REPURPOSED DRUGS TARGETING CANCER SIGNALING PATHWAYS: CLINICAL INSIGHTS TO IMPROVE ONCOLOGIC THERAPIES

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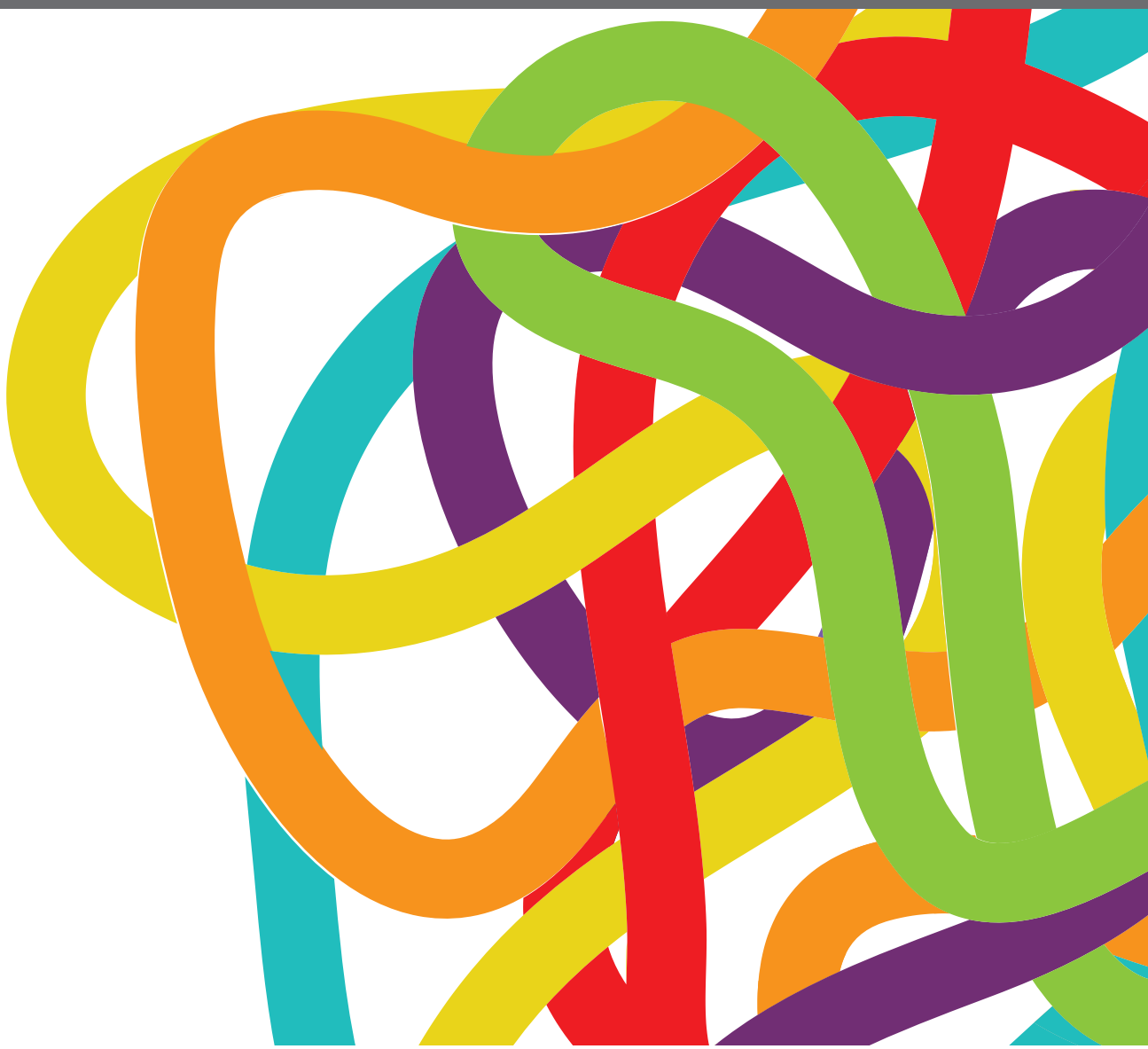

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REPURPOSED DRUGS TARGETING CANCER SIGNALING PATHWAYS: CLINICAL INSIGHTS TO IMPROVE ONCOLOGIC THERAPIES

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Editorial: Repurposed Drugs Targeting Cancer Signaling Pathways: Clinical Insights to Improve Oncologic Therapies

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Editorial on the Research Topic

Repurposed Drugs Targeting Cancer Signaling Pathways: Clinical Insights to Improve Oncologic Therapies

The analyses of drug development from different standpoints show how resource-intensive this process is, with two main disadvantages: time and cost. It takes 11 to 14 years to develop a pharmaceutical product, with a financial cost currently estimated around 161-1,800 million dollars (1). Bypassing these limitations has made drug repositioning one of the most burgeoning areas in pharmacology over the past decade. Drug repositioning finds new applications of existing drugs by testing them against diseases unrelated to their initial use; so, the availability of complete data on pharmacology, formulation, safety, and adverse effects reduces development time and cost. However, it also has disadvantages: managing patents, intellectual property, investment, market demand, and even production technology (2). Regardless, drug repositioning poses a fascinating challenge with the potential to improve human health, in particular for the treatment of various types of cancer, but also a complex challenge in the legal and regulatory fields (3).

Drug repurposing has captivated the cancer research community due to the increasing demand for new anticancer drugs. Although there are several treatments, such as chemotherapy and targeted therapies, cancer is characterized by the eventual development of resistance or lack of response to these drugs and medications, making the design of new drugs against cancer a flourishing area of study. In this regard, drug repositioning is an attractive research area that has gained tremendous popularity. Oncology has taken advantage of existing, well-characterized, widely-used, non-cancer drugs and successfully tested them as anticancer agents (4–6). To get an overall picture, we searched the Medline (pubmed.ncbi.nlm.nih.gov) and ClinicalTrials.gov (clinicaltrials.gov) databases for

reports indexed under "cancer" and "drug repositioning". We found an evident increase the number of studies and clinical trials focused on drug repurposing in cancer since 2010 in both databases (**Figure 1** and **Supplementary File 1**) . Thus, we confirm that this strategy has yielded enriched oncology's vision for treatment of cancer patients. Use of drugs approved for diabetes and hypertension, such as metformin and statins, for cancer clearly exemplifies this. Statins improved the survival in lymphoma patients (4). Metformin improved survival in type 2 diabetic patients with ovarian cancer (5). Also, beneficial anticancer effects or metformin has been shown in breast cancer patients (6). On the other hand, hydralazine, which is a typical antihypertensive drug, is employed in metastatic cervical and ovary cancer phase III clinical trial (NCT00532818 and NCT00533299, respectively). Likewise, is employed in phase II clinical trial that including breast cancer patients (NCT00395655) and in solid tumors to overcome chemotherapy resistance (NCT00404508). However, the dark side of non-oncological drugs, i.e., without single-agent activity in cancer, carries therapeutic failure risk. For instance, in preclinical studies of chloroquine, a drug originally intended to prevent or treat malarial infections, as anticancer drug have shown positive therapeutic effect; nevertheless, parameters, doses, animal models and tumor types differ strongly between studies, complicating the interpretation of the results and highlighting the need for further clinical investigations (7).

Despite the efforts made, further research is still required to advance interventional approaches as well as to accelerate the introduction of drug repositioning in the clinic, improving treatments for cancer patients. The topic is vast, and the scientific community is focused on repositioning drugs to expand and improve cancer treatment. We appreciate the interest of the researchers to participate in this topic, in which ten manuscripts were collected (one original research and nine reviews). The research presented in this topic provide valuable information and insights on novel therapeutic options for cancer with existing drugs, facilitating their use in clinical practice. A short description of these manuscripts follows.

In their original research, Medina Jiménez and Monroy-Torres implemented an individualized nutritional intervention in cervical cancer patients treated simultaneously with radiotherapy. They reported the effect of personalized nutrition on the maintenance of muscle mass, weight, hemoglobin levels, and a decrease in gastrointestinal adverse effects, favoring the radiotherapy treatment outcome. Moreover, the authors suggested implementing an individualized nutritional intervention in cervical cancer patients treated with repurposing drugs to improve their efficacy and, therefore, the quality of life of oncology patients.

Martinez-Escobar et al., in their review, recommended the use of drug repurposing combined with CRISPR-dCas9-based artificial transcription factors (ATFs), as a viable alternative cancer treatment to reduce mortality. Strikingly, CRISPR-dCas9-based ATFs can manipulate DNA and modify target genes, activate tumor suppressor genes, silence oncogenes and tumor resistance mechanisms for targeted therapy. In cancer research, it is imperative to identify new drug combinations that generate synergistic effects and thereby achieve more efficient therapies.

Another strategy to treat cancer is nanomedicine, which is based on glycosylated nanoparticles (NPs). NPs can carry both cancer-targeting molecules and drugs and deliver them precisely, avoiding the severe side effects derived from nonspecific drug delivery in standard chemotherapy treatments. They bind to receptors overexpressed by tumor cells, such as lectin receptors, glucose transporters (GLUT), and glycosylated immune receptors of programmed cell death. In this regard, Torres-Pérez et al. reviewed crucial nanomedicine innovations to discover more specific cancer receptors and new glycan-based ligands or repurposed drugs against these receptors as potential opportunities for cancer therapy, prevention, pathological imaging, and theranostics.

Regarding drug resistance, Hu and Carraway discussed the role of cationic amphiphilic drugs such as antidepressants, antibiotics, antiarrhythmics, and diuretics to be repurposed to trigger lysosomal cell death (LCD) and lysosomal membrane

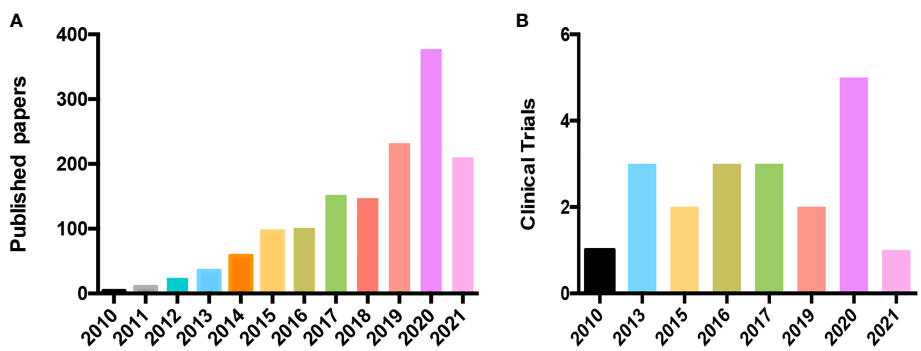

FIGURE 1 | Drug repositioning in cancer has increased its relevance and popularity in recent years. **(A)** Papers published in PubMed in the last decade **(B)** Clinical Trials of the last decade.

permeabilization (LMP) within therapy-resistant tumor cell populations in their review.

Repositioning molecular strategies to fight cancer are also being studied. Montaño-Samaniego et al. reviewed gene therapy targeted toward cancer- and tumor-specific promoters. The authors focused on cancer suppressors and suicide genes by employing diverse experimental strategies, such as prevention of tumor angiogenesis, gene silencing, and gene-editing technology. The authors concluded that emerging novel recombinant DNA technologies and gene therapies respond to the need for new treatments in cancer. Nevertheless, further studies are needed to analyze its use in combination with other therapies in clinical practice.

Montalvo-Casimiro et al. summarized the use of epidrugs, which are novel epigenetic regulators that present new therapeutic candidates against cancer. The development of epidrugs, such as 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine), Hydralazine, Vorinostat (SAHA), and Valproic acid, are proposed to enhance epigenetic therapy in cancer contributing to the development of precision medicine.

Recent advances in the application of computational molecular biology and bioinformatic approaches were discussed by Hernández-Lemus and Martinez-García. In their review, the authors emphasize the importance of the use of both high-throughput-omics data analyses and mining of extensive, well-annotated databases, which should be supplemented with experimental data and clinical validation. These interdisciplinary approaches represent a comprehensive methodology to combat some of the challenges during anticancer drug repurposing.

Cortés et al. centered their review on discussing the advantages of the knowledge of the cellular and molecular mechanisms of skin cancer, which have provided essential information for drug repurposing for this disease. The authors emphasize that the evidence from ongoing clinical trials in this regard is limited. Therefore, the authors invite researchers to expand on this topic and comment that the addition of nano-formulations could improve the efficacy of drugs to treat cancer; thus, this approach will allow repurposing known drugs to treat skin cancer.

On the other hand, Llaguno-Munive et al. provide a comprehensive review on glioma, the most common and aggressive primary tumor of the central nervous system. The authors discussed the repositioning of mifepristone, an antiprogestin, as an adjuvant drug to treat high-grade gliomas. Indeed, its effectiveness against cancer is already being analyzed in clinical trials. Also, the authors summarized previous findings that reported a synergistic action when mifepristone is combined with cisplatin or temozolomide plus radiation in cancer. Mifepristone is a repositioned drug that promises improved therapeutic efficiency and more prolonged patient survival.

Acute lymphoblastic leukemia (ALL) is known to present resistance to conventional treatment and glucocorticoids (GC). In their work, Olivas-Aguirre et al. reviewed the pharmacological strategies that reverse GC resistance. Among them, the repositioned drugs tigecycline, cannabidiol, tamoxifen, and some anthelmintics showed promising results. The authors proposed that these medications should be considered for inclusion in chemotherapeutic protocols to treat GC-resistant ALL.

Although establishing repositioned drugs appears to be a rapid strategy, several studies are undeniably required before their use in clinical practice, albeit with less time and financial resources. This topic showed that a broad range of therapeutic strategies, which span from bioinformatics analyses to cutting-edge molecular technologies, such as liposomes and CRISPR-Cas9, constitute powerful tools that are currently used in the clinic to assess the success of repositioned drugs in supplementing conventional cancer therapies.

AUTHOR CONTRIBUTIONS

AC-P conceived the topic. AC-P and CP-P wrote the original draft of this editorial. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Epidrug Repurposing: Discovering New Faces of Old Acquaintances in Cancer Therapy

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Gene mutations are strongly associated with tumor progression and are well known in cancer development. However, recently discovered epigenetic alterations have shown the potential to greatly influence tumoral response to therapy regimens. Such epigenetic alterations have proven to be dynamic, and thus could be restored. Due to their reversible nature, the promising opportunity to improve chemotherapy response using epigenetic therapy has arisen. Beyond helping to understand the biology of the disease, the use of modern clinical epigenetics is being incorporated into the management of the cancer patient. Potential epidrug candidates can be found through a process known as drug repositioning or repurposing, a promising strategy for the discovery of novel potential targets in already approved drugs. At present, novel epidrug candidates have been identified in preclinical studies and some others are currently being tested in clinical trials, ready to be repositioned. This epidrug repurposing could circumvent the classic paradigm where the main focus is the development of agents with one indication only, while giving patients lower cost therapies and a novel precision medical approach to optimize treatment efficacy and reduce toxicity. This review focuses on the main approved epidrugs, and their druggable targets, that are currently being used in cancer therapy. Also, we highlight the importance of epidrug repurposing by the rediscovery of known chemical entities that may enhance epigenetic therapy in cancer, contributing to the development of precision medicine in oncology.

Keywords: epidrugs, drug repurposing, cancer therapy, cancer, epigenetic inhibitors, epigenetics

INTRODUCTION

Since the turn of the century, epigenetics has become an important research area in human diseases study, where genetic mutations have been classically understood as the main cause in the development of human pathologies (1). The term epigenetics involves a wide variety of mechanisms that cells use to regulate the transcription of their DNA without changing its

genetic material (2). Whether an epigenetic modification has a facilitating or inhibiting role in the gene expression depends on the chemical nature of the mark that is placed over the chromatin, and the type of modification that is set down on the proximal environment of these genes (3). Thus, epigenetics shapes a regulatory complex that bridges the gap between genetic sequences and actionable mutations. Due to current knowledge about these epigenetic mechanisms, the importance of this regulatory system has become more evident and it has led to the understanding that epigenetic alterations are some of the main mechanisms underlying many human diseases such as cancer, which arises through aberrant genetic and epigenetic alterations, both of which have a key role in malignant transformation, tumor progression and prognosis (4).

Nowadays, it is known that as cancer progresses, there are genetic aberrations that make tumors highly prone to developing resistance to therapies (5). Emerging data on cancer-associated epigenetic alterations have shown that epigenetic modifications leading to drug resistance may be the cue for individual variation in chemotherapy response, having the potential to be reversible using epigenetic therapy (6). The possibility to reprogram the cancer epigenome is becoming a promising target therapy for both, treatment development and reversibility of drug resistance. Which focuses on the development of pharmacological compounds that can reprogram the epigenetic landscape to enhance chemotherapy response (7).

For a few years, the design of therapeutic strategies has been a growing field of query for single-target epigenetic drugs (epidrugs); however, the traditional epidrug discovery pathway is time-consuming and expensive (8, 9). Hence, a promising strategy for epidrug development is based on tracing novel potential epi-targets in previously approved drugs through a process called drug repositioning or repurposing (10, 11). Epidrug repurposing allows exploring a wide diversity of molecular combinations in multifactorial diseases such as cancer, where combinational epigenetic therapies are likely to be more effective than monotherapy to overcome chemotherapy resistance (9). This review focuses on the emerging area of epidrug repurposing, highlighting strategies to enhance cancer therapy. To further understand this, we will discuss the main mechanisms and elements involved in epigenetic alterations in cancer and its relevance in cancer therapy response.

Background in Epigenetics

Epigenetics is the term coined by Conrad Hal Waddington seventy-six years ago, to refer to the molecular mechanisms that may exert their influence on gene expression that do not involve alterations in its gene code. Through these, an organism can develop and adapt its phenotype to environmental changes (12). Over time, many definitions of Epigenetics have arisen (13); however, we can understand epigenetics as reversible chemical modifications of DNA and histone proteins (epimarks) that regulate specific functions in chromatin remodeling without altering the DNA sequence (14). Epimarks are associated with the transcription and function of a gene, that may change the cellular phenotype or its functional patterns in response to a particular context, across different developmental stages, cellular differentiation, or maintenance of tissue-specific cell lineages (15).

At the molecular level, epigenetic machinery is composed mainly of three interconnected components working synergistically in the chromatin organization levels, which include DNA methylation, histone post-translational modifications, and regulatory non-coding RNAs (ncRNAs) (14, 16). In the nucleus, chromatin can exist in two physical and functional states: heterochromatin (condensed chromatin), which is associated with transcriptional repression; and euchromatin (relaxed chromatin), associated with transcriptional activation (17) (**Figure 1**). The organizational states of the chromatin are highly regulated by epigenetic mechanisms involving nucleosome, which is the basic packaging unit of chromatin, composed by an octamer of histone proteins (two dimers of H2A-H2B and a tetramer of H3-H4 histones) (**Figure 1A**), that constitutes a compact structure with 147 base pairs of DNA turned almost twice around it (17, 18). N-terminal tails of histone proteins can acquire post-translational modifications through multiple mechanisms including phosphorylation, ubiquitination, methylation/demethylation, and acetylation, the latter being the most studied. Histone and direct DNA modifications constitute "the epigenetic code": an interplay between epigenetic factors and positive and negative feedback mechanisms that regulate it (18). Therefore, understanding the main mechanisms in the field of epigenetic research and their role in disease development is essential in its application in cancer therapy.

DNA Methylation

Methylation on DNA's cytosine is the most broadly studied epigenetic modification in humans. It encompasses a reaction defined as "the covalent transfer of a methyl group to the C-5 position of a cytosine ring of DNA" (15, 19). Generally, in mammals, DNA methylation occurs predominantly—but not exclusively—in the context of genomic regions called CpG islands, which are formed by clusters of CpG dinucleotides, and it's catalyzed by a group of enzymes called DNA methyltransferases (DNMTs). These enzymes transfer a methyl group from the donor molecule S-adenosylmethionine (SAM) to the fifth carbon of a cytosine residue to form 5-methylcytosine (5mC) (18, 19) (**Figure 1B**). This covalent modification is able to inhibit DNA transcription; either through the steric hindrance imposed by the methyl group which prevents transcription factors from binding DNA (18–20), or by the recruitment of proteins with methyl-CpG-binding domains (MBD). These proteins also contain domains able to recruit histone-modifying and chromatin-remodeling complexes to the methylated sites, forming repressor complexes that enhance the silencing state on that chromatin region (21). Three different DNMTs generate and maintain methylation patterns. DNMT1 is the methyltransferase enzyme specialized in the maintenance of previously placed methylation patterns, and DNMT3a & DNMT3b are instead involved in the establishment of *de novo* methylation patterns over DNA (18, 22, 23).

DNA methylation patterns occur in different regions of the genome. Alterations in these patterns lead to diseases (18). For

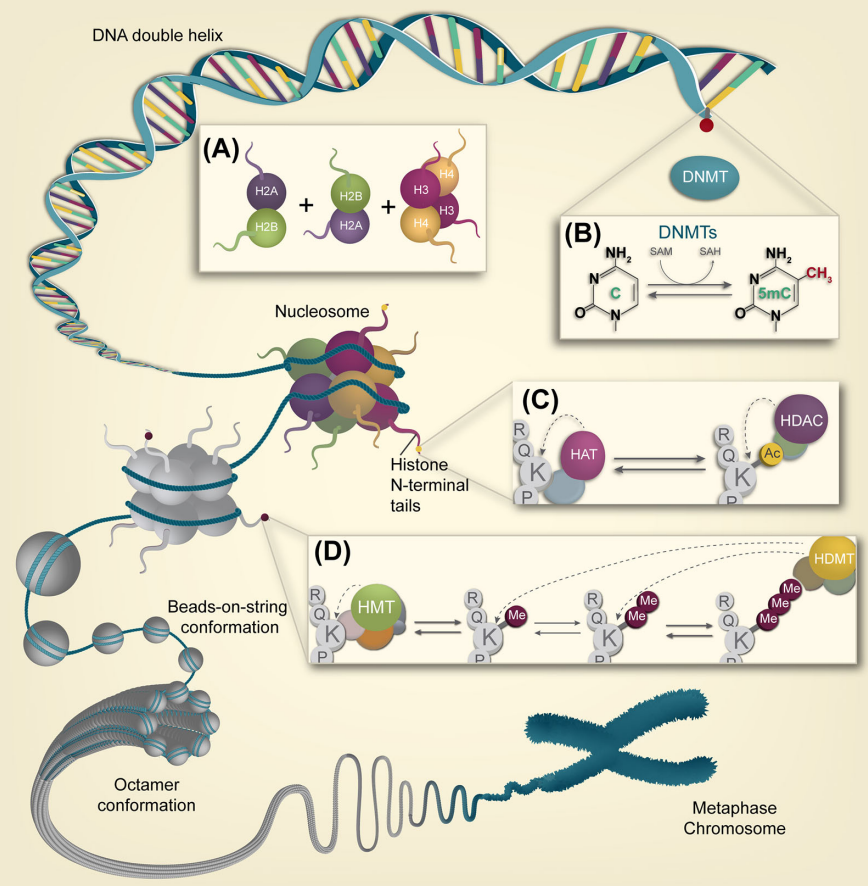

FIGURE 1 | Overview of the epigenetic landscape. Different compaction levels of chromatin are depicted, from naked DNA to the metaphasic chromosome. **(A)** Two dimers of H2A-H2B and a tetramer of H3-H4 histones are required for nucleosome assembly, the chromatin's basic packaging unit **(B)** DNA methylation is a process carried out by DMNTs in CpG dinucleotides, particularly on CpG islands. This dynamic epigenetic mark can be reversed by enzymatic conversion. **(C)** Histone acetylation is performed on lysine residues by HAT enzyme complexes. In contrast, histone lysine deacetylation is carried out by HDACs enzyme complexes. **(D)** Histone lysine methylation is carried out by HMT complexes. Lysines can be processively methylated from mono to di and trimethylation.

instance, gene promoters which are mainly embedded in CpG islands (70%) are normally unmethylated, thus allowing transcription. Aberrant hypermethylation patterns of these gene regulatory elements lead to transcriptional inactivation and are tumor-type specific as well as a common hallmark of cancer (9). Alternatively, during diseases, other alterations occur, like the demethylation of the gene body. Such alteration allows transcription to be initiated at several incorrect sites. In consequence, DNA hypomethylation at specific regions can activate the aberrant expression of genes, some of which could behave as proto-oncogenes (18). Finally, as aforementioned, alterations of hypermethylated patterns in repetitive sequences can promote the activation of transposable elements and chromosomal instability, both phenomena being also correlated with carcinogenesis and metastasis (6).

However, the reactions that lead to altered patterns of DNA methylation can potentially be reversible and restored through DNMT inhibitors (DNMTi: see below) that contain nucleoside derivatives and non-nucleoside analogs, some of them have been highly researched and shown promise in cancer therapies (24).

Histone Post-Translational Modifications

Another axis of the epigenetic machinery, closely associated with DNA methylation, are the covalent post-translational modifications of nucleosomal histones. Through the addition of chemical groups at specific sites within the amino- or carboxy-terminus of each histone, different functional consequences influencing chromosome structure can be elicited. Chromatin is functionally divided into actively transcribed euchromatin and transcriptionally inactive heterochromatin, which finally regulates the accessibility to genomic DNA and has a role in the control of gene expression (18, 25). The principal histone proteins modifications include methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, and ribosylation, from which methylation and acetylation are the most common and characterized, and generally occur in the proximity of promoter and enhancer genomic regions (26). Each histone

residue can undergo one or more modifications, which have different effects depending on which residue is modified, giving rise to crosstalk between the different marks, constituting "the histone code" altogether (18).

Multiple enzymes catalyze histone post-translational modifications with specific catalytic activity based on each histone tail's amino acids that can act as their substrates. Most of these modifications are reversible. There are specialized enzymes that can remove each type of covalent modification. Histone acetyltransferases (HATs) and deacetylases (HDACs) control acetylation, as well as histone methyltransferases (HMTs) and demethylases (HDMs) coordinate histone methylation. Acetylation and deacetylation of histones are among the most studied reversible, followed by methylation and demethylation of histone lysines (17, 27).

Due to the importance of histone epimarks in gene regulation and cellular function, aberrant histone post-translational modifications may change gene expression patterns and cause human pathologies (6). Thus, it is of great importance to understand the reversible nature of these marks as an advantageous alternative for the treatment of diseases where epigenome deregulation is one of the hallmarks.

Histone Acetylation and Deacetylation

Histone acetylation has a key role in many biological processes (cell cycle regulation, alternative splicing, nuclear transport, among others) (28). It can promote relaxed states of the chromatin (euchromatin) and favor gene transcription, while deacetylation exerts the opposite effect, generating heterochromatin domains that can inhibit transcription (2). Two families of enzymes with reverse functions control the feedback regulation between acetylation/deacetylation of histones: histones acetyltransferases (HATs or KATs) and histones deacetylases (HDACs) (2). HATs catalyze the transfer of acetyl groups to lysine-amino-terminal residues using acetyl-CoA as a donor; this reaction neutralizes the positive charge of the Lys (17, 29) (**Figure 1C**). As a result, the interaction between the histone and the DNA is weakened, forming an opening domain in chromatin, leading to exposure of DNA sequences and their transcription (2, 28). HATs are divided in three families based on their catalytic domain's functional and structural identity, which bears the acetyltransferase activity for the recognition of acetyl-lysine residues (17). Several HATs associate with other protein complexes and subunits to selectively modify the different histones; however, p300/CBP is probably the most extensively studied HAT, since it is capable of acetylating all four histones along with many other coactivator or corepressor transcriptional complexes (30).

In contrast, HDACs remove acetyl groups from lysine residues through different reactions that reestablish the positive charges on histone tails, increasing its interaction with DNA and stabilizing the chromatin in place (2, 28) (**Figure 1C**). The histone deacetylase family includes 18 members (31), divided into two groups based on their enzymatic activity: Zn^{2+}-dependent enzymes, which include classes I, II, and IV HDACs, exert their function through hydrolytic catalysis; and NAD+ cofactor-dependent enzymes, that include class III sirtuins (SIRTs), with a catalytic mechanism of nucleophilic substitution for histone deacetylation (28).

Both HATs and HDACs play a key role in the maintenance and regulation of chromatin accessibility, leading gene expression regulation, among other mechanisms. Histone acetylation global imbalance is one of the prominent alterations in the diseased state and a hallmark of many tumor types, where HDACs have been found overexpressed (32) or mutated (33). Additionally, abnormal genomic events such as translocations, mutations, or deletions in HAT- and acetylation readers-related genes may occur during cancer development (18). As a result, aberrant acetylation-related proteins contribute to the progression of the disease. For instance, germline mutations and overexpression of HDACs have been observed in various cancers, resulting in a global loss of histone acetylation and the consequent silencing of tumor suppressor genes (34). Also, it has been observed that reduced lysine 16 acetylation (H4K16ac), as well as the loss of acetylation of histone 3 (H3ac) are also hallmarks of human cancer (35, 36). Furthermore, HATs and HDACs are targeted to transcriptionally-active genes by phosphorylated RNA polymerase II through the recruitment of effector proteins with specialized reader domains (18), suggesting that the mechanistic switch between acetylation/deacetylation can be manipulated and restored by specific drugs inhibiting key enzymes by targeting their catalytic reaction (HATi and HDACi; see below).

Histone Methylation and Demethylation

Histone methylation occurs on arginine (R) and lysine (K) residues, and it is catalyzed by HMTs (or KMTs and RMTs) that use S-adenosyl-l-methionine (SAM) as a methyl donor group (**Figure 1C**). Lysine methyltransferases are divided into two broad groups based on the presence or the absence of a SET domain (Su(var)3-9, Enhancer-of-zeste, and Trithorax): SET-domain containing methyltransferase family and DOT1-domain lysine N-methyltransferase (37, 38).

KMTs can transfer three methyl groups onto lysine residues, prompting mono, di, and, trimethylation (me1, me2 and, me3 respectively) (17) (**Figure 1D**). The association of an active or repressive transcriptional state depends on the number of methyl groups and in the position of the lysine residue in the histone amino acid sequence. A repressed chromatin state (heterochromatin, constitutive, or facultative), correlates with methylation of H3K9me2,3, H3K27me2,3, and H4K20me3, while methylation of H3K4me2,3, H3K9me1, H3K27me1, H3K20me1, and H3K36me1 are associated with transcriptionally active chromatin (euchromatin) (17, 39). Besides, histone methylation also has an important role in DNA repair, DNA replication, alternative splicing, and chromosome condensation (18). Histone demethylases HDMs (or KDMs) can revert these modifications (**Figure 1D**), divided into two different families with distinct enzymatic mechanisms: KDM1A/LSD1 amine oxidase family, dependent on flavin adenine dinucleotide (FAD) as a cofactor; and the KDM2A/B dioxygenase family, which contain a Jumonji C (JmjC) domain and are iron Fe (II) and α-ketoglutarate-dependent to accomplish histone demethylation through methyl groups oxidation (40). The readers of methylated lysine residues

consist of various proteins with specialized domains that can recognize these modifications (17).

Besides the global loss of acetylation and DNA hypomethylation, the deregulation of histone methylation/demethylation can lead to chromosome instability (18). It has been suggested that the aberrant expression of both histone methyltransferases and demethylases genes is the main cause of an altered distribution of histone methylation marks. Deregulation of histone methylation patterns can become a driver for mutations in many types of tumors (15). For instance, cancer cells have a global loss of activation marks, such as H4K20me3; along with a gain of methylation in repressive marks, such as H3K9me and H3K27me, as well as the monomethylation of H3K4me (35, 36) which are associated with DNA hypermethylation of silenced genes. The basal patterns of histone methylation are essential for establishing a permissive euchromatic state, allowing the expression of tumor suppressor genes. Therefore, its alteration results in the repression of some of these genes and oncogene aberrant expression (18, 35). Instead, instability of the methylation/demethylation mechanistic switch can promote proliferation and neoplastic transformation in several cancer types (41–43).

Epigenetic Alterations in Cancer and Cancer Therapy

As mentioned before, the cancer epigenome is characterized by global changes in DNA methylation, disruptions in histone posttranslational modification patterns, and alterations of normal chromatin-modifying enzymes expression (18, 36) (**Figure 2**) [see review (44)]. Accordingly, these changes can promote the disruption of cellular homeostasis in precancerous cells through the deregulation of genes implicated in cancer initiation and progression (4); for instance, those genes associated with apoptosis resistance, proliferation, invasive potential, and genomic instability, as well as genes correlated to therapeutic response (45, 46). Thus, the relationship between genetic disruptions and epigenetic abnormalities are mutually beneficial in order to drive cancer development and could be playing a key role in individual differences displayed by patients in the way they respond to therapies in both toxicity or treatment efficacy (15, 46, 47). Multiple studies demonstrate that reversing epigenetic patterns through *de novo* epidrugs and epidrug repurposing can resensitize cancer cells to chemotherapy (48–50).

Principles of Epigenetic Therapy

Increasing understanding of epigenetic mechanisms and their importance in disease has led to the development of therapeutic interventions targeting epigenetic modulatory mechanisms. Due to the chemical reversibility nature of DNA methylation and histone posttranslational modifications, epigenetic proteins can be druggable targets by means of small-enzymatic inhibitors that aim for the restoration of the aberrant epigenetic machinery and hold the potential for reverting epigenetic signatures in cancer (14).

Epigenetic drugs (epidrugs) are chemical agents that modify the structure of DNA and chromatin, facilitating disruption of transcriptional and post-transcription changes, primarily by controlling the enzymes required for their establishment and maintenance, reactivating the tumor suppressor and DNA repair genes that are epigenetically silenced (51). Lately, epigenetic therapy has taken relevance in the field of oncology, where epidrugs have been successfully used in treatment, mostly in combination with standard chemotherapy (52).

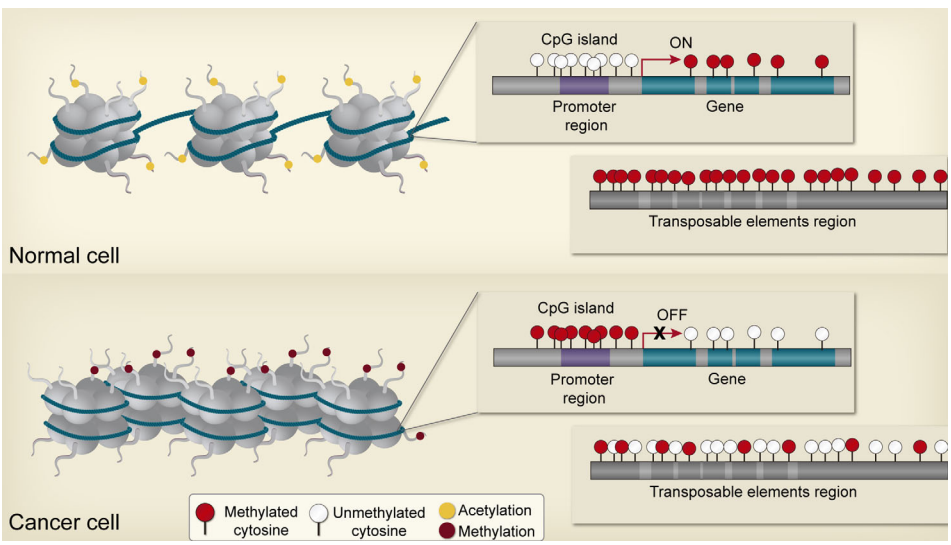

FIGURE 2 | Epigenetic alterations in cancer cells. In non-neoplasic cells, CpG islands of tumor suppressor gene promoters are generally unmethylated and acetylated, resulting in transcriptional activation and expression. In contrast, non-coding regions and repetitive elements are hypermethylated, ensuring chromosome stability. Gene bodies are normally methylated, enhancing transcription. Neoplasic cells are characterized by global hypomethylation and local CpG island hypermethylation, especially at tumor suppressor gene promoters, resulting in aberrant transcription and genomic instability.

Epidrugs (with one-target, as well as repurposed epidrugs; see below) that are designed based on these principles can exert direct cytotoxic effects over malignant cells (14, 46), function as sensitizers in complementary therapies (53, 54), or can be used to overcome epigenetically-acquired drug resistance against the limits of chemotherapy efficacy, as there are the dynamic associations between epigenetic pattern changes and resistance to therapeutic regimes for cancer (50, 52, 55). New epidrugs compounds are continually being tested for cytotoxicity, pharmacological parameters, and a better understanding of their mode of action; in both preclinical research (*in vitro* and *in vivo*) as well as in clinical trials. Epigenetics therapy is enhanced by a combination of laboratory and clinical data. The US Food and Drug Administration (FDA) has approved many epigenetic treatments and used them for treating cancer (6).

Epidrug Generations

Historically, molecules designed to inhibit the catalytic function of epigenetic factors have not only resulted in the reduction of the targeted enzymatic activity but also the appearance of indirect modifications of the transcription of large gene sets (56). Several epigenetic protein families have similar cofactors and co-substrates, similar epidrugs could target several epigenetic protein families. Some compounds can inhibit the functionality of a whole family of epigenetic proteins (**Table 1**).

The quest for finding epigenetic inhibitors led to the **first generation of epidrugs,** characterized by a meager degree of selectivity (57). Epidrugs of the first generation include DNMTi and HDACi, some of which have already been approved to treat hematological malignancies (58). DNMTi are pyrimidine analogs incorporated into DNA during replication and form covalent DNA adducts that cause DNA damage response activation and eventually lead to apoptosis. This was not without cytotoxic implications (3, 59). On the other hand, first generation HDACi are molecules that inhibit the Zn^{2+} dependent HDAC enzymes, except for sirtuin inhibitors, which inhibit a specific class of histone deacetylases that depend on NAD+ to perform their catalytic activity (59).

First-generation inhibitors represented many undesirable pharmacokinetic properties and poor target selectivity, resulting in the need for the creation of **second-generation epidrugs**, which included DNMTi (such as zebularine and guadecitabine), and HDACi (including hydroxamic acid, belinostat and panobinostat, tucidinostat and valproic acid) with improved physiological properties (59).

The second generation of epidrugs was characterized by strong academic research accompanied by industrial drug discovery to find molecules that resembled first generation epidrugs. The hypothesis was that molecules with more potent inhibitor action and fewer side-effects could be found. Another thing to consider was pharmacokinetics: first generation epidrugs had poor bioavailability, were more active within non pH physiological ranges, and were targets of cellular deaminases, which ultimately meant a short half-life for these compounds.

Ultimately, the **third generation of epidrugs** reflected that epigenetic factors could write, delete, or read epigenetic marks in the form of protein complexes. Therefore, a deeper understanding of epigenetic protein's interactome is essential for the design of highly selective epidrugs (57). Epi-drugs of third generation includes, among others, histone methyltransferase inhibitors (HMTi), histone demethylase inhibitors (HDMi), and bromodomain and extra-terminal domain inhibitors (BETi) (59).

DNMT Inhibitors

DNA methylation inhibitors intercalate between DNA base pairs and suppress the CpG dinucleotide's methylation, especially important at CpG islands. These inhibitors can be classified as DNMTi nucleoside analogs and non-nucleoside analogs (60) (**Figure 3**). DNMTi cytidine analogs are usually chemically unstable, and because of their similarity to cytidine, DNA and RNA polymerases identify both compounds and add them into growing nucleic acid chains, therefore hampering their selectivity (61).

Since the first DNMTi discovery (azacytidine), the number of inhibitors of DNMT has increased exponentially. The CHEMBL database reports 841 compounds tested for DNMT1 inhibition (CHEMBL1993), 258 compounds for DNMT3A (CHEMBL1992), and 80 compounds for DNMT3B (CHEMBL6095) (62) (**Table 1**, DNMTi section).

Among azacytidine derivatives, 5-aza-2'-deoxycytidine gained importance in the clinic, commonly known as "Decitabine". Decitabine contains DNA sugar deoxyribose and is only integrated into DNA, while azacytidine allows for both RNA and DNA incorporation (14). Of note, Azacitidine and decitabine have both the same action mechanism. They both behave as a suicide substrate, trapping DNMTs after metabolic conversion and incorporation into DNA (3).

Guadecitabine is a hypomethylating agent of the second generation whose active metabolite is decitabine. Guadecitabine holds an amazing property: it is not a cytidine deaminase substrate, thus improving its selectivity. This drug has shown promise in treatments and recently tested in a Phase II clinical trial for treating non-intensive chemotherapy candidates with AML (63).

In 2004, azacytidine became the first medication approved by the FDA for all stages of myelodysplastic syndrome, a bone marrow disorder with a high risk of AML progression, characterized by irregular blood cell development, followed by decitabine in 2006 (64). These two drugs are currently used as first-line MDS therapy when other therapies are insufficient (14) (**Table 2**, DNMTi section).

As mentioned before, DNMTs have two substrates, the methyl group donor cofactor SAM and the methylated cytosine. Non-nucleoside DNMTi includes analogs of the methyl donor S-adenosyl-L-methionine (SAM) and small molecules that interact with the active site of the enzyme DNMT (**Figure 3**). Indeed, it is possible to obtain potent DNMT inhibitors by designing substrate analogs and connecting them (65). This strategy has resulted in the most effective way to inhibit DNMTs and reactivate genes in cancer cells by promoting demethylation (60). Many forms of these derivatives have shown remarkable results in many models of cancer and other human diseases. These include hydralazine,

TABLE 1 | Current inhibition assays performed for different epigenetic factors.

Type of inhibitor		Epigenetic Factor	Acronym	CHEMBL ID	Inhibitor molecules
DNMTi		DNA (cytosine-5)-methyltransferase 1	DNMT1	CHEMBL1993	841
		DNA (cytosine-5)-methyltransferase 3A	DNMT3A	CHEMBL1992	258
		DNA (cytosine-5)-methyltransferase 3B	DNMT3B	CHEMBL6095	80
HDACi	**HDACi (Zn dependent)**	Histone deacetylase 1	HDAC1	CHEMBL325	6434
		Histone deacetylase 6	HDAC6	CHEMBL1865	4701
		Histone deacetylase 8	HDAC8	CHEMBL3192	2420
		Histone deacetylase 3	HDAC3	CHEMBL1829	2043
		Histone deacetylase 2	HDAC2	CHEMBL1937	2003
		Histone deacetylase 4	HDAC4	CHEMBL3524	1279
		Histone deacetylase 7	HDAC7	CHEMBL2716	521
		Histone deacetylase 11	HDAC11	CHEMBL3310	503
		Histone deacetylase 5	HDAC5	CHEMBL2563	460
		Histone deacetylase 10	HDAC10	CHEMBL5103	419
		Histone deacetylase 9	HDAC9	CHEMBL4145	348
	SIRTi (NAD+ dependent)	NAD-dependent deacetylase sirtuin 1	SIRT 1	CHEMBL4506	2073
		NAD-dependent deacetylase sirtuin 2	SIRT 2	CHEMBL4462	2839
		NAD-dependent deacetylase sirtuin 3	SIRT 3	CHEMBL4461	634
		NAD-dependent deacetylase sirtuin 5	SIRT 5	CHEMBL2163183	250
		NAD-dependent deacetylase sirtuin 6	SIRT 6	CHEMBL2163182	221
		NAD-dependent deacetylase sirtuin 7	SIRT 7	CHEMBL2163184	10
HMTi	**KMTi**	Histone-lysine N-methyltransferase, H3 lysine-9 specific 5	KMT1D	CHEMBL6031	238
		Histone-lysine N-methyltransferase, H3 lysine-9 specific 3	G9A	CHEMBL6032	92523
		Histone-lysine N-methyltransferase MLL	MLL1	CHEMBL1293299	17219
		Histone-lysine N-methyltransferase EZH2	EZH2	CHEMBL2189110	1243
		Histone-lysine N-methyltransferase, H3 lysine-79 specific	DOT1L	CHEMBL1795117	344
		Histone-lysine N-methyltransferase SETD7	SETD7	CHEMBL5523	204
		Histone-lysine N-lysine methyltransferase SETD8	SETD8	CHEMBL1795176	98
		Histone-lysine N-lysine methyltransferase SMYD2	SMYD2	CHEMBL2169716	84
		Histone-lysine N-methyltransferase SMYD3	SMYD3	CHEMBL2321643	54
		Histone-lysine N-methyltransferase SUV39H1	SMYD2	CHEMBL2169716	84
		Histone-lysine N-methyltransferase EZH1	EZH1	CHEMBL2189116	32
		Histone-lysine N-methyltransferase SUV39H2	SUV39H2	CHEMBL1795177	21
		Histone-lysine N-methyltransferase NSD2	NSD2	CHEMBL3108645	20
		Histone-lysine N-methyltransferase SETDB1	SETDB1	CHEMBL2321646	14
		Histone-lysine N-methyltransferase SUV420H2	SUV420H2	CHEMBL2321644	12
		Histone-lysine N-methyltransferase SETD2	SETD2	CHEMBL3108647	11
		Histone-lysine N-methyltransferase, H3 lysine-36 and H4 lysine-20 specific	NSD1	CHEMBL3588738	10
		Histone-lysine N-methyltransferase PRDM9	PRDM9	CHEMBL3588737	10
		Histone-lysine N-methyltransferase SUV420H1	SUV420H1	CHEMBL2321645	9
		Histone-lysine N-methyltransferase MLL3	MLL3	CHEMBL2189113	7
		Histone-lysine N-methyltransferase NSD3	NSD3	CHEMBL3108646	7
		Histone-lysine N-methyltransferase ASH1L	ASH1L	CHEMBL3588739	6
		Histone-lysine N-methyltransferase SETMAR	SETMAR	CHEMBL2189111	3
		Histone-lysine N-methyltransferase MLL2	MLL2	CHEMBL2189114	2
		Histone-lysine N-methyltransferase MLL4	MLL4	CHEMBL2189112	2
		Histone-lysine N-methyltransferase SETD1B	SET1B	CHEMBL4105837	1
		Histone-lysine N-methyltransferase SETD1A	SETD1A	CHEMBL4105954	1
	RMTi	Histone-arginine methyltransferase CARM1	CARM1	CHEMBL5406	201
		Protein-arginine N-methyltransferase 1	PRMT1	CHEMBL5524	528
		Protein arginine N-methyltransferase 6	PRMT6	CHEMBL1275221	139
		Protein arginine N-methyltransferase 3	PRMT3	CHEMBL5891	138
		Protein arginine N-methyltransferase 5	PRMT5	CHEMBL1795116	91
		Protein arginine N-methyltransferase 7	PRMT7	CHEMBL3562175	25
HDMi	**JmjC**	Probable JmjC domain-containing histone demethylation protein 2C	JHDM2C	CHEMBL3792271	1
		Histone lysine demethylase PHF8	PHF8	CHEMBL1938212	136
		Lysine-specific demethylase 2A	KDM2A	CHEMBL1938210	128
		Lysine-specific demethylase 2B	KDM2B	CHEMBL3779760	333
		Lysine-specific demethylase 3A	KDM3A	CHEMBL1938209	87
		Lysine-specific demethylase 3B	KDM3B	CHEMBL3784906	9
		Lysine-specific demethylase 4A	KDM4A	CHEMBL5896	51948
		Lysine-specific demethylase 4B	KDM4B	CHEMBL3313832	73

(Continued)

TABLE 1 | Continued

Type of inhibitor		Epigenetic Factor	Acronym	CHEMBL ID	Inhibitor molecules
		Lysine-specific demethylase 4C	KDM4C	CHEMBL6175	878
		Lysine-specific demethylase 4D	KDM4D	CHEMBL6138	53
		Lysine-specific demethylase 4D-like	KDM4E	CHEMBL1293226	110
		Lysine-specific demethylase 5A	KDM5A	CHEMBL2424504	621
		Lysine-specific demethylase 5B	KDM5B	CHEMBL3774295	469
		Lysine-specific demethylase 5C	KDM5C	CHEMBL2163176	147
		Lysine-specific demethylase 6A	KDM6A	CHEMBL2069164	29
		Lysine-specific demethylase 6B	KDM6B	CHEMBL1938211	203
		Lysine-specific demethylase 7	KDM7A	CHEMBL2163177	35
	LSD	Lysine-specific histone demethylase 1	KDM1A	CHEMBL6136	1710
		Lysine-specific histone demethylase 1B	KDM1B	CHEMBL1938208	62
BETi	**Bromo and Extra terminal Domain**	Bromodomain-containing protein 1	BRD1	CHEMBL2176774	121
		Bromodomain-containing protein 2	BRD2	CHEMBL1293289	570
		Bromodomain-containing protein 3	BRD3	CHEMBL1795186	474
		Bromodomain-containing protein 4	BRD4	CHEMBL1163125	4864
		Bromodomain testis-specific protein	BRDT	CHEMBL1795185	119

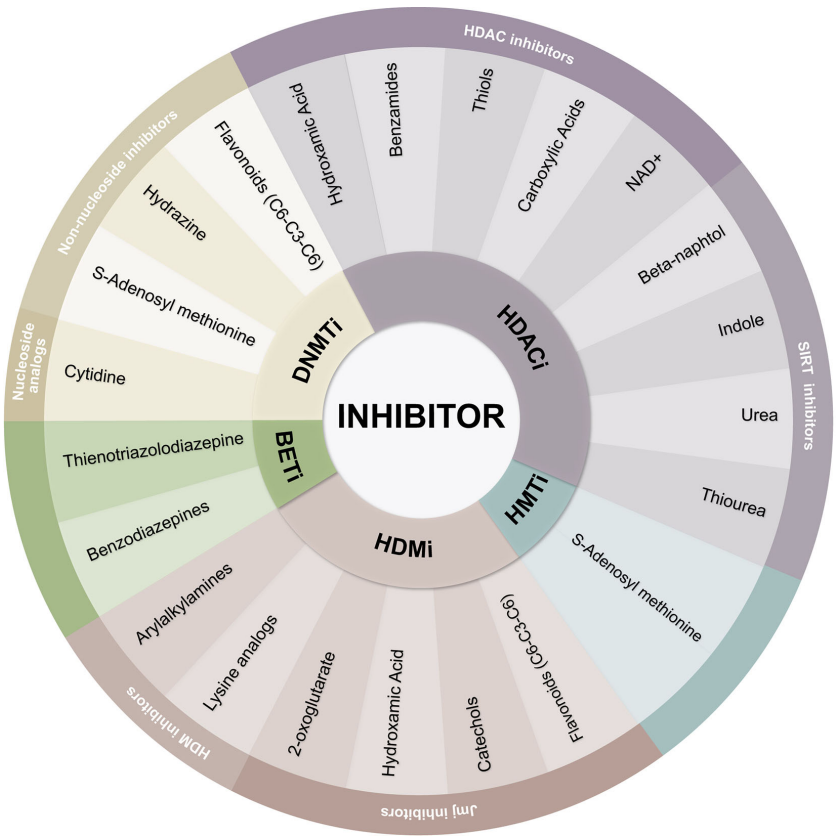

FIGURE 3 | Classification of epigenetic inhibitors. Epigenetic inhibitors are classified as DNMTi, HDACi, HMTi, HDMI, and BETi. The chemical nature of each inhibitor defines the affinity of its targets.

EGCG, RG108, MG98, and disulfiram (66–71) (**Table 2**, DNMTi section). MG98 is a second-generation phosphorothioate antisense oligodeoxynucleotide that inhibits translation effects of DNMT1 mRNA but has no apparent impact on tumors (72).

Despite preclinical evidence indicating a potentiating chemotherapy cytotoxic activity of HDAC inhibitors and DNMT inhibitors, clinical outcomes have been discouraging: three of the five main combination randomized trials were

TABLE 2 | Overview of epigenetic inhibitors currently in clinical trials for cancer therapies.

Inhibitor	Mechanism of Action	Functional Molecule or Chemical Group	Examples	CAS	Clinical Trials				
					Phase Studies				Conditions
					I	II	III	IV	
DNMTi	Nucleoside analogs: Cytidine analogs incorporate into DNA instead of cytidine, covalently linking the enzyme and leading to DNMT degradation	Cytidine	Azacytidine	320-67-2	272	350	58	7	MDS, CML, AML, glioma, prostate cancer, pancreatic cancer, ovarian cancer, metastatic melanoma.
			Decitabine	2353-33-5	189	240	51	7	CML, AML, MDS, prostate cancer, thyroid cancer.
			Guadecitabine	929901-49-5	15	23	3	0	AML, MDS, HCC, CMML, ovarian cancer, urothelial carcinoma, colorectal cancer, peritoneal cancer
			5-fluoro-2'-deoxycytidine	10356-76-0	3	1	0	0	AML, MDS, Head and Neck Neoplasms, Lung Neoplasms, Urinary Bladder Neoplasms, Breast Neoplasms
			4'-thio-2'-deoxycytidine	134111-30-1	2	0	0	0	Currently establishing the safety, tolerability, and MTD in patients with refractory solid tumors.
	Non-nucleoside inhibitors either block the DNMTs enzyme catalytic site, interact with enzyme recognition of target sequences or are SAM cofactor competitors.	S-Adenosyl methionine	Sinefungin	58944-73-3	0	0	0	0	NA
		Hydrazine	Hydralazine	86-54-4	6	16	13	12	ovarian cancer, cervical cancer, refractory solid tumors, breast cancer.
		Flavonoids (C6-C3-C6)	Epigallocatechin-3-gallate	989-51-5	18	44	14	3	Adenocarcinoma of the prostate, head and neck cancer, colon cancer, pancreatic cancer, breast cancer, lung cancer, bladder cancer, colorectal cancer, prostate cancer.
HDACi	HDACi are molecules capable of Zinc trapping that bind to the zinc-containing catalytic domain of HDACs and supress their deacetylase enzymatic activity	Hydroxamic Acid	Vorinostat	149647-78-9	165	149	9	0	Rhabdomyosarcoma, Leiomyosarcoma, Lymphoma, melanoma, Lung carcinoma, lung cancer, head and neck cancer, leukemia, breast cancer, MDS, ovarian cancer, glioblastoma, pancreatic cancer, breast cancer.
			Trichostatin A	58880-19-6	1	0	0	0	Relapsed or Refractory Hematologic Malignancies
			Belinostat	866323-14-0	32	25	0	0	MDS, Non-Hodgkin lymphona, mantle cell lymphoma, diffuse large B-cell lymphoma, breast cancer, ovarian cancer, lung cancer, glioblastoma, AML, ATLL, bladder cancer, liver cancer,
			Panobinostat	404950-80-7	87	78	7	1	AML, MDS, lung cancer, gliosarcoma, prostate cancer, multiple myeloma, CMML, breast cancer, pancreatic cancer.
			dacinostat	404951-53-7	0	0	0	0	NA
			givinostat	497833-27-9	5	15	2	0	chronic lymphocytic leukemia, multiple myeloma, hodgkin's lymphoma.
		Benzamides	Entinostat	209783-80-2	40	37	2	0	breast cancer, prostate adenocarcinoma, renal cell carcinoma, lymphoma, MDS, melanoma, lung cancer, AML, colorectal cancer, pancreatic cancer
			mocetinostat	726169-73-9	14	15	0	0	urothelial carcinoma, Hodgkin lymphoma, Head and Neck cancer, MDS, lung cancer, melanoma.
		Thiols	Romidepsin	128517-07-7	55	57	5	0	T cell lymphoma, glioma, multiple myeloma, CTCL, leukemia, astrocytoma, pancreatic cancer, lung cancer, thyroid cancer, prostate cancer, male breast cancer, renal cancer, bladder cancer.
		Carboxylic Acids	Valproic acid	1069-66-5	85	115	90	89	AML, MDS, Head and Neck cancer, SCC, glioma, bladder cancer, sarcoma, glioblastoma, leukemia, breast cancer, lung cancer.
			Butyric Acid	107-92-6	1	3	2	0	schyzofrenic disorders

(Continued)

TABLE 2 | Continued

Inhibitor	Mechanism of Action	Functional Molecule or Chemical Group	Examples	CAS	Clinical Trials				
					Phase Studies				Conditions
					I	II	III	IV	
			Phenylbutiric Acid	1821-12-1	20	30	3	2	colon cancer, leukemia, gastric cancer, MDS.
			Pivanex	122110-53-6	1	3	0	0	melanoma, lung cancer, leukemia.
	SIRTi are small molecules, many of them recently discovered by cell-based screening assays, with multiple inhibition mechanisms including reactivity with chemical intermediates, non-competitive inhibition with substrate and uncompetitive inhibition with NAD+.	NAD+	Nicotin	54-11-5	0	0	0	0	NA
		beta-naphtol	sirtinol	410536-97-9	0	0	0	0	NA
			splitomicin	1384339	0	0	0	0	NA
			salermide	1105698-15-4	0	0	0	0	NA
			cambinol	14513-15-6	0	0	0	0	NA
		indole	EX-527	49843-98-3	0	1	0	0	Endometriosis
			oxyndole	59-48-3	0	0	0	0	NA
		urea	suramin	129-46-4	8	12	3	0	lung cancer, breast cancer, adrenocortical carcinoma, renal cancer, prostate cancer, bladder cancer, multiple myeloma, head and neck cancer.
		thiourea	tenovin	380315-80-0	0	0	0	0	NA
HMTi	HKMTi are SAM like molecules and molecules that directly inhibits the enzyme S-adenosyl-L-homocysteine hydrolase or interact with the cofactor binding pocket of KMTs	S-Adenosyl methionine	Sinefungin	58944-73-3	0	0	0	0	NA
			EPZ004777	1338466-77-5	0	0	0	0	NA
			EPZ-5676	1380288-87-8	4	2	0	0	AML, MDS, leukemia
			EPZ004777	1338466-77-5	0	0	0	0	NA
			Valemetostat	1809336-39-7	1	1	0	0	leukemia, lymphoma, prostate cancer, renal cancer.
			tazemetostat	1403254-99-8	11	10	2	0	B cell lymphoma, prostate cancer, mesothelioma, Non Hodgkin lymphoma, tissue sarcoma, Bladder cancer, sinonasal carcinoma, follicular lymphoma.
	Most HRMT inhibitors are molecules which occupy and inhibit the SAM pocket, the substrate pocket, or both.	S-Adenosyl methionine	GSK3326595	1616392-22-3	2	0	0	0	neoplasms
HDMi	HDM inhbitors are molecules that inhibit monomine oxidases family of enzymes or that are substrate mimics (lysine analogs).	Arylalkylamines	Phenelzine	51-71-8	4	2	0	0	breast cancer, prostate cancer.
			Tranylcypromine	155-09-9	6	3	1	3	AML, MDS
			Pargyline	306-07-0	0	0	0	0	NA
		Lysine analogs	propylhydrazine	5039-61-2	0	0	0	0	NA
	JmjC inhibitors are derivates of 2OG, hydroxamic acids, catechols and flavonoids.	2-oxoglutarate	N-oxalylglicine	5262-39-5	0	0	0	0	NA
		Hydroxamic Acid	Methylstat	1310877-95-2	0	0	0	0	NA
		Catechols	Hematoxylin	517-28-2	0	0	0	0	NA
			Caffeic acid	331-39-5	3	1	3	1	esophagus cancer
		Flavonoids (C6-C3-C6)	Myricetin	529-44-2	0	0	0	0	NA
			Baicalein	491-67-8	0	2	0	0	Influenza
			Epigallocatechin-3-gallate	989-51-5	18	44	14	3	Adenocarcinoma of the prostate, head and neck cancer, colon cancer, pancreatic cancer, breast cancer, lung cancer, bladder cancer, colorectal cancer, prostate cancer.
BETi	BET inhibitors are derivates of benzodiazepines that take up the hydrophobic región of BET enzymes which binds acetylated lysines.	Thienotriazolodiazepines	JQ1	1268524-70-4	0	0	0	0	NA
			CPI-203	1446144-04-2	0	0	0	0	NA
			OTX015	202590-98-5	5	2	0	0	AML, glioblastoma, breast cancer, lung cancer, prostate cancer.
		Benzodiazepines	CPI-0610	1380087-89-7	3	2	0	0	Myeloma, lymphoma, leukemia, MDS.
			Molibresib	1260907-17-2	2	1	0	0	lymphoma, NUT carcinoma,

stopped because of ineffectiveness or disadvantaged toxicity profiles compared to chemotherapy alone (59). The possible role of DNMT inhibitors remains unclear, but in conjunction with other therapies, these agents may theoretically still be of use.

There is a good scientific justification for combining DNMT inhibitors with HDAC inhibitors since both hypermethylated DNA and hypoacetylated histones are associated with closed chromatin states that repress gene expression by independent mechanisms. Further studies should be carried out into the efficacy of this combination at different dosages and durations of treatment. To date, hundreds of clinical trials have studied the effects of anti-DNA methylation therapy on different cancers.

HDAC Inhibitors

The development of the first HDACi commenced with the finding that erythroleukemia murine cells differentiated in the presence of dimethyl sulfoxide (DMSO). Later, chemical analogs that could make similar interactions as DMSO were studied (56). This was the case of vorinostat (SAHA), a molecule capable of metal coordination and hydrogen bonding. Interestingly, natural compounds inhibitors of HDACs (trichostatin A and trapoxin A) were found to chemically resemble vorinostat at the hydroxamic acid moiety. The mechanism of action of these compounds inhibits HDACs by reversibly binding to Zn^{2+} in the enzyme's active site. Since the discovery of vorinostat, a lot of new activity assays are performed every day with inhibitor compounds (62) (**Table 1**, HDACi section).

Zinc binding is essential for the inactivation of most HDACs (56). As mentioned before, the Zn-binding hydroxamic moiety has proven to be one of the most successful inhibitors, and thousands of synthetic HDAC inhibitors with this moiety have been reported. Many of these inhibitors have focused primarily on optimizing the pharmacokinetics of vorinostat and trichostatin A (**Figure 3**; **Table 2**, HDACi section).

Currently, vorinostat therapy clinical applications have been applied to neurological conditions and, surprisingly, to reactivating chronic viral infection (73). Therapies for HIV-1 patients do not kill the virus entirely because it may be latent in reservoirs of CD4 + cells (74). Epigenetic mechanisms regulate viral latency, and so, clinical trials to test the effect of vorinostat therapy in reactivation of HIV-1 viral latency are currently being performed.

This optimizing focus led to the design of the hydroxamic acid containing HDACi, such as belinostat, dacinostat, givinostat, and panobinostat. The latter being the only HDACi with approval within the EU. As single agents, these molecules have shown limited efficacy, but when in combination with DNMTi, they have shown to be more effective, especially in patients with solid tumors (75, 76). Other metal-binding functional groups have been of great interest to this group. This is the case of thiols, benzamides, and carboxylic acids (56). Examples of these functional groups can be found in the drugs: romidepsin, entinostat, mocetinostat, and short-chain fatty acids, such as sodium butyrate, Pivanex, phenylbutyric acid, and valproic acid (**Figure 3**; **Table 2**, HDACi section).

Unlike hydroxamic acid analogs, short-chain fatty acids occupy an acetate escape tunnel, which may have a zinc-binding function or compete with an acetate group released in the deacetylation reaction. These are the least potent type of HDACi (77). The benzamide inhibitor class consists of a chemical moiety capable of contacting specific amino acids in the HDAC core tube active site, with or without zinc ion binding (78). These inhibitors are active at micromolar levels. The antiproliferative and cytotoxic activity has been shown by entinostat against several tumor cell lines *in vitro*. Entinostat is a clinical trial available orally active inhibitor (79) (**Figure 3**; **Table 2**, HDACi section).

Currently, the discovery of sirtuin inhibitors (SIRTi) is an ongoing quest in which most compounds are still under preclinical investigation (80). Most efforts have been driven toward the discovery of SIRT1 and SIRT2 inhibitors. SIRT1 inhibitors have been proposed for treating cancer, for they have shown to inhibit TNBC cell growth, survival, and tumorigenesis (56, 81). Nicotinamide is the only inhibitor of sirtuin currently used in solid tumor clinics (82). SIRTi can be categorized as β-naphthols (sirtinol, splitomicin, salermide, and cambinol), indoles (EX-527 and oxindole), and urea (suramin and tenovin) (83) (**Figure 3**; **Table 2**, SIRTi section).

HDACi have many biological effects due to changes in patterns of histone acetylation and many non-histone proteins, including proteins involved in gene expression control, extrinsic and intrinsic apoptosis pathways, the progression of the cell cycle, redox pathways, mitotic division, DNA repair, cell migration and angiogenesis (56). Whether selective inhibition of HDACs will be beneficial as anti-cancer agents over broader-acting HDACi is a question that remains unanswered (56).

Histone Methyltransferase Inhibitors

HMTs are enzymes that add up to three methyl groups to lysine (KMTs) or arginine (RMTs) residues in histone proteins (84). Lysine methylation may either activate or silence gene transcription depending on the lysine residue involved (85). Nearly 100 KMTs have been described which use the SAM molecule as the methyl donor (14). SAM-like molecules, such as sinefungin, compete with SAM for its binding site (**Figure 3**). These molecules are inhibitors of all SAM using enzymes, like HMTs (14). KMT drug discovery heavily relies on their cofactor binding pocket, which has structural characteristics convenient for inhibitor interaction and makes these enzymes appealing for the design of small molecular inhibitors for interference (80). Examples of HMTi can be found in drugs such as EPZ004777, EPZ-5676, DZNep, pinometostat, and tazemetostat. Pinometostat and tazemetostat are selective DOT1L and EZH2 inhibitors, respectively (**Table 2**, HMTi section).

Both inhibitors are of interest in some types of cancer because DOT1L is a KMT involved in abnormal methylation of H3K79 and expression of HOX genes that cause leukemia (Copeland et al., 2013), while elevated expression of the KMT, EZH2, is associated with many forms of cancer due to hypermethylation of H3K27 which facilitates transcriptional silencing (80). Also, in B-cell-lymphoma patients, EZH2 mutations occur with a frequency of approximately 15-20 percent in either tumor type, particularly in diffuse large-B cell-lymphomas and follicular lymphomas (86, 87). These modifications contribute to the

more effective trimethylation of H3K27 by the mutant form of this protein (88). Preclinical studies showed that EZH2 inhibitors induced the arrest of proliferation, differentiation, and eventual apoptosis of DLBCL cells. These results were stronger in DLBCL cells that bear EZH2 mutations, but they also occurred in EZH2-wild-type DLBCL cells (89).

While several small molecule inhibitors have been developed for PRMTs with adequate potency, most PRMT inhibitors' selectivity remains to be improved. Therefore, the detection of PRMT inhibitors involves further analysis of novel approaches (i.e., allosteric control) (90). Three PRMT inhibitors, including PRMT5 inhibitor GSK3326595 (**Table 2**, HRMTi section), and JNJ-64619178 as well as PRMT1 inhibitor GSK3368715 have entered clinical trials so far. PRMT inhibitors with novel action mechanisms and strong drug-like properties will shed new light on developments in drug discovery and development of PRMTi (87, 90). The number of inhibitor assays reported on CHEMBL database against the enzymatic activity of the HMTs increases everyday (62) (**Table 1**, HMTi section).

Histone Demethylase Inhibitors

Significant progress has been made in the development of JmjC-KDM inhibitors since the first inhibitors were identified in 2008 (91). The vast majority enter the catalytic domain and inhibit the enzyme's activity by chelating the active site Fe (II), interfering with the 2OG binding. Because of the similarity between JmjC-KDMs' active site pockets, it has proved difficult to achieve selectiveness in the broad superfamily of 2OG dioxygenases (92). The recent availability of JmjC-KDM crystal structures has encouraged medicinal chemistry efforts and has made it possible for the JmjC-KDMs to produce many chemical candidates. Examples of these inhibitors include hydroxamate derivatives, pyridinedicarboxylate derivatives, N-oxalyl amino acid derivatives, and agents which interfere with metal binding (71) (**Figure 3**; **Table 2**, HDMi section).

In 2004, Professor Yang Shi first described LSD1 and discovered that it had significant biological functions in a wide variety of biological processes, including cancer (93). During carcinogenesis, in AML and SCLC, elevated levels of LSD1 were observed (94). Pharmacological LSD1 inhibition with small molecules has shown that it suppresses the division, proliferation, invasion, and migration of cancer cells (95). LSD1 thus becomes an evolving clinical target for anticancer therapy. Many LSD1 inhibitors, including natural products, peptides, and synthetic compounds, have been identified.

The similarity of LSD demethylases with monoamine oxidases (MAOs) has started the quest for repurposing MAO inhibitors to find inhibitors for these types of enzymes. Initially approved by the FDA for the treatment of mood and anxiety disorders (96), the MAO inhibitor tranylcypromine (TCP) was found to be able to inhibit its homolog LSD1 moderately by forming covalent adducts (97). As a result, many MAO inhibitors (MAOi) such as pargyline, phenelzine, and tranylcypromine have been shown to inhibit HDM KDM1A (80) (**Figure 3**; **Table 2**, HDMi section). New studies are now ongoing in clinical trials with some TCP-based LSD1 inhibitors alone or combined therapy with other therapeutic agents for treating cancer (98).

Bromo and Extra Terminal Domain Inhibitors

Bromodomains are protein motifs present in several epigenetic readers including BET family, that recognize and bind to acetylated lysine residues located on histone tails. BETs consist of two bromodomains and an extra-terminal region. The BET family includes the Bromodomain testis-specific protein (BRDT), BRD2, BRD3, and BRD4 (99). BETs lead to malignancies production and progression by stimulating and enhancing the expression of main oncogenes such as *MYC* (100). Indeed, when treated with the inhibitor JQ1, BET inhibition resulted in *MYC* downregulation, which resulted in decreased levels of mRNA and protein in mouse MLL-fusion leukemia cells (101).

In various forms of cancers, including breast, neuroendocrine, ovarian, rhabdomyosarcoma, and glioma, preclinical studies of BET inhibitors have shown their efficacy (87). They disrupt the recognition by BET-containing reader proteins of acetylated lysine residues in histones, a mark associated with active transcription (102). The mechanism of BETi relies on the fact that the region that binds acetyl-lysine is hydrophobic and can be taken up by small hydrophobic molecules that specifically target this catalytic site. Examples of these inhibitors can be found in Thienotriazolodiazepines (JQ1, CPI-203, OTX015) and Benzodiazepines (CPI-0610 and molibresib) (**Figure 3**; **Table 2**, BETi section).

Preliminary clinical trials have demonstrated that BET inhibitors cannot induce long-lasting cytotoxic effects in human cancers when administered as single agents (103). Nevertheless, the potential of combinations with other epigenetic therapies is important (104). Although BET inhibitors' toxicity may reduce such combinations, HDACi studies indicate that combinations with reduced doses may be effective, possibly reducing toxicity. This also reflects on the number of inhibitor assays for BRDs (62) (**Table 1**, BETi section).

The Basis for Drug Repurposing

Although epigenetic therapy has proven to be remarkably effective, epidrug discovery remains as a traditional "*de novo*" drug discovery pathway, which has significant disadvantages such as high costs, time consuming, and low success rate (105, 106) (**Figure 4**). An answer that addresses these problems and could speed up epidrugs in the clinic has arisen from the relatively recent idea of using known drugs for new targets, commonly known as drug repurposing (DR). This approach has gained considerable popularity, emerging as an interesting approach in cancer therapy research and many fields within medicine (107).

DR is the discovery process of finding new medical uses of a preexisting drug which was previously approved for another indication, withdrawn from the market due to adverse effects or disapproved for failing to prove its efficacy and safety (11, 107) (**Figure 4**).

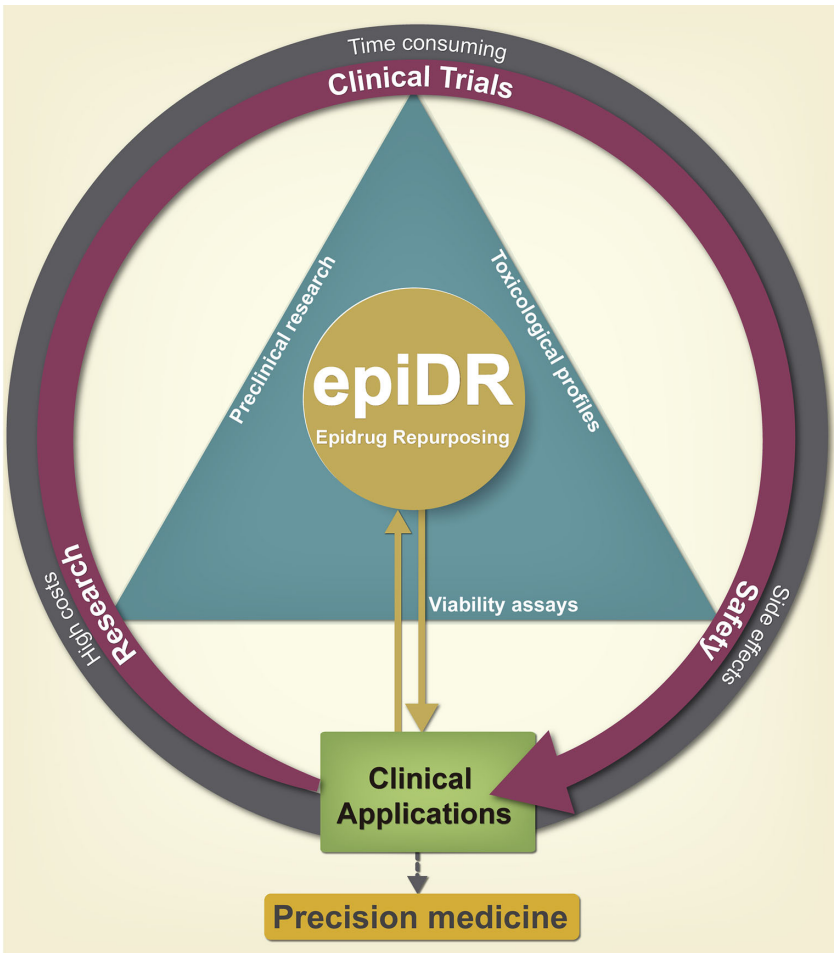

FIGURE 4 | Advantages of pharmacological epi-drug repurposing in clinical applications. Drug repurposing serves as a shortcut reducing the time of incorporating a drug into the clinic; since the preclinical phase has already been carried out previously, giving a second chance to old drugs. Initially, it reduces the cost of development and toxicity research, which leads to greater cost-benefit efficiency for the pharmaceutical industry by generating a new cancer therapy. The repositioning of epi-drugs is a promise for the generation of new drugs of precision medicine.

This approach includes the selection of drugs with promising repurposing potential and it also has important advantages over the "*de novo*" drug discovery processes. Previously assessed drug safety significantly reduces both costs and time for making these drugs readily available for use in the clinic (108, 109).

Historically speaking, repurposing of medications was mainly fortuitous; if an off-target effect or newly discovered target was detected, it was sure for it to be targeted for commercial usage. Examples of this are shown in drugs like sildenafil citrate, whose repurposing for erectile dysfunction was not based on a systemic approach, nor was thalidomide repurposing for erythema nodosum leprosum (ENL) and multiple myeloma, which are still the most promising examples of DR (107). Sildenafil was first formulated as an antihypertensive medication. However, after Pfizer reprofiled it for erectile dysfunction therapy and sold it as Viagra, it held the lead market share in erectile dysfunction medications in 2012, with global sales totaling more than 2 billion (110). Thalidomide, an antiemetic first sold in 1957, was discontinued within four years due to its notorious association with teratogenic defects in infants born to mothers who took the drug during their first trimester of pregnancy (107). However, the efficacy of thalidomide, first in ENL and decades later in multiple myeloma has been successfully demonstrated. Ever since, thalidomide has achieved considerable market success for treating multiple myeloma and has also contributed to the production and authorization of many more effective formulations, such as lenalidomide, which had $8.2 billion in worldwide revenues in 2017 (111).

These achievements have led to the implementation of systematic approaches to detect repurposable substances (109). The field of DR is fascinating, and its importance reflects in the vast number of drug projects of pharmaceutical companies that already have several candidate molecules that, although successful in phase I, they did not prosper in Phase II or III clinical trials. This gives rise to the existence of several known molecules, which are relatively safe to use in the clinic. Hence,

this large reservoir of molecules provides a vast niche for the search for repositionable drugs, which is much larger than the set of approved drugs (112).

A DR approach usually consists of three phases before the target drug is taken into further development: The selection of a target molecule for a specific indication, analysis of the drug impact in preclinical models, and the evaluation of the effectiveness in clinical trials in phase II, when enough adequate safety results are available from phase I tests. These methods can be classified into computational approaches and experimental approaches, which are now both being widely used synergistically. DR is encompassed within these two large fields, focused on clinical evidence (109).

Experimental approaches include **binding assays** for the identification of novel target interactions. These types of assays come from proteomic methods, like affinity chromatography and mass spectrometry are used to detect novel targets of existing drugs (113); and **phenotypic screening**, which are approaches based on *in vitro* or *in vivo* models of disease screening of compounds can indicate clinical potential (114). These approaches offer testing in a relevant biochemical context by performing *in vitro* assays with live cells (115, 116). The evolution of *in vitro* screening has led to systematize drug discovery, allowing ultra-high-throughput screening, analyzing up to 10,000 compounds per day (116, 117); however, the main limitation of these methodologies are the high costs of the required infrastructure, as well as nonspecific results (8).

Computational methods include the study of large sets of data (e.g., gene expression, chemical composition, genotype or proteomic data or electronic health records) that lead to the development of reprofiling hypotheses (118). Computational approaches include: **signature matching**, which results for comparing a drug signature such as its transcriptomic, structural or adverse effect profile to that of another pharmaceutical product or disease phenotype (119); **molecular docking**, a structural computational strategy focused to predict complementarity of the binding site between a drug and a receptor (120); **genetic association**, a high throughput analysis of genes associated with a disease which can turn out to be potential targets for drugs (121); **pathway mapping**, another approach that analyses biological pathways in order to develop networks of drugs or disorders based on patterns in gene expression, disease biology, protein interactions or GWAS data to better classify repurposable candidates (122); **retrospective clinical analysis,** a systematic review of electronic health records, data from clinical trials and surveillances post-marketing could be useful identifying repurposable drugs; and **novel sources**, which is the combination of large-scale in-vitro drug screens with genomic data, electronic health records and self-reported patient data represents new ways to repurpose drugs (123, 124).

In sum, these approaches allow multiple manners for conducting DR. However, these methodologies applications need to be taken with caution, as many of them seem to be reductionist (117, 125). Numerous strategies are now coupling drug networks with computational analysis to characterize different diseases' metabolic pathways. These efforts aim to identify drugs acting not only on a single target but also on a whole network of proteins (126, 127). In every computational approach, experimental validation is compulsory since the actual methods are not 100% accurate.

HTS (High-Throughput Screening) is the most common approach in DR of epidrugs, and most of them are designed to inhibit catalytic sites of epigenetic writer enzymes (128). Computational methods, such as virtual screening, aim to efficiently discover novel active compounds against epigenetic factors (8). The increasing attention on epigenetic targets as an opportunity for DR provides high expectations. Next, we will summarize the current efforts in epidrug repurposing for cancer therapy.

Available Databases Focused on Exploration and Recompilation of DR Research

Nowadays, there is a large amount of information available focused on the search and annotation of drugs to be repurposed and the drugs that currently have research that supports their proposed new uses. Some public databases such as ChemBL, DrugBank, and DrugCentral are repositories of bioactivity data and drug chemical structures. These databases summarize multiple indications and chemical drug-target interactions. More specifically, the FDA-approved epidrugs are gathered in several databases focused on tested epidrugs and provides information about annotation tools (**Table 3**, Section Epidrugs). These databases are useful because they facilitate the integration of epidrug datasets obtained from experimental and computational approaches, reducing the manual search of information, and helping to increase collaboration on the field.

Other databases that aim to summarize the current efforts and latest frontiers in DR research are the REPOHub, repoDB, and the Project Repethio; these include clinical trials, pre-clinical tools for annotations, and information resources. Unlike the previous ones, these databases focus on gathering and matching the results from both predictive tools and experimental or clinical trials, resulting in faster results on drugs that could be repurposed (**Table 3**, Section Drug Repurposing). Tanoli et al., 2020 summarize the types of data available through multi-database exploration focused on DR (142). Currently, the ReDO project (Repurposing Drugs in Oncology) is probably the only database focused on assembling DR for cancer targets. And it has played a crucial role in the development of research for new drugs to cancer therapy with the DR approach.

Epidrug Repurposing in Cancer (Epi-DR)

The interest in oncological DR has emerged as a response to the declining productivity of oncological drug development (143) and as a source of low-cost treatments to meet the increased demands for novel treatments, in efforts to overcome chemoresistance and reduce the development time of *de novo* drugs (144).

Some widely used and well-known drugs for cancer therapy are examples of epi-DR, with an effect on epigenetic targets, and are either currently FDA-approved or under clinical

TABLE 3 | Some databases and tools that summarize the current knowledge on DR.

Category	Database name	Link	Key features	Reference
Drug-target interactions and bioactivity databases	ChEMBL	ebi.ac.uk/chembl/	Provides bioactivity data, structures and properties, clinical trials and drug annotations references for diseases	(62)
	PubChem	pubchem.ncbi.nlm.nih.gov/	Provides chemical structures and physical properties, bioactivity information, current patents, toxicity and safety; among others	(129)
	DrugCentral	drugcentral.org/	Provides chemical structures, chemical entities action, drug mode of action, dosage and pharmacological indications	(130)
	DrugTargetCommons (DTC)	drugtargetcommons.fimm.fi/	Bioactivity data, protein classification, assays and clinical trials data and disease gene associations for many proteins	(131)
	DrugBank	drugbank.ca/	Matches drug bioactivity information with drug-target physiological information	(132)
Epigenetic drugs databases	HEDD	hedds.org/index.jsp	Integration of experimental epigenetic drug datasets, provides information from target-disease, and tools from high-throughput screening	(133)
	HISTome2	actrec.gov.in/histome2	Provides histone proteins data and 127 epidrugs that have been categorized by modifier type; and advanced tools for histone modifier-drug prediction	(134)
	dbEM	crdd.osdd.net/raghava/dbem	Provides epigenetic modifiers data in normal and cancer genomes; and information for 54 drug molecules against different epigenetic proteins	(135)
Drug Repurposing databases	PROMISCUOUS	bioinformatics.charite.de/promiscuous	Provides an exhaustive set of drugs (25,000), experimental assays and annotations from protein relationships	(136)
	REPO Hub	clue.io/repurposing	Repurposing library that assemble a collection of 4,707 compounds, experimentally confirmed, clinical trials and annotations based on literature-reported targets	(137)
	RepurposeDB	repurposedb.dudleylab.org	Provides a summarize on drug repositioning studies reported on public databases. Assemble a repertoire of drugs, drug targets and associated disease indications	(138)
	repoDB	apps.chiragjpgroup.org/repoDB	Provides information from 1,571 compounds, both approved and failed drugs; as well as computational repositioning tools	(139)
	Project Repethio	het.io/repurpose	Provides a compilation of 3394 repurposing candidates based on computational predictions	(140)
Drug Repurposing in cancer databases	ReDO project	redo-project.org/	Provides a curated list of 270 drugs with pre-clinical and clinical evidence of anti-cancer action	(141)

development (145). The first repurposed drugs as an anticancer epidrug in the field were the 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine) (146). At first, these drugs were both approved by the FDA to treat myelodysplastic syndromes due to their antimetabolic effects on *in vitro* assays in cancer cells (146). However, the toxicity shown by 5-azacytidine led to other chemotherapeutic regimens being preferred (146); later, it was found that azacytidine and decitabine could both inhibit DNA methylation and were incorporated by tumor cells and also in myelodysplastic syndromes (146–148).

DNMT Inhibitors

The natural compound **Harmine** downregulates the expression of DNMT1, which results in reactivation of the p15 tumor suppressor gene in AML. Future studies are expected to assess if Harmine can be considered a potential therapy for AML and if it can be used as a single agent or adjuvant (149). **Chlorogenic acid** is a polyphenol coffee that has been found to suppress DNMT1. Its inhibitory activity derives from a chemical change resulting in increased S-adenosyl-L-homocysteine (SAH) production. Chlorogenic acid has been shown to inhibit DNMT1, using breast cancer cell lines, which lowers DNA methylation (150).

Laccaic acid A is a direct, competing DNMT1 natural compound inhibitor that reactivates genes silenced by promoter DNA methylation synergistically with 5-azadC in breast cancer cells (151). **Procaine** is a promising treatment with growth-inhibiting and DNA-hypomethylation effects in cancer cells. Especially in gastric cancer where its antiproliferative and apoptotic effects have been proven (152). Its well-defined, safe use as a local anesthetic, with well-known pharmacology, should promote procaine to pre-clinical trials (153). **Procainamide**, a derivative of procaine, hinders the enzymatic activity of DNMT1 by directly reducing the enzyme affinity for both DNA and S-adenosyl-L-methionine. It would be important to analyze whether procainamide, a fairly stable non-nucleoside inhibitor of DNMT1, will prevent cancer from arising (154).

A computer-based search for similarities between a database of approved drugs and 5-aza-2'-deoxycytidine has recently been detected as an ideal candidate for DR. **Mahanine**, a plant derived alkaloid, was shown to induce DNMT1 and DNMT3B proteasomal degradation by inactivating Akt, which in turn restored RASSF1A expression in prostate cancer cells. Mahanine then represents a possible therapeutic agent for advanced prostate cancer when RASSF1A expression is inhibited (155).

Hydralazine, approved as an antihypertensive, is a non-nucleoside DNMTi that interacts with the binding domain of DNMTs, and can decrease DNMT1 and DNMT3A mRNA expression and protein levels in T cell leukemia cell lines (156). In advanced cervical cancer, bladder, and cervical cancer cell lines, respectively (157, 158), hydralazine induces DNA demethylation and decreases DNMT activity. Also,

hydralazine, combined with magnesium valproate, is an opportunity to reverse imatinib resistance in patients with several malignancies, including lung (NCT00996060), cervical (NCT00404326), and locally advanced breast (NCT00395655) cancers, as well as different solid tumors which are refractory to current therapies (159–161) (NCT00404508). **Olsalazine,** an FDA approved anti-inflammatory agent, has proven its hypomethylating and very low cytotoxicity effects in cell-based screen tests (162).

Mithramycin A, an antibiotic with potent antitumor activity, binds to sequences of GC-rich or CG-rich DNA and upregulates tumor suppressor genes' expression by reducing the methylation of their promoters through binding and depleting the DNMT1 protein in lung cancer cells (163). **Nanaomycin A**, an anthracycline antibiotic, has demonstrated selectivity to DNMT3B in biochemical assays. Dock modeling strategies suggest that nanaomycin A is capable of binding DNMT3B's catalytic site. Treatment of the human tumor lines of the colon, lung, bone marrow with nanaomycin A demonstrated substantial genomic demethylation. While it is unclear if anthracyclines will be a successful choice for clinical DR due to certain long-term cardiotoxicity concerns, Nanaomycin A is the first non-SAH DNMT3B-selective compound that offers valuable biochemical properties for additional studies (164).

Disulfiram is an alcohol aversive drug that has been approved by the FDA for more than 60 years for treating alcohol abuse. It allows acetaldehyde to accumulate in the blood by inhibiting ALDH (165). Disulfiram's anticancer activity is mediated by its ability to suppress DNMT1 and through the reactivation of epigenetically silenced genes such as *APC* and *RARB* in prostate cancer cell lines (70) (**Table 4**, Section 1).

Peptides are small proteins made up of fewer than 50 amino acids. Such compounds have several roles in the human body and can modulate epigenetic pathways, raising the exciting possibility of peptide-based therapy. Such peptides may be endogenous, or food derived. **Amyloid beta (Aβ),** the central component of Alzheimer's senile plaque (AD), reduces global DNA methylation but increases DNA methylation in the Neprilysin gene promoting region, an Aβ-degrading enzyme (189). Soluble Aβ oligomers decrease intracellular glutathione levels by hampering cysteine uptake, followed by a global decrease in DNA methylation (174). **BCM7 and GM7** are food derived peptides produced by hydrolytic casein and gliadin digestion. They decrease cysteine absorption through opioid receptor activation in neuronal and gastrointestinal cells. This reduction is followed by an increase of oxidized glutathione and an increase in DNA methylation (175, 176) (**Table 4**, Section 1).

Dual DNMT and HDAC Inhibitors

In most cancer types, altered DNMT and HDAC activity is observed (190). Therefore, some repurposed drugs that inhibit both DNMT and HDAC enzymes could improve efficacy over one-target agents (**Table 4**, Section 2).

Berberine, an isoquinoline alkaloid derived from *Berberis vulgaris* (191) and used to treat bacterial, parasitic, and fungal infections, has been repurposed as a DNMT and HDAC dual inhibitor (192). In multiple myeloma cell lines, berberine treatment showed downregulated DNMT1 and DNMT3A expression, restoring p53 expression through DNA hypomethylation (193). Berberine also inhibits Class I and II HDACs in lung cancer cell lines, down-regulates gene expression, and increases histone H3 and H4 acetylation (194). **EGCG** is a polyphenol found in green tea (*Camellia sinensis*) and is a known anti-inflammatory compound (195). It has recently been proposed as an inhibitor of DNMT by direct interaction with the catalytic site of DNMT (186–188). EGCG reduces cell growth and increases apoptosis in renal carcinoma cells through the upregulation of TFPI-2. In skin carcinoma cells, EGCG increases the levels of acetylation of histone H3 and histone H4 lysine residues through HDAC inhibition, leading to the upregulation of tumor-suppressor genes (188) (**Table 4**, Section 2). **Resveratrol** is a natural polyphenolic compound found in grapes and berries (196), and it has been proposed as a dual inhibitor of both DNMTs and HDACs. In breast cancer cell lines, resveratrol inhibits both HDAC and DNMT1 activity, decreases histone H3 lysine 27 methylation, and increases its acetylation (182–184). In thyroid cancer cell lines, treatment with resveratrol showed resensitization to therapy when in combination with retinoic acid through the demethylation of CpG sites at promoter regions of CRABP2 gene (185); the effect of resveratrol as a repurposed cancer drug was also investigated in clinical trials (NCT00256334, NCT01476592, NCT00433576). Finally, **parthenolide** is a terpenoid compound, isolated from *Tanacetum parthenium*, with anti-inflammatory properties. Parthenolide downregulates HDAC1 gene expression (179) and increases histone acetylation (177, 180). It reverses drug resistance in some cancer cell lines (178) and restores silenced gene expression through a decrease in DNA methylation levels (181) (**Table 4**, Section 2).

HDAC Inhibitors

As previously mentioned, the use of HDACi among the chemotherapeutic agents is growing (**Table 5**, HDACi). Hydroxamic and carboxylic acids are being studied as potential HDACi; for instance, drugs like **Vorinostat (SAHA)**, approved for psoriasis treatment, and **Valproic acid** (anticonvulsant) are currently included in several clinical trials against different types of cancers (236). A complete overview about clinical trials in some of the most studied HDACi repurposed, such as **Vorinostat**, **Valproate**, **Belinostat, Panobinostat,** and cyclic peptide **Romidepsin** is available (236) (**Table 5**, HDACi).

Compounds with HDACi potential have been found in plants. **Ginseng** (*Panax ginseng*) is a popular plant extract commonly used in South Korea and traditional Chinese medicine, which contains several compounds (ginsenosides) with pharmacological properties (144). **Platycodi radix** (*Platycodon grandiflorum*), commonly known as balloon flower, is used to treat many diseases related to obesity in East Asia (237). Recently, Byun and cols. demonstrated that ginseng and platycodi have significant HDACi activity in Lung Carcinoma cell lines, thus upregulating p21 gene expression and promoting cell death (204). **HC toxin** is a cyclic tetrapeptide derived from a plant-fungal parasitic-association between *Helminthosporium carbonum* (ascomycetes) and its

TABLE 4 | Current DNMTi and DNMT-HDAC dual inhibitors repurposed drugs with applications in cancer therapy [*modified from Moreira-Silva et al. (9)].

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
Section 1. DNMT inhibitors							
Non-nucleoside analogs	**Hydralazine**	Anti-hypertensor	DNMT1	Four high-affinity interaction points with DNMT1 through the residues Lys 162 and Arg 240 within the enzyme active site.	T-Cell Leukemia cells	Increases LFA-1 expression inhibits T Cells ERK pathway phosphorylation, decreases DNMT enzyme activity, and decreases DNMT1 and DNMT3A protein levels. Reduces *de novo* methylation due to greater affinity to hemi methylated substrates (target of DNMT1),.	(156, 166)
					Breast Cancer cells	*In vivo* induces DNA demethylation and increases	(167)
					Bladder Cervical Cancer cells	expression of ER as well as RARb, p12 and p16 *in vitro*.	(157)
					Prostate Cancer cells	Increases apoptosis, inhibits RGFR pathway, thus induces cell cycle arrest. Decreases DNMT1, DNMT3a and b protein levels. Upregulates p21 which decreases promoters DNA methylation and induces histone acetylation.	(156)
					Cervical Cancer cells	Induces APC expression, inhibits cell growth, induces cell cycle arrest and apoptosis. Promotes DNA demethylation.	(158)
	Disulfiram (DSF)	Alcohol aversive	DNMT1	DSF could interfere with the catalytic activity of DNMT1 by reacting with a citosine ring via thiol group of catalytic site of DNMT1.	Prostate Cancer cells	Reduces global 5mC content, through inhibition of DNMT1 activity on hemimethylated substrates. Decreases methylation in APC and RARB gene promoters, thus increasing re-expression. Inhibits growth and clonogenic survival of prostate cancer cell lines.	(70)
	Procainamide	Cardiac arrythmias	DNMT1	Partially competitive inhibitor of DNMT1 that interacts with the binding pocket of the enzyme	Prostate Cancer cells	Promotes GSTP1 CpG island hypomethylation, thus induces GSTP1 re-expression in LNCaP cells *in vitro* and in *in vivo* assays	(168)
					Breast Cancer cells	Induces DNA demethylation increases expression of ER RARb; also induces re-expression of p12 and p16 (*in vitro*).	(169)
					Colon Cancer cells	Greatly reduces affinity for hemi-methylated DNA and SAM in catalysis, reduces global 5mC content, thus reduces gene-specific hypermethylation at promoter CpG islands.	(154)
					Non-small Cell Lung Cancer cells	Inhibits DNMT activity and decreases promoter demethylation of WIF-1, restoring WIF-1 expression, thus downregulating the Wnt pathway	(169)
	Procaine (PCA)	Anesthesic for spinal block	DNMT1, DNMT3A	Interacts with the binding pocket of the enzyme inhibiting catalytic activity (non-nucleoside),	Breast Cancer cells	Demethylates densely hypermethylated CpG islands, reduces 5mC DNA content by 40%, restoring gene expression of RARβ2, and has growth-inhibitory effects, causing mitotic arrest	(153)
					Gastric Cancer cells	Inhibits DNMT1 and 3A activity through molecular docking in the catalytic binding site, disrupting the binding of DNMT to DNA. Reduces proliferation, induces apoptosis, and restores expression of CDKN2A and RARb	(152) (170)

(Continued)

TABLE 4 | Continued

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
					Hepatocellular Carcinoma cells	DNA demethylation and silenced gene reactivation of p16, HAI-2/PB, and NQO1. Promotes cell cycle arrest and reduces viability. Also shown significant reduction in tumor volume *in vivo*.	
					Non-small Cell Lung Cancer cells	Inhibits DNMT activity, causing promoter demethylation of WIF-1, thus restores WIF-1 expression and downregulation of Wnt pathway	(169)
Antibiotic	**Nanaomycin A**	Anthracycline antibiotic	DNMT3B	Interaction with active site of DNMT3B in specific a.a. (Glu697 Arg731 Arg733) of enzyme binding pocket, thus promoting a molecular docking in DNMT3B that inhibits enzymatic activity	Colon Cancer cells Lung Cancer cells Bone marrow cells	Decreases DNMT1, 3A, 3B expression. Inhibits DNMT3B activity promoting reactivation of RASSF1A. Reduces cell proliferation and viability	(164)
	Mithramycin A (MMA)	Hypercalcemia drug, and antineoplastic agent	DNMT1	Possibly interferes with DNMT1 binding at the CpG region in TSG promoters through binding DNMT1 protein or might be a form a complex between MMA, DNMT1 and double-stranded DNA	Lung Cancer cells	Inhibits DNMT1 activity and decreases protein level. Decreases CpG methylation on SLIT2 and TIMP-3 promoters, inducing re-expression. Inhibits invasor pehotype thus prevents metastasis	(163)
Polyphenol	**Chlorogenic acid**	Natural Compound (not approved)	DNMT1	Increases SAH formation inhibiting DNA methylation through COMT mechanism (non-competitive),	Breast Cancer cells	Inhibits DNMT1 activity, reduces methylation of the promoter region of the RARb gene	(150)
	Harmine	Natural Compound (not approved)	DNMT1	Not described	Myeloid Leukemia cells	Decreases DNMT1 gene expression, induces p15 promoter demethylation. Also decreases proliferation and promotes cell cycle arrest in G0/G1 phase	(149)
	Laccaic acid A (LCA)	Natural Compound (not approved)	DNMT1	DNA-competitive DNMT inhibitor through competition for the oligonucleotide substrate	Breast Cancer cells	Inhibits directly DNMT1, also have effects on DNMT3A, 3B inhibition, and reactivates genes silenced by promoter methylation (CEACAM5, DHRS3, RGS16)	(151)
	Mahanine	Natural Compound (not approved)	DNMT1, DNMT3B	Induces proteasomal degradation of DNMT1 and DNMT3B	Prostate Cancer cells	Inhibits DNMT activity, increases expression of RASSF1A and inhibits cyclin D1. Induces proteosomal degradation on DNMT1 and DNMT3B through Akt inactivation, thus facilitates demethylation of RASSF1A promoter and increases its expression	(155, 171)
	Genistein	Natural compound; isoflavone	DNMT	Inhibits DNