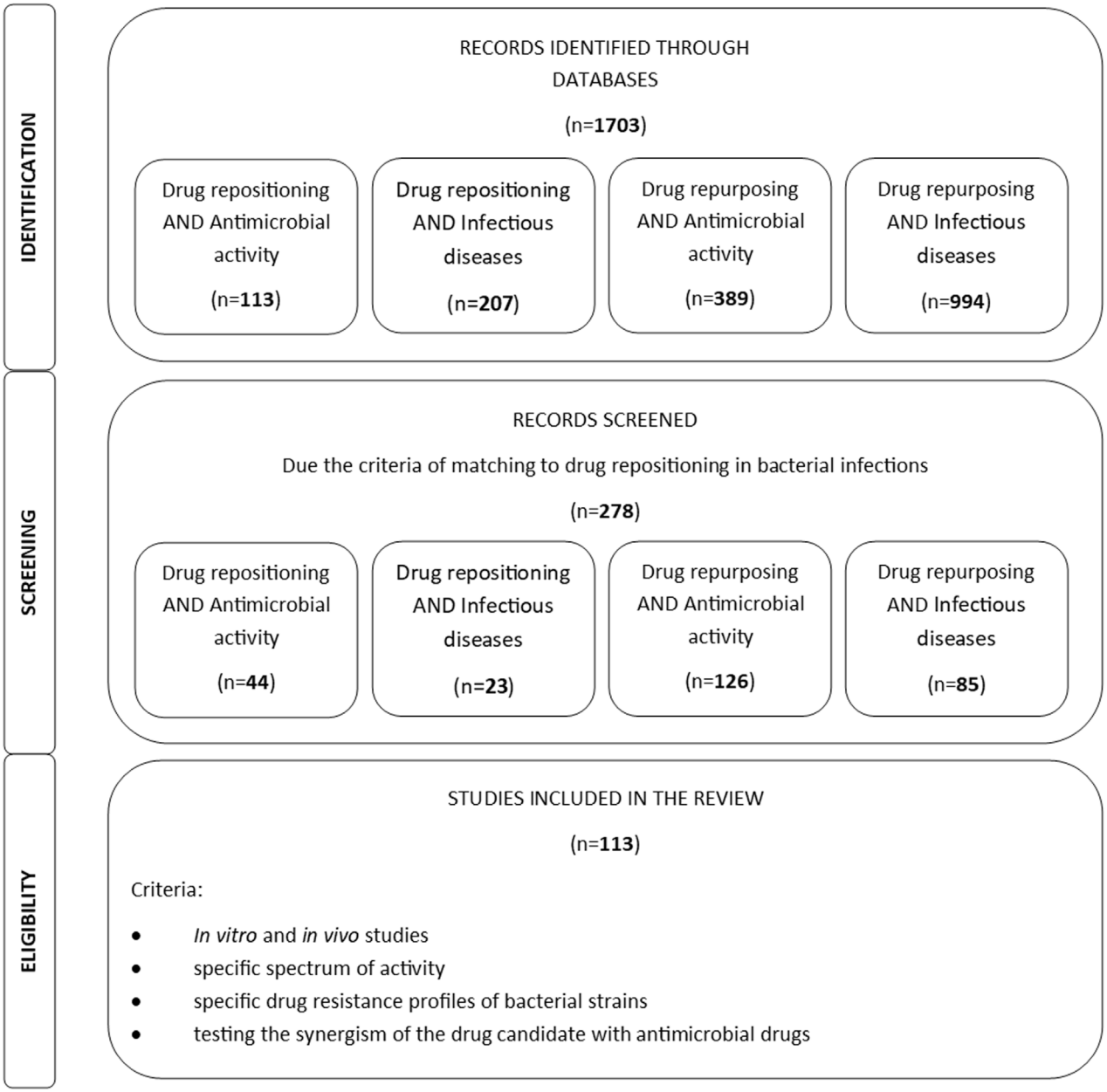

FIGURE 1
Results of the applied article search strategy.

(Younis et al., 2017). Anti-inflammatory drugs, antidepressants, antihypertensives, statins, proton pump inhibitors have been recognized as potential candidates in the process of drug repositioning in the treatment of infectious diseases (da Rosa et al., 2022).

2.1 Medicines used in oncology

2.1.1 Cytostatics

Candidates for antibacterial drugs include cytotoxic and anticancer drugs. However, due to their cytotoxicity or genotoxicity profile, they can be considered as having high clinical value, mainly in the treatment of infections in oncological patients (Gouveia et al., 2023).

One of the concerned anticancer substances is floxuridine, a derivative of 5-fluorouracil, which has demonstrated antibacterial activity against *S. aureus* strains, including methicillin resistance *S. aureus* (MRSA), and *S. epidermidis*. It is important in research on this drug to optimize the structure of the drug molecule so that it is not activated in human cells and does not have a cytotoxic effect on them (Thangamani et al., 2015; Younis et al., 2017; Cheng et al., 2019; Hua et al., 2022). Ethyl bromopyruvate, a derivative of 3-bromopyruvic acid, demonstrated antibacterial activity against strains of the ESKAPE group. The authors of the study proved that 24-h

bromopyruvate therapy can achieve the same effects as vancomycin therapy. Additionally, this compound was responsible for inhibition of iron uptake, by *Mycobacterium tuberculosis* (Kumar et al., 2019a).

It has also been proven that oncological drugs can be effective in combination therapy with antibiotics. Mitoxantrone, in combination with colistin was effective in the treatment of *P. aeruginosa* infections and biofilm control (Torres et al., 2018). In turn, mitotane with polymyxin B resulted in an increased antibacterial activity against isolates resistant to this antibiotic and carbapenem-resistant *A. baumannii*, *P. aeruginosa*, and *K. pneumoniae*. In this combination, polymyxin B, due to its action on the outer membrane of Gram-negative bacterial cells, allowed the oncological drug to penetrate into them (Tran et al., 2018).

Research on the repositioning of this group of drugs in infectious diseases is currently under investigation in the preclinical phase. At present, clinical trials on the repositioning of anticancer drugs mainly concern the treatment of Alzheimer's disease. They are believed to have the ability to regulate processing and reduce the aggregation of amyloid plaques (Advani and Kumar, 2021). It has been shown that the use of these drugs in oncological diseases reduced the risk of developing dementia (Ancidoni et al., 2021).

2.1.2 Tyrosine kinase inhibitors

The development of tyrosine kinase inhibitors (TKIs) represents one of the most significant milestones in the medicine of the 21st century. The first TKI, imatinib, was designed to target the BCR-Abl hybrid protein, produced in patients with Philadelphia-chromosome-positive chronic myelogenous leukemia. Since the introduction of imatinib, the application of TKIs has been ever-expanding, but has addressed mainly oncological patients (Thomson et al., 2023). Several signaling pathways have gained attention as potential targets for repurposing of TKIs for other diseases; these include pulmonary arterial hypertension (PAH), as a platelet-derived growth factor receptor, or mast/stem cell growth factor receptor kit (c-KIT), can contribute to inflammation and proliferation processes being involved in the pathogenesis of the disease. The results from experimental studies with imatinib were promising, and oral application yielded improvements in exercise capacity and hemodynamics in clinical trials; however its further development was limited by systemic side effects with subdural hematomas (Weatherald et al., 2020). Currently, research is underway on the repositioning of TKIs in the treatment of Human Immunodeficiency Virus (HIV) infection. *In vitro* studies have shown that TKIs have the ability to modulate the immune response and have antiviral activity against not only HIV-1 virus but also Cytomegalovirus (CMV), Monkeypox virus (MPV), Varicella zoster virus (VZV), and filoviruses. The greatest hopes are associated with the repositioning of TKIs in viral diseases due to the inhibition of the escape of viruses from infected cells, thus reducing the spread of infection (Ananthula et al., 2018; Climent and Plana, 2019; Vigón et al., 2020; Rodríguez-Agustín et al., 2023).

Research on the antibacterial activity of TKIs is currently in the preclinical phase; however, studies have identified two candidates. Fostamatinib was considered a molecule with anti-tuberculosis activity. It inhibits the activity of protein kinases (serine/threonine-protein kinase) found in *M. tuberculosis* and *M. bovis*. This drug was found to kill infected macrophages and did not show cytotoxicity (Rodrigues et al., 2020). Ponatinib also demonstrated antibacterial activity against planktonic cells and biofilm-forming *Streptococcus mutans* (Saputo et al., 2018).

2.1.3 Hormonal drugs—selective estrogen receptor modulators

Selective estrogen receptor modulators (SERMs) are used in the treatment of cancer and in the prevention of osteoporosis in postmenopausal women. Examples of SERMs are tamoxifen, toremifene, raloxifene, and clomiphene. In addition to its listed indications, tamoxifen is also used to treat infertility and reduce the risk of breast cancer in women. These drugs compete with estrogen and prevent its binding to the estrogen receptor, thus inhibiting its stimulating effect. They also induce apoptosis and regulate the expression of oncogenes and growth factors. Moreover, they also demonstrate various pleiotropic neuroprotective, cardioprotective, and antimicrobial effects (Garg et al., 2020).

The SERM clomiphene demonstrated antibacterial activity against strains of *Bacillus subtilis*, *E. faecium* and methicillin-resistant staphylococci. It is believed to act by influencing a bacterial cytoplasmic enzyme important for the synthesis of teichoic acid. Its inhibition leads to impaired cell wall synthesis of Gram-positive bacteria. In the case of Gram-negative bacteria, clomiphene have been found to have an effect in combination with colistin. In these bacteria, this SERM member appears to influence the synthesis and transport of lipopolysaccharide precursors (Younis et al., 2017; Torres et al., 2018).

Toremifene has also demonstrated antibacterial activity. When applied in combination with polymyxin B, it is characterized by a strong antibacterial effect against *P. aeruginosa* bacterial cells, which form biofilms and are usually resistant to these antibiotics. Toremifene induced depolarization of the cytoplasmic membrane, increased permeabilizing activity and stimulated the production of reactive oxygen species in bacterial cells. It is believed that by influencing the permeability of bacterial cell membranes and lipopolysaccharides, polymyxins enable SERMs to better penetrate microbial cells. Unlike the previously-mentioned SERMs, tamoxifen interacts with lipids in bacterial membranes, causing structural changes that may result in cell lysis. Its analogues, however, induce the outflow of ions from Gram-negative bacterial cells and lead to the loss of their membrane potential (Hussein et al., 2017). In addition, SERM drugs have also shown activity against viruses, fungi, and parasites (Johansen et al., 2013; Garg et al., 2020). One clinical study assessed the safety and effectiveness of tamoxifen in combination with amphotericin B and fluconazole in the treatment of cryptococcal meningitis (NCT03112031), but failed to obtain satisfactory results.

Potential mechanisms of action of the drugs listed in this section are presented in Figure 2 (Johansen et al., 2013; Younis et al., 2017; Saputo et al., 2018; Torres et al., 2018; Tran et al., 2018; Garg et al., 2020; Advani and Kumar, 2021; Li et al., 2023).

2.2 Medicines used in gastroenterology

Proton pump inhibitors (PPI), typically used in the treatment of gastric hyperacidity, stomach ulcers, gastroesophageal reflux and

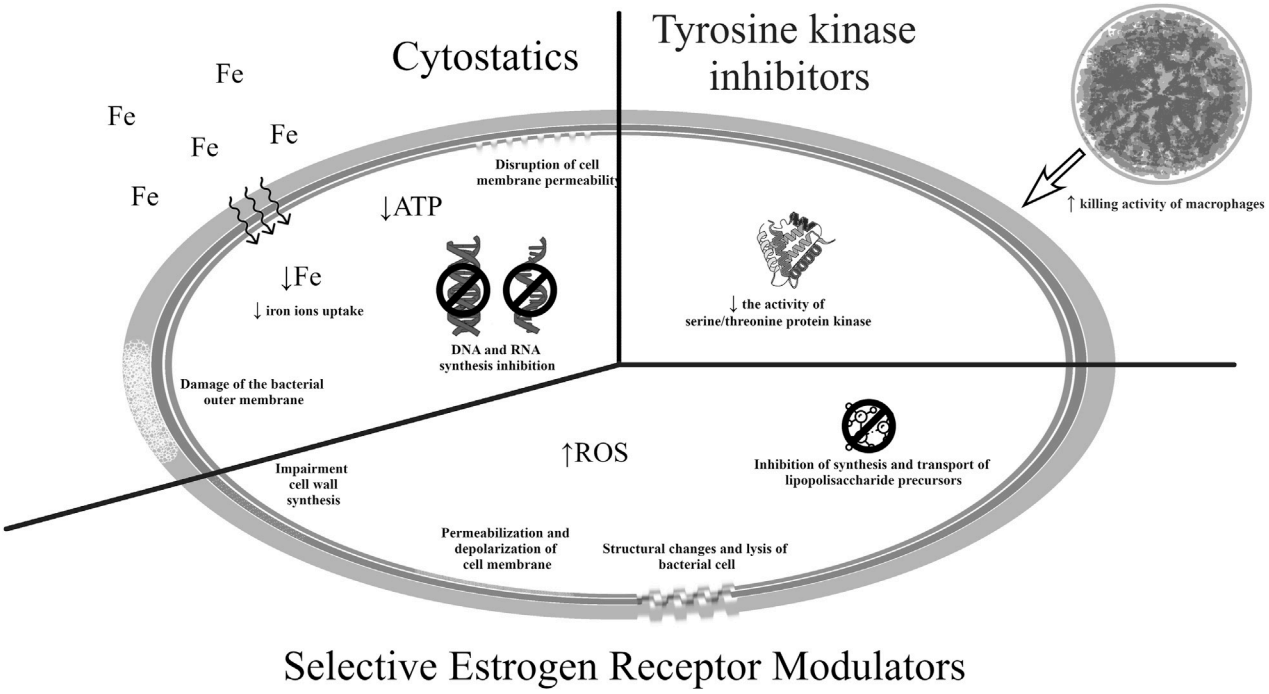

FIGURE 2
Potential mechanisms of antibacterial action of medicines used in oncology.

gastritis, also inhibit the growth of bacteria, fungi and viruses. They have demonstrated antibacterial activity against Gram-positive bacteria (*S. aureus*, *E. faecalis*) and a number of Gram-negative bacteria (*P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *E. cloacae*, *U. urealyticum*), as well as *M. tuberculosis*. Molecules with antimicrobial activity include esomeprazole, lansoprazole, omperazole, and pantoprazole (Foletto et al., 2021a; da Rosa et al., 2022). It has been proven that PPIs inhibit the activity of fluoroquinolone efflux pumps in *S. aureus* strains, which increases the activity of, among others, levofloxacin, ciprofloxacin and norfloxacin in staphylococcal infections (Thangamani et al., 2015). Additionally, when used in therapy, they may also interrupt the respiratory chain and deplete energy resources, as demonstrated in studies on mycobacteria (Rodrigues et al., 2020).

Currently, the possibility of repositioning proton pump inhibitors is under examination in many preclinical and clinical studies. One clinical trial involved the use of esomeprazole to reduce organ failure in sepsis (NCT03452865), but no results have been posted so far. This molecule has been shown to inhibit the secretion of TNF-α and IL-1β. In studies in animal models, a single dose of esomeprazole protected against endotoxic shock. Other studies include the possibility of using PPIs in cancer treatment due to their ability to inhibit fatty acid synthase, induce cancer cell apoptosis, and inhibit the proton pump of cancer cells (Ikemura et al., 2017; Spugnini and Fais, 2020). Clinical trials are currently in the recruitment phase (NCT04930991, NCT04337580).

The opioid loperamide, a drug that inhibits gastrointestinal motility is poorly absorbed and well tolerated. It is used in the treatment of infectious and non-infectious chronic and acute diarrhea (Brown, 2015; Zhang et al., 2023). Due to its mechanism of action, i.e., mediating the intracellular calcium concentration by blocking calcium channels and activating opioid receptors, it is also a candidate for relieving pain, controlling anxiety or reducing insulin resistance (Juárez et al., 2018; Barrientos et al., 2021). As an agent with antibacterial potential, loperamide has demonstrated synergism with several classes of antibiotics in the treatment of bacterial infections causing intestinal infections (*in vitro* studies). Its use increased the effectiveness of tetracyclines, cephalosporins, as well as polymyxin B and colistin against Gram-negative bacteria. The molecule has been recognized to influence the deformation of bacterial cells and the permeability of their cell membrane, which may allow the antibiotic to accumulate. For this reason and due to the pharmacokinetic properties of this drug, some authors claim that loperamide can be used in the treatment of intestinal infections (Brown, 2015; Zhang et al., 2023). Moreover, *in vitro* tests have demonstrated bactericidal activity against the tuberculous strain *M. tuberculosis* and the non-tuberculous *M. bovis* BCG, *M. terrae* and *M. smegmatis*. In addition to activating bactericidal mechanisms, this drug also caused an immune response, influencing the activity of macrophages and the inflammatory process (Juárez et al., 2018; Barrientos et al., 2021). Research on the antibacterial effect of loperamide is currently in the preclinical phase.

Figure 3 shows the potential mechanisms of action of the drugs mentioned in this section (Thangamani et al., 2015; Juárez et al., 2018; Saputo et al., 2018; Barrientos et al., 2021; Zhang et al., 2023).

It can be assumed that drugs used in gastroenterology may potentially be repositioned as supportive therapy for antibiotic treatment in infectious diseases of the gastrointestinal tract and sepsis.

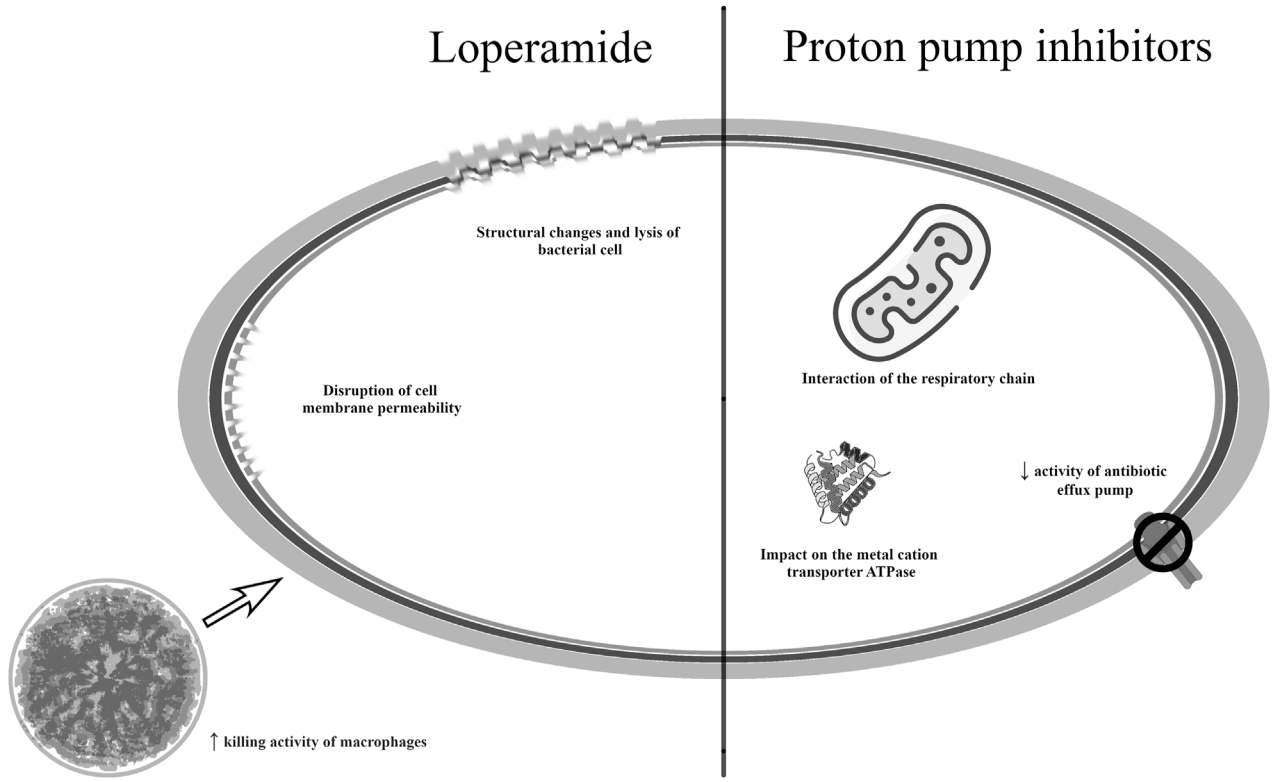

FIGURE 3
Potential mechanisms of antibacterial action of medicines used in gastroenterology.

2.3 Medicines used in therapy against pain

2.3.1 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most frequently-prescribed therapeutic groups, with the "basic" mode of action covering the inhibition of COX enzymes and reduction of release of prostaglandins (PGs) and thromboxane (TxA). While inhibition of COX isoforms has been demonstrated as a primary mechanism for the efficacy of the NSAIDs, preclinical and clinical data have revealed the presence of additional COX-independent mechanisms. These include the inhibition of various transcription factors: activator protein 1 (AP-1) and nuclear factor-kappa B (NF-κB) regulating the expression of various pro-inflammatory cytokines (TNFα, IL-1β, IL6) and mediators (NO, PGE2, ICAM-1, VCAM-1) inhibiting signaling pathways involved in inflammatory processes (MAPK and PI3K/AKT) modulating nuclear receptors (PPAR-γ and PPAR-δ) inhibiting leukocyte and neutrophil adherence, inhibiting matrix metalloproteinases (MMP2 and MMP9) or inhibiting cAMP-specific phosphodiesterase (PDE) IV. Some of these mechanisms may support the rationale for *in vitro*/*in vivo* studies on the antiproliferative, antimetastatic, antiangiogenesis, and neuroprotective effects of NSAIDs. Due to these multifactorial effects, they may represent promising candidates for repurposing for chemoprevention and cancer treatment (Ozleyen et al., 2023).

Antibacterial activity has been demonstrated, among others, by acetylsalicylic acid, diclofenac, ibuprofen, celecoxib and fenamic acids. While indomethacin, meloxicam, naproxen, nimesulid, and paracetamol have also demonstrated antibacterial activity, the possibility of using them in the treatment of infections requires further research (Foletto et al., 2021a). Acetylsalicylic acid (ASA) inhibited the production of alpha toxin and negatively influenced the expression of genes responsible for adhesion to components of the host extracellular matrix in *S. aureus* strains, including methicillin-resistant ones (Barbarossa et al., 2022). ASA has been found to have a synergistic effect with vancomycin in an animal model of endocarditis (Brown, 2015). Currently, studies are underway to use the indicated anti-inflammatory drug to reduce mortality in HIV-infected tuberculosis patients (NCT04145258, phase 3 clinical trial, currently recruiting) (Maitre et al., 2022). Another example of an NSAID drug that has demonstrated antimicrobial activity is ibuprofen. It inhibited the growth of *E. coli* and *P. aeruginosa*, as well as *S. aureus* strains under low pH conditions. Additionally, ibuprofen also reduced the rate of bacterial cell accumulation during biofilm formation by *P. aeruginosa* cells (Barbarossa et al., 2022). An ongoing clinical trial, currently in the recruitment phase, is focusing on the use of acetylsalicylic acid and ibuprofen as adjunctive therapy in the treatment of both sensitive and multidrug-resistant tuberculosis (NCT04575519).

Diclofenac is another molecule of interest for studies on drug repurposing due to its properties to inhibit biofilm formation on the dentin after topical usage (Barbarossa et al., 2022). Studies have also shown that it might inhibit the number of viable *E. coli* and *Mycobacterium* cells when given in combination with streptomycin, and to have an antibacterial effect against *Listeria*

spp. strains when given with gentamycin (Brown, 2015; Foletto et al., 2021a). *In vitro* studies also found combined diclofenac and oxacillin to be effective against MRSA strains. The use of these two substances led to apoptosis of bacterial cells through damage to the cell membrane and breaks in the DNA (Queiroz et al., 2021). Diclofenac also inhibited the multiplication of MDR clinical isolates of *E. coli*, *A. baumannii*, *S. aureus*, and *S. epidermidis* (Foletto et al., 2021a); however, its antibacterial effects were only observed at very high doses (Brown, 2015). Research is currently underway on its new derivatives with better properties (Hamed et al., 2023; Tolba et al., 2023). So far, the use of diclofenac has also been considered in the treatment of various types of cancer (Pantziarka et al., 2016).

Celecoxib has demonstrated antibacterial activity against both Gram-negative and Gram-positive bacterial strains. It improved the sensitivity of *M. smegmatis* and MRSA strains to antimicrobial therapy by increasing the uptake of antibiotics by bacterial cells; this was observed when the drug was used either locally or systemically. Depending on the dose used, celecoxib could also inhibit RNA, DNA and proteins synthesis and reduce the level of pro-inflammatory cytokines (Bak and Krupa, 2023). Interestingly, it has also been shown to be effective against *Francisella tularensis* strains causing tularemia (Chiu et al., 2009). Research conducted to determine effective and safe doses of celecoxib in antibacterial therapy identified structural analogues with strong antimicrobial activity. These molecules were able to improve the effectiveness of polymyxins against multidrug-resistant Gram-negative bacteria and were active against MRSA strains (Krishnamurthy et al., 2019). Current clinical trials are focused on evaluating the activity of celecoxib in the treatment of tuberculosis (NCT02602509, completed, no study results) and in viral suppression in chronic hepatitis B (NCT05256823, pre-recruitment). In addition to its antimicrobial activity, celecoxib has also shown good results as a possible drug in anticancer therapy (Bak and Krupa, 2023).

Fenamic acid derivatives, tolfenamic acid, flufenamic acid, and mefenamic were effective against *Neisseria gonorrhoeae* strains. Importantly, these compounds did not affect commensal *Lactobacillus* spp. Fenamic acid derivatives showed better properties in reducing the intracellular load of *N. gonorrhoeae* in infected cells compared to ceftriaxone and reduced the expression of pro-inflammatory cytokines in cervical cells (Seong et al., 2020).

The use of nonsteroidal anti-inflammatory drugs in the treatment of infections has great potential due to the possibility of use in both local and systemic therapy. However, preclinical and clinical phase studies will also have to determine a safe dose. Combination therapy with NSAIDs and antibiotics has demonstrated synergy and seems to be a good solution.

2.3.2 Drugs used in general and local anesthesia

The drugs used in general and local anesthesia inhibit the activity of the nervous system by suppressing synaptic transmission; this is believed to result from the intensification of inhibitory processes, or suppression of stimulatory processes associated with such activity. Administration thus causes reversible loss of consciousness, amnesia, immobility and analgesia (Tesoro et al., 2020; Hogarth et al., 2023). Due to the interaction with γ-aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA), glycine, glutamic acid, nicotinic acetylcholine receptors and, equally importantly, with voltage-dependent sodium, potassium, and calcium channels, it blocks the depolarization of the nerve cell membrane (Yang et al., 2024). In addition to their anesthetic effect, some drugs from this group, such as ketamine, propofol or lidocaine, have demonstrated *in vitro* anticancer activity by preventing cell proliferation and inducing apoptosis (Zhou et al., 2020; Wu et al., 2022). Research is also underway on the possibility of their use in the treatment of anorexia nervosa (NCT04714541) or postpartum depression (NCT03927378, NCT05907213).

Some anesthetic drugs also have antimicrobial activity. *In vitro*, studies have confirmed the effectiveness of ketamine against strains of *P. aeruginosa*, *E. faecalis*, *S. pyogenes*, *S. epidermidis* and *S. aureus*, including those resistant to methicillin (Begec et al., 2013; Coutinho et al., 2021; Sokolowska et al., 2023). The drug is believed to induce apoptosis by altering the integrity of the cell membrane and damaging bacterial DNA. It has also demonstrated affinity for sortase A, which is an important virulence factor in staphylococci. This enzyme is responsible for the acquisition of iron from host cells, the adhesion of bacteria to the extracellular matrix of the host, and the evasion of its immune system. A similar mechanism of antibacterial activity occurs in the case of lidocaine and procaine, these damage the bacterial cell membrane, leading to changes in permeability and ion outflow within the bacterial cell (Coutinho et al., 2021). An ongoing clinical trial (NCT04843982, in the recruitment phase) aims to evaluate the anti-inflammatory effect of one of the ketamine stereoisomers in the acute phase of sepsis. Research on the possibility of repositioning anesthetic drugs in the treatment of infectious diseases is currently in the preclinical phase.

The potential mechanisms of antimicrobial action of drugs used in pain therapy are presented in Figure 4 (Chiu et al., 2009; Begec et al., 2013; Maitre et al., 2022; Ferrer-Luque et al., 2023; Tabatabaeifar et al., 2023).

2.4 Medicines used to treat cardiovascular disorders

Cardiovascular diseases are often treated using β-adrenergic receptor antagonists and calcium channel blockers. β-adrenergic receptor antagonists (β-blockers) weaken the action of catecholamines, causing a decrease in chronotropy and inotropy (Pascual et al., 2016; Goldfine et al., 2023). The calcium channel blockers include dihydropyridines (e.g., amlodipine) and non-dihydropyridines (e.g., diltiazem or verapamil). Non-dihydropyridines are characterized by greater selectivity towards cardiac calcium channels. Dihydropyridines act on vascular smooth muscle cells, leading to their dilation; however, loss of selectivity may result in lower cardiac contractility (Goldfine et al., 2023). Medicines used to treat cardiovascular disorders are also being tested for their anticancer and anti-inflammatory potential (Qasim et al., 2020; Liang et al., 2021).

The calcium channel blocker amlodipine, in addition to its antihypertensive effect, has also shown antibacterial activity by inhibiting β-lactamases (Hua et al., 2022). Among the non-selective β-adrenergic receptor antagonists, carvedilol demonstrated synergistic effects when used in combination with gentamicin or with amlodipine against strains of the same species (Ugurel and Turgut-Balik, 2023).

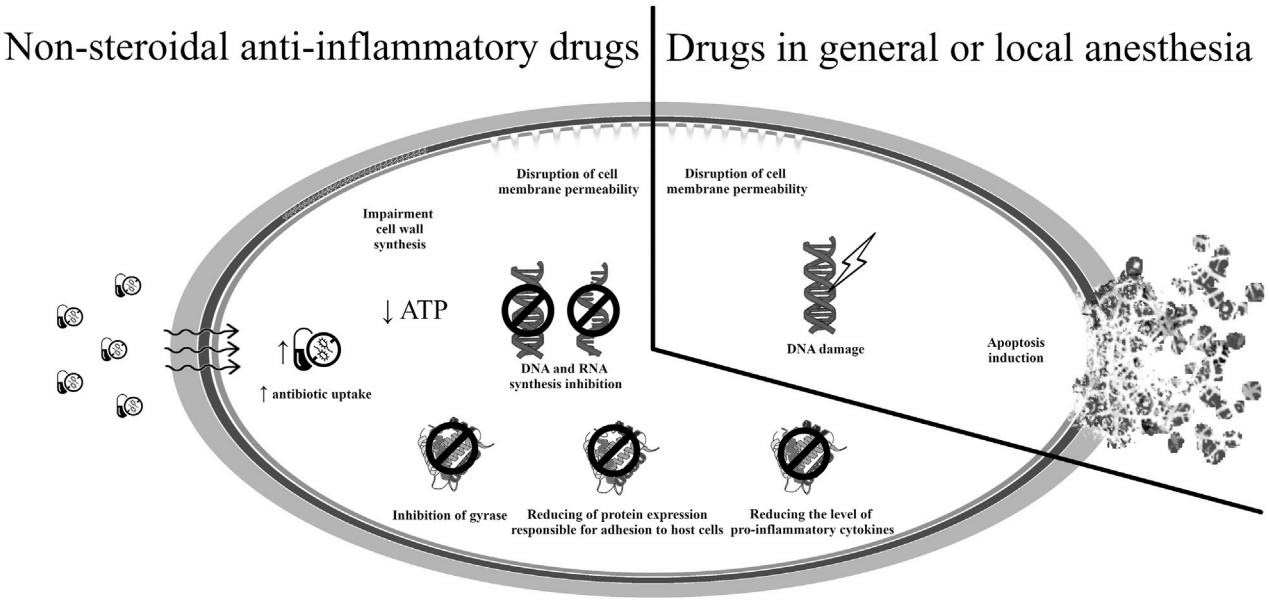

FIGURE 4
Potential mechanisms of antibacterial action of medicines used in therapy against pain.

Recent studies have shown that calcium channel blockers, such as lacidipine, nifedipine, and verapamil, could inhibit the growth of Gram-positive and Gram-negative microorganisms including *S. aureus* and *Vibrio cholerae* (Hua et al., 2022). Verapamil is an anti-hypertensive and antiarrythmic drug with a broad spectrum of antibacterial activity. Its effectiveness against *P. aeruginosa*, *S. aureus*, and *M. tuberculosis* strains has been demonstrated, as well as the ability to reduce the resistance of *S. aureus* strains to fluoroquinolones (Thangamani et al., 2015; Foletto et al., 2021a). Verapamil exerts an antituberculosis effect by inhibiting antibiotic efflux pumps and intensifying the action of drugs typically used in the treatment of tuberculosis; this may be an indication for combination therapy (Singh and Chibale, 2021). This enhanced effect has been demonstrated for rifampicin, isoniazid, ethambutol and fluoroquinolones (Rodrigues et al., 2020). Additionally, the molecule reduced the tolerance of both susceptible and drug-resistant mycobacterial strains to bedaquiline, clofazimine, and moxifloxacin (Adams et al., 2014; Andries et al., 2014). However, very high concentrations of verapamil are required to achieve therapeutic concentrations in *M. tuberculosis* infections, indicating that further research is needed on more effective and selective structural analogues (Singh et al., 2014; Padmapriyadarsini et al., 2023). It also showed antifungal activity against various *Candida* spp. and was effective in inhibiting infections caused by influenza A viruses, in which regulation of calcium concentration played a key role in inhibiting viral protein transport and maturation (Jayaseelan and Paramasivam, 2020; Scorzoni et al., 2020). Preclinical studies indicate that drugs used in cardiovascular diseases have a wide spectrum of antimicrobial activity; however further research is necessary to determine the appropriate doses of the drugs in these indications.

The mechanisms of antibacterial action of the drugs described in this section are presented in Figure 5 (Zawadzka et al., 2019; Singh and Chibale, 2021; Abdel-Karim et al., 2022; Hua et al., 2022; Barbosa et al., 2023).

2.5 Medicines used in neurological therapy and psychiatry

2.5.1 Antipsychotics

The first generation of typical antipsychotic drugs, e.g., including haloperidol, fluphenazine, thioridazine and loxapine, were first used in the 1950s. The act by regulating the neurotransmitters in the brain, most important by blockading the D2 dopamine receptor (Weston-Green, 2022). Atypical antipsychotics, such as olanzapine or quetiapine, are characterized by high affinity for 5-HT2 receptors. Drugs in this group are considered capable of binding to multiple therapeutic targets, resulting in multiple mechanisms of action. They interact not only with neurotransmitter receptors, but also have the ability to suppress NF-kB, deregulate cyclins, or phosphorylate β-catenin (Awuah et al., 2023).

Antipsychotics are used to treat mental disorders and psychoses, especially schizophrenia and bipolar disorder (Vitiello et al., 2009; Lin et al., 2022). Research indicates the possibility of their use as complementary drugs in anticancer therapy, and this has been the subject of several clinical trials (Vlachos et al., 2021). The anticancer effect may result from modulation of various signaling pathways as well as autophagy, and influencing changes in cell membrane permeability and cell metabolism (Rácz and Spengler, 2023). For example, chlorpromazine and thioridazine have been included in clinical trials as an adjunct in cancer treatment, as well as in the treatment of migraine (NCT05190315, NCT05967442) (Aslostovar et al., 2018; Cohen and Friedman, 2021).

Among the antipsychotics, flupentixol, thioridazine, and prochlorperazine have demonstrated antibacterial activity. They may act by inhibiting the enzymes influencing the bacterial cell membrane and antibiotic efflux pumps (Barbarossa et al., 2022; Hua et al., 2022). It has been demonstrated that phenothiazines can reduce the ability of microorganisms to adhere to endothelial cells

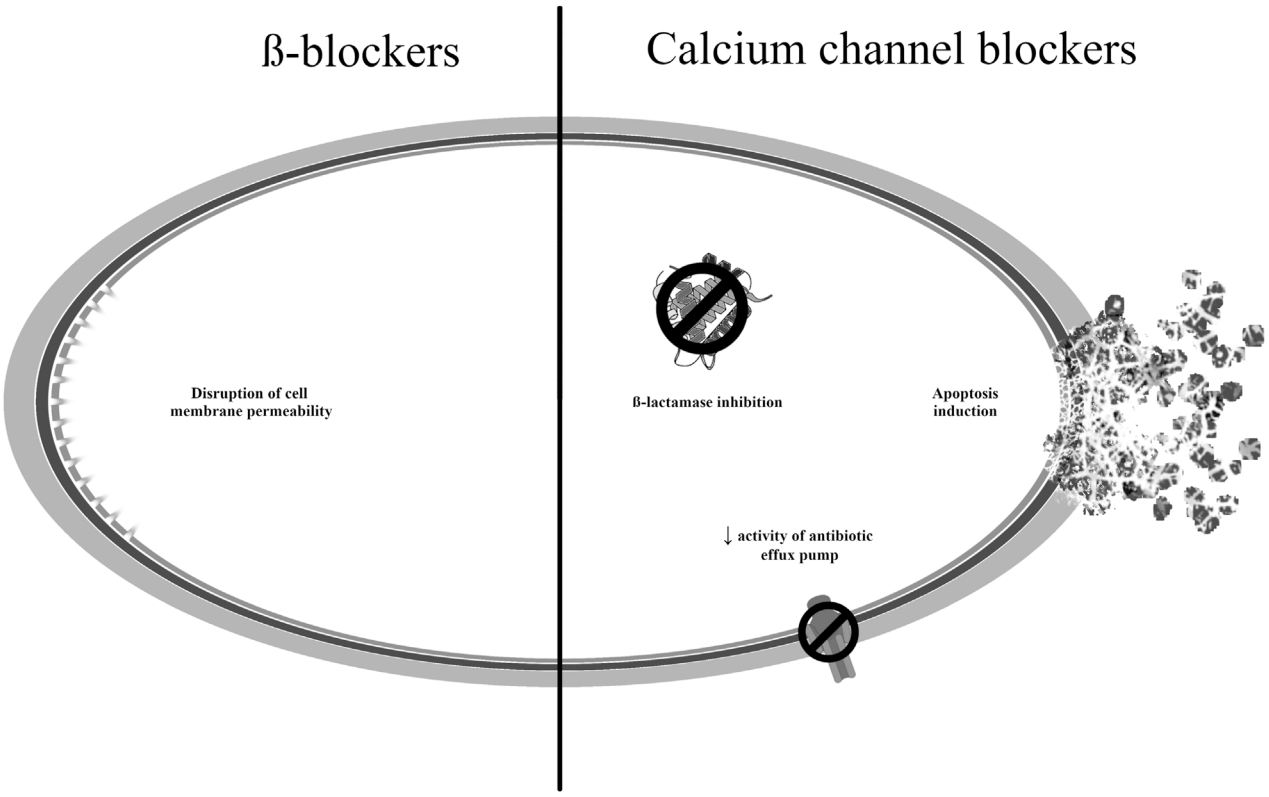

FIGURE 5
Potential mechanisms of antibacterial action of medicines used to treat cardiovascular disorders.

and can interfere with the action of ATPases, which inhibits bacterial replication (Barbarossa et al., 2022). Prochlorperazine was found to have antibacterial activity against Gram-negative and Gram-positive bacteria by infuencing the functioning of efflux pumps. Flupentixol, in turn, reduced the membrane potential in *S. aureus* cells (Hua et al., 2022). Importantly, a study on urinary catheters found chlorpromazine to be effective against the bacterial species most commonly implicated in urinary tract infections, i.e., *E. coli*, *P. mirabilis* and *Klebsiella* spp.; hence it may be suitable for coating urological instruments and preventing urinary tract infections (Sidrim et al., 2019).

Interestingly, thioridazine, withdrawn from treatment of schizophrenia due to cardiotoxicity, turned out to be effective against methicillin-sensitive and methicillin-resistant strains of *S. aureus*, *Enterococcus* spp., and *M. tuberculosis*, *M. avium* (Kristiansen et al., 2007; Deshpande et al., 2016; Ruth et al., 2020). Also, among the phenothiazine derivates, it also the most promising candidate for use as an antituberculosis drug; it inhibits the action of efflux pumps and increases the killing activity of macrophages. The antimycobacterial effect was associated with the inhibition of potassium and calcium channels, which led to a decrease in pH in the phagolysosome, activation of hydrolases, and subsequent destruction of *M. tuberculosis* cells (Rodrigues et al., 2020). The levorotatory form showed better antibacterial activity, both *in vitro* and *in vivo*; racemic thioridazine was found to reduce the ability of Gram-positive and Gram-negative bacteria to invade cell lines (Foletto et al., 2021a). However, care should be taken to minimize its negative effects on the central nervous system and cardiotoxicity. Hence, there is currently considerable interest in identifying suitable thioridazine analogues or changing the form of the drug, e.g., encapsulation of the drug in nanoparticles (Rodrigues et al., 2020). Currently, research on the repositioning of antipsychotic drugs in the treatment of infectious diseases is in the preclinical phase.

2.5.2 Antidepressants

The most commonly prescribed antidepressants include selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs). They improve mood and anxiety disorders by inhibiting the transporters responsible for the reuptake of norepinephrine and serotonin. Currently, antidepressants are typically used to treat neuropathic pain in oncological patients. Studies in animal models indicate their analgesic effect is associated with their potential to achieve high noradrenaline concentrations through their action on α2-adrenergic receptors. Dopamine and serotonin may also enhance the analgesic effects of norepinephrine. Additionally, TCAs also inhibit the production of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (Asensi-Cantó et al., 2022; Rácz and Spengler, 2023). They can also inhibit cancer cell growth by blocking their molecular pathways. These phenomena support the rationale for further trials on the usage of TCAs in cancer diseases.

Antidepressants, particularly serotonin reuptake inhibitors (SSRIs), have also been recognized as potential candidates for drug repurposing for treating infectious diseases. For example,

sertraline has been demonstrated to enhance the activity of fluoroquinolones and aminoglycosides against *S. aureus* strains, and its antimicrobial effect in combination with gentamicin and erythromycin, has also been evaluated against *P. aeruginosa* and *E. coli*. Sertraline has also shown antibacterial activity against *Helicobacter pylori* strains in a concentration-dependent manner, as well as possible synergism with amoxicillin, clarithromycin, tetracycline, and metronidazole, used to eradicate *H. pylori*. The potential mechanism could involve the inhibition of bacterial protein translation and interference with bacterial efflux pumps (Barbarossa et al., 2022). In another study, sertraline increased the effectiveness of polymyxin B against strains of other Gram-negative species such as *A. baumannii*, *E. coli*, and *K. pneumoniae* (Otto et al., 2019), as well as against strains of *Enterococcus* spp. resistant to vancomycin (Foletto et al., 2021a). The bactericidal effect of sertraline against the reference strains *S. aureus*, *E. faecalis*, *B. cereus*, and *E. coli* was found to be further enhanced by combining it with disulfiram, used in the treatment of alcoholism. Disulfiram was metabolized by bacterial cells to diethyldithiocarbamate (DDTC), which has antibacterial properties. Additionally, disulfiram has a proteolytic effect due to its ability to chelate ions (Serafin et al., 2020). Clinical trials also included combination therapy of fluconazole and sertraline in the treatment of early disseminated cryptococcal infection (NCT03002012). However, when high doses of sertraline were used, the clinical trial was terminated due to high rate of adverse events (Boulware et al., 2020). *In vitro* studies have demonstrated synergistic and additive effects of SSRIs in combination with azoles. Sertraline had the best antifungal activity against *Cryptococcus* spp.; it was believed to act by damaging the mitochondrial membrane and increasing the production of reactive oxygen species, leading to apoptosis (da Silva et al., 2023).

In turn, fluoxetine showed activity against MRSA strains, vancomycin-resistant *Enterococcus* spp. strains, and *A. baumannii* strains, which was probably caused by changes in the integrity of bacterial plasma membranes and damage to their DNA, leading to bacterial cell death (Foletto et al., 2020; Foletto et al., 2021a; Foletto et al., 2021b). Both fluoxetine and paroxetine showed improved efficacy in combination with ciprofloxacin against both Gram-negative and Gram-positive microorganisms, compared to ciprofloxacin alone. When the antidepressant was combined with this fluoroquinolone, its potency against MDR-resistant strains of *E. coli*, *A. baumannii*, *K. pneumoniae*, and *E. faecium* was eightfold increased (Foletto et al., 2020; Foletto et al., 2021b). SSRIs can affect a number of processes regulating the biosynthesis of products important for microorganisms, regardless of absorption into bacterial cells. The mechanism of action may also be based on the inhibition of bacterial efflux pumps (de Sousa et al., 2018; Foletto et al., 2020; Foletto et al., 2021a) or on disturbing bacterial cell wall synthesis and preventing bacterial cell division (Basha et al., 2018). Moreover, fluoxetine affected plasma membrane exopolysaccharides integrity and induced DNA damage, which may lead to bacterial cell apoptosis (Neto et al., 2019; Foletto et al., 2021b).

In turn, amitriptyline, a TCA, had antibacterial activity against carbapenemase-producing strains of *K. pneumoniae*. A synergistic effect was also obtained when combined with colistin, i.e., the "last resort" antibiotic, and tetracyclines (Barbarossa et al., 2022; Ugurel and Turgut-Balik, 2023).

2.5.3 Medicines used in epilepsy

Valproic acid, used in the treatment of epilepsy and bipolar disorders, stimulates the formation of autophagosomes *in vitro*. Its has been found to enhance the clinical effects of isoniazid and rifampicin in the treatment of tuberculosis, as demonstrated in studies on human cell lines. The antimycobacterial activity of valproic acid may result from the ability to inhibit succinic semialdehyde dehydrogenase, associated with the process of aerobic respiration. Valproic acid also influences the metabolism of fats, thus regulating the fluidity of cell membranes, and reduce the amount of substrate for the production of prostaglandins (Rodrigues et al., 2020). It is currently also the subject of several clinical trials regarding its effectiveness in the treatment of neuropathic pain or anticancer therapy (NCT01928849) (Caponigro et al., 2016; Iannelli et al., 2020).

2.5.4 Medicines used in neurodegenerative diseases

Entacapone and tolcapone are able to reversibly inhibit catechol-O-methyltransferase (COMT), and are hence used in the supportive treatment of Parkinson's disease. These drugs have also shown antituberculosis activity (Maitra et al., 2015; Beklen et al., 2021). The possible mechanism of action against *M. tuberculosis* strains might be based on their ability to inhibit the synthesis of mycolic acid: a compound necessary for the construction of the mycobacterial cell wall. Its loss leads to the death of bacterial cells (Maitra et al., 2015; Sharma et al., 2023). Unlike isoniazid, entacapone and tolcapone do not require enzymatic activation, so there is no possibility of mutations that can develop resistance (Maitra et al., 2015).

Memantine, a drug used to treat Alzheimer's disease, has also shown antibacterial properties. It acts by inhibiting N-methyl-d-aspartate (NMDA) receptors in the central nervous system (Johnson and Kotermanski, 2006). Moreover, the discovery of NMDA receptors in non-neuronal tissues, such as the heart, lungs, and kidneys, suggests it may have value across a broader therapeutic spectrum (Zakariaa et al., 2023). Memantine, used in combination with ampicillin, blocked the inflammatory response to bacterial infection caused by *E. coli*. Studies indicate that memantine affects the α7 nAChR receptor: an inflammation regulator also controlled by bacterial cells. It also reduces the intracellular survival of bacteria, both during bacteremia and in the later phase of meningitis caused by *E. coli*. Memantine may make it more difficult for the pathogen to penetrate the blood-brain barrier, as evidenced by studies in animal models. Its ability to inhibit the development of meningitis is correlated with the degree of bacteremia development. The proposed doses of this drug showed neuroprotective properties and did not have a toxic effect on human cells (Yu et al., 2015).

The mechanisms of action of antibacterial drugs used in neurological diseases and psychiatry are presented in Figure 6 (Johnson and Kotermanski, 2006; Marchi et al., 2015; Caponigro et al., 2016; Basha et al., 2018; de Sousa et al., 2018; Saputo et al., 2018; Neto et al., 2019; Foletto et al., 2020; Beklen et al., 2021; Maitre et al., 2022).

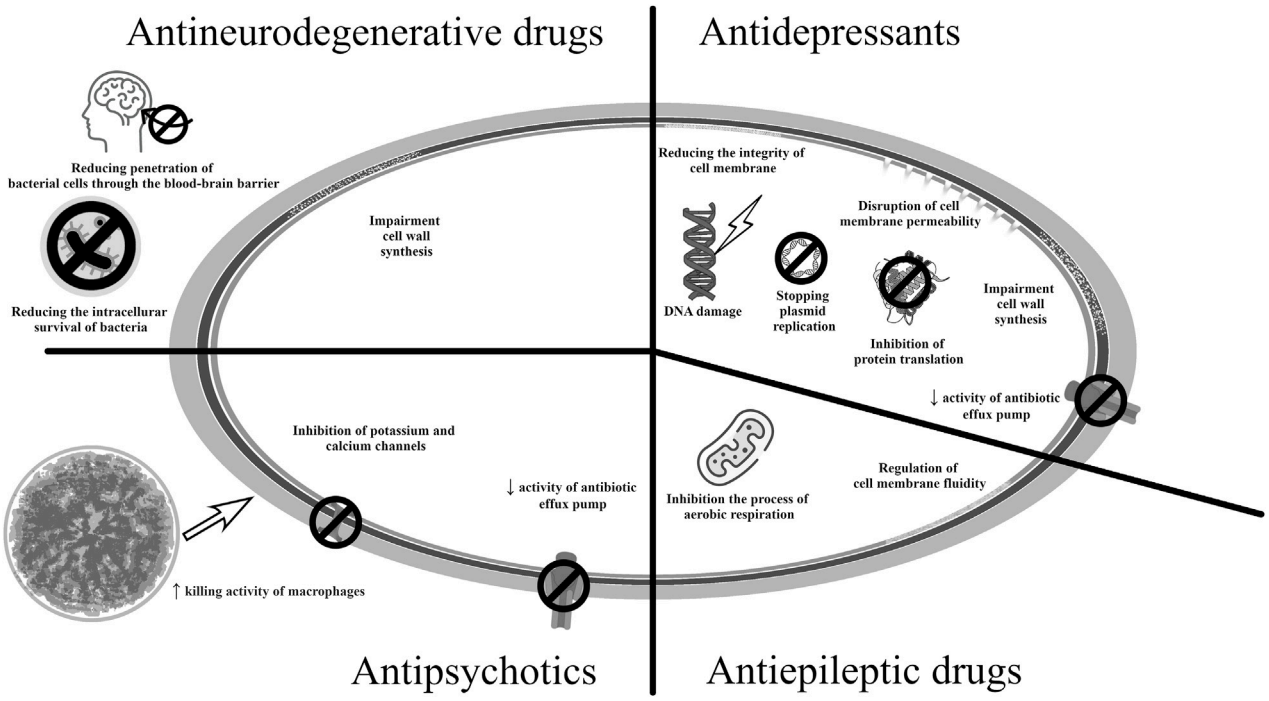

FIGURE 6
Potential mechanisms of antibacterial action of medicines used in neurological therapy and psychiatry.

2.6 Medicines used in parasitic infections

Antiprotozoal drugs have also demonstrated antibacterial effects. One of them is pentamidine, which disrupts cellular metabolism and affects the metabolism of nucleic acids, folic acid and proteins. Pentamidine has been proven to treat infections caused by multidrug-resistant Gram-negative bacilli by affecting the integrity of the outer bacterial membrane (Hua et al., 2022).

Despite being currently used as an anthelmintic drug in veterinary medicine, salicylanilide derivatives have also shown good effects in antimicrobial therapy. Among these, niclosamide is used to treat tapeworm and roundworm infections and has been widely studied as an antimicrobial drug. It has demonstrated high activity against strains of Gram-positive bacteria, MRSA, and vancomycin-resistant *E. faecium* (Domalaon et al., 2019). Niclosamide is also considered as a candidate for repositioning in cancer; it has been shown to inhibit the Wnt/β-catenin pathway, which is important for embryogenesis and cell differentiation, and is involved in the proliferation of cancer tissue. However, it is difficult to maintain the concentrations of the drug within the therapeutic range (Schweizer et al., 2018). Niclosamide disrupt the proton flow within the cell membrane, which also inhibits the growth of *H. pylori*. The drug has demonstrated synergistic effects with colistin in the case of resistant strains *A. baumannii* and *K. pneumoniae* (Barbarossa et al., 2022).

Colistin has also displayed synergy with the salicylanilide derivates oxyclozanide, rafoxanide, and closantel against multidrug-resistant strains of *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, *E. coli*, and *E. cloacae*. The development of derivatives with better pharmacokinetic properties and a better toxicity profile could influence the effectiveness of therapy for infections caused by multidrug-resistant Gram-negative bacilli (Tran et al., 2016; Domalaon et al., 2019).

Ivermectin has antiparasitic activity due to its ability to activate a glutamate-gated chloride channel, which does not occur in vertebrate organisms. It is effective against nematodes and arthropods (Hu et al., 2023). In vertebrates, it has the ability to inhibit type A γ-aminobutyric acid and glycine receptors and activate the acetylcholine receptor in brain nerve cells. Because of its mechanism of action, ivermectin is being investigated for its repurposing in the treatment of disorders associated with alcohol abuse or epilepsy. However, a significant problem is the poor ability of this molecule to penetrate the blood-brain barrier (Loescher, 2023). The justification for its use is based on the proven anti-inflammatory effect due to the inhibition of pro-inflammatory cytokines, and antiparasitic effect on demodex (Baranska-Rybak and Kowalska-Oledzka, 2019). Ivermectin is also among the antiparasitic drugs with antibacterial properties. *In vitro*, its activity has been demonstrated against *S. aureus* strains and the biofilm they create (Ashraf et al., 2018). Ivermectin itself has a bacteriostatic effect, while its analogues may have a bactericidal effect. It is indicated that structural analogues destroy the bacterial cell wall, additionally interact with cell membranes and influence their permeability. Moreover, they have very good anti-biofilm properties against MRSA (Tan et al., 2021). Among antiparasitic drugs, thiabendazole, as a representative of benzimidazoles, also showed antitubercular activity. This drug has the ability to inhibit *M. tuberculosis* cell division. It is also indicated that this compound has the ability to inhibit the enzymes succinate dehydrogenase and

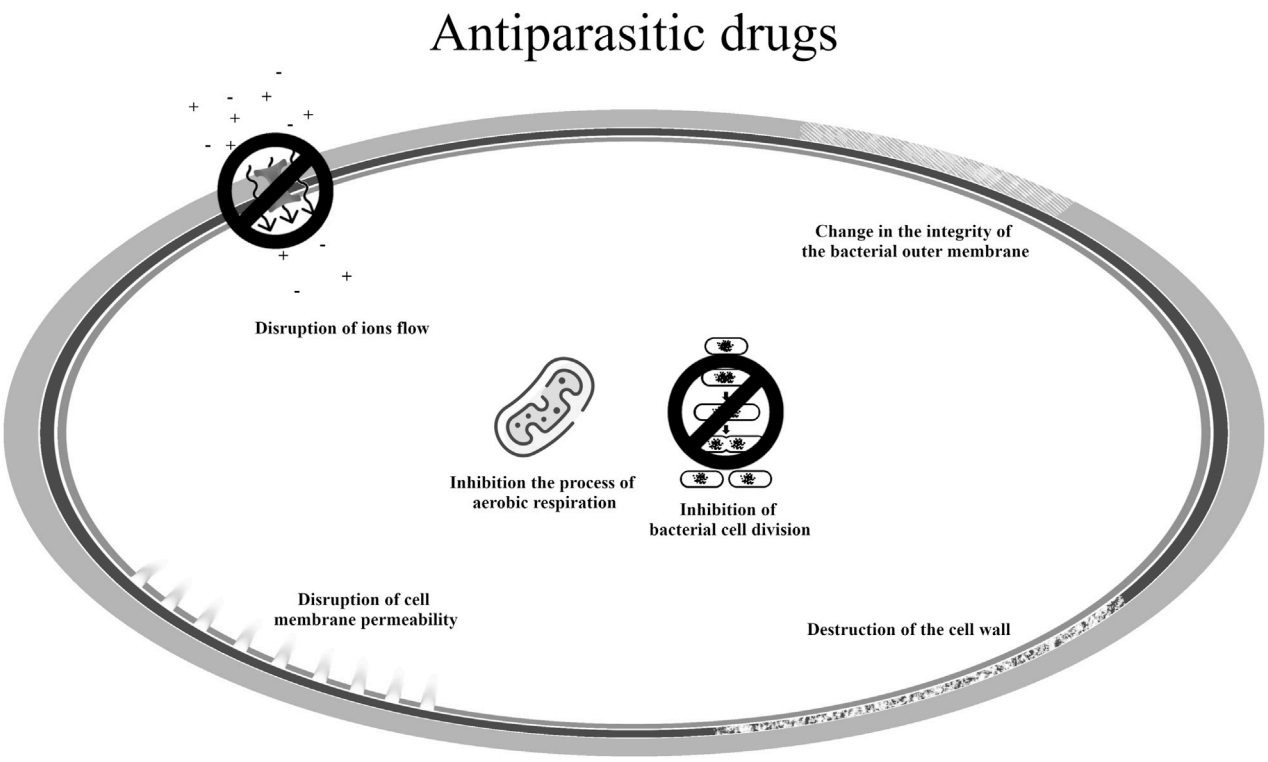

FIGURE 7
Potential mechanisms of antibacterial action of medicines used in parasitic infections.

fumarate reductase, which are involved in the aerobic respiration of tuberculous mycobacteria (Rodrigues et al., 2020).

The mechanisms of action of antiparasitic drugs in the new indication are summarized in Figure 7 (Rodrigues et al., 2020; Tan et al., 2021; Hua et al., 2022; Hu et al., 2023).

2.7 Medicines used in metabolic disorders

2.7.1 Antidiabetic drugs

One of the antidiabetic drugs that inhibit the growth of bacteria is metformin, for which the possibility of repositioning is being investigated in many indications (Gadducci et al., 2016; Matsuoka et al., 2021; Agostini et al., 2022). While metformin has remained the first-line medication to treat type 2 diabetes mellitus (T2DM), its mechanisms of action are complex and not fully understood. The molecule has been recognized to act through activated protein kinase (AMPK)-dependent and AMPK-independent mechanisms in the liver, where it controls hepatic glucose production. The current understanding of the molecular mechanisms of metformin action focuses on the other sites of its pharmacological activity, and consequently, recent years have brought an increasing interest to repurpose metformin for the treatment of cancer, age-related diseases, inflammatory diseases or COVID-19 (Foretz et al., 2023). Metformin has also been successfully repositioned in the treatment of polycystic ovary syndrome.

The use of metformin and Triton X-100 allowed for a reduction in cell viability and a reduction in the virulence potential of *E. faecalis* (Barbarossa et al., 2022). It has been suggested that the antibacterial effect may be related to the rupture of the inner bacterial membrane (Foletto et al., 2021a). *In vivo* studies also found that metformin increases phagocytosis by macrophages in the case of *E. coli* infection and reduces the severity of the disease in the case of *M. tuberculosis* infection (Brown, 2015). It has also been shown that in the case of tuberculosis, the use of metformin inhibits cell necrosis and contributes to tissue regeneration (Guler and Brombacher, 2015). Metformin also promotes autophagy in infected macrophages and limits the development of mycobacteria by positively influencing the production of reactive oxygen species (Vyas et al., 2022). For this reason, the molecule is currently in clinical trials for the treatment of tuberculosis (NCT05215990, NCT04930744). These are phase 1 and phase 2 interventional studies aimed at assessing whether metformin will have an impact on lung damage and the duration and effectiveness of therapy in tuberculosis. Other studies are ongoing to assess the effect of metformin on mortality in patients with and without diabetes and on inflammatory markers in sepsis and septic shock (NCT05572060, pre-recruitment; NCT06181422; pre-recruitment; NCT05979038, in the recruitment phase—phase 2 and 3 clinical trials).

Acarbose, which inhibits α-glucosidase activity in the small intestine, also has many additional effects beyond its effectiveness in diabetes. *In vitro* studies have shown that it reduces the inflammation, oxidative stress and platelet activation observed in the course of atherosclerosis (Chan et al., 2016). In *in vitro* studies, acarbose showed activity against *M. tuberculosis*: its addition allowed the doses of isoniazid and ethambutol to be halved

(Sharma et al., 2023). Additionally, at high concentrations, acarbose was able to inhibit biofilm formation by *M. tuberculosis* when combined with ethambutol and isoniazid (Kumar et al., 2019b). Similarly, it was found to be effective against respiratory infections caused by *P. aeruginosa* strains in a type 2 *in vitro* diabetes model; treatment anti-inflammatory and antimicrobial effects and reduced mortality (Liu et al., 2023).

2.7.2 Hypolipemic drugs

Statins are a group of drugs used in the treatment of hypercholesterolemia and in the prevention of atherosclerosis. They are responsible for inhibiting the enzyme hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is involved in the synthesis of cholesterol in the liver (Foletto et al., 2021a). In recent years, HMG-CoA reductase inhibitors (HMGRI, statins) have emerged as the most important class of lipid-lowering agents. Clinical trials have confirmed the beneficial effects of statins in cardiovascular disorders, in primary and secondary prevention settings, and in asymptomatic subjects with a high cardiovascular risk (Jasinska et al., 2007; Jiang et al., 2021). They are prescribed, among others, after myocardial infarction and ischemic stroke, and in the treatment of atherosclerosis or coronary artery diseases (Murphy et al., 2020). It is well-known that statin pleiotropy provides various beneficial effects in addition to their lipid-lowering properties, such as improved endothelial dysfunction and better nitric oxide bioavailability; treatment also has antioxidant effects, anti-inflammatory and immunomodulatory properties, and has been found to stabilize atherosclerotic plaques and inhibit cardiac hypertrophy. In addition, statins have demonstrated anti-tumor properties, which have attracted particular attention for repurposing (Jasinska et al., 2007; Rohilla et al., 2016; Jiang et al., 2021). Some studies have shown HMGRI to have potential efficacy in dementia and Alzheimer's disease, non-alcoholic fatty liver disease, and due to their immunomodulatory and antioxidant properties, also in rheumatoid arthritis (Murphy et al., 2020; Aminifar et al., 2023).

A wealth of evidence shows that people taking statins could have a lower risk of bacterial infections and better survival during infection. They are less exposed to community-acquired blood infections caused by *S. aureus* strains. Additionally, the risk of developing sepsis was also lower. *In vivo* studies have shown that simvastatin treatment is beneficial in lung infections caused by *S. aureus* strains: it lowered inflammatory marker levels and reduced mortality in animal models (Evans and McDowell, 2021). It has been proposed that the drugs exert their antibacterial activity by promoting apoptosis, and the mechanism of action is unrelated to the inhibition of HMG-CoA reductase (Foletto et al., 2021a).

Antibacterial effects were also demonstrated by atorvastatin and pitavastatin. It has been proposed that they act by breaking down the structures of teichoic acid and reducing the number of alanine residues on the surface of Gram-positive bacteria cells, which would reduce the ability of bacterial cells to form a biofilm (Barbarossa et al., 2022). Atorvastatin, lovastatin, and simvastatin have demonstrated significant inhibition of bacterial growth in biofilms, including *S. mutans* (Saputo et al., 2018).

Simvastatin also showed synergy of action with silver ions against MRSA strains and against *E. coli* producing β-lactamases with an extended substrate spectrum. Treatment resulted in distorted and lysed bacterial cells associated with a change in membrane permeability (Barbarossa et al., 2022). In an animal model of diabetes, simvastatin also accelerated wound healing and angiogenesis, influencing the control of inflammation by limiting the production of TNF-α and IL-6. Therefore, it may be an adjunct drug in the treatment of skin infections caused by MRSA strains (Thangamani et al., 2015). Atorvastatin was similarly active against both Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*A. baumannii*, *E. aerogenes*, *E. coli*) (Foletto et al., 2021a). Atorvastatin is currently involved in clinical trials for the treatment of bronchiectasis (NCT01299194), a chronic lung disease characterized by thick secretions and frequent respiratory infections. In most patients, it leads to chronic bacterial colonization.

M. tuberculosis can use cholesterol contained in host macrophages for infection and its own survival (Su et al., 2021). Therefore, the presence of higher cholesterol levels may be a predisposing factor to tuberculosis, and reducing its level may help limit the entry of bacteria to macrophages. Simvastatin, in combination with rifampicin, isoniazid, and pyrazinamide, has been shown to be effective against infections caused by *M. tuberculosis*. These combinations show increased mycobacterial killing, a decrease in the number of colony-forming units in the lungs, and a shorter time to obtain a pure culture. Additionally, the inflammatory process was also found to be regulated by increased secretion of the cytokines IL-12, IL-1β, and IL-10 (Sharma et al., 2023). *In vivo* models have shown that statins shorten the cure time for tuberculosis and have a beneficial effect on changes in the lungs. They also have the ability to inhibit T cell activation induced by *M. tuberculosis* antigens. Additionally, their use significantly reduces the rate of tuberculosis recurrence by almost 50%. As a consequence, several clinical trials are currently being conducted on the effectiveness of statins as an adjuvant therapy in tuberculosis (NCT04504851, NCT04721795, completed, no trial results; NCT06199921, in the recruitment phase). Atorvastatin is also being considered for treatment to reduce inflammation after TB treatment (NCT04147286, in the recruitment phase). These clinical trials on the use of statins in the treatment of tuberculosis are currently in phase 2 and 3 of clinical trials.

The antibacterial mechanism of antidiabetic anh hypolipemic drugs is shown in Figure 8 (Brown, 2015; Brindha et al., 2016; Guerra-De-Blas et al., 2019; Evans and McDowell, 2021; Vyas et al., 2022).

2.8 Other examples of non-antimicrobial drugs with antimicrobial activity

Among drugs used externally, ciclopirox has antibacterial effect. It inhibited the growth of *A. baumannii*, *E. coli*, and *K. pneumoniae*, regardless of any antibiotic resistance of the strains. The probable mechanism of action results from the possibility of inhibiting the synthesis of lipopolysaccharide in Gram-negative bacteria, a structure that protects bacteria against various substances, including antibiotics. Hence, treatment could sensitize gram-negative bacteria to some groups of antibiotics used in infections with Gram-positive bacteria (Brown, 2015). Cyclopirox is currently

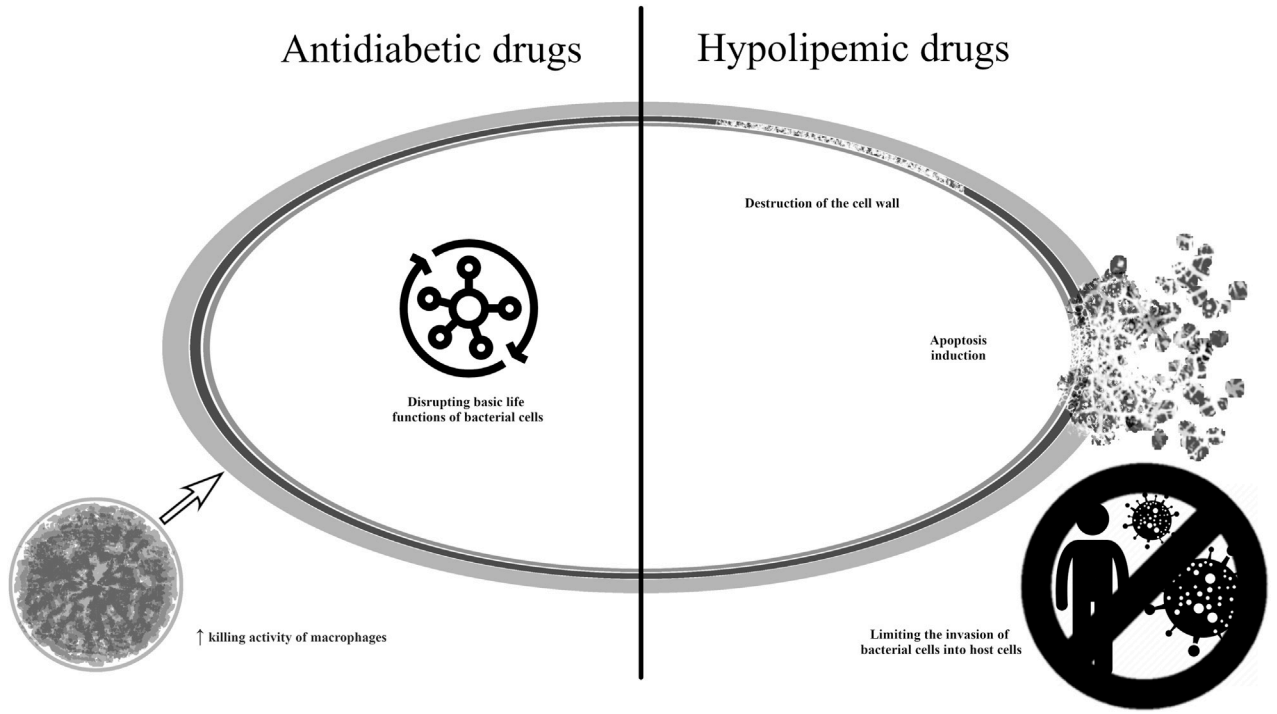

FIGURE 8
Potential mechanisms of antibacterial action of medicines used in metabolic disorders.

used only topically, but due to new potential applications, including in the treatment of cancer, research is underway on the possibility of oral use (Weir et al., 2011). Among the drugs used externally, zinc pyrithione, an antiseborrheic substance, has demonstrated bacteriostatic properties against streptococci and staphylococci. Its antibacterial effect is believed to be related to the ability to chelate metals and transport them through bacterial membranes (Saputo et al., 2018).

Ebselen is an organoselenium compound studied for its antioxidant, anti-inflammatory, and cytoprotective properties (Maslanka and Mucha, 2023). This molecule displayed high antibacterial activity against strains of Gram-positive bacteria, including *S. aureus* resistant to methicillin, vancomycin, and linezolid. Additionally, it also increased survival in cases of sepsis in animal models. Ebselen also demonstrated activity against strains of vancomycin-resistant enterococci (VRE) and streptococci (Younis et al., 2015; Maslanka and Mucha, 2023). Ebselen also inhibited the production of toxins, α-hemolysin, and Panton-Valentine leukocidin, important virulence factors in the pathogenesis of *S. aureus*. It also reduced the survival of *S. aureus* and *E. faecium* bacterial cells in biofilms. When used topically, ebselen reduced the level of pro-inflammatory cytokines in skin infections caused by strains of *Staphylococcus*. It also showed synergistic activity in combination with many antibiotics, linezolid, clindamycin, vancomycin, chloramphenicol, erythromycin, rifampicin, and gentamicin (Maslanka and Mucha, 2023). The combination of ebselen with isoniazid could also bring good therapeutic effects also against antituberculosis drug-resistant strains of *M. tuberculosis* (Padiadpu et al., 2016; Sharma et al., 2023).

Ebselen inhibits bacterial thioredoxin reductase; this prevents the bacteria from reducing disulfides in many substrates, thus disrupting the synthesis of DNA and cellular proteins and inducing oxidative stress inside bacterial cells (Maslanka and Mucha, 2023). In intestinal infections, ebselen also has the ability to protect human cells against *Clostridioides difficile* toxins, which are responsible for the destruction of human intestinal cells and tissue damage. In such cases, ebselen treatment promotes better regeneration of the intestinal microbiome and reduces the risk of repeated infections (Bender et al., 2015; Garland et al., 2020).

Much of the repositioning research in this area has focused on auranofin, which has been used for several decades to treat rheumatoid arthritis. Studies have shown the drug to have bactericidal activity against *N. gonorrhoeae* strains, without any activity against commensal lactic acid bacteria. It could be an effective response to the growing resistance demonstrated by *N. gonorrhoeae* strains to recommended antibiotics. Importantly, it has been found to inhibit the secretion of the pro-inflammatory cytokine IL-8 by cervical cells and have a long-lasting post-antibiotic effect. Auranofin has also exhibited antibacterial activity against MRSA and VRE strains, and *C. difficile* causing ulcerative colitis (Elkashif and Seleem, 2020).

Auranofin has demonstrated weak activity against gram-negative bacteria, which may be due to the inability of the drug to penetrate the outer cell membrane. However, the combination of auranofin with colistin, which has permeabilizing properties, causes strong activity against Gram-negative bacterial cells, including *P. aeruginosa*, that form a biofilm (Torres et al., 2018). Additionally, good effects against clinical isolates of *K. pneumoniae*,

A. baumannii, *P. aeruginosa*, *C. freundii*, *E. cloacae*, and *E. coli* with MDR resistance were obtained by the combination colistin-ceftazidime-auranofin and colistin-rifabutin-auranofin. It has been shown that adding a third drug to combination therapy with antibiotics may be beneficial in the treatment of infections with multidrug-resistant strains. Auranofin was also active against *S. pneumoniae* strains with MDR-type resistance (Sun et al., 2016).

Auranofin has recently been granted orphan drug status by the FDA for the treatment of amoebiasis in humans. This confirms the importance of drug repurposing also in the treatment of infections (Younis et al., 2015). Auranofin was also tested in HIV-1 eradication (NCT02961829). It was well tolerated in combination with antiviral drugs, and no serious adverse events were detected during the clinical study. This drug affected the viral reservoir by reducing the total viral DNA in blood cells (Diaz et al., 2019). The use of auranofin is also being considered as adjunctive therapy for lung cancer (NCT01737502) and giardiasis (NCT02736968).

The immunosuppressive drug cyclosporine A demonstrated antimicrobial activity against *M. tuberculosis*. It inhibited biofilm formation by *M. tuberculosis* cells and a synergistic effect when combined with ethambutol and isoniazid. Its role in bacterial activation in latent tuberculosis has also been analyzed: administartion was found to improve the availability of bacterial cells for anti-tuberculosis drugs, increasing their effectiveness. The use of a minimal concentration of cyclosporine A that inhibits the growth of *M. tuberculosis* strains in combination with anti-tuberculosis drugs could minimize its immunosuppressive effect (Kumar et al., 2019b). In addition to preventing transplant rejection, the clinical range of cyclosporine has been expanded to include autoimmune diseases such as severe rheumatoid arthritis, psoriasis, nephrotic syndrome, severe atopic dermatitis and uveitis. Cyclosporine is used to treat many eye diseases, e.g., dry eye syndrome, posterior blepharitis, spring and atopic keratoconjunctivitis. It has also demonstrated therapeutic effects against ulcerative colitis. In recent years, the drug has been found to possess special cardioprotective and neuroprotective properties in *inter alia* myocardial infarction, traumatic brain injury or stroke. However, the use of cyclosporine in these indications involves the use of high doses (Guada et al., 2016).

As mentioned earlier, disulfiram has also been shown to have antibacterial activity against strains of *M. tuberculosis*, including strains with MDR and XDR resistance types, as well as against non-tuberculous mycobacteria *M. fortuitum* and *M. abscessus* (Maitra et al., 2015; Saputo et al., 2018; Das et al., 2019). These species of nontuberculous mycobacteria are most often isolated from cases of lung, skin, or lymphatic system infections in immunocompetent people and may also cause disseminated infections in immunocompromised people (Gharbi et al., 2021). It has demonstrated synergy with drugs used in standard therapy, and reduced the bacterial load in macrophages more effectively than amikacin; it also minimized the number of mycobacterial cells in the kidneys in animal models of neutropenic bacteremia (Das et al., 2019).

The mechanisms of action of the drugs presented in this section are presented in Figure 9 (Younis et al., 2015; Saputo et al., 2018; Abutaleb and Seleem, 2020; Feng et al., 2021; Kobatake et al., 2021; Barbarossa et al., 2022; Maslanka and Mucha, 2023).

3 Future implications and limitations

Increasing antibiotic resistance among both Gram-negative and Gram-positive bacteria has prompted the search for new antimicrobial compounds. One direction of research involves repositioning drugs with known safety profiles. The data on the number of publications presented in Figure 1 indicate that increasing attention is being paid to the potential of drug repositioning in infectious diseases.

Figure 10 summarizes and presents the mechanisms of action of repositioning drug candidates in infectious diseases. Most of the drugs discussed in this review cause structural changes within the bacterial cell and its metabolism; when used as combined therapy, an important mechanism of their action also involves inhibiting bacterial efflux pumps for antibiotics. Some of the drugs presented in this work, such as the SSRIs, show several mechanisms of action.

Many drugs used to treat diseases other than infections have antibacterial properties and synergism with antibiotics. In the face of the growing number of multidrug-resistant strains, combination therapy may prove to be the most effective solution, reduce the risk of antibiotic resistance and be the answer to the problem of antibiotic resistance. One of the important discoveries in this area is the combination of β-lactam antibiotics with non-antibiotic β-lactamase inhibitors, e.g., clavulanic acid, tazobactam or vaborbactam and relebactam, discovered in recent years. Additionally, the synergistic combination may extend the usefulness of well-established antibiotics in therapy. Table 1 summarizes the synergistic pairs of repositioning drug candidates and antimicrobial drugs presented in this manuscript.

However, research is needed to ensure that the transferred drug reaches its expected site of action and to limit drug-related side effects while maintaining its antimicrobial activity. An additional problem is also possible drug interactions that could occur between drugs showing synergism and drugs of patients with concomitant diseases. In the case of some compounds, such as acarbose or some antidepressants, the impact of the repositioned drug on the patient's microflora is also important. As a result of combining different classes of drugs in therapy, the increase in antibiotic resistance may also accelerate (Tarín-Pelló et al., 2023).

Many of the presented drugs exhibit antibacterial activity at concentrations higher than those available in human serum. However, many of them may affect the activity of the human immune system, which would indicate the possibility of using such drugs as adjuvants in the treatment of infections. Among the drugs described, those used in metabolic disorders (e.g., seem to have the greatest repositioning potential due to their well-known pleiotropic effects, pharmacological profile and side effects) in fact, preclinical and clinical studies seem to indicate that this group of drugs has the greatest potential for repositioning in new indications. Selective serotonin reuptake inhibitors also show great promise in repositioning themselves in the treatment of infectious diseases due to their diverse mechanisms of antimicrobial action.

Although the presented examples of drug candidates for repositioning and their activity profile raise great hopes, it should be emphasized that most of the studies are *in vitro* studies, which do not allow drawing far-reaching conclusions. Moreover, more detailed research is needed, including *in vivo* animal studies and

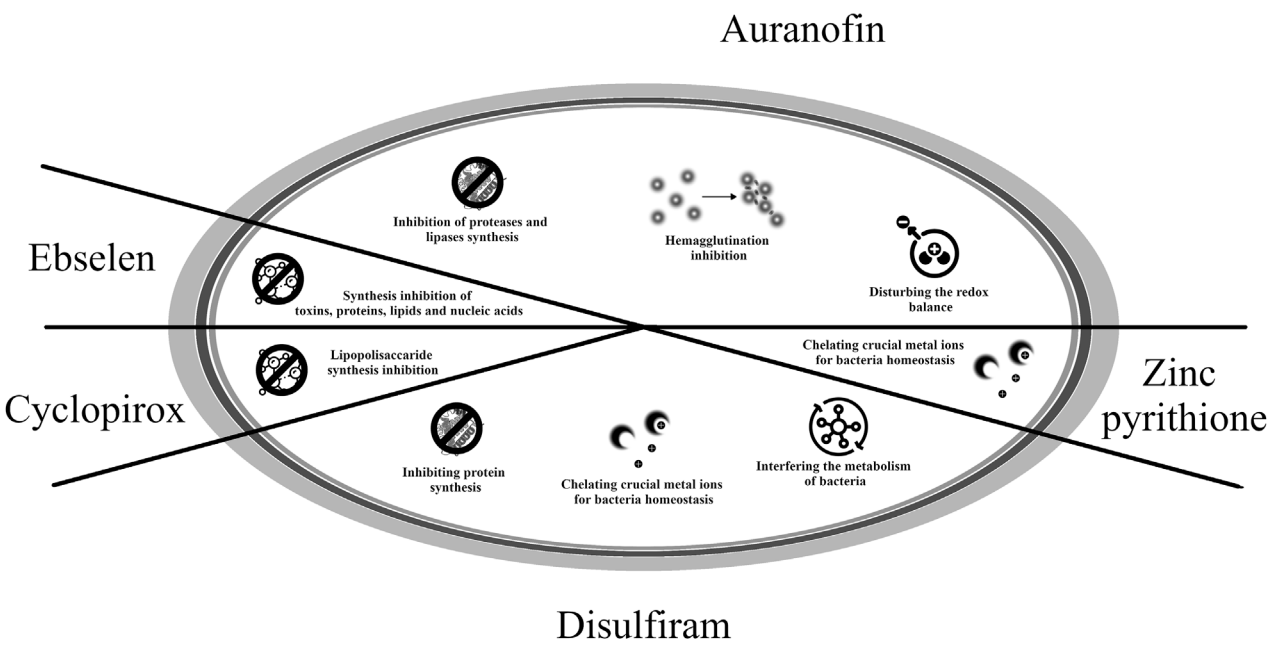

FIGURE 9
Potential mechanisms of action of repositioned drug candidates in infectious diseases—ciclopirox, zinc pyrithione, ebselen, auranofin and disulfiram.

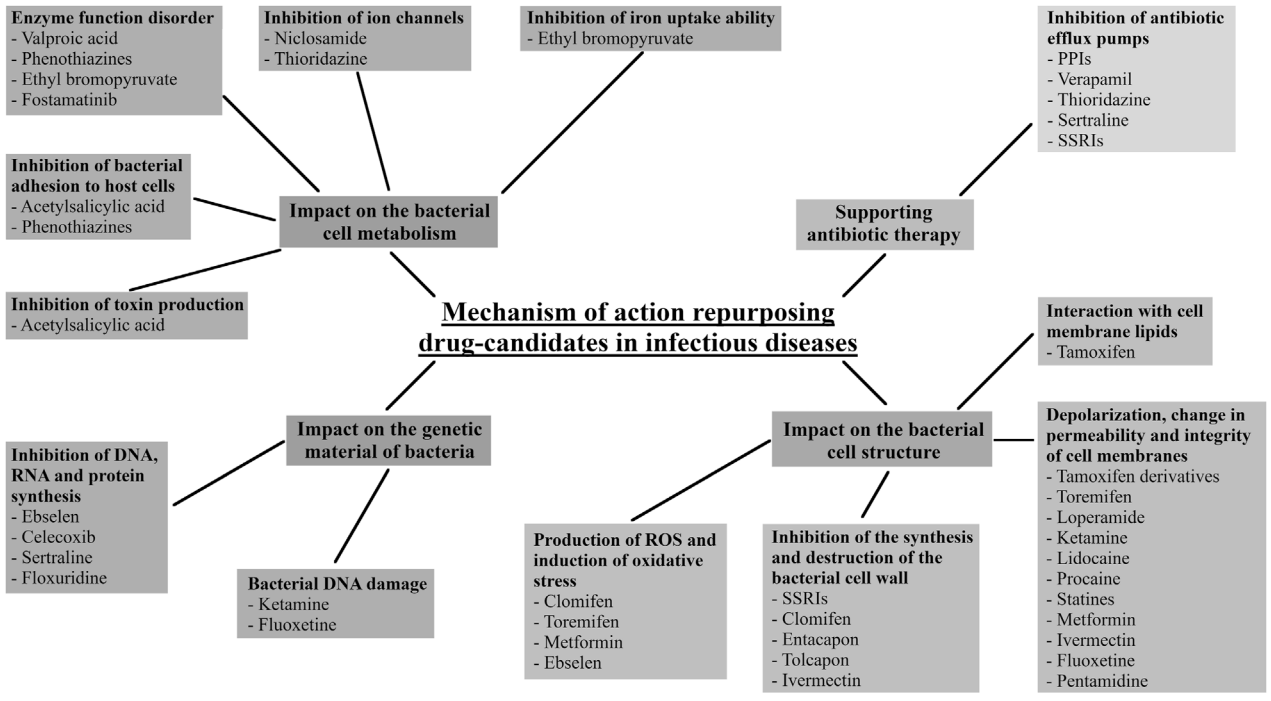

FIGURE 10
Mechanisms of action repurposing drug-candidates in infectious diseases.

clinical trials. They will answer questions about the precise pharmacokinetics, pharmacodynamics, effectiveness, stability and safety of the new drug. The effectiveness of the new, repositioned drug is also related to the selection of its appropriate dose and form of the drug, taking into account the possible side effects it may cause. In the era of increasing antibiotic resistance, it is also reasonable to determine whether a new compound may cause resistance to itself or increase resistance to antibiotics used in therapy. Ongoing screening

TABLE 1 Synergistic combinations of candidates for new antibacterial drugs with existing antimicrobial drugs.

Category	Group of drugs	Name of the repositioned drug	Synergistic antimicrobial drug	Spectrum of activity	Ref.
Medicines used in oncology	Cytostatics	Mitoxantrone	Colistin	*P. aeruginosa*	Torres et al. (2018)
		Mitotane	Polymyxin B	*A. baumannii, P. aeruginosa, K. pneumoniae*	Tran et al. (2018), Gontijo et al. (2022)
	Selective estrogen receptor modulators	Toremifene	Polymyxin B	*P. aeruginosa*	Hussein et al. (2017)
Medicines used in gastroenterology	Proton pump inhibitors	Omeprazole	Erythromycin	*S. aureus*	Mahfouz et al. (2023)
	Synthetic piperidine derivative	Loperamide	Minocycline	*Salmonella* sp.	Brown (2015)
		Loperamide	Novobiocin	*E. coli*	Brown (2015)
Medicines used in therapy against pain	NSAIDs	Acetylsalicylic acid	Vancomycin, cefuroxime, chloramphenicol	*S. aureus* (include MRSA)	Brown (2015); Chan et al. (2017), Barbarossa et al. (2022)
		Acetylsalicylic acid	Linezolid	*Staphylococcus* sp.	Maarouf et al. (2021)
		Ibuprofen	Cefuroxime, chloramphenicol	*S. aureus* (include MRSA)	Chan et al. (2017)
		Ibuprofen	Linezolid	*Staphylococcus* sp.	Maarouf et al. (2021)
		Ibuprofen	Ceftazidime	*P. aeruginosa*	Chen et al. (2023)
		Diclofenac	Streptomycin	*E. coli, Mycobacterium* spp.	Foletto et al. (2021a)
		Diclofenac	Gentamicin	*Listeria* spp.	Brown (2015)
			Oxacillin	*S. aureus* (include MRSA)	Queiroz et al. (2021)
		Celecoxib	Polymyxin B	*S. aureus* (include MRSA)	Krishnamurthy et al. (2019)
Medicines used to treat cardiovascular disorders	Calcium channel blockers	Amlodipine	Tetracycline	*A. baumannii*	Ugurel and Turgut-Balik (2023)
		Amlodipine	Imipenem	*A. baumannii*	Hu et al. (2018)
		Verapamil	Amikacin, tigecycline, cefoxitin	*M. abscessus*	Mudde et al. (2022)
	β-blockers	Carvedilol	Gentamicin	*A. baumannii*	Ugurel and Turgut-Balik (2023)
		Carvedilol	Ciprofloxacin	*S. aureus*	Zawadzka et al. (2019)
Medicines used in neurological therapy and psychiatry	Antidepressants	Sertraline	Fluoroquinolones, aminoglycosides	*S. aureus*	Barbarossa et al. (2022)
		Sertraline	Gentamicin, erythromycin	*P. aeruginosa, E. coli*	Barbarossa et al. (2022)
		Sertraline	Amoxicillin, clarithromycin, tetracycline, metronidazole	*H. pylori*	Barbarossa et al. (2022)
		Sertraline	Polymyxin B	*A. baumannii, E. coli, K. pneumoniae, Enterococcus* spp.	Otto et al. (2019), Foletto et al. (2021a)
		Fluoxetine, Paroxetine	Ciprofloxacin	*E. coli, A. baumannii, K. pneumoniae, E. faecium*	Foletto et al. (2020), Foletto et al. (2021b)
		Amitriptyline	Colistin	*K. pneumoniae*	Barbarossa et al. (2022), Ugurel and Turgut-Balik (2023)
Medicines used in epilepsy		Valproic acid	Isoniazid, rifampicin	*Mycobacterium* spp.	Rodrigues et al. (2020)

(Continued on following page)

TABLE 1 (*Continued*) Synergistic combinations of candidates for new antibacterial drugs with existing antimicrobial drugs.

Category	Group of drugs	Name of the repositioned drug	Synergistic antimicrobial drug	Spectrum of activity	Ref.
Medicines used in neurodegenerative diseases		Memantine	Ampicillin	*E. coli*	Yu et al. (2015)
Medicines used in parasitic infections	Salicylanilide derivatives	Niclosamide	Colistin	*A. baumannii, K. pneumoniae*	Barbarossa et al. (2022)
		Oxyclozanide, rafoxanide, closantel	Colistin	*P. aeruginosa, A. baumannii, K. pneumoniae, E. coli, E. cloacae*	Tran et al. (2016), Domalaon et al. (2019)
Medicines used in metabolic disorders	Antidiabetic drugs	Acarbose	Isoniazid, ethambutol	*M. tuberculosis*	Sharma et al. (2023)
	Statins	Simvastatin	Rifampicin, isoniazid, pyrazinamide	*M. tuberculosis*	Skerry et al. (2014)
Other drugs		Ebselen	Mupirocin, fusidic acid, retapamulin, daptomycin	*Staphylococcus* spp.	Maslanka and Mucha (2023)
		Ebselen	Isoniazid	*M. tuberculosis*	Padiadpu et al. (2016), Sharma et al. (2023)
		Auranofin	Azithromycin, ceftriaxone, cefixime, tetracycline	*N. gonorrhoeae*	Elkashif and Seleem (2020)
		Auranofin	Colistin	*P. aeruginosa*	Torres et al. (2018)
		Auranofin	Colistin and ceftazidime, colistin and rifabutin	*K. pneumoniae, A. baumannii, P. aeruginosa, C. freundii, E. cloacae, E. coli*	Sun et al. (2016)
		Cyclosporine A	Ethambutol, isoniazid	*M. tuberculosis*	Kumar et al. (2019b)

using patient isolates and drug candidates, also taking into account possible synergism with existing antibiotics, may identify treatments for severe infections. Additionally, it will reduce the time, costs and risks associated with the introduction of new antibacterial substances. It also brings hope given the scarcity of effective therapeutic options for treating infectious diseases.

4 Conclusion

The process of drug repurposing can be a rapid and effective method for discovering new antibacterial substances, as well as a solution to the problem of increasing antibiotic resistance, especially in the face of the small number of drug discoveries. This procedure is faster compared to *de novo* development of new antimicrobials. The review presents current data on the potential for repositioning drugs from various therapeutic groups in infectious diseases and summarizes the chance of their synergistic use in combination with antibiotics. The spectrum of their action and the possibility of use in the case of infections caused by multidrug-resistant bacteria were also taken into account.

Much research is still needed to identify potential repositioning candidates in infectious bacterial diseases in preclinical and clinical studies. Nevertheless, this research is necessary and antibacterial therapy in the treatment of infections, often caused by multidrug-resistant strains of bacteria, using repositioned drugs is promising and has great commercial potential.

Author contributions

PG: Conceptualization, Writing–original draft. AB: Writing–original draft. MJ-S: Supervision, Writing–review and editing.

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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.

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Repurposing effect of cardiovascular-metabolic drug to increase lifespan: a systematic review of animal studies and current clinical trial progress

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With the increase in life expectancy, aging has emerged as a significant health concern. Due to its various mechanisms of action, cardiometabolic drugs are often repurposed for other indications, including aging. This systematic review analyzed and highlighted the repositioning potential of cardiometabolic drugs to increase lifespan as an aging parameter in animal studies and supplemented by information from current clinical trial registries. Systematic searching in animal studies was performed based on PICO: "animal," "cardiometabolic drug," and "lifespan." All clinical trial registries were also searched from the WHO International Clinical Trial Registry Platform (ICTRP). Analysis of 49 animal trials and 10 clinical trial registries show that various cardiovascular and metabolic drugs have the potential to target lifespan. Metformin, acarbose, and aspirin are the three most studied drugs in animal trials. Aspirin and acarbose are the promising ones, whereas metformin exhibits various results. In clinical trial registries, metformin, omega-3 fatty acid, acarbose, and atorvastatin are currently cardiometabolic drugs that are repurposed to target aging. Published clinical trial results show great potential for omega-3 and metformin in healthspan.

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KEYWORDS

aging, drug repositioning, cardiovascular, metabolic, lifespan, animal model, clinical trial

1 Introduction

Aging is a complex and inexorable process that correlates with a decrease in capability status and physiological functions thus eventually leading to the amelioration of healthspan and shortening of lifespan. Growing evidence showed that aging was found to be an irreversible risk factor for multiple comorbid, including diabetes (Chentli et al., 2015),

cardiovascular disease (North and Sinclair, 2012; Rodgers et al., 2019), neurodegenerative disease (Xia et al., 2018), and cancer (Berben et al., 2021). Global Burden of Disease Study revealed that the mortality rates were higher in older adult populations since various degenerative diseases have been detected in these populations (Roth et al., 2018).

The recent innovation of medical science has greatly empowered our understanding of the molecular mechanisms of aging and developed new potential approaches for deferring the aging process (Campisi et al., 2019). Numerous aging interventions, including gerotherapeutics were shown to increase lifespan and prevent the occurrence of chronic disorders linked to aging (Partridge et al., 2020).

Cardiovascular and metabolic drugs are frequently repurposed due to their diverse molecular mechanism in many diseases (Ishida et al., 2016; Schubert et al., 2020). With various molecular mechanisms found in the aging process, cardiometabolic drugs possess the potential to delay aging. For instance, aspirin and statin are potentially beneficial for cancer (Zaleska et al., 2018; Ahmadi et al., 2020; Wang et al., 2021), or the pleiotropic effect of metformin in cancer, cardiovascular disease, and dementia in diabetic patients (Barzilai et al., 2016). Of note, aspirin and metformin could extend the lifespan of rodents (Strong et al., 2008; Martin-Montalvo et al., 2013). However, other reviews usually focus on *in vitro* scoring, 3D protein structures, orthology relationship, and drug binding, all of which require additional validation through *in vivo* study and clinical trial (Ziehm et al., 2017; Dönertaş et al., 2018). Therefore, our systematic review primarily focused on animal studies, with additional consideration given to clinical trials and their protocols.

Dramatic growth in the variety of longevity medicines that are being identified from animal studies is not always successfully translated to clinical applications (de Magalhães, 2021). Aspirin treatment failed to prevent mortality and morbidity in healthy older adult people and potentially increased the hemorrhagic risk in those people (McNeil et al., 2018b; 2018a). In parallel, metformin could not prolong the lifespan in *drosophila* and rather increased the mortality in female mice (Slack et al., 2012; Anisimov et al., 2015). Moreover, the clinical trials of metformin, such as MILES (Metformin In Longevity Study), showed the enhancement of longevity-related gene expressions, but the valid molecular mechanisms by which metformin facilitates this activity remain unknown (Mohammed et al., 2021).

This systematic review will summarize and analyze the evidence of cardiovascular and metabolic drugs from pre-clinical animal studies and recent clinical trials and highlight the rationale for the use of the repurposing potential of cardiometabolic drugs to increase lifespan in animal studies.

2 Materials and methods

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (Page et al., 2021). The study protocol can be observed on The International Prospective Register of Systematic Reviews (PROSPERO) database: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=457358. This systematic review of animal studies aims to determine the effect size and mechanism underlying lifespan increase. Following this, a search was conducted on the ICTRP (International Clinical Trial Registry Platform) clinical trial registry using the identified cardiometabolic drug from the animal studies.

2.1 Study eligibility criteria

We selected all interventional animal studies that met specific inclusion and exclusion criteria for our study using the PICO framework. The P stands for "animal," I for "cardiometabolic drugs," C for "no cardiometabolic drug," and O for "lifespan." Any animal model (natural or gene-modified animal to induce aging) is included in the Population. To replicate the natural aging process in humans, we exclude any intervention or induction that induces any disease apart from aging throughout the animal's lifetime. Yeast lifespan studies were also excluded because they are not a proper model for human aging studies (Zadrag et al., 2008).

As for intervention, we include all healthy animals who are given any routine cardiometabolic drug as part of the intervention at any time of their life until the animal is dead. Cardiometabolic drugs that are not currently approved by the FDA (Food and Drug Administration) or stated in the AHA (American Heart Association) and ADA (American Diabetes Association) cardiometabolic drug list are excluded (AHA, 2020; 2023; ADA, 2023). Any intervention during animal life that can cause a difference in their lifespan, such as an unnatural diet, is also excluded. We also exclude the comparator other than placebo because it will be a source of bias. Treatment other than intervention should be the same.

The primary outcome of this study is median or mean lifespan. In the absence of median or mean lifespan information, we would still consider including an article on cardiometabolic drugs if it included a Kaplan-Meier curve. The secondary outcome in this systematic review is healthspan, which consists of cardiometabolic, neurodegenerative, musculoskeletal, and neoplasm outcomes. Any other outcome that will impact animal health is also included. We also include any laboratory parameters that relate to lifespan and healthspan.

2.2 Search strategy

We conducted animal systematic literature search based on PICO "animal", "cardiometabolic drugs", and "lifespan". Each cardiometabolic drug was searched individually based on the AHA and ADA cardiometabolic drug lists (AHA, 2020; 2023; ADA, 2023). Our complete search strategies from four databases (Pubmed, Embase, Web of Science, and Scopus) are detailed in Supplementary Table S1. We considered all animal studies regardless of language and year of publication.

Clinical trial registries in lifespan and healthspan were also searched in ICTRP (International Clinical Trials Registry Platform). We considered conducting a search at ICTRP for all cardiometabolic drugs found in animal trials. Our search strategies consist of "cardiometabolic drug name" and "aging". Completed clinical trial registries are manually searched for the

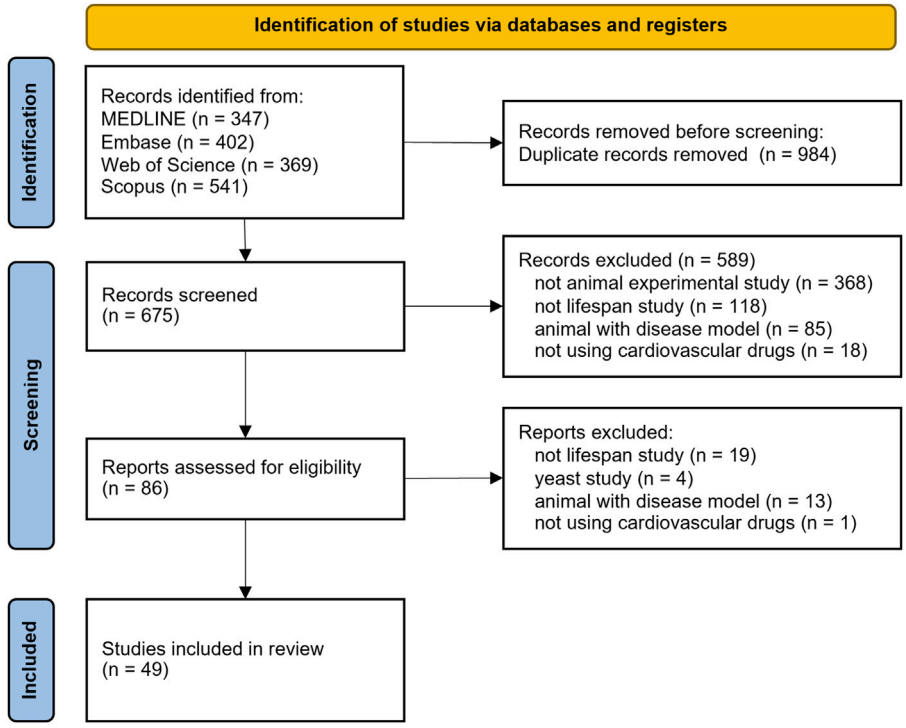


FIGURE 1
PRISMA flow chart search strategy.

full text. All clinical trial results will be described as a narrative review.

2.3 Study selection

Based on our search, all found studies are collected and managed in Mendeley Desktop version 1.19.8 (Glyph & Cog LLC, 2020). The software will automatically delete any duplicates. Furthermore, we manually identified and excluded other duplicates that cannot be detected by the software. Two independent authors (HH and AJB) screened all non-duplicate titles and abstracts according to inclusion and exclusion criteria; further discrepancies were discussed with a third author (ML). We recorded all reasons for excluded records as outlined in Figure 1.

We obtained all full text of included studies based on title and abstract screening by searching or buying the full text. Unobtainable full text was requested from the corresponding author. We excluded the unobtainable full text if the corresponding author did not respond. All full text eligibility was evaluated by two independent authors (HH and AJB) in accordance with inclusion and exclusion criteria; any discrepancies were resolved through consultation with a third author (ML).

2.4 Data extraction and management

All included animal study data were obtained based on PICO:

- Method: study design, year of study, number of study locations
- Animal model: species, gender, species strain/gene-modification, total animal used
- Intervention: drug name, dose, age at treatment initiation, comparator (placebo)
- Outcome: primary and secondary outcome
- Notes: funding and conflict of interest of the study

Two authors (HH and AJB) individually extracted the data and other potential data related to the results. We resolved the disagreement by consensus with the third author (ML). The results of this consensus were input to a word processor, and another author (NGK) double-checked all data input. If any changes were made, the other first three authors were asked about the appropriateness.

2.5 Risk of bias assessment

We used SYRCLE's risk of bias tool for animal studies to assess the risk of bias in this study (Hooijmans et al., 2014). This tool consists of six domains (selection, performance, detection, attrition, reporting, and other bias) and ten questions based on animal intervention study potential of bias. We also used RoB 2 tool for assessing the risk of bias in published clinical trial registry studies (Sterne et al., 2019). Three independent authors (HH, AJB, and NGK) individually searched and discussed all potential biases of all included studies.

2.6 Measures of treatment effect

This systematic review of treatment effect is based on its primary outcome, lifespan. Lifespan in animal studies is commonly stated as

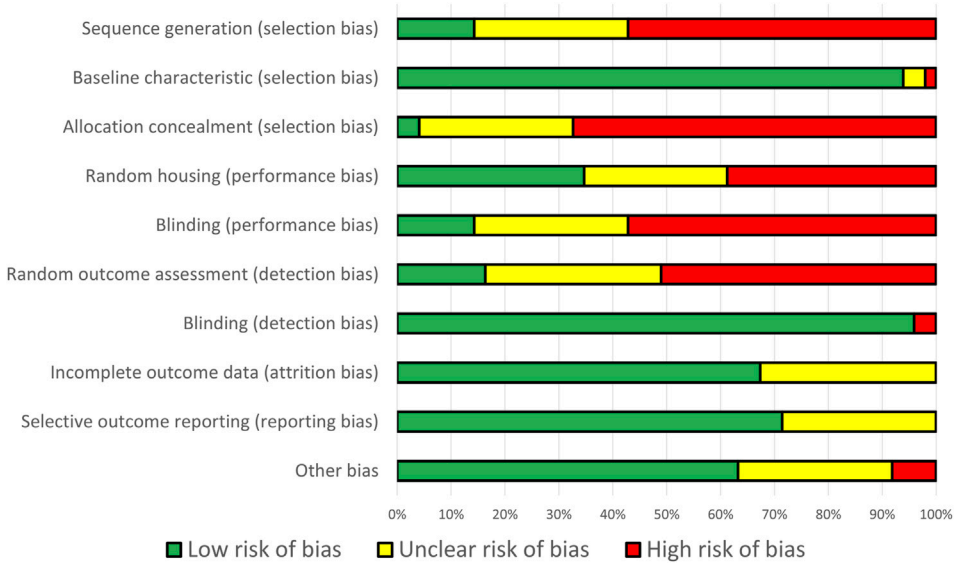


FIGURE 2
Percentage of bias risk across all domains from 49 identified studies.

the increase in percentage compared to control. We do not intend to proceed with a meta-analysis of this systematic review due to the numerous heterogeneities present in the study, including different cardiometabolic drugs, drug dosages, and animal models. As a result, meta-analysis is deemed unsuitable for the present study design.

3 Results

A comprehensive search across four databases yielded a total of 675 studies. Search results for each cardiometabolic drug can be seen in Supplementary Table S2. We identified 49 studies after applying the inclusion and exclusion criteria outlined in the methods section. The complete PRISMA flowchart for this study is illustrated in Figure 1. All included animal trials were evaluated based on PICO.

Sequence generation, allocation concealment, random housing, blinding, and random outcome assessment were all found to have a significant risk of bias. Although random housing is impractical for smaller animals, concealment and blinding are critical for animal research. Nevertheless, the potential for bias in these studies could be mitigated because lifespan is an objective parameter. Additional operator-dependent healthspan parameters, such as muscle size, may lead to bias in the absence of adequate blinding and concealment. The comprehensive RoB assessment of each study is detailed in Supplementary Table S3. The summary of RoB result is illustrated in Figure 2.

A diverse range of animals, including rats, mice, common fruit flies, roundworms, and silkworms, were utilized in these studies. Drug exposure starts at various stages of life in animals. Prolonged drug exposure yields more favorable results regarding extending lifespan (Espada et al., 2020; Strong et al., 2022).

Diverse drug concentrations also exhibit distinct impacts on the extension of lifespan. Research on captopril and metformin has demonstrated that while an appropriate dose of cardiometabolic drugs appears to extend lifespan, higher doses can shorten it (Martin-Montalvo et al., 2013; Anisimov et al., 2015; Onken et al., 2022; Egan et al., 2023). Our full list of extraction data can be seen in Supplementary Table S4. We summarized the data in Table 1.

3.1 The lifespan extension effect of cardiovascular drugs

Nine cardiovascular drugs in prolonging lifespan (acetazolamide, aspirin, captopril, enalapril, hydralazine, metolazone, metoprolol, nebivolol, and verapamil) were found to extend lifespan significantly, while the other two (candesartan and ramipril) did not show the same effect. Some drugs (hydralazine, metolazone, and verapamil) only have been tested in *Caenorhabditis elegans*. Therefore, further higher animal studies are needed.

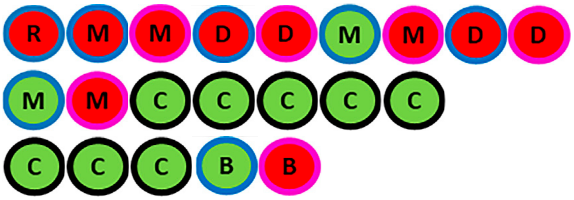
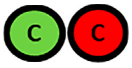
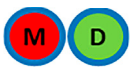
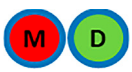
Aspirin was successfully shown as a lifespan-extending compound in *C. elegans*, *drosophila*, and mice (Ayyadevar et al., 2013; Danilov et al., 2015; Strong et al., 2008; Wan et al., 2013). However, one study found that aspirin failed to extend the lifespan in *C. elegans* with glp-1 mutation (Huang et al., 2013). An additional interesting discovery pertains to the fact that certain cardiovascular drugs within the same class, ACE inhibitors (ACE-I), exhibit distinct characteristics in terms of prolonging lifespan. Ramipril lacks the ability to induce an extension in lifespan, but not in captopril and enalapril (Santos et al., 2009; Kumar et al., 2016; Spindler et al., 2016; Egan et al., 2023). Meanwhile, candesartan as Angiotensin Receptor Blocker (ARB) failed to extend the lifespan in mice (Harrison et al., 2021).

TABLE 1 Summary of animal trials finding.

Drug(s)	Study found	References
Cardiovascular drugs		
Acetazolamide	M	Leibrock et al. (2016)
Aspirin	M M D D C C C	Strong et al. (2008), Strong et al. (2008), Ayyadevara et al. (2013), Huang et al. (2013), Wan et al. (2013), Danilov et al. (2015), Danilov et al. (2015)
Candesartan	M M M	Spindler et al. (2016), Harrison et al. (2021), Harrison et al. (2021)
Captopril	M M C C	Kumar et al. (2016), Strong et al. (2022), Strong et al. (2022); Egan et al. (2023)
Enalapril	R	Santos et al. (2009)
Hydralazine	C C	Dehghan et al. (2017), Dehghan et al. (2019)
Metolazone	C	Ito et al. (2021)
Metoprolol	M D	Spindler et al. (2013), Spindler et al. (2013)
Nevibolol	M D	Spindler et al. (2013), Spindler et al. (2013)
Ramipril	M	Spindler et al. (2016)
Verapamil	C	Liu et al. (2020)
Antidiabetic drugs		
Acarbose	M M M M M M M M C	Harrison et al. (2014), Harrison et al. (2014), Strong et al. (2016); Strong et al. (2016), Harrison et al. (2019), Harrison et al. (2019); Smith et al. (2019), Smith et al. (2019), Banse et al. (2023)
Canaglifozin	M M	Miller et al. (2020), Miller et al. (2020)
Dapaglifozin	C	Onken et al. (2022)
Glibenclamide	C	Mao et al. (2022)
Glimepiride	C	Mao et al. (2022)
Glipizide	C	Onken et al. (2022)
Linagliptin	M	Hasegawa et al. (2017)

(Continued on following page)

TABLE 1 (*Continued*) Summary of animal trials finding.

Drug(s)	Study found	References
Metformin		Onken and Driscoll (2010), Smith et al. (2010), Slack et al. (2012), Slack et al. (2012), Cabreiro et al. (2013), Martin-Montalvo et al. (2013), De Haes et al. (2014), Anisimov et al. (2015), Anisimov et al. (2015), Strong et al. (2016), Strong et al. (2016), Chen et al. (2017), Abrat et al. (2018), Abrat et al. (2018); Song et al. (2019), Song et al. (2019), Espada et al. (2020), Zhu et al. (2021), Onken et al. (2022), Xiao et al. (2022), Cedillo et al. (2023)
Nateglinide		Onken et al. (2022)
Pioglitazone		Jia et al. (2022), Onken et al. (2022)
Rosiglitazone		Xu et al. (2020)
Sitagliptin		Onken et al. (2022)
Dyslipidemia drugs		
Clofibrate		Brandstädt et al. (2013)
Fenofibrate		Brandstädt et al. (2013)
Lovastatin		Andreas et al. (2020)
Niacin		Preuss et al. (2011), Yang et al. (2019)
Omega-3 PUFA		Spindler et al. (2014), Champigny et al. (2018)
Simvastatin		Spindler et al. (2012), Spindler et al. (2016)
Drugs combination		
Acarbose + Rapamycin		Strong et al. (2022), Strong et al. (2022)
Metformin + Rapamycin		Strong et al. (2016), Strong et al. (2016)
Ramipril + Simvastatin		Spindler et al. (2016)

One circle indicates one study result; a study with various doses is denoted as one circle. R: rat (*Rattus norvegicus*), M: mouse (*Mus musculus*), B: silkworm (*Bombyx morii*), C: roundworm (*Caenorhabditis elegans*, *Caenorhabditis brigisae*, or *Caenorhabditis tropicalis*), D: common fruit fly (*Drosophila melanogaster*). The blue border indicates male, pink indicates female, and black indicates hermaphrodite roundworm. Green fill indicates a positive effect in increasing lifespan, whereas red fill indicates no effect in increasing lifespan. References are consecutively arranged according to the circle.

3.2 The lifespan extension effect of dyslipidemia drugs

Many drugs that aim to increase lifespan and are linked to dyslipidemia have been tested on different organisms. Niacin (nicotinic acid) was discovered to increase the lifespan of *C. elegans* at a concentration of 600 nmol, but not at 100 and 200 nmol (Yang et al., 2019). It also extended the lifespan of Zucker Fatty rats (Preuss et al., 2011). Simvastatin has been shown to increase lifespan in *Drosophila* but not in mice (Spindler et al., 2012; 2016). Furthermore, the combination of simvastatin and ramipril extended the lifespan of mice (Spindler

TABLE 2 Current completed and ongoing clinical trial in repurposing cardiometabolic drug for aging.

Clinical trial identifier	Year of registration	Study name	Drug name (dose)	Drug administration length	Total subject (enrollment)	Total subject (finished)	Participant age	Availability of result
NCT00996229	2009	Effects of Dietary Interventions on the Aging Brain	Omega 3 (2.2 g/d)	26 weeks	Exp: 40 Con: 40	Exp: 22 Con: 27	50–75	Yes, (Külzow et al., 2016)
NCT02102724	2014	Fish Oil for HIV-Related Inflamm-aging and Immune Senescence	Omega 3 (1.6 g/d)	12 weeks	Exp: 18 Con: 19	Exp: 16 Con: 18	40–70	Yes, (Swanson et al., 2018)
NCT02953093	2016	Study of acarbose in Longevity (SAIL)	Acarbose (no data in dose)	10 weeks	Crossover trial: 10	NA	60–100	No
NCT02865499	2016	Acarbose Anti-aging Effects in Geriatric Subjects (Substudy B & C)	Acarbose (300 mg/d)	8 weeks	Pre and post-study: 8	6	70–95	Yes, available on https://clinicaltrials.gov/study/NCT02865499
NCT04386577	2017	Effects of Vitamin D and Omega-3 Supplementation on Telomeres in VITAL	Omega 3 (840 mg/d)	208 weeks	Exp: 250 Con: 250	NA	Men > 50 Women >55	No
NCT03228550	2017	Omega-3 Fatty Acids and Exercise on Mobility and Cognition in Older Women (MOBILE)	Omega 3 (1.16 g/d)	24 weeks	Exp: 15 Con: 15	Exp: 12 Con: 13	>60	Yes, (Fairbairn et al., 2020)
NCT02432287	2018	Metformin in Longevity Study (MILES)	Metformin (1.7 g/d)	6 weeks	Crossover trial: 16	14	>60	Yes, (Kulkarni et al., 2018)
NCT04264897	2020	Antecedent Metabolic Health and Metformin Aging Study (ANTHEM)	Metformin (1.5 g/d)	12 weeks	Exp: 74 Con: 74	NA	40–75	No, protocol published at (Kumari et al., 2022)
NCT04536870	2020	Statins in Reducing Events in the Elderly (STAREE) Heart Sub-study (STAREE-HEART)	Atorvastatin (40 mg/d)	162 weeks	Exp: 500 Con: 500	NA	>70	No, protocol published at (Zoungas et al., 2023)
EUCTR 2021-003299-15-ES	2021	Metformin vs. placebo for reversal of accelerated biological aging in persons living with HIV 50 years	Metformin (850 mg/d)	96 weeks	Exp: 60 Con: 60	NA	>50	No

Dose of omega 3: 1,320 mg EPA and 880 mg DHA (Külzow et al., 2016); 800 mg EPA, 600 mg DHA, and 200 mg other omega-3 fatty acids (Swanson et al., 2018); 465 mg EPA and 375 DHA; dietary supplements (1,000 mg DHA, 160 mg EPA, 1 mg folic acid, 124 phosphatidylserine, 240 mg *G. biloba*) (Fairbairn et al., 2020). Doses are arranged respectively based on all omega-3 studies. Exp: experimental total subject. Con: control total subject.

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et al., 2016). Lovastatin also extended the lifespan of *C. elegans* (Andreas et al., 2020). Fenofibrate increased lifespan in a dose-dependent manner, while clofibrate only extended lifespan at a concentration of 10 μM in *C. elegans* (Brandstädt et al., 2013). An interesting study on omega-3 found that it significantly extends the lifespan of *Drosophila* but appears to reduce the lifespan of mice, although the result was not statistically significant (Spindler et al., 2014; Champigny et al., 2018). The results highlight that the lifespan extension effects of cardiometabolic drugs vary depending on the species.

3.3 The lifespan extension effect of antidiabetic drugs

Anti-diabetic medications were the most used drugs repurposed for aging. Three antidiabetic medications (acarbose, canagliflozin, and rosiglitazone) have been found to significantly extend lifespan, with acarbose being the most extensively researched. Positive effects are predominantly seen in male animals, whereas research has been unsuccessful to prolong lifespan in females. Additionally, metformin was found to be insignificant in rat (Smith et al., 2010) and beneficial in only two out of six rodent studies (Cabreiro et al., 2013; Zhu et al., 2021). Every study conducted on metformin in *C. elegans* is significant. It demonstrates that the effect of metformin on life expectancy varies by species. Metformin study in various *Caenorhabditis* species also showed that metformin's beneficial effects on lifespan are limited to *C. elegans* but not in *Caenorhabditis briggsae* or *Caenorhabditis tropicalis* (Onken et al., 2022).

3.4 The lifespan extension effect of drug combination

Several studies employing drug combinations, including ramipril and simvastatin, acarbose and rapamycin, and metformin and rapamycin, were identified. Ramipril or simvastatin alone do not increase lifespan in mice, but the combination of these medications significantly extends lifespan Simvastatin may blunt insulin sensitivity, while both simvastatin and ramipril induce hypercholesterolemia and hypertriglyceridemia (Spindler et al., 2016). A significant increase in lifespan has also been observed when rapamycin is combined with metformin or acarbose. We cannot ascertain whether these interactions are additive or synergistic, but it is speculated that these anti-diabetics may prevent hyperglycemia due to rapamycin administration by enhancing insulin sensitivity (Strong et al., 2016; 2022). Of note, rapamycin has been shown in a meta-analysis study of laboratory mice that may significantly increase the lifespan (Swindell, 2016). In summary, drug combination trials may be regarded as prospective areas of research in the field of lifespan.

3.5 Completed and ongoing clinical trial of cardiometabolic drug in aging

We identified 14 of the 44 study registries discovered in ICTRP that met our inclusion and exclusion criteria. We obtained data from these registries on 12 healthy elderly individuals and 2 patients with HIV. Search result is detailed in Supplementary Table S5. We discovered clinical trial registries for six metformin, four omega-3 fatty acids, two acarbose, one fenofibrate, and one atorvastatin. The results of the ten registries are detailed in Table 2, of which results for four have been published. We put the other four registries in Supplementary Table S6 due to unknown, terminated, or withdrawn status. One study was terminated due to recruitment being difficult and not achieved.

We identified three published clinical trial results in omega-3 (Külzow et al., 2016; Swanson et al., 2018; Fairbairn et al., 2020) and one in metformin (Kulkarni et al., 2018). Supplementary Table S7 shows a summary of clinical trials study risk of bias. In one study, the proportion of smokers in the control group was significantly greater than in the experimental group (Swanson et al., 2018). The other study has a dropout rate of over 20%, which lowers its significance result (Külzow et al., 2016).

4 Discussion

The breakthrough of gerotherapeutic as medication that molecularly targets the aging has become an emerging new era for overcoming shortened lifespans and preventing age-related pathologies (Couteur and Barzilai, 2022). Moreover, cardiovascular and metabolic pharmacology have been evaluated as candidates for gerotherapeutics in both preclinical and clinical models (Williams and Kim, 2003; Barzilai et al., 2016).

This systematic review has compiled the lifespan extension effect of cardiovascular and metabolic pharmacological interventions in animal models. The animal models, particularly in rodents, with human pathology phenotypes and yeast models were excluded from this study to maintain the quality of this review. The gold standard of drug identification with lifespan extension study in rodent models has been reviewed elsewhere (Spindler, 2012). As mentioned in this review, long-lived and healthy rodents, such as F1 hybrid mice, are ideally recommended for longevity drug screening. Several drugs that successfully extended the lifespan were mostly reported in short-lived or pathological mice models such as obese or diabetic mice. The failure of reproductivity data of these lifespan extension compounds in healthy rodents is likely due to the consequence of using pathological rodent models. Therefore, we did not include those models in this systematic review. We also excluded the lifespan studies that used yeast (*Saccharomyces cerevisiae*) since this model is not an appropriate model for representing aging in humans (Zadrag et al., 2008).

4.1 Cardiovascular drugs

The presence of chronic and low-grade inflammation phenotype is strongly associated with the process of aging. An *in vivo* study using a chronic inflammation mice model demonstrated accelerated aging and reduced regeneration capacity in the mice (Jurk et al., 2014). Moreover, anti-inflammation therapy such as Non-Steroid Anti-Inflammatory Drugs (NSAID) prevents the senescence phenotype. Additionally, Cyclooxygenase 2 (COX-2) and Prostaglandin E2 (PGE2) were identified to be involved in

inflammation-mediated senescence (Martien et al., 2013). These data suggested the potential role of aspirin in delaying the aging process.

This systematic review summarized that aspirin was well conserved as a lifespan-extending compound in *C. elegans*, *drosophila*, and mice (Ayyadevar et al., 2013; Danilov et al., 2015; Strong et al., 2008; Wan et al., 2013). Only one study in *C. elegans* showed the non-beneficial effects of aspirin for extending the lifespan. The failure might probably be due to the use of glp-1 mutant *C. elegans* as the worm model in this study (Huang et al., 2013). It is thought that GLP-1, a master regulator of germline development and longevity, is essential for the effects of aspirin on metabolism and lifespan extension in *C. elegans*. Therefore, the disruption of GLP-1 function will highly affect the effect of aspirin (Kenyon, 2010). In *C. elegans* studies, lifespan extending effect of aspirin can be explained by the activation of ampk and DAF-16/FOXO signaling pathway and oxidant stress prevention (Ayyadevar et al., 2013; Wan et al., 2013). Moreover, aspirin could downregulate Pkh2-ypk1-lem3-tat2 pathway in *drosophila* and act as an anti-inflammation compound in mice (Strong et al., 2008; Danilov et al., 2015). However, in healthy older adult population, aspirin failed to minimize mortality and morbidity and might have raised the risk of bleeding in such individuals (McNeil et al., 2018b; 2018a). This evidence showed the translational challenge of the use of aspirin in aging humans.

Studies on ACE-I have yielded conflicting results regarding its ability to extend lifespan, whereas all studies on ARB have shown no significant impact on prolonging lifespan. Candesartan failed to prolong the lifespan in UM-HET3 mice (Harrison et al., 2021). A study showed captopril extended lifespan in the dose at 2.5 mM (preferable dose) and 3.2 mM in *C. elegans* (Kumar et al., 2016). Another study demonstrated that captopril has a lifespan-extending effect at doses of 1.6, 2.5 (preferred dose), and 3.8 mM. However, this study revealed the toxicity of captopril in a dose of 7.6 mM. It might be because the drug dose in *C. elegans* should be lower than 3.8 mM (Egan et al., 2023). In the rodent models, captopril extended lifespan in female UM-HET3 mice (Strong et al., 2022). However, ramipril failed to extend the lifespan but was able to extend the lifespan when combined with simvastatin in C3B6F1 mice (Spindler et al., 2016). A detailed explanation will be given in the next section. Meanwhile, enalapril increased lifespan in Wistar rats by reducing leptin levels and ACE activity and enhancing the genes that involved lipid storage and antioxidant properties (Santos et al., 2009). A detailed explanation of these discrepancies in results was not shown in those studies but the different use of model organisms might explain the rationale explanation of these data.

The diuretic drugs, such as metolazone and acetazolamide extended the lifespan of *C. elegans* and Klotho hypomorphic (kl/kl) mice, respectively. Metolazone upregulates mitochondrial chaperone and activates mitochondrial unfolded protein response (UPRmt) to extend lifespan in worms, while acetazolamide inhibits osteoinductive signaling, ameliorates calcification markers, and reduces aldosterone and ADH levels in kl/kl mice (Ito et al., 2021; Leibrok al., 2015). Even though the lifespan was greatly increased by nearly 201% after acetazolamide treatment in kl/kl mice, the clinical translation into humans of this drug remains challenging since acetazolamide is widely applied as an eye drop for glaucoma treatment (Lusthaus and Goldberg, 2019). Hydralazine extended the lifespan in *C. elegans* in the optimal dose of 100 μM by activating SIRT1/SIR-2.1 and NRF2/SKN-1 signaling pathway, thus maintaining the mitochondrial function (Dehghan et al., 2017; 2019).

Anti-hypertensive medicines, such as beta-blockers (metoprolol and nebivolol) and verapamil were analyzed in this study. Sympathetic overdrive and overactivity in beta adrenergic receptors were found in aging organisms and led to age-associated cardiac failure (Lakatta, 1993; Swynghedauw et al., 1995). Metoprolol and nebivolol extended lifespan in both *drosophila* and mice by decreasing G proteins stimulation and reducing PKA activity in the heart after beta adrenergic receptor blockade. These drugs may also reduce tumor mass in mice (Spindler et al., 2013). Verapamil at the dose of 100 and 400 μM also increased the lifespan in *C. elegans* by reducing the calcineurin gene and enhancing LGG-1/LC3 expression level as the autophagy genes (Liu W et al., 2020).

4.2 Dyslipidemia drugs

The lifespan-extending effect has been identified in statin. Simvastatin increased the lifespan in *drosophila*, with the most significant impact observed at a dose of 0.24 mM, while lower or higher doses did not show the same effect (Spindler et al., 2012). This study found that simvastatin decreased Ras protein isoprenylation and reduced growth factor receptor signaling pathways to prolong the lifespan in *drosophila*. Another study also demonstrated that lovastatin extended the lifespan of *C. elegans* in a dose-dependent manner by preventing the accumulation of aging pigment and inhibiting the Jun N-terminal Kinase (JNK-1) pathway (Andreas et al., 2020). Interestingly, the beneficial effect of simvastatin alone could not be translated into mice. However, when combined with ramipril, the two drugs were able to prolong the lifespan by inhibiting AT1R signaling-mediated NAD(P)H oxidase inactivation, thus decreasing oxidative stress following ramipril administration (Spindler et al., 2016). Concerns were raised about hypertriglyceridemia and hyperglycemia when combining simvastatin and ramipril due to potential unexpected effects similar to those seen with a 40% calorie restriction diet and rapamycin administration (Spindler et al., 2016).

Niacin extended the lifespan in *C. elegans* only under high dose (Yang et al., 2019) and in Zucker Fatty rats (Preuss et al., 2011). In the worms study, it is suggested that niacin may raise intracellular nicotinamide adenine dinucleotide (NAD+) levels and maintain sirtuin-saturating concentrations to prolong lifespan (Yang et al., 2019). Moreover, fibrate treatment, specifically fenofibrate, extends lifespan in a dose-dependent manner by activating NHR-49 (an orthologue of Peroxisome Proliferator-Activated Receptor Alpha (PPAR-α) in mammals) to induce mitohormesis in *C. elegans* (Yang et al., 2019). We identified the conflicting data of lifespan-extending effect of omega-3 in *drosophila* and mice. Omega-3 may increase lifespan in *drosophila* males by increasing antioxidant enzymes and maintaining mitochondrial metabolism (Champigny et al., 2018). In contrast, this compound has rather shortened the lifespan in mice. The mechanism is not fully understood but it is speculated that the anticoagulant effects of omega-3 may

induce bleeding risk and omega-3 can suppress $CD8^+$ activation, thus inducing tumor progression in mice (Spindler et al., 2014; Champigny et al., 2018).

4.3 Anti-diabetic drugs

Metformin has been extensively studied in aging, including in lifespan studies. A total 15 metformin lifespan studies have been summarized in this review, including eight worm studies, two *drosophila* studies, four mice studies, and one rat study (Onken et al., 2022). Interestingly, the lifespan-extending effect of metformin is diverse among species. In worms, the beneficial effect of metformin was found when given at 0 days at the L4 larvae stage even though the therapeutic dose varied among studies (Onken et al., 2010; Cabreiro et al., 2013; Chen et al., 2017; Xiao et al., 2022; Cedillo et al., 2023). Metformin increased lifespan when administered at doses of 10, 25, and 50 mM starting at the L4 larvae stage from day 1, and at a dose of 50 mM from day 4. Moreover, the shortening of lifespan was detected when the metformin started at 10 days at the L4 larvae stage. This is probably due to the mitochondrial dysfunction caused by metformin toxicity at this stage (Espada et al., 2020). Another peculiar finding investigated by De Haes et al., 2014 that this study showed the lifespan-extending effect could happen when treating *C. elegans* with metformin 50 mM at the L1 stage and the adult phase only (De Haes et al., 2014). On the other hand, the advantageous effects of metformin on lifespan was found limited to *C. elegans* but not *C. briggsae* or *C. tropicalis* suggesting that metformin works in the specific target organism (Onken et al., 2022).

In contrast with the metformin effect in worms, metformin rather reduced lifespan in *drosophila* (Slack et al., 2012; Abrat et al., 2018). Metformin at the dose of 100 mM in male and more than 25 mM in females may induce the shortening of lifespan in *drosophila*. These phenomena could be caused by the starvation-like phenotype and intestinal fluid imbalance due to overactivity AMPK signaling induced by metformin intoxication (Slack et al., 2012). Similarly, another study also found this unexpected phenotype although it speculated that the starch diet used in this study might disrupt metabolic homeostasis in *drosophila* (Abrat et al., 2018). Altogether, these data showed the unexpected effects of metformin in this organism.

Metformin has no beneficial effect on lifespan in F344 male rats and rather decreases the body weight of this rat (Smith et al., 2010). Moreover, the gender-specific lifespan effect of metformin has been observed in mouse models. Metformin extended lifespan in male 129/SV (100 mg/kgBW/day) and C57BL/6 mice (0.1% w/w). Renal toxicity occurred in C57BL/6 mice when treated with the dose at 1% w/w (Montalvo et al., 2013; Anisimov et al., 2015). Moreover, metformin decreased lifespan at 129/SV and C57BL/6 female mice, even when administered at the similar dose in the male study (100 mg/kgBW/day) (Montalvo et al., 2013; Zhu et al., 2021). The administration of metformin in female mice may result in an elevated level of cardiac stress indices, such as Myh7/Myh6, Nppa, and Nppb, which can account this event (Zhu et al., 2021). Another fact revealed that a combination of metformin 1,000 ppm and rapamycin 14 ppm prolonged lifespan in both male and female UM-HET3 mice even though the metformin 1,000 ppm was not sufficient to promote this phenotype. As mentioned previously, this mechanism can be interpreted by the hypothesis that metformin improves glucose homeostasis by enhancing the insulin sensitivity that is perturbated by rapamycin (Strong et al., 2016).

Among all anti-diabetic drugs, acarbose has been identified as the most consistent compound for extending lifespan in rodents, but it failed to prolong the lifespan of various types of worms (Banse et al., 2023). Studies showed that acarbose at a dosage of 1,000 ppm had positive effects on C3D2F1/J or CByB6F1/J mice when treated from 8 months old, and on UM-HET3 mice when treated from 4, 8, 9 months old, respectively (Harrison et al., 2014; 2019; Strong et al., 2016; Smith et al., 2019). This lifespan-extending effect might be due to the change in microbiome composition and fecal Short-Chain Fatty Acids production. The increase of FGF21 and reduction of IGF-1 plasma levels may also be involved as the molecular mechanism of lifespan-extension phenotype in acarbose (Harrison et al., 2014; 2019; Smith et al., 2019). When started at 16 months old, acarbose at the dose of 1,000 ppm extended its lifespan in male mice only. However, when combined with rapamycin at the dose of 14.7 ppm, acarbose was able to extend the lifespan of both genders, even when the initial treatment started at 16 months old (Strong et al., 2022). This synergistic effect might be explained by the insulin-sensitizing effect of acarbose could neutralize the hyperglycemia condition caused by rapamycin. These studies collectively suggest the potential role of translating acarbose for extending lifespan in aging humans.

Thiazolidinediones (TZD), such as pioglitazone and rosiglitazone, have been reviewed in this study. Pioglitazone extended lifespan at concentrations of 0.1 and 0.5 mM but failed when given less than 0.1 or at 2 mM in *C. elegans* (Jia et al., 2022; Onken et al., 2022). This effect is due to the activation of DAF-16/FOXO and SKN-1/NRF2 Signaling Pathways while inhibiting insulin/insulin-like signaling (IIS) and reproductive signaling pathways, as well as the activation of dietary restriction-related pathway (Jia et al., 2022). Unfortunately, no further studies in larger organisms than worms have been identified in TZD. Similar to TZD, the lifespan study of sulphonylureas (chlorpropamide, glibenclamide, glimepiride, and glipizide) has been limited to *C. elegans* only (Mao et al., 2022; Onken et al., 2022). Our systematic review showed that all sulphonylureas, except for glipizide, increased lifespan at different doses. The increases of the mitochondrial electrical potential and SDH activity in Complex II, and mitochondrial reactive oxygen species (mtROS) play the molecular mechanism of this lifespan-extending effect (Mao et al., 2022). In addition, nateglinide was unlikely to shorten the lifespan in *C. elegans* and *C. tropicalis* although the mechanism remains unknown (Onken et al., 2022).

Sodium-glucose transport Protein 2 (SGLT2) Inhibitors showed different results between worm and mice studies (Miller et al., 2020; Onken et al., 2022). When administered at a maximum dose of 100 μM, dapagliflozin did not increase the lifespan of various types of worms (Onken et al., 2022). However, when given at a dose of 180 ppm to UM-HET3 male mice starting at 7 months old, canagliflozin extended their lifespan, but not that of the female mice. The valid mechanism has not been established yet, but it speculated that canagliflozin enhances fatty acids and ketones

metabolism, suppresses the TORC1 signaling pathway, and increases AMPK activity in liver tissue (Miller et al., 2020). The effect of lifespan extension of Dipeptidyl Peptidase 4 (DPP 4) Inhibitors such as sitagliptin and linagliptin have been investigated in various types of worms and Klotho$^{-/-}$ mice, respectively (Hasegawa et al., 2017; Onken et al., 2022). The analysis of sitagliptin at a maximal dose of 100 μM in worms found that lifespan extension phenotype has only happened in *C. elegans* and *C. tropicalis*, but not in *C. briggsae* (Onken et al., 2022). On the other hand, linagliptin extended lifespan in Klotho$^{-/-}$ mice model by enhancing phosphorylation activities of Akt, eNOS, and CREB in the brain. However, the treatment of linagliptin in this model induced hyperglycemia status and increased body weight (Hasegawa et al., 2017). Consistent with our findings, another review of the potential of gerotherapeutic drugs revealed that SGLT-2 exhibits superior efficacy in extending preclinical lifespan compared to metformin (Kulkarni et al., 2022).

4.4 Lesson from animal aging models

According to our findings, five distinct species were utilized in the aging drug repositioning study. Worms, flies, mice, and rats are the most frequently utilized in aging trials, respectively. These species are utilized on account of their properties in easy handling, short generation times, availability of standardized strain, and high quality in genomic and transcriptomic sequencing data (de Magalhães, 2021; Holtze et al., 2021).

Roundworms (*C. elegans*) are predominantly employed in aging trials because of their simple cultivation and brief life cycle and life span (two to 3 weeks). Furthermore, 50% of *C. elegans* genes are present in the human genome (Taormina et al., 2019). *D. melanogaster*, a higher animal frequently used in lifespan studies, possesses four pairs of chromosomes and functional orthologues for sixty percent of the genes implicated in human diseases. This characteristic renders the fruit fly a more suitable subject for lifespan studies (Taormina et al., 2019). Additionally, our research uncovered one article that utilized silkworm (*Bombyx mori*) as an animal model (Song et al., 2019). An additional noteworthy characteristic of this model is its profusion of three to six larval instars, in contrast to three larval instars in *D. melanogaster*. This increased the plasticity of lifespan extension (Song et al., 2017).

Mice contain almost 99% human orthologue genes, making them one of the most appropriate for human models. However, their studies are more complex and challenging because they are higher animals. Additionally, higher animals possess advantageous system organs, including but not limited to the musculoskeletal apparatus, endocrine system, and immune system, which can be modified to target drugs of action selectively (Taormina et al., 2019). Rats, similar to mice, are a fascinating species to investigate in the context of lifespan. Rats are more prone to developing cardiovascular and renal diseases, rendering them more disease-prone in comparison to mice. However, rats have a lower cancer incidence (74%–88% compared with 83%–95% in mice). These characteristics indicate that rats have a narrower margin for the prevalence of cardiovascular, cancer, and renal diseases in humans (Carter et al., 2020). However, in metabolic-focused research, such as insulin resistance, mice are preferable to rats due to their extensive use and the well-established development of transgenic mice for insulin resistance (Berglund et al., 2008). Thus, research on the aging of mice and rats should be considered more representative of the human condition.

Interpretation bias can arise from species variation caused by specific characteristics of the species being studied, leading to inaccurate generalizations (Holtze et al., 2021). For instance, sirtuin extends the lifespan of yeast through Sir2-mediated mechanisms (Kaeberlein et al., 1999), but this effects not well replicated in higher animal models (Park et al., 2013). Our study further supports a distinct attribute of species by revealing that metformin can prolong the lifespan of *C. elegans* while diminishing it in *C. tropicalis* (Park et al., 2013). Hypothetically, this distinction occurred due to the distinction between the epithelial boundary and the *Caenorhabditis cuticle*, which distinguishes the ability of metformin to penetrate *Caenorhabditis* cells (Holden-Dye and Walker, 2014; Onken et al., 2022).

Utilizing exclusively normal strains of animals may occasionally give rise to an additional issue. Normal animal strains typically result in restricted genetic diversity, which may not consistently apply to clinical applications in the considerably more heterogeneous human population (de Magalhães, 2014). As an illustration, C57BL/6 mice, which have been utilized in 70% of published animal studies, exhibit a higher incidence of lymphoma and increased vulnerability to metabolic dysregulation (Ward, 2006; Mitchell et al., 2015). Information derived from a solitary inbred strain might lack generalizability to the entire species. Moreover, the genetic uniformity that ensues from the breeding of strains is not indicative of the human population (Mitchell et al., 2015). As a result, genetically modified animals can occasionally serve to advance our understanding of genetic diversity.

Our systematic review also incorporates genetically modified mice that demonstrate premature aging; however, we do not incorporate animals with disease models. Several Klotho mouse studies were included in this systematic review (Leibrock et al., 2016; Hasegawa et al., 2017). Over 2 decades ago, Klotho was implemented as a gene modification in an aging model. The phenotype of these mice klotho modified includes frailty, vascular calcification, cardiovascular disease, and multiple organ degeneration (Kuro-o et al., 1997). Furthermore, recent studies have demonstrated that klotho serum levels play a role in the aging process and physical function of humans (Arroyo et al., 2023). It is noteworthy that human klotho serum levels exhibited a U-shaped curve. In participants with low Klotho serum, the phenotypic age acceleration decreased significantly with increasing serum Klotho, whereas it increased in participants with high Klotho serum (Li et al., 2023). However, the mechanism of this phenomenon is still unclear.

Gender differences in the lifespan-extending effect likely happened in some of the studies. Most drugs have been tested and proven effective in male mice but not in female mice. For instance, anti-diabetic drugs are more effective in male than female mice. The mechanism behind this phenomenon is not completely understood, but research suggests that certain drugs may interact with sex hormones and impact the reproductive organs of a particular gender (Garratt, 2020). Another concern that needs to be addressed is determining the optimal timing for administering longevity compounds to animal models. Additional research is

needed to clarify the gender- and time-specific impacts of gerotherapeutics.

Selecting the correct therapeutic dosage is crucial to avoid a false negative outcome or unforeseen intoxication. It is advisable to utilize the dosage specified in previous literature or to modify the therapeutic dosage based on a human study. Pharmacokinetic variations among organisms should be taken into account to establish the correct dosage, especially for long-term use in lifespan studies (Spindler, 2012).

Various side effects, including severe ones, have been identified in this study, such as the bleeding risk and malignancy phenotype that can be found in omega-3 and the risk of renal failure or mitochondrial dysfunction that might occur in metformin treatment (Montalvo et al., 2013; Spindler et al., 2014; Espada et al., 2020; Zhu et al., 2021). The interplay between drugs and lifestyle variables is complex and needs careful deliberation. Cardiometabolic and antidiabetic medications offer significant benefits in clinical settings. Their integration with exercise and dietary therapies may yield diverse results. Although statin is generally beneficial for reducing cholesterol levels and minimizing the risk of cardiovascular events, this medicine has been connected with muscle-related adverse effects, such as myalgia, which can hinder physical performance during exercise (Parker et al., 2013). Of note, statin might potentially diminish the beneficial impacts of exercise on muscle adaptability and mitochondrial function. While Beta-blocker provides cardiovascular protection by reducing heart rate, their effect on exercise tolerance and performance is frequently detrimental, which may discourage physical activity in patients. Metformin, an important therapy for type 2 diabetes, has undergone substantial research to explore its potential as an anti-aging medication. However, metformin may potentially impede the beneficial effect of aerobic exercise on cardiorespiratory fitness and insulin sensitivity by reducing the mitochondrial adaptations to exercise (Konopka et al., 2019). Additionally, diets rich in fiber may hinder the absorption of certain medicines, including statin and beta blocker, thus decreasing their effectiveness as well (Jenkins et al., 2000).

4.5 Future direction in repurposing cardiometabolic drugs for aging

Our systematic review of animal study results indicates that several drugs have the potential to enhance lifespan. However, as this is solely an animal study, its impact may vary in human studies. Moreover, certain animals can exhibit a better representation of human characteristics compared to other animals. Overall, rats and mice exhibit a stronger weight of evidence compared to *Drosophila*, whereas *C. elegans* demonstrates the lowest weight of evidence based on gene orthologue (Holtze et al., 2021). Hence, it is important to carry out human clinical trials on this subject. Further discussion will focus on the latest developments in human clinical trials for cardiometabolic drugs that are related to improving both healthspan and lifespan.

The anti-aging effects of metformin have been the subject of extensive animal and human testing as part of the TAME (Targeting Aging with Metformin) initiative (Barzilai et al., 2016). Three thousand nondiabetic adults aged 65 to 80 will participate in the TAME clinical trial, a 6-year double-blind, randomized, placebo-controlled study. Metformin slow-release 1,500 mg will be administered (AFAR, 2023). IL-6, TNFα-receptor I or II, CRP, GDF15, insulin, IGF1, cystatin C, NT-proBNP, and hemoglobin A1c biomarkers will be utilized in this investigation, as they have been demonstrated to be the most accurate predictors of numerous biological aging processes (Justice et al., 2018). Hopefully, these biomarkers can also be implemented in future human aging research.

The Antedecendent Metabolic Health and Metformin (ANTHEM) Aging Study is an additional noteworthy clinical trial on metformin and aging. Hundreds of participants in a shorter period will be enrolled so that results can be anticipated before TAME. It will assess changes in insulin sensitivity and mitochondrial transport system by skeletal muscle biopsy (Kumari et al., 2022). We also noted the finished Metformin in Longevity Study (MILES), which demonstrated that after 6 weeks of administration to older adults, metformin regulated numerous metabolic and nonmetabolic pathways in skeletal muscle and subcutaneous adipose tissue. Metformin exerted its effects not solely on metabolic genes and pathways but also on DNA repair genes in muscle and mitochondrial genes in adipose tissue (Kulkarni et al., 2018).

However, our systematic review of animal trials indicates that metformin does not consistently extend life expectancy. This is the first systematic review to examine animals without disease induction. Other meta-analyses conducted on animal trials indicate that the efficacy of metformin is limited to *C. elegans* when administered early in life, while its effects vary among the other model organisms (Parish and Swindell, 2022). This raises further inquiries, such as whether the TAME and ANTHEM study will yield favorable outcomes considering its heterogeneous impact on the other animal species.

We also notice an ongoing clinical trial on metformin in patients with HIV (EUCTR 2021-003299-15-ES). An individual afflicted with HIV is subjected to a multitude of stressors, including the virus, antiretroviral medications, and substances misused, all of which have the potential to trigger premature cellular senescence (Cohen and Torres, 2017). Therefore, HIV-positive individuals are incorporated into this clinical trial registry review as they exhibit early cellular aging. This study employs the potentially effective Epigenetic Age Acceleration (EAA) for its primary outcome, which is intended to predict lifespan (Joyce et al., 2021).

Three metformin registries appear incomplete for various reasons, including participant assignment difficulties. Additionally, we identified studies with dropout rates exceeding 20%, which diminished the conclusions' reliability. Hopefully, future clinical trials involving older individuals will incorporate improved recruitment and retention strategies and more efficient planning and execution. One of the solutions is illustrated in the cited source (Chaudhari et al., 2020).

Besides metformin, omega-3 fatty acids have been shown to reduce telomere attrition via antioxidant effect, decreased proinflammatory markers, and direct action on telomeres based on *in vitro* and *in vivo* studies; these effects have promising implications for health and longevity (Ogłuszka et al., 2022). In addition, levels of long-chain omega-3 fatty acids were found to be inversely associated with mortality in the Framingham Heart Study (Harris et al., 2018).

Results from three studies concerning omega-3 in healthy subjects have been published. Although omega-3 fatty acids have no discernible

impact on immunosenescence pathway (Swanson et al., 2018), they have been found to influence cognitive function in healthy humans positively (Külzow et al., 2016; Fairbairn et al., 2020). While the precise mechanism by which omega-3 fatty acids influence cognitive function remains unknown, they do regulate the expression of genes encoding enzymes involved in homocysteine metabolism, amino acids that correlate to neuronal senescence (Huang et al., 2012; Tawfik et al., 2021).

Omega-3 dose is also a challenge in the aging trial. No fixed EPA and DHA dose combination proves to increase lifespan. A systematic review also showed that no fixed dose has been established in the cognitive area. It only shows that significantly altered neurophysiological function or brain morphology can be achieved with prolonged omega-3 administration (Dighriri et al., 2022). One study also uses the combination of folic acid, phosphatidylserine, and *Gingko biloba*, thereby augmenting the treatment effect bias (Külzow et al., 2016). In conclusion, omega-3 fatty acids may extend the cognitive healthspan of healthy individuals. However, additional research employing rigorous methodologies is required to determine whether it extends lifespan.

Two registries (NCT02865499 and NCT02953093) are evaluating the fecal microbiome of healthy older individuals after acarbose treatment (8 and 10 weeks). One of these registries also analyses gene expression in abdominal adipose tissue and muscle tissue. None of the results has been published yet. Animal studies indicate that acarbose is hypothesized to extend lifespan through modifications to the gut microbiome and an increase in short-chain fatty acids (SCFAs), including propionate (Smith et al., 2019). As a result, the gut microbiome serves as a reliable biomarker for the effect of acarbose on longevity. Nevertheless, it is desirable that future research incorporates larger sample sizes and more prolonged acarbose administration periods to interpret the effect size more accurately.

Statins for Extension of Disability-Free Survival and Primary Prevention of Cardiovascular Events Among Older People (STAREE) is also an intriguing study in the aging field. This investigation comprises the STAREE-HEART and STAREE-MIND substudies (Harding et al., 2023; Zoungas et al., 2023). STAREE-HEART will examine the incidence of atrial fibrillation and global longitudinal strain (GLS) in healthy older adults taking 20 mg of atorvastatin daily over a 3-year follow-up period. Following a 4-year follow-up period, STAREE-MIND will assess brain aging parameters and investigate the correlation between changes in brain imaging and cognitive impairment. Hopefully, the findings of this research will shed light on the impact of atorvastatin on healthy subjects' cardiovascular and neurological aging.

Several included articles incorporated cardiometabolic drugs that were discontinued in human application due to safety concerns (Brandstädt et al., 2013; Cabreiro et al., 2013; Mao et al., 2022; Cedillo et al., 2023). These are bezafibrate (hepatotoxicity), tolbutamide (cardiovascular mortality), chlorpropamide (hypoglycemia), and phenformin (lactic acidosis). Animal lifespan research aims to identify the most effective medication for extending human health and lifespan. So, we encourage further lifespan research to avoid the use of discontinued drugs, particularly when safety concerns arise.

4.6 Limitations

Our study has several limitations. First, our search was limited to drugs that extend lifespan, not healthspan. As a result, articles that do not provide follow-up of the animal until death were excluded. Second, we excluded animals with disease models, as they are inappropriate for our PICO. These two limitations may result in less comprehensive cardiometabolic drug mechanisms in aging. Third, our search criteria exclusively included drugs that have received approval from the FDA, which has been established for their efficacy and safety in treating cardiometabolic disease. Hopefully, it can also be safe as future potential human lifespan-extending drugs. Fourth, most of the medications in rodents studies were given orally. However, the majority of these studies did not explicitly mention whether the medications were administered orally or mixed with chow. Additionally, drug concentrations for *C. elegans* and *Drosophila* studies are commonly used in the millimolar (mM) range format. This approach is in agreement with the established protocols in aging research when evaluating pharmacological interventions in these model organisms. Lastly, regarding clinical trials, our search criteria in ICTRP are limited to the keyword "aging". Some clinical trial registries may repurpose cardiometabolic drugs for degenerative conditions but not use aging as their keyword. It is possible that such research may not be included in this systematic review.

5 Conclusion

Metformin, omega-3 fatty acid, acarbose, and atorvastatin are currently cardiometabolic drugs repurposed to target aging in clinical trials. Our systematic review of animal trials identified several additional cardiometabolic drugs that could potentially extend life expectancy. We strongly advise other researchers to initiate clinical trials of these drugs in the context of aging, given the significant concern that this will become in the coming years. Additional animal experiments utilizing wild-strain animals to evaluate the effects of gerotherapeutics are also recommended.

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 authors.

Author contributions

AB: Conceptualization, Writing–original draft, Writing–review and editing, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization. HH: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. ML: Methodology, Supervision, Validation, Writing–review and editing. NK: Data curation, Investigation, Writing–review and editing. RM: Data curation, Investigation, Writing–review and editing. IF: Data curation, Investigation, Writing–review and editing. MF: Data curation, Investigation, Writing–review and editing. AJ: Data curation, Investigation, Writing–review and editing.

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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.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1373458/full#supplementary-material

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Drug repurposing to tackle parainfluenza 3 based on multi-similarities and network proximity analysis

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Given that there is currently no clinically approved drug or vaccine for parainfluenza 3 (PIV3), we applied a drug repurposing method based on disease similarity and chemical similarity to screen 2,585 clinically approved chemical drugs using PIV3 potential drugs BCX-2798 and zanamivir as our controls. Twelve candidate drugs were obtained after being screened with good disease similarity and chemical similarity ($S > 0.50$, $T > 0.56$). When docking them with the PIV3 target protein, hemagglutinin-neuraminidase (HN), only oseltamivir was docked with a better score than BCX-2798, which indicates that oseltamivir has an inhibitory effect on PIV3. After the distance (Z_{dc}) between the drug target of 14 drugs and the PIV3 disease target was measured by the network proximity method based on the PIV3 disease module, it was found that the Z_{dc} values of amikacin, oseltamivir, ribavirin, and streptomycin were less than those of the control. Thus, oseltamivir is the best potential drug because it met all the above screening requirements. Additionally, to explore whether oseltamivir binds to HN stably, molecular dynamics simulation of the binding of oseltamivir to HN was carried out, and the results showed that the *RMSD* value of the complex tended to be stable within 100 ns, and the binding free energy of the complex was low (–10.60 kcal/mol). It was proved that oseltamivir screened by our drug repurposing method had the potential feasibility of treating PIV3.

KEYWORDS

parainfluenza 3, drug repurposing, chemical similarity, disease similarity, network proximity

Introduction

There is no specific antiviral treatment for parainfluenza (PIV) illness. Most people with PIV illness will recover on their own. However, PIVs can also cause more serious illnesses in children and adults older than 65 years, including bronchitis, bronchiolitis, and pneumonia (Moscona, 2005; Schmidt et al., 2011). Among PIVs, parainfluenza 3 (PIV3) is the most prevalent subtype, and it is not only a most common cause of acute respiratory infections in infants, but it also causes severe respiratory symptoms in older adults,

immunocompromised patients, and transplant recipients (Branche and Falsey, 2016). Despite causing serious health problems, there is currently no clinically approved drug or vaccine for PIV3 (Contreras et al., 2021; Rafeek et al., 2021). PIV3 infects its target cells through the coordinated action of the hemagglutinin-neuraminidase receptor-binding protein (HN) and the fusion envelope glycoprotein, which together comprise the molecular fusion machinery (Palmer et al., 2014; Van Den Bergh et al., 2022). Peptide-fusion protein inhibitors that target the fusion envelope glycoprotein are challenging to utilize in clinical settings because of limitations such as high manufacturing costs, low oral bioavailability, and severe injection site reactions caused by the immunogenicity of virus-derived peptides (Aggarwal and Plemper, 2020). Current preclinical development and clinical trials for treating PIV3 primarily focus on inhibitors that target the HN protein (Chibanga et al., 2019; Rota et al., 2023).

At present, the common PIV3 drug research and development is carried out on HN inhibitors using virtual screening by molecular docking. Molecular docking is one of the most commonly used strategies in structure-based drug design (Parihar et al., 2022; Tayubi and Madar, 2023) and has been widely employed in the development of anti-PIV3 drugs (Indumathi et al., 2019; Bhasin et al., 2022). Zanamivir successfully docked with HN, and it has been proven to have anti-PIV3 effects *in vitro*, but is not suitable for clinical use due to its relatively high IC50 (Bailly et al., 2016). In the current clinical studies of PIV3, BCX-2798 has been shown to inhibit HN in mouse models, and this research has only recently progressed to the clinical stage. Further trials are necessary before it can be practically applied (Alymova et al., 2005). However, these classical screening methods often ignore the progression of the disease, and drug safety also needs to be fully assessed.

Recently, drug repurposing strategies, with the advantage of drug safety, have become strong approaches to the research and development of antiviral drugs (Kumar et al., 2021; Sonkar et al., 2021). The disease similarity and chemical similarity were calculated to uncover associations between diseases and drugs for use in drug repurposing (Li et al., 2018; Yin et al., 2023; Pushpakom et al., 2019). Interestingly, zanamivir and BCX-2798 are both derived from Neu5Ac2en, the neuraminidase (NA) inhibitor (Alymova et al., 2004). Their ability to inhibit PIV3 suggests that structurally similar compounds often share similar physicochemical properties and biological activities (Durai et al., 2020). Apart from structure-based virtual screening (Khan et al., 2023), the network-based drug-disease proximity method that uncovers the relationship between drug targets and disease modules is widely used (Yildirim et al., 2007; Zhou et al., 2020), and it is utilized not only for predicting the potential side effects but also for repurposing approved drugs for new indications (Guney et al., 2016; Wang et al., 2021; Xi et al., 2023).

Therefore, this research focuses on the following: 1) multi-similarity methods to understand the intrinsic link between diseases and drugs, 2) the structural characteristic link of potential drugs, and 3) the network proximity of disease targets and drug targets to understand drugs related to the occurrence and onset of diseases. We combined disease and chemical similarity methods to screen candidate drugs against PIV3. Molecular docking and network proximity methods were used to identify the best anti-PIV3 drug. Finally, the binding ability and stability of candidate drugs and key disease targets in a dynamic environment were evaluated using molecular dynamics simulation.

Methods

Our approach involves collecting data, calculating disease similarity to PIV3, and assessing chemical similarity to potential existing drugs for PIV3. We further screen through molecular docking and assess network proximity between the drug and PIV3. Finally, we conduct molecular dynamics simulations and calculate the binding free energy. The workflow is shown in Figure 1.

Data collection

The Medical Subject Headings (MeSH) for PIV3 were obtained from https://nlmpubs.nlm.nih.gov/projects/mesh/2022/meshtrees/ and were used to calculate the disease similarity of PIV to all other diseases in the MeSH database that contain Medical Subject Headings. We obtained the approved drugs (molecular weight <500) from the DrugBank database (https://go.drugbank.com) as candidate drugs for drug repurposing. Zanamivir and BCX-2798 (potential therapeutic drugs for PIV3) were considered two controls.

Disease targets associated with PIV3 were obtained by using "*parainfluenza*"as a search term from GeneCards (https://www.genecards.org/), OMIM (https://www.omim.org/), and DisGeNET (https://www.disgenet.org/), excluding non-coding RNA proteins. The drug targets were sourced from the DrugBank database and DGIdb (https://dgidb.org). All data were downloaded on 26 March 2024. Disease genes and drug targets were sorted out with the same format in UniProt using Python 3.9.

Multi-similarity analysis

Multi-similarity analysis, including disease therapy similarity and drug chemical similarity, was used to virtually screen all candidate drugs. The disease therapy similarity is calculated based on the directed acyclic graph (*DAG*), which is constructed using grid descriptors (Wang et al., 2007). The disease PIV3 are represented by $DAG_{(H)} = \{T_H, E_H\}$, where T_H is the set of ancestor nodes containing node PIV3 and E_H represents the set of edges from the parent node to the child node. In $DAG_{(H)}$, the contribution of a certain node N to PIV3 ($SV_H(\mathrm{n})$) is calculated as Equation 1:

$$SV_H(\mathrm{n}) = \begin{cases} 1 & if\ n = H \\ max\left\{\Delta^* SV_H(\mathrm{n}') \middle| n'echildren\ of\ n\right\} & if\ n \neq H \end{cases} \quad (1)$$

The semantic similarity of PIV3 and disease A ($S_{(H,A)}$) is calculated by Equation 2. If $S_{(H,A)}$ >0.5, it indicates that there is a good similarity between these two diseases.

$$S_{(H,A)} = \frac{\sum_{n \in N(H) \cap N(A)} (SV_H(\mathrm{n}) + SV_H(\mathrm{A}))}{SV(H) + SV(A)} \quad (2)$$

The Tanimoto coefficient (T) was calculated to assess the chemical similarity of all drugs. After the MACCS fingerprints of

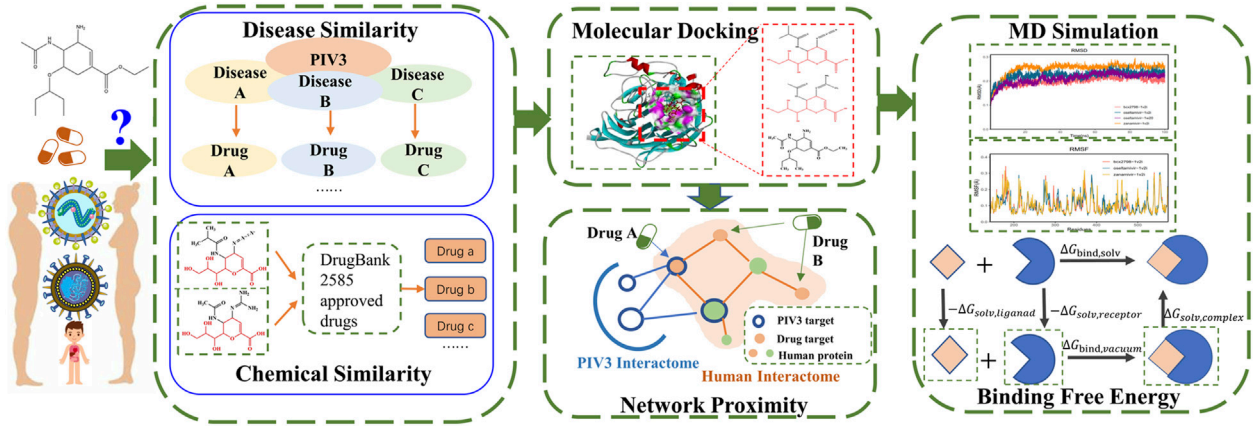

FIGURE 1
Workflow of PIV3 drug repurposing.

all drugs described by PaDEL (Yap, 2011), T is calculated using Equation 3:

$$T = \frac{c}{a + b - c} \tag{3}$$

where a and b are the numbers of MACCS fingerprint bits of drug A and B, respectively, and c is the number of fingerprint bits that two drugs have the same value on the MACCS fingerprint bits. If one drug has *bigger* T, the drug is a potential candidate.

The drugs obtained through the multi-similarity method are the initial candidates for the next round of screening.

Molecular docking

After the candidates were screened by multi-similarity analysis, we employed molecular docking to assess the docking and interaction between the screened drugs and the key target HN (PDB ID: 1V2I) (Indumathi et al., 2019). We utilized a literature-based method to identify the binding sites due to the absence of binding ligands in the original PDB structure. The key amino acids ARG192, ASP216, GLU409, ARG424, ARG502, TYR530, and GLU549 were designated as active sites (Lawrence et al., 2004; Mizuta et al., 2014; Bhasin et al., 2022). Molecular docking analyses were conducted on the selected candidates and two controls with HN using Discovery Studio 2019 (DS 2019).

Network proximity analysis

Meanwhile, the network proximity calculation will be performed to assess the shortest path lengths between the candidate drug targets and PIV3 disease module (Wang et al., 2022; Tang et al., 2023). This evaluation will determine if the candidate drugs have the potential to treat PIV3.

PIV3 disease targets were distributed throughout the protein–protein-interaction (PPI) network (Menche et al., 2015; Dai et al., 2020), forming the PIV3 disease module and constructing the largest connected component (LCC) within the PIV3 disease module. The shortest path length (d_c, Equation 4) of the PIV3 disease target set (V) and drug target set (T) is as follows:

$$d_c(V,T) = \frac{1}{|T|}\sum_{t\in T} min_{v\in V}\, d(v,t) \tag{4}$$

Using the average $\mu_d(V,T)$ and standard deviation $\sigma_d(V,T)$ of the reference distribution between randomly selected protein groups, the calculated distance is converted into the average relative distance between drugs and PIV3 diseases. Equation 5 is for calculating the relative average shortest distance between V and T. If Z_{dc} <0, which means that the distance between the test drug and the PIV3 disease module is less than the value of the reference distance, then the test drug is a potential candidate for anti-PIV3 (Morselli Gysi et al., 2021).

$$Z_{dc} = \frac{dc - \mu_{dc}(V,T)}{\sigma_{dc}(V,T)} \tag{5}$$

Molecular dynamics simulation

After completing the aforementioned screening methods, we identified drugs that met all the screening requirements and conducted molecular dynamics (MD) simulations of the drugs and HN complexes using GROMACS 2022.3.

Our simulation utilized the CHARMM36 force field and TIP3P solvent (Nayar et al., 2011), with the addition of a 0.15 mol/L NaCl solution to mimic physiological conditions. The steepest descent method was chosen for energy minimization (Koulgi et al., 2021). To replicate a physiological environment, a Langevin thermostat with a pressure of 1 atm and a temperature of 310 K was applied. The particle mesh Ewald (PME) algorithm was employed for calculating long-range interactions (Darden et al., 1993; Gu et al., 2011). Moreover, the simulation employed a step size of 2 fs/step, with a total of 50,000,000 steps, resulting in a simulating duration of 100 ns. Post-simulation, an analysis was conducted on the root-mean-square deviation (*RMSD*), root-mean-square fluctuation (*RMSF*), and radius of gyration (*RG*) of the complex.

TABLE 1 Results of drug repurposing.

No.	Drug	*T* (zanamivir)	*T* (BCX-2798)	*CE* (kcal/mol)	Z_{dc}
1	Streptomycin	0.67	0.56	−19.26	−0.95
2	Acarbose	0.64	0.60	—	−0.11
3	Cytarabine	0.64	0.56	−7.59	2.40
4	Streptozocin	0.63	0.72	−19.26	−0.69
5	Amikacin	0.63	0.59	—	−2.47
6	Fosdenopterin	0.63	0.56	−22.88	1.21
7	N-acetylglucosamine	0.61	0.57	−21.62	0.80
8	Oseltamivir	0.61	0.57	−24.69	−1.45
9	Plazomicin	0.61	0.59	—	−0.27
10	Peramivir	0.61	0.50	−18.90	—
11	Telbivudine	0.60	0.56	−8.05	—
12	Riboflavin	0.59	0.56	−14.98	0.12
13	Regadenoson	0.57	0.56	10.01	1.21
14	Ribavirin	0.54	0.57	10.85	**−2.27**
[a]	Zanamivir	[a]1.00	0.77	−31.10	−0.93
[a]	BCX-2798	0.77	[a]1.00	−23.05	—

[a]Represents the control, and *CE* is CDOCKER energy calculated by DS.

Additionally, the binding free energy for each frame trajectory in the last 10 ns (90–100 ns) of the MD simulation was computed using the MMPBSA.py script and the MMGBSA.py (Valdés-Tresanco et al., 2021). The total binding energy is denoted as ΔG, and the Equations 6–8 for calculating ΔG are as follows:

$$\Delta\text{Gsol} = \Delta G_{NP} + \Delta G_{P} \quad (6)$$

$$\Delta\text{EMM} = \Delta G_{vdw} + \Delta G_{ele} + \Delta G_{int} \quad (7)$$

$$\Delta\text{G TOTAL} = \Delta\text{G sol} + \Delta\text{EMM} \quad (8)$$

where ΔGsol represents the solvation free energy and ΔEMM represents the gas phase energy. ΔEMM is also equal to the sum of van der Waals energy (ΔG_{vdw}), internal energy (ΔG_{int}), and electrostatic energy (ΔG_{ele}), whereas ΔGsol is equal to the sum of polar solvation free energy (ΔG_{P}) and non-polar solvation free energy (ΔG_{NP}).

Results

Data collection

In this research, 2,585 approved drugs were obtained from the DrugBank database (see Supplementary Table S1A) as candidates. Subsequently, the chemical similarity between the controls (zanamivir and BCX-2798) and the 2,585 candidates were calculated, respectively. The results are listed in Supplementary Table S1B, C. At the same time, 324 PIV3 targets were compiled from the GeneCards, OMIM, and DisGeNET databases (see Supplementary Table S2).

Multi-similarity analysis

First, we calculated the semantic similarity of 1,364 diseases to PIV3 (see Supplementary Table S3). Only three diseases were identified with an $S > 0.5$ to PIV3, including respiratory syncytial virus (RSV) ($S = 0.78$), Ebola virus ($S = 0.55$), and Orthomyxoviridae ($S = 0.51$). Till now, to treat these three diseases, there were only four clinically approved drugs, which are zanamivir, oseltamivir, ribavirin, and peramivir.

After the chemical similarity of 2,585 candidates to the two controls were calculated, it was found that only 12 drugs had $T > 0.50$, as shown in Table 1 and Supplementary Figure S1. It means that only 12 drugs had higher structure similarity to the controls. From Table 1, the chemical similarity between zanamivir and BCX-2798 is significant ($T = 0.77$). When we observed their molecular structures, in Supplementary Figure S1, it was noticed that they share the same pyranic acid core, polyhydroxy side chain, and amide side chain. Similar to the control, most of the screened drugs contain oxygen-containing hexatomic rings, carboxyl groups, and amide structures. These functional groups may serve as crucial structures for inhibiting the activity of PIV3 and warrant further exploration.

Therefore, because two of the four candidates belonged to the 12, there were 14 selected candidates based on disease similarity ($S_{(H,A)} > 0.5$) and chemical similarity ($T > 0.56$) (as shown in Table 1).

Drug candidates docking to HN

The 14 candidates were molecularly docked with HN (PDB ID: 1V2I), and their docking energy values are listed in Table 1. It was

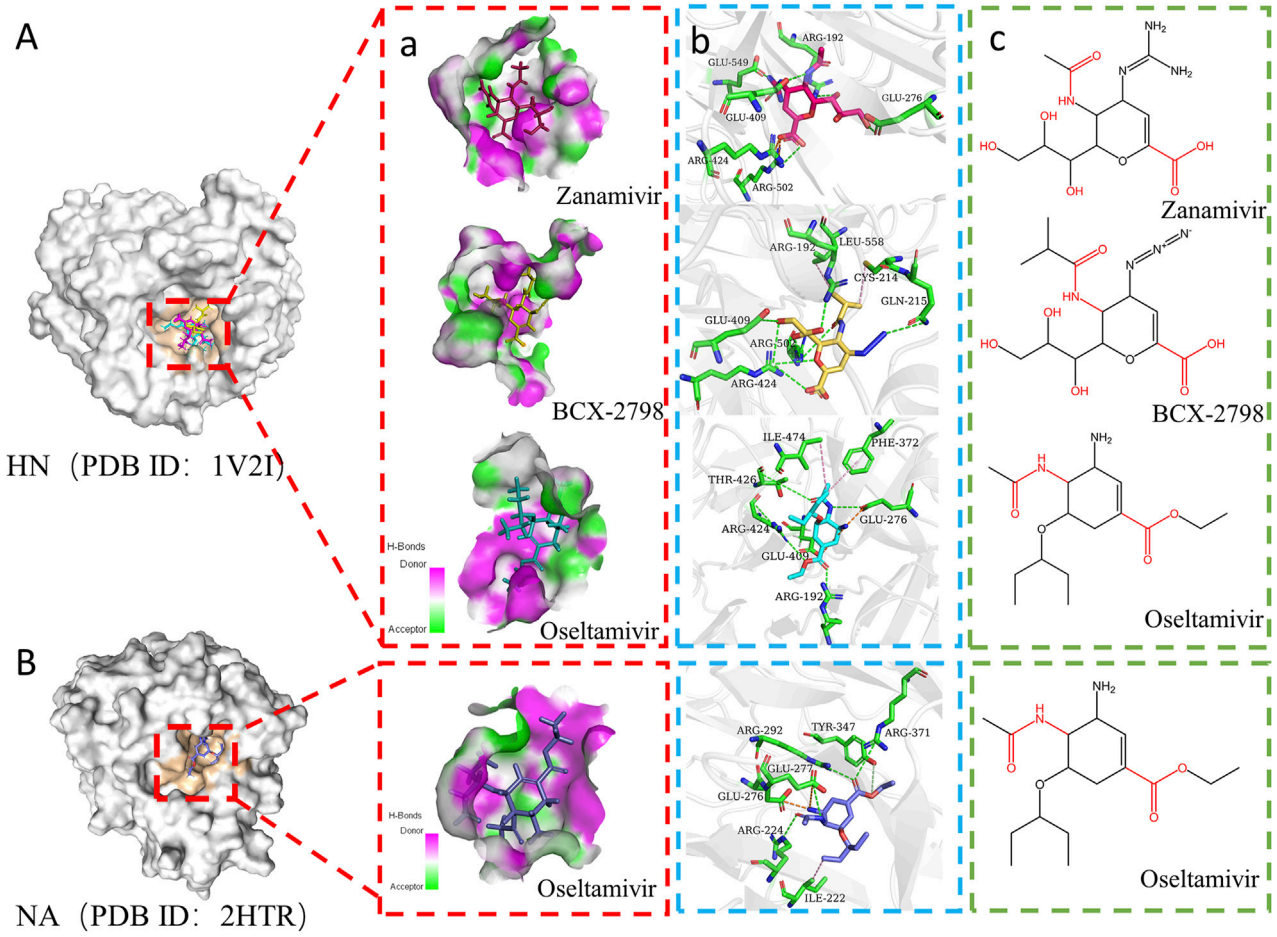

FIGURE 2
Interaction modes of the three drugs docked to different proteins. **(A)** Interaction diagram of three small molecules with HN (PDB ID:1V2I). **a)** Pocket diagram of zanamivir, BCX-2798, and oseltamivir at the same binding site. Pink areas represent amino acids in pockets as hydrogen bond donors. The green area represents the amino acid in the pocket as the hydrogen bond acceptor. The red stick is zanamivir, the yellow stick is BCX-2798, and the blue stick is oseltamivir. **b)** Interaction between three small molecules and key amino acid residues in binding pockets. The green stick is the key amino acid residue. The green dotted line represents hydrogen bonds, the orange dotted line represents electrostatic attraction, and the pink dotted line represents hydrophobic interaction. **c)** Chemical structure diagram of three molecules. **(B)** Interaction diagram of oseltamivir with NA (PDB ID:2HTR).

found that only oseltamivir (*CE* = −24.69 kal/mol) had a lower docking energy value than BCX-2798 (*CE* = −23.05 kal/mol), which indicated that oseltamivir bound to HN more stably than BCX-2798. Among all complexes, the top three drugs with stronger interactions were zanamivir, oseltamivir, and BCX-2798, so oseltamivir was the best among all the candidates. Visualization was carried out using DS 2019 and PyMol 2.5.0, as illustrated in Figure 2. We observed that all three compounds were stably bound to the pocket groove. Zanamivir was located in the shallow part of the binding pocket, revealing good flexibility and more options for binding poses. It was the same for BCX-2798. Oseltamivir occupied the deeper areas of the binding pocket, showing that oseltamivir had low flexibility, but it formed a more tightly bound complex. Oseltamivir was a good potential inhibitor of HN.

Furthermore, the intermolecular interactions of three complexes were observed and analyzed, and the contribution of key amino acid residues in binding pockets was explored Figures 2A, 3A. As shown in Figure 2A, in the three complexes, there were hydrogen bonding, electrostatic attraction, hydrophobic interaction, and some other intermolecular forces between small-molecule compounds and key amino acid residues, which promoted the stability of intermolecular binding. Most of the hydrogen bonds and electrostatic attraction in the oseltamivir–HN complex were contributed by ARG and GLU residues, similar to the two control–HN complexes. Especially, the amino of oseltamivir was bound to GLU276, amide was bound to THR426, and carboxyl groups were bound to ARG192 and ARG424 of HN. Compared with the controls, since the alkylation of oseltamivir increased the hydrophobicity of the carboxyl side chain, it can be found that there were some Pi–alkyl interactions with ILE474 and PHE372 in oseltamivir, leading to more stable binding. Therefore, it can be concluded that oseltamivir has a strong bond–bond interaction with amino acids around the HN protein-binding pocket.

Oseltamivir was a successful classical drug designed case when it was considered a neuraminidase (NA) enzyme inhibitor in influenza viruses (Davies, 2010). Then, we compared the molecular docking results of oseltamivir and NA (PDB ID: 2HTR) (Russell et al., 2006) to explore the interaction between oseltamivir and PIV3. As shown

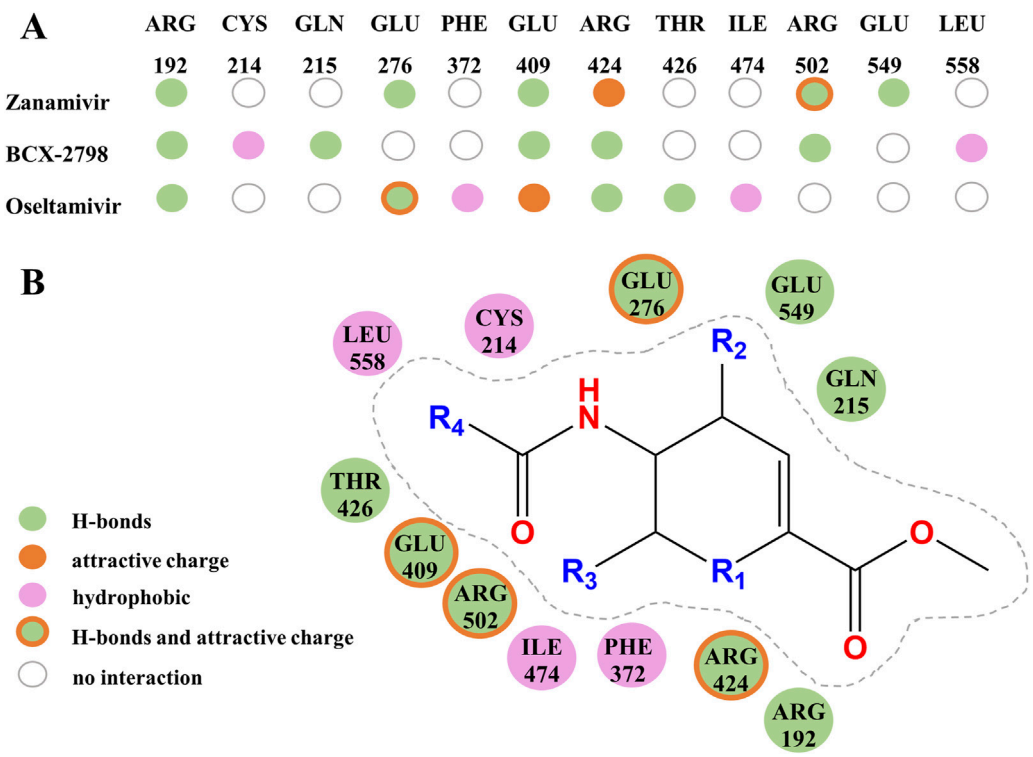

FIGURE 3
Amino residues and structural contributions of three drugs' docking with HN. **(A)** Key amino acids combine with three kinds of small molecules to produce various interactive contributions. **(B)** Contribution of important chemical structures and groups to the formation of various interactions.

in Figure 2B, the side-chain amides, carboxyl groups, and amino groups of oseltamivir played crucial roles in inhibiting HN, similar to inhibiting NA (Laborda et al., 2016). Particularly, the acetamido fragment was well-accommodated within the cavity-binding domain both in HN and NA (Guillon et al., 2014). Thus, oseltamivir has the potential to inhibit PIV3.

Based on the contribution of amino residues binding to the pocket in Figure 3A and the common parent nucleus of three compounds (zanamivir, oseltamivir, and BCX-2798), the structural formula of potential lead compounds is derived, as shown in Figure 3B. From Figure 3B, the main structural differences among the three compounds are in R1, R2, R3, and R4. Among them, R1 mainly formed hydrogen bonds through residue binding, R2 formed hydrogen bonds and electrostatic interactions, and R3 and R4 could form hydrogen bonds and hydrophobic interactions with residues, respectively. From the analysis of the binding situation of the three molecules, for example, at the R1 position, BCX-2798 and zanamivir both have oxygen substitution groups, which can form a strong binding effect, while oseltamivir does not, indicating that the contribution to the binding energy here is little. R2 and R4 exhibited good binding affinity, in which the functional groups at the R2 position of the three drugs differed but all contained amines, and all substituted functional groups were alkyl at the R4 position. It indicated that R2 and R4 both made great contributions, but there were few differences among the three drugs. However, at the R3 position, they are all oxygen-substituted long-chain fatty acid groups with good binding affinity. However, it is an alkoxy group in oseltamivir, while the others have hydroxyl groups, making it easy to form more hydrophobic interactions. This indicates that the contribution of this position is greater than that of R1 when the leading needs optimization for designing drugs. The side-chain amide and carboxyl groups of oseltamivir, along with the two controls, play a crucial role in facilitating complex binding. Various substituents (guanidine, azide, and amino) at the C4 position (R2) interact with crucial amino acids to facilitate intermolecular binding. As shown in Figure 3B, the guanidine group of zanamivir, the azide group of BCX-2798, and the amino group of oseltamivir are strongly bound to GLU549, GLN215, and GLU276 through hydrogen bonding or electrostatic interactions, respectively. In addition, as shown in Figure 2B, oseltamivir docks to neuraminidase (NA) at the amino acid residue that also binds to these key groups (amino, carboxyl, and amides). Thus, oseltamivir may be a good choice for anti-PIV3 treatment with good docking results.

Network proximity analysis of the candidates to PIV3

To further explore the potential candidates, we applied network proximity analysis to confirm it. A total of 324 disease targets were identified, with 314 targets prominently located in the PPI, to form the PIV3 disease module (Z = 18.71, P< 0.0001, randomly 1,000 times). In the PIV3 disease module, 231 targets formed the core disease module, and a total of 223 targets with Z = 6.66 and *p-value* = 3.6×10^{-10} formed the largest connected component

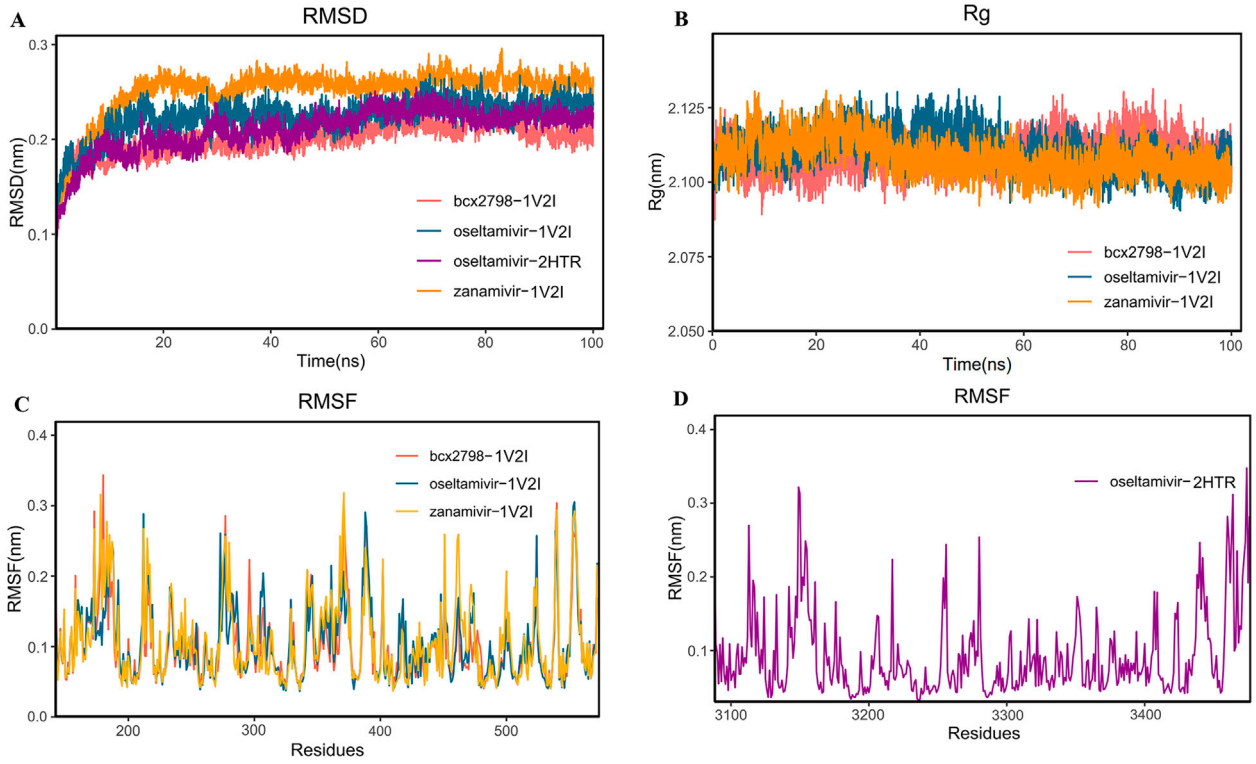

FIGURE 4
Molecular dynamics simulation analysis diagram of the complex at 100 ns. **(A)** *RMSD* analysis; **(B)** *RG* analysis; **(C, D)** *RMSF* analysis (red represents the bcx-2798–1V2I complex, yellow represents the zanamivir–1V2I complex, blue represents the oseltamivir–1V2I complex, and purple represents the oseltamivir–2HTR complex).

(LCC), as shown in Supplementary Figure S2. It indicates that the localization of the LCC is significant in this disease module, which guarantees the formed PIV3 module is of good quality. Hence, network proximity analysis was based on this PIV3 disease module.

The targets of these 14 drugs were collected from the DrugBank and DGIdb databases to calculate network proximity (see Supplementary Figure S4). Then, the network proximity of the 14 candidates to the PIV3 disease module was calculated separately, and the results are also listed in Table 1. Because BCX-2798 has not yet been approved for clinical use, the related targets have not yet been identified, and the drug targets of telbivudine and peramivir cannot be located in the PPI, only 12 candidates had Z_{dc} values. Table 1 shows only four drugs, streptomycin, amikacin, oseltamivir, and ribavirin, with lower Z_{dc} than that of zanamivir (the control, $Z_{dc} = -0.93$). It means that these four drugs including oseltamivir were better candidates for anti-PIV3 treatment according to the network proximity analysis.

Molecular dynamics simulation analysis

Combining the above three screening methods, we arrived at the results, of which only oseltamivir met all the aforementioned anti-PIV3 drug screening requirements. We used *RMSD* to assess the binding stability of the complexes formed by oseltamivir, zanamivir, and BCX-2798 with 1V2I over a period of 100 ns. As shown in Figure 4A, the *RMSD* of the oseltamivir–1V2I complex gradually stabilized after 40 ns, and the average *RMSD* of the oseltamivir–1V2I complex (0.22 nm) was significantly lower than that of the zanamivir–1V2I complex (0.25 nm) in the control group, indicating that it exhibited better structural stability. All complexes including oseltamivir–1V2I demonstrate good structural stability.

To assess the compactness of the complex structure, the *RG* values were also calculated for the three above complexes and listed in Figure 4B. As shown in Figure 4B, each set of complexes remained significantly compact throughout the 100-ns simulation. The average *RGs* of the oseltamivir–1V2I complex and the two control complexes were the same, 2.11 nm. The oseltamivir–1V2I complex was similar to the two control complexes with a smaller *RG* in higher density, which led to a more stable system structure. As shown in Figure 4C, the *RMSF* illustrates the flexibility of the molecule (Awan et al., 2024), and the average *RMSF* of zanamivir is 0.11 nm, while the mean *RMSFs* of both BCX-2798 and oseltamivir were 0.10 nm. Furthermore, in Figure 4C, the three complexes show similar fluctuations in residue levels in different regions, leading to improved conformational optimization during substrate binding. Lower *RMSF* values may indicate active sites or binding sites, as these regions tend to become more stable after binding ligands. In oseltamivir–HN, the *RMSF* values of the key residues GLU276 (0.12 nm), GLU409 (0.13 nm), ARG424 (0.08 nm), THR426 (0.10 nm), and ARG502 (0.10 nm) at the

TABLE 2 Binding energies of three complexes (kcal/mol).

Parameters	Zanamivir	BCX-2798	Oseltamivir
MMPBSA			
ΔG_{vdw}	−32.95	−12.65	−20.70
ΔG_{ele}	−144.42	−83.30	−20.87
ΔG_{P}	163.58	86.40	35.16
ΔG_{NP}	−3.63	−1.98	−4.19
ΔGgas	−177.37	−95.95	−41.57
ΔGsol	159.95	84.43	30.97
ΔG TOTAL	−17.42	−11.52	−10.60
MMGBSA			
ΔG_{vdw}	−33.17	−14.76	−21.42
ΔG_{ele}	−139.74	−93.21	−13.80
ΔG_{P}	154.27	94.82	24.82
ΔG_{NP}	−4.56	−2.55	−4.60
ΔGgas	−172.92	−107.97	−35.22
ΔGsol	149.70	92.27	20.22
ΔG TOTAL	−23.21	−15.71	−15.00

binding pocket were very close to the average *RMSF* (0.10 nm), indicating that the binding between oseltamivir and the protein was relatively stable. Both the *RMSD* and *RG* of oseltamivir–HN indicate that the system is stable. The *RMSF* also shows stable residues near the ligand binding site, leading us to consider the complex binding to be stable.

Simultaneously, the binding free energies of the three complexes were calculated by using MM/PBSA and MM/GBSA, thereby determining the ligands' binding affinity at the protein active site (Bashir et al., 2023), and the results are shown in Table 2. The binding energy values obtained from the two algorithms are quite similar. The total binding free energies of oseltamivir–1V2I (−10.60 kal/mol) and the two controls were less than −9.55 kal/mol (normal threshold for strong binding), indicating strong binding ability for both compounds. Therefore, oseltamivir was a good candidate according to results of molecular dynamics simulation.

Discussion

In this article, we explored an integrated drug repurposing method, including disease similarity and chemical similarity as multi-similarity analysis approaches, molecular docking and molecular dynamic simulation methods as structure-based screening approaches, and network proximity analysis, in which we quantified the network distance between the disease module of PIV3 and the drug targets to probe the potential anti-PIV3 drugs. In addition, according to this drug repurposing protocol, we confirm that oseltamivir is the best potential anti-PIV3 drug.

In our research results, a similar pattern was observed when oseltamivir inhibited PIV3 and influenza A. As is known, zanamivir is a successful structural design drug for NA inhibitor, and oseltamivir is designed using zanamivir as the lead compound. Among them, the side-chain amides, carboxyl groups, and amino groups play a crucial role in inhibiting the influenza A virus (Laborda et al., 2016; Tao et al., 2022), and the acetamido fragment can be well-accommodated within the cavity-binding domain (Guillon et al., 2014). In our study, oseltamivir showed good chemical similarity to zanamivir and BCX-2798. Their shared side-chain amide and carboxyl group result in similar interactions when bound to HN using molecular docking, respectively. HN can accommodate larger acyl groups in the C5 binding region (Guillon et al., 2014), and the amides of all three complexes provide hydrogen bonds for protein binding. In the oseltamivir–HN complex, the amide also forms hydrogen bonds with THR426, which greatly contributes to the stable binding of the complex. This advantage distinguishes it from other complexes. The carboxylic acid side chain of oseltamivir forms hydrogen bonds with the surrounding arginine (ARG192 and ARG424), which enhances the binding stability between molecules.

Though some unique structures of oseltamivir can also facilitate its binding to HN, various substituents at position C4 (e.g., guanidine, azido, amide, and amino group) can enter the cavity around the residue of the active site of PIV3, thereby inhibiting the activity of PIV3 (El-Deeb et al., 2014; Rota et al., 2023). Specifically, the guanidine group of zanamivir is tightly bound to the hydrogen bond of GLU549, and the azido group of BCX-2798 can form hydrogen bonds with GLN215. The addition of the azido group significantly inhibits the activity of PIV (Chibanga et al., 2019). The amino group at the C4 position of oseltamivir enhances the electron-donating capability of nitrogen ions. This enhancement allows for the formation of an electrostatic attraction with GLU276 and GLU409, thereby increasing the charge attraction and binding force between molecules. Esterification of the oseltamivir side-chain carboxyl group leads to the elongation of the backbone, enabling it to penetrate deeper into the grooves of the binding site, thereby increasing the tightness of the binding complex. Therefore, the utilization of the chemical similarity method can offer more effective information for the development of anti-PIV3 drugs. Additionally, employing molecular docking in conjunction with molecular dynamics simulation methods can enhance the exploration of the structural interactions.

Searching for drugs based solely on chemical structure makes it difficult to consider the genetic regulatory processes of the disease itself. The synergistic functions of a large number of genes during disease initiation and progression were considered in this study. The association between PIV3 and oseltamivir based on genes was explored using network proximity analysis. The proximity can also be used to define the similarity between two drug candidates and covered several drug–disease associations. If a drug is proximal to the PIV3 disease, it is more likely to be effective than a distant drug. It indicated that the oseltamivir targets are more closely linked to the PIV3 disease module than those of the control (zanamivir). The network proximity of candidate targets to the PIV3 disease genes provides special insights into the candidate mechanism of action, uncovering the patho-biological components targeted by

candidates, and improves the feasibility and interpretability for drug repurposing.

However, if some drugs cannot be localized within the PPI network due to the incomplete human interactome network, these drugs may not be screened by network proximity. Repurposed drugs may skip phase-I clinical trials, but they still need to undergo phase-II and phase-III trials to assess their efficacy against new diseases. Although there are some limitations to this study, this approach allows for the rapid identification of potential therapeutic agents to mitigate the effects of emerging epidemics.

While computational methods are robust, clinical data or experimental validation of oseltamivir's inhibitory effect on PIV3 would significantly strengthen the findings. The clinical data were collected from the Second Affiliated Hospital of Chongqing Medical University, the Third Affiliated Hospital of Chongqing Medical University, and the Children's Hospital of Chongqing Medical University by searching on Yidu Cloud (https://www.yiducloud.com.cn/, from January 2016 to June 2024). There were 539 PIV3 cases, including 479 cases of children and infants under 6 years of age, 14 cases of individuals aged 7–17 years, 18 cases of individuals over 60 years old, and only 28 adult cases. Till now, there were no clinically approved antiviral drugs for treating PIV3 infection. Among 539 PIV3 patients, most mainly received expectorant cough medicine and nebulizer therapy without antiviral treatment, 203 patients received ribavirin treatment, and 17 patients were treated with oseltamivir. Of those 17 patients who were administered off-label oseltamivir, the patients did not undergo further treatment according to the medical records. This suggests that oseltamivir has a certain efficacy in treating PIV3 in clinical settings, aligning with the findings of our result. Given that most PIV3-infected patients are children, ribavirin can lead to severe adverse clinical reactions such as hemolytic anemia and teratogenic mutations (Sinclair et al., 2017). Oseltamivir is deemed safe for children over 1 year of age as an influenza treatment and has been FDA-approved for infants and young children over 14 days old for enhanced safety (Malosh et al., 2018). Therefore, we recommend considering oseltamivir for clinical application.

Conclusion

Oseltamivir is screened as a potential anti-PIV3 drug through a variety of drug repurposing methods as it has been considered an effective drug in clinical off-label medications for anti-PIV3 treatment in some children's hospitals in recent years. Oseltamivir exhibited high similarity to potential PIV3 inhibitors (zanamivir and BCX-2798), demonstrated strong binding ability to the key target protein HN, and was closely related to the PIV3 disease module. Therefore, oseltamivir fulfilled all the screening requirements and emerged as the most effective anti-PIV3 drug. The molecular docking results revealed that oseltamivir and PIV3 were bound through hydrogen bonding, electrostatic attraction, and hydrophobic interactions. In particular, the amides, carboxyl, and amino groups in oseltamivir are important structures for inhibiting PIV3. This multi-similarity drug repurposing method will be a feasible reference for other disease and drug repurposing research.

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.

Author contributions

XC: Data curation, Formal Analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing–review and editing. BZ: Conceptualization, Validation, Writing–review and editing, Data curation. XJ: Data curation, Methodology, Writing–review and editing. HZ: Methodology, Formal Analysis, Software, Writing–review and editing. AY: Formal Analysis, Data curation, Investigation, Writing–review and editing. TZ: Formal Analysis, Funding acquisition, Methodology, Validation, Writing–review and editing. CZ: Formal Analysis, Methodology, Validation, Writing–review and editing. XL: Data curation, Resources, Writing–review and editing. YZ: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Validation, Writing – original draft, Writing–review and editing.

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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.

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Supplementary material

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β-blockers and statins: exploring the potential off-label applications in breast, colorectal, prostate, and lung cancers

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Despite advances in cancer treatment, current cancer incidence and prevalence still demand multimodal treatments to enhance survival and clinical outcomes. Drugs used in cardiology, such as β-blockers and statins have gained attention for their potential roles in oncology. This review focused on their possible complementary use in solid tumors, including breast, colorectal, lung, and prostate cancers. The involvement of the autonomic nervous system in promoting tumor growth can be disrupted by β-blockers, potentially hindering cancer progression. Statins, known for their pleiotropic effects, may also inhibit cancer growth by reducing cholesterol availability, a key factor in cell proliferation. We will provide an update on the impact of these therapies on cancer treatment and surveillance, discuss the underlying mechanisms, and explore their effects on the heart, contributing to the growing field of cardio-oncology.

KEYWORDS

drug repositioning, solid cancer, β-blockers, statin, survival

Introduction

Cancer cases were estimated at 19.3 million, with 10 million deaths globally in 2020. The burden of cancer continues to raise concern, with 28.4 million cases projected by 2040, representing a 47% increase compared to 2020 (Sung et al., 2021). Breast and prostate cancers, followed by lung, bronchus, and colorectal cancers, are the most prevalent across both sexes (Sung et al., 2021). Over the last decade, significant progress has been made in understanding the hallmarks of cancer development and treatment. These advances have improved cancer therapy and extended survival rates. Currently, cancer treatments include surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy. The use of existing drugs has also emerged as a promising strategy in the fight against cancer. In this context, drug repositioning involves finding new applications for approved or investigational drugs beyond their original medical indications (Ashburn and Thor, 2004). The advantages of repurposing drugs include time savings, cost-effectiveness, safety, and efficiency, given their well-known pharmacological properties and toxicity profiles (Malik et al., 2023). Especially in the field of cardio-oncology, drug repositioning is emerging as a multifactorial therapy within the cancer continuum, as it may not only prevent or mitigate treatment-related side effects but also improve survival and reduce all-

cause mortality (Lee et al., 2020; Attar et al., 2022). In this context, β-blockers and statins have been considered promising repurposing options for cancer treatment due to their clinical benefits and safety. Importantly, these drugs offer overlapping benefits in both cardiovascular and oncology outcomes, as they may minimize cardiovascular complications associated with cancer therapies. For example, the use of β-blockers in breast cancer (BC) treatment has been associated with reduced cardiotoxicity and decreased overall and cardiovascular mortality (Attar et al., 2022; He et al., 2022). Similarly, these benefits extend to statin treatment (Lee et al., 2020; Tailor et al., 2021). Notably, the reduction of low-density lipoprotein cholesterol (LDL-C) plasma concentrations helps to lower the cardiovascular disease (CVD) burden in the cancer population (Mohamed et al., 2024). This is particularly significant since one of the leading causes of death in cancer survivors is CVD (Zaorsky et al., 2017).

Mechanistically, increased sympathetic nervous activity may promote cancer progression (Stavropoulos et al., 2020), which is a target for use of β-blockers. Supporting this, solid tumors have been observed to signal new innervation, attracting nerve growth to the cancer (Li et al., 2022). Regarding statins, solid tumors require more structural support for development, and cholesterol, a structural lipid, may play a role in promoting this growth (Xiao et al., 2023; Halimi and Farjadian, 2022). This study aims to provide a deeper examination and exploration of the potential role of statins and β-blockers in different populations to improve cancer outcomes during treatment.

In this review, we will now explore the impact of β-blockers and statins on overall and disease-free survival in the conventional treatment of breast, colorectal, lung, and prostate cancers. These cancers were selected due to their high prevalence and mortality rates in both sexes globally (Sung et al., 2021), as well as the potential interactions of β-blockers and statins with standard therapies. Our review will encompass data from observational studies, primarily retrospective analyses, and randomized clinical trials within the cancer continuum. Our goal is to provide further insight into their role in oncology, encouraging their consideration as part of integrated cancer treatment strategies.

β-blockers and statins: the role against breast cancer cardiotoxicity

BC is the most common neoplasia in women and it is markedly characterized by heterogeneous molecular profiles, based on hormonal receptors and human epidermal growth factor receptor-2 (HER2) status. The conventional multimodal therapy used for BC patients includes surgical tumor resection, chemo, and radiotherapy (Loibl et al., 2021) and has successfully resulted in a growing number of survivors. However, this population is at risk of developing treatment-induced CVD compared to healthy controls, especially those treated with anthracycline agents (i.e., doxorubicin, daunorubicin, epirubicin, idarubicin, and mitoxantrone). Cancer survivors who underwent chemotherapy have increased resting heart rate (Lakoski et al., 2015), and higher circulating norepinephrine levels (Nousiainen et al., 2001), which are suggestive of sympathetic overdrive. Because β-adrenergic receptors (β-AR) also may play a role in invasion and cancer progression (Hiller et al., 2020), β-AR antagonists, also called β-blockers, are emerging as novel anticancer therapeutics. For BC therapy, β-blockers are an evolving intersection of the cardiovascular and oncology fields and represent a strategy to counteract treatment-induced cardiotoxicity.

β-blockers are a group of antihypertensive agents with diverse mechanisms of action. Overall, they antagonize adrenergic stimulation of β-AR according to receptor selectivity (β1 and β2-AR), exhibit intrinsic sympathomimetic activity, vasodilating properties, and modulate metabolism (Gorre and Vandekerckhove, 2010). Importantly, only the subtypes β1 and β2-AR encompass the scope of this review.

Because the heart has a larger proportion of β1-AR compared to β2-AR, specifically in a 70/30 ratio (Gorre and Vandekerckhove, 2010), the effect of β-blockade on the heart is heterogeneous. Stimulating β1 leads, cyclic adenosine monophosphate (cAMP)-dependent intracellular pathways, to positive inotropic, chronotropic, lusitropic and dromotropic effects (Triposkiadis et al., 2009); but also, stimulating β2-AR in arterial walls results in vasodilation.

Thus, β-blockers are stratified in "generations" according to their pharmacological properties. The first-generation of β-blockers is the non-selective, blocking both β1 and β2 receptors, such as propranolol. The second-generation agents include β-blockers with higher affinity for β1-AR, which is often referred to as "cardio"-selectivity. Examples include atenolol, metoprolol, bisoprolol and betaxolol. The third generation of β-blockers exhibit varied selectivity for β1-AR (do Vale et al., 2019). They may stimulate β2-AR leading to vasodilation, nitric oxide synthesis and anti-inflammatory properties (Triposkiadis et al., 2009; Bakris, 2009). Additionally, vasodilation may also be mediated by blocking alpha1-AR and activating β3-AR. The third generation β-blockers include agents like pindolol, carvedilol and nebivolol (do Vale et al., 2019).

Many RCTs addressed cardiotoxicity in BC by evaluating left ventricular ejection fraction (LVEF). Considering the cancer *continuum*, at the pre-chemotherapy stage, starting lisinopril and bisoprolol (β_1-AR antagonist) 24 h before the first cycle of chemotherapy reduced the decline in LVEF and prevented anthracycline-induced cardiotoxicity in women with advanced BC (Wihandono et al., 2021). Endorsing it, LVEF was unchanged in the carvedilol (β_1/β_2 -AR antagonist) group but decreased in the control arm (Nabati et al., 2017). During chemotherapy, administering carvedilol was shown to prevent doxorubicin-induced cardiotoxicity compared with a placebo. Beyond LVEF, strain and strain-rate function, which are more sensitive indices to detect early stages of myocardial dysfunction, were examined. Even though these measures worsened in the control group following chemotherapy, they were unchanged in subjects who received carvedilol (Tashakori et al., 2016). Carvedilol administered daily for 6 months during chemotherapy maintained LVEF and fractional shortening within normal limits for all patients before and after anthracycline treatment, indicating a cardioprotective effect (Elitok et al., 2014). Starting candesartan or carvedilol within 1 month after initiating doxorubicin-containing chemotherapy, and maintaining it throughout the treatment might have prevented an early decrease in LVEF (Lee et al., 2021). The MANTICORE 101-Breast Study examined women with HER2-positive early BC on adjuvant therapy with trastuzumab. It was observed that perindopril and

bisoprolol protected against cancer therapy-related declines in LVEF; nonetheless, trastuzumab-mediated left ventricular remodeling could not be prevented by this drug protocol (Elitok et al., 2014). Another study showed that β-blockers protect against cardiotoxicity following trastuzumab and anthracycline-containing medication treatment (Gao et al., 2023). Besides, continuous use of β-blockers was associated with a lower heart failure incidence in patients with BC under trastuzumab treatment (Seicean et al., 2013). The concomitant use of β-blockers with angiotensin-converting enzyme receptors was associated with a recovery of LVEF during months 3–12 of adjuvant trastuzumab therapy (Oliva et al., 2012). Using metoprolol (β_1-AR antagonist) and candesartan concomitantly with epirubicin-based anthracycline therapy increased circulating cardiovascular biomarkers without association with a decline in ventricular function in early BC women. In this same study, troponin levels were attenuated by metoprolol, but not candesartan (Gulati et al., 2017). HER2-positive BC women showed that cardiotoxicity-free survival was longer on both lisinopril and carvedilol treatment compared to placebo (Guglin et al., 2019). Complementing these findings, the Prevention of Cardiac Dysfunction During Adjuvant Breast Cancer Therapy (PRADA) study suggested that a cardioprotective approach might not be required in patients without preexisting CVD (Heck et al., 2021). Regarding the post-chemotherapy stage, one study pointed out higher LVEF in the β-blocker group compared with placebo (Shah et al., 2019).

Summarizing, there is evidence that β-blockers prevent chemotherapy-induced cardiotoxicity and improve or contribute for maintenance of LVEF and better survival. Carvedilol, a third generation β-blocker which has additional alpha-blockade and vasodilating properties, was the most cited intervention used during chemotherapy and led to cardioprotective effects with preservation of LVEF. In addition, the combined use of β-blockers with angiotensin-converting enzyme receptors was associated with LVEF recovery during cancer therapy. Importantly, β-blockers exhibit only minor side effects and may prove an effective, safe, and inexpensive anticancer repurposing strategy, being a feasible alternative to integrate BC treatment.

Another potential drug repurposing candidate for cancer are statins. Statins belong to a class of drugs that may be divided into two types, according to their solubility in the lipidic environment, being either lipophilic or hydrophilic. The solubility may characterize the statins' capacity to be incorporated into the liver. Lipophilic statins can be incorporated by passive diffusion (i.e., atorvastatin, simvastatin, fluvastatin, and pitavastatin), while hydrophilic statins require active transport (i.e., pravastatin and rosuvastatin) (Minichsdorfer et al., 2022). Statins act by inhibiting 3-hidroxi-3-methyl-glutaril-CoA redutase HMG-CoA reductase, which reduces cholesterol synthesis in the liver, thereby decreasing blood cholesterol carried by LDL-C. The survival rates associated with statin treatment in BC patients may be related to their chemopreventive effects on attenuating cardiotoxicity. The impact of statin treatment on primary and secondary prevention of CVD is well known and it reduces chemotoxicity resulting from BC treatment (Mohamed et al., 2024). In BC patients, the prophylactic use of 40 mg of atorvastatin, a lipophilic statin, for 6 months may prevent cardiac dysfunction resulting from anthracycline-based therapy (Mohamed et al., 2024). Similar results were observed in BC patients under treatment of rosuvastatin, hydrophilic statin (Nabati et al., 2019). Corroborating these data, HER2+ BC patients under statins who were receiving trastuzumab, with or without anthracyclines, displayed a lower risk for cardiotoxicity (Calvillo-Arguelles et al., 2019). A study showed that LVEF did not change with statin treatment, whereas in the placebo groups, it decreased over time (Mohamed et al., 2024; Nabati et al., 2019; Calvillo-Arguelles et al., 2019). In contrast, another study could not endorse the results, and showed that treatment based on 40 mg of atorvastatin did not improve cardiovascular function. The possible reason for this may be the heterogeneity of cancers addressed, since the study included breast, lymphoma, leukemia, sarcoma, and thymoma; also, these patients were more prone to develop complications, with a higher risk for treatment-induced toxicity (Thavendiranathan et al., 2023). Like β-blockers, statins seem safe and beneficial against cardiotoxicity. In addition, they might have a positive effect on overall survival in cancer patients, as discussed below. Because cholesterol may serve as substrate for growth and development of cancer cells; and considering that statins inhibit cholesterol production, placing these drugs as an adjuvant treatment hold promise to improve survival (Xiao et al., 2023; Halimi and Farjadian, 2022).

β-blockers and breast cancer: survival analysis

Retrospective observational studies indicated that β-blockers improved BC-related clinical outcomes, regardless of β-blocker selectivity (Botteri et al., 2013; Choy et al., 2016; Gillis et al., 2021; Powe et al., 2010; Sørensen et al., 2013). A meta-analysis study that addressed β-blockers, angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II receptor blockers (ARB) use showed that β-blockers improved BC specific survival for women treated at the time or in the year across BC diagnosis; however, the association of ACEi/ARB did not yield to disease free and overall survival. In this study, between-study heterogeneity was assessed to investigate outcomes for propranolol vs other β-blockers. The antiangiogenetic and antimetastatic property of β-blockers, in particular propranolol, was endorsed (Raimondi et al., 2016). Another meta-analysis, which did not specify β-blocker selectivity, found that β-blocker use after diagnosis but not before diagnosis is beneficial for the survival of cancer patients (Zhong et al., 2016). The positive effects of β-blockers on survival were confirmed also for patients with triple-negative BC (TNBC), with the most prevalent drugs used being selective β-blockers, mainly metoprolol; followed by atenolol (Melhem-Bertrandt et al., 2011). BC-specific mortality and presenting with advanced tumors were significantly lower for propranolol users (Barron et al., 2011). A lower prevalence of cardiovascular toxicity regardless of the time of initiation of β-blockers (Martins Carvalho et al., 2023) and reduced risk of cardiovascular mortality upon using metoprolol, atenolol, and carvedilol Tabassum et al. (2024) have been reported. The ROSE/TRIO-012 Study examined survival and clinical benefits in patients with HER2-negative advanced BC treated with docetaxel in combination with ramucirumab or placebo. The use of β-blockers was associated with BC specific survival only in patients with TNBC, with hazard ratios of 0.66 (95% CI: 0.47–0.91) for use of any type of β-blocker, 0.24 (95% CI:0.06–0.96) for use of non-selective β-blocker, and 0.71 (95% CI:0.51–0.99) for use of selective β-blockers (Løfling et al., 2022).

However, other studies failed to confirm associations between β-blocker therapies and improved outcomes. Studies that stratified the population between β-blocker users and non-users showed that the observed poor disease-free survival did not endorse β-blockers antitumor effects (Cardwell et al., 2016; Kim et al., 2017; Li et al., 2020; Sakellakis et al., 2014). Likewise, another study that was not able to address the selectivity of β-blockers could not point to improved mortality deaths and recurrence (Li et al., 2020). Also, using perioperative β-blockers (selectivity not specified) did not improve cancer-specific survival before and after index surgical resection for the breast (Musselman et al., 2018). Contrarily, β-blockers were linked to poorer progression-free and overall survival based on their use before cancer diagnosis, with the most prevalent prescribed drugs being atenolol, followed by propranolol and another β-blocker (Shah et al., 2011). Using propranolol or non-selective β-blockers was not associated with improved survival (Cardwell et al., 2016). Corroborating, it was shown that trastuzumab concomitantly with any dose of a β-blocker (propranolol, carvedilol, atenolol, or bisoprolol) potentially had negative outcomes in women with HER2-positive advanced BC (Hsieh et al., 2023). Nonetheless, in a study that compared β-blocker users to non-users, death was associated with short-term (0–3 months) β-blocker therapy, indicating a protective effect associated only with long-term use (i.e. 3 years +) (Scott et al., 2022).

Overall, RCT studies showed that using β-blockers was associated with cardioprotective effects for women with BC receiving anthracyclines over the cancer *continuum* and showed promising results for improved survival in BC, regardless of the selectivity of the drug, when reported. In general, results are summarized in Table 1.

Retrospective studies, however, were unclear concerning the use of β-blockers in the BC approach. These conflicting outcomes might be explained by methodological limitations. Previous studies fail reporting the time of medication use. Besides, there was scarce information on between β-blockers and concurrent use of other medication for chronic diseases, such as diabetes or dyslipidemia. Additionally, because these studies normally address data of women from Europe or North America, the association between β-blockers use and prognosis in BC should also consider women with other ethnicities because BC incidence and prevalence differ among populations globally. It is crucial to point out that most retrospective studies were unable to evaluate the selectivity of β-blockers and how it would yield to different prognosis since this data were rarely retrieved. So, it is recommended to investigate future studies to identify the doses, exposure, and selectivity of β-blockers repurposed in cancer.

TABLE 1 Effect of β-blockers on breast cancer survival.

Author, year	Survival outcome
Botteri et al. (2013)	↑
Choy et al. (2016)	↑
Gillis et al. (2021)	↑
Powe et al. (2010)	↑
Sørensen et al. (2013)	↑
Raimondi et al. (2016)	↑
Zhong et al. (2016)	↑ - Only when used after cancer diagnosis
Melhem-Bertrandt et al. (2011)	↑
Barron et al. (2011)	↑
Tabassum et al. (2024)	↑
Løfling et al. (2022)	↑ - Only in TNBC
Cardwell et al. (2016)	↔
Kim et al. (2017)	↔
Li et al. (2020)	↔
Sakellakis et al. (2014)	↔
Musselman et al. (2018)	↔
Shah et al. (2011)	↓
Hsieh et al. (2023)	↓
Scott et al. (2022)	↓ Short-term use (0–3 months) ↑ Long-term use (>3 years)

↑: increase; ↓: decrease; ↔: unchanged.

Statins and breast cancer: survival analysis

Similarly to β-blockers, statins are widely known for their cholesterol-lowering effects, have also garnered attention for their possible benefits in cancer therapy. The use of statins is mainly directed at the control of blood cholesterol concentrations. Total cholesterol levels are associated with BC mortality, and so, its reduction and control via statin treatment may directly impact the patient's survival (Murto et al., 2023). Beyond the recurrence risk (Jia et al., 2023), improvement of BC survival was demonstrated in reviews and meta-analysis (Jia et al., 2023; Wu et al., 2015; Beckwitt et al., 2018), but also in cohort studies (Li et al., 2019; Borgquist et al., 2019). Data from 1.3 million of patients from UK Biobank show the associative protector role of statin on the prognosis of BC patients. Also, statin appears to have the ability to convert a cold to hot tumor (Li et al., 2024). It means the increased capacity to respond to anticancer therapies. Corroborating with these data, women who were receiving statins for 6 months before BC diagnosis presented a lower risk of all-cause mortality compared with non-users, which was mainly attributed to cancer-related rather than cardiovascular death. Moreover, the reduction of cancer-related deaths was time-dependent on the statin treatment (Chang et al., 2023). Endorsing it, retrospective studies showed that using statins for more than 5 years was associated with higher overall and disease-free survival in comparison to non-statin users in BC, regardless of neoplasia type (Li et al., 2019; Sim et al., 2022).

In the Austrian Breast and Colorectal Cancer Study Group (ABCSG) trial 18, postmenopausal women with hormone receptor-positive BC who were receiving adjuvant denosumab or placebo were examined. In the treatment arm, which was taking hydrophilic statin, disease-free survival was worse in comparison to non-users; however, after correcting for confounders such as age, ethnicity, and body-mass index, and others, the hydrophilic statin's detrimental effect was attenuated. On the other hand, lipophilic statins, such as atorvastatin, simvastatin, fluvastatin and pitavastatin, did not lead to any effects on survival (Minichsdorfer et al., 2022).

TABLE 2 Effect of statins on breast cancer survival.

Author, year	Survival outcome
Murto et al. (2023)	↑
Jia et al. (2023)	↑
Wu et al. (2015)	↑
Beckwitt et al. (2018)	↑
Li et al. (2019)	↑
Borgquist et al. (2019)	↑
Chang et al. (2023)	↑
Sim et al. (2022)	↑
Minichsdorfer et al. (2022)	↔

↑: increase; ↓: decrease; ↔: unchanged.

Despite scarce data, statins-based long-term treatment appears to be beneficial for BC women, especially before diagnosis, according to Table 2. Further studies should explore the clinical outcomes regarding statin solubility. For now, new strategies need to be considered to replace statins as primary prevention in the BC scenario. Statins off-label repositioning is attractive within the BC context because they may mitigate chemotherapy-related cardiotoxicity and display some benefits on overall survival.

Another neoplasia that raises concern is colorectal cancer (CRC), which is increasingly affecting younger patients, with rising incidence and mortality rates.

β-blockers and colorectal cancer

CRC is a significant health concern worldwide, characterized by its high incidence and mortality rates (Bray et al., 2024). Despite advancements in treatment strategies, managing CRC remains challenging, particularly in the advanced stages of the disease (Cercek et al., 2020; Chen et al., 2023).

Several preclinical studies have provided insights into the potential benefits of β-blockers in CRC (Mohammadpour et al., 2021; Qiao et al., 2019; Qiao et al., 2021). Despite promising preclinical data, clinical evidence regarding the efficacy of β-blockers in CRC remains somewhat limited and inconclusive. Cui and colleagues showed an increase in overall survival in patients with CRC using β-blockers regardless of chemo or radiotherapy regimen, time of β-blocker use, and stage of the disease (Cui et al., 2019). However, many other retrospective studies have failed to replicate these findings (Musselman et al., 2018; Shah et al., 2011; Ekestubbe et al., 2022; Hicks et al., 2013; Holmes et al., 2013; Numbere et al., 2017; Zhang et al., 2022).

Interestingly, some studies that stratified patients by stage of the disease show promising results. Balakrishnan et al. showed that the use of β-blockers is associated with reduced mortality in patients with stage I-III of CRC (Balkrishnan et al., 2021). Jansen and colleagues evaluated CRC patients of different stages of the disease, and only patients at stage IV showed benefits from the use of β-blockers (Jansen et al., 2012; Jansen et al., 2014).

TABLE 3 Effect of β-blocker on colorectal cancer survival.

Author, year	Survival outcome
Mohammadpour et al. (2021)	↑
Qiao et al. (2019)	↑
Qiao et al. (2021)	↑
Cui et al. (2019)	↑
Musselman et al. (2018)	↔
Shah et al. (2011)	↔
Ekestubbe et al. (2022)	↔
Hicks et al. (2013)	↔
Holmes et al. (2013)	↔
Numbere et al. (2017)	↔
Zhang et al. (2022)	↔
Balkrishnan et al. (2021)	↑
Jansen et al. (2012)	↑
Jansen et al. (2014)	↑
Kocak et al. (2023)	↑

↑: increase; ↓: decrease; ↔: unchanged.

The type of primary cancer therapy seems to also alter β-blocker benefits. Giampieri et al. showed that the association of β-blockers and chemotherapy in metastatic CRC patients led to improvement in overall survival, but when the patients were also treated with bevacizumab, the opposite effect was observed (Giampieri et al., 2015). However, the number of patients receiving therapy in this study was small (nine) and the conclusion might not be representative of the population. In a larger study with metastatic CRC patients receiving chemotherapy and immune checkpoint inhibition, β-blockers showed a beneficial effect on overall survival and progression-free survival (Kocak et al., 2023).

In the context of CRC, β-blocker-based treatment led to mixed results, with studies reporting benefits, whereas others reporting neutral outcomes, according Table 3. However, no study pointed out the worsening in patient's condition upon β-blockers use. Therefore, we endorse that more studies are needed to clarify the real effect of β-blocker on CRC survival.

Statins and colorectal cancer

Statins appear to be beneficial and safe for use during CRC treatment. In combination with other non oncology-related drugs, such as aspirin and anti-inflammatory, statins may improve patient survival (Bardou et al., 2010). In cohort studies, statin treatment displays interesting results. When initiated before CRC diagnosis, but not after, statins were associated with improved cancer-specific survival (Gray et al., 2016). In another cohort with a mean duration of 5.6 years, statin use was associated with a lower risk of CRC-related mortality and, also, all-cause mortality (Sun et al., 2023).

Conversely, the effects of statin treatment on survival could not be confirmed. A multi-center study with 269 patients previously

TABLE 4 Effect of statin on colorectal cancer survival.

Author, year	Survival outcome
Gray et al. (2016)	↑ - When initiated before cancer diagnosis
Sun et al. (2023)	↑
Lim et al. (2015)	↔
Krens et al. (2014)	↔
Ng et al. (2011)	↔

↑: increase; ↓: decrease; ↔: unchanged.

TABLE 5 Effect of β-blocker on prostate cancer survival.

Author, year	Survival outcome
Grytli et al. (2013)	↑
Lu et al. (2015)	↑
Kaapu et al. (2016)	↔
Assayag et al. (2014)	↔
Posielski et al. (2021)	↔

↑: increase; ↓: decrease; ↔: unchanged.

treated for metastatic CRC, showed that 7 months of using 40 mg of simvastatin did not improve survival (Lim et al., 2015). Corroborating, two trials that investigated the mutation of KRAS protein, which is a widely studied oncogene associated with malignancies, found no impact of statins on survival in metastatic CRC patients, even though statins often inhibit the expression of KRAS phenotype (Krens et al., 2014; Ng et al., 2011).

Results are presented in Table 4, but as previously observed with BC patients, the association between statin use and survival benefits appears to be associated with the exposition of drugs during the time. Long-term use of statins, mainly, when started before cancer diagnosis (Sun et al., 2023; Dobrzycka et al., 2020; Siddiqui et al., 2009) leads to better clinical outcomes. Thus, when started before or even during treatment, statins have the potential to prevent side effects of CRC treatment (Dobrzycka et al., 2020). Additional studies, mainly retrospective, need to better investigate the time-dependent effect of statin treatment on CRC to reinforce the drug-repositioning on primary prevention. Interestingly, none of the trials reported impairments in patient's health following statin treatment. Based on the above, it can be suggested that statins are safe and do not lead to significant adverse effects in CRC treatment.

β-blockers and prostate cancer

Prostate cancer (PC) is the second most common neoplasia among men and the fifth leading cause of death (Sung et al., 2021). β-blockers are particularly interesting to be used during PC therapy because the activation of β-adrenergic receptors is related to angiogenesis, proliferation, and migration of tumor cells (Malik et al., 2023). Although research suggests a role for these receptor pathways in prostate neoplasia, outcomes on β-blockers and prostate remain unclear.

The following evidence was retrieved from observational studies using population-based cancer registries and hospital electronic medical records. Men under sotalol, (non-selective blocker of β_1 with antiarrhythmic properties), had a decreased risk of PC according to the duration of sotalol use (Kaapu et al., 2015). β-blocker treatment was associated with a 10% lower risk for all PC in models adjusted for age and race. Importantly, this therapeutic benefit was lost when adjusting it for the history of CVD (Rodriguez et al., 2009). In a study with ethnically diverse men, participants were stratified according to the risk of PC. Atenolol (β_1-AR selective antagonist) was associated with a 38% reduction in odds of incident PC compared to men not taking a β-blocker. In addition, taking atenolol for 3–5 years was associated with a substantial reduction in intermediate and high-risk disease (Zahalka et al., 2020). β-blockers were associated with reduced PC-specific mortality, but no effect was reported on all-cause mortality (Grytli et al., 2013; Lu et al., 2015).

Conversely, the Finnish Randomized Study of Screening for Prostate Cancer showed no general protective effects against PC death with digoxin usage, sotalol, and antiarrhythmic drugs in general, before or after PC diagnosis (Kaapu et al., 2016). Hazard ratios of mortality outcomes associated with post-diagnostic use of β-blockers in men diagnosed with non-metastatic PC revealed that β-blockers, regardless of selectivity, were not associated with a decreased risk of PC and all-cause mortality (Assayag et al., 2014). Even in patients at high-risk PC on androgen deprivation therapy, using any β-blocker was not associated with improved survival (Posielski et al., 2021). In addition, the use of antihypertensive drugs was associated with an increased risk of PC. However, because the authors addressed distinct categories of antihypertensive drugs separately and in combination, this outcome may be related to underlying hypertension (Siltari et al., 2018).

We could not retrieve RCT data in our search. Taken together, the retrospective studies on PC mainly addressed outcomes such as risk and mortality of cancer and shared conflicting results, according to Table 5. While studies point out that β-blocker-based treatment was associated with a decreased risk of developing PC, others report no protective effects for mortality and risk; and one study positively associated β-blocker and PC. Future research will be required to determine whether β-blockers have differential effects as a function on patients' survival.

Statins and prostate cancer

The risk of fatal PC was lower in statin users than in non-users (Craig et al., 2022). Adopting statin therapy before PC diagnosis did not impact survival, however, when starting it following diagnosis, statins were associated with decreased PC-related mortality. The decreased risk was dose-dependent and observed especially among patients undergoing hormone therapy (Murtola et al., 2017). Statin outcomes are often linked to a better prognosis, regardless of whether it is hydrophilic or lipophilic (Cao et al., 2023). A study reported good prognosis and overall survival in men with castration-resistant metastatic PC (Hamilton et al., 2021). In general, prospective studies endorse positive results concerning the effect of statin treatment on PC mortality (Gutt et al., 2010; Joentausta et al., 2019; Kollmeier et al., 2011; Van Rompay et al., 2019; Wu et al.,

2019; Yu et al., 2014). These benefits are independent of the type of treatment, such as surgery or hormone therapy, and cancer grade. Additionally, similar to findings in other previously described cancers such as CRC and BC, patients who are taking statins before diagnosis experience a greater beneficial effect against PC (Yu et al., 2014).

In a study with over one thousand patients who were initiating androgen deprivation therapy (ADT), statin therapy was related to reduced overall mortality (Hamilton et al., 2021). Likewise, statin treatment also had positive effects on a population-based cohort study that involved 4,428 men and had a 6.3-year follow-up. The treatment initiated after ADT was associated with improved PC prognosis and survival (Peltomaa et al., 2021).

Statin treatment is widely supported in the literature as a strategy against PC-related and overall mortality, as shown in Table 6. Statins prescription needs to be reconsidered by clinicians aiming for better cancer-related prognosis but also in the environment of primary prevention, such as controlling risk factors for CVD.

TABLE 6 Effect of statin on prostate cancer survival.

Author, year	Survival outcome
Craig et al. (2022)	↑
Murtola et al. (2017)	↑ - Only after taking after diagnosis
Cao et al. (2023)	↑
Hamilton et al. (2021)	↑
Gutt et al. (2010)	↑
Joentausta et al. (2019)	↑
Kollmeier et al. (2011)	↑
Van Rompay et al. (2019)	↑
Wu et al. (2019)	↑
Yu et al. (2014)	↑
Peltomaa et al. (2021)	↑

↑: increase; ↓: decrease; ↔: unchanged.

β-blockers and lung cancer

Lung cancer (LC) remains one of the most prevalent and deadly forms of cancer worldwide, with a high mortality rate despite advances in treatment (Bray et al., 2024). It often presents in advanced stages, challenging successful treatment (Nilsson et al., 2020). However, recent research has shed light on potential adjunct therapies that could improve overall survival rates. In this sense, β-blockers, commonly used to manage hypertension and heart-related conditions (Nilsson et al., 2020) can be a promising adjuvant therapy for cancer. Several studies have suggested a potential link between β-blocker use and improved overall survival in LC patients. One hypothesis is that β-blockers may exert anti-tumor effects by inhibiting signaling pathways involved in cancer progression and metastasis (Schuller and Al-Wadei, 2012; Yan et al., 2023). Additionally, β-blockers might mitigate the harmful effects of stress on tumor growth, as stress hormones can promote tumor development and resistance to treatment (Yan et al., 2023).

Despite promising preliminary evidence, the relationship between β-blockers and LC outcomes remains complex and not fully understood. Musselman et al. (2018) performed a retrospective analysis comparing patients who were exposed to β-blockers and those who were not before and after undergoing surgical resection for breast, lung, and colorectal cancer. Despite the extensive scope of this population-based study, no correlation was found between perioperative β-blocker exposure and enhanced cancer-specific survival rates in individuals with breast, lung, or CRC (Musselman et al., 2018). These results corroborate another retrospective study that evaluated perioperative the use of β-blockers in the overall survivor and progression-free survival (Cata et al., 2014). Other retrospective studies have explored the correlation between β-blocker usage and survival in LC. However, findings from these studies indicate that the administration of β-blockers before and after diagnosis did not change the overall survival of LC patients (Holmes et al., 2013; Coelho et al., 2020; Udumyan et al., 2020; Weberpals et al., 2017). Interestingly, studies comparing the use of β-blockers with some specific therapies have shown promising results. Wang et al. (2013) showed that the incidental use of β-blocker in patients with non-small cell lung cancer (NSCLC) that did radiotherapy is associated with progression-free survival, distant metastasis-free survival, disease-free survival, and overall survival. These results corroborate the findings of Chaudary and colleagues, who observed in a retrospective study the association of β-blockers with chemo and radiotherapy in patients with NSCLC led to increased overall survival and distant metastasis-free survival (Chaudhary et al., 2019).

In general, studies have reported conflicting results, as shown in Table 7, highlighting the need for further research to elucidate the mechanisms underlying any potential benefits, especially through prospective studies, since almost all studies published so far are retrospective. Factors such as dosage, duration of use, and selectivity of β-blocker used and combination with other cancer therapies may also influence outcomes, calling for careful consideration in future studies. If β-blockers indeed prove to enhance overall survival in LC patients, they could represent a readily available and affordable adjunct therapy with the potential to positively impact patient care. However, rigorous clinical trials are needed to confirm these findings and establish clear guidelines for their use in the oncological setting. Similar results were observed in respect to statin and LC, which are necessary for more studies, and also, with the combination of both drugs.

Statins and lung cancer

Statins are under-prescribed for patients with LC. Importantly, in LC patients, atherosclerotic disease is the leading cause of death (Tailor et al., 2021). Therefore, prevention of dyslipidemia and its adequate treatment are important strategies to prevent atherosclerotic CVD in cancer patients, and statin adherence is associated with reduced all-cause, cancer-related and cardiovascular mortalities (Lee et al., 2020).

Drug repositioning is already widely adopted in LC treatment. Using diverse drugs such as metformin, aspirin, and statin in combination is protective against LC mortality. In addition, statin alone reduces LC mortality in a dose-dependent manner (Kang et al.,

TABLE 7 Effect of β-blockers on lung cancer survival.

Author, year	Survival outcome
Musselman et al. (2018)	↔
Cata et al. (2014)	↔
Holmes et al. (2013)	↔
Coelho et al. (2020)	↔
Udumyan et al. (2020)	↔
Weberpals et al. (2017)	↔
Wang et al. (2013)	↑
Chaudhary et al. (2019)	↑

↑: increase; ↓: decrease; ↔: unchanged.

TABLE 8 Effect of statin on lung cancer survival.

Author, year	Survival outcome
Kang et al. (2021)	↑
Nguyen et al. (2020)	↑
Ung et al. (2018)	↑
Cardwell et al. (2015)	↑
Lee et al. (2017)	↔
Lee et al. (2023)	↔
Seckl et al. (2017)	↔
Han et al. (2011)	↑

↑: increase; ↓: decrease; ↔: unchanged.

2021). In the first line treatment, with epidermal growth factor receptor kinase inhibitors, statin was associated with prolonged life survival in LC patients (Nguyen et al., 2020).

Retrospectively, the post-diagnosis treatment with simvastatin and atorvastatin was associated with extended survival in NSCLC patients (Ung et al., 2018). In general, statin reduced rates of cancer-specific mortality, especially simvastatin, which is a lipophilic drug (Cardwell et al., 2015). However, in a RCT addressing patients with both NSCLC and SCLC, statin therapy did not appear to improve prognosis. In a phase II trial with afatinib plus 40 mg/day of simvastatin or afatinib alone, with 68 previously treated patients with advanced non-adenocarcinomatous NSCLC, it was not possible to establish additional clinical benefits for using statins in survival (Lee et al., 2017). In the phase II trial with SCLC, which examined the effectiveness of irinotecan plus cisplatin with and without 40 mg/day of simvastatin in 125 patients, survival was not improved (Lee et al., 2023). Another phase III, randomized, double-blind, placebo-controlled trial with treatment of 40 mg/day of pravastatin added to first-line standard chemotherapy (etoposide plus cisplatin or carboplatin) in SCLC, included 846 patients. Despite safety, pravastatin treatment did not result in better survival (Seckl et al., 2017). Conversely, in another RCT, simvastatin in combination with gefitinib improved survival in NSCLC patients in comparison to those who were not receiving statin treatment (Han et al., 2011).

Overall, clinical trials do not support the effectiveness of statins during LC treatment on survival, according to Table 8, although they are safe for prescription. Depending on the cancer stage and differences in protocol treatment, it is suggested that statins do not aggregate benefits for LC patients; however, concerning overall survival, statins might play a role in reducing CVD-related mortality.

Other considerations and future perspectives

This study has limitations. Some studies did not fully detail study design, type of drug (i.e., selectivity, solubility) and dose used, mainly when it comes to retrospective studies. Both drugs, β-blockers, and statins, have potential to modulate prognosis in different cancer populations. The combined use of different β-blockers and statins appear to be effective during the chemo- and radiotherapy treatment, regardless of their pharmacological class, as they also improve survival in cancer patients. Addressing alternative interventions that incorporate the combination of β-blockers and statins may enlighten the understanding on how to achieve better survival and disease-free survival.

Regarding side effects, both drugs may lead to undesirable consequences, although they tend to be mild, and few patients report them. Depending on the dose, class, and generation, statins are known to cause myalgia, which may lead patients to discontinue treatment (Selva-O'Callaghan et al., 2018). Similarly, β-blockers may lead to hypotension, resulting in dizziness and weakness (Koracevic et al., 2022), especially when the dose is not adequately adjusted for the patient. These side effects can affect treatment for dyslipidemia and hypertension, preventing patients without these chronic conditions from benefiting from the anticancer effects of the medications studied. It is important to note that these drugs are generally safe for long-term use worldwide. However, we cannot overlook the cytotoxic effects of β-blockers, which can disrupt healthy cells (Kavakcioglu Yardimci et al., 2021). While these effects may seem negative, they can enhance the ability to induce apoptosis in cancer cells. Additionally, statin therapy appears to improve the effectiveness of chemotherapy by increasing drug concentration within the cells (Ahmadi et al., 2018), suggesting a potential for repurposing these drugs for cancer treatment.

Conclusion

Using β-blockers and statins are safe during breast, colorectal, lung, and prostate cancers treatment. β-blockers seem to lead to better clinical outcomes for breast cancer, whereas statins were positively associated with greater outcomes in breast, colorectal and prostate treatment, with additional benefits for patients with PC. Much of the intersection between CVD and the cancers discussed in this review share similarities in predisposing risk factors, such as hypertension and dyslipidemia. Thus, controlling these factors either with β-blockers and statins use and/or changes in lifestyle, is a relevant strategy for better cancer survival. As in the treatment of chronic conditions, oncological disease also requires continuous management, so the benefits of being exposed enough to complementary drugs can be translated into clinical outcomes. Therefore, long-term use of β-blockers and statins may influence a better prognosis in cancer survival.

In respect to future directions, the safety, low-cost and effectiveness observed with the use of β-blockers and statins should encourage future trials, which need to enrolled patients with chronic disease and observe, during a follow, the hypothesis if the β-blockers and statin treatment and the ability to control the disease, such blood pressure and LDL-C concentrations, may influence cancer diagnosis and outcome. In addition, future reports need to detail the drug dosage, time of treatment and different classes of β-blockers and statins. Also, though by Mendelian randomization study, a determinant factor is understanding the role of living with these risk factors for a long time may be mandatory, or not, for the survival in the cancer cases.

Author contributions

PGSB: Conceptualization, Data curation, Supervision, Writing–original draft, Writing–review and editing, Visualization. JV: Writing–original draft, Writing–review and editing. AG: Writing–original draft, Writing–review and editing. PCB: Writing–original draft, Writing–review and editing, Data curation, Supervision, Visualization, Conceptualization.

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The authors show the possibility to use old drugs, such as β-blockers and statin, in the challenge scenario to be addressed to solid tumor treatment.

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

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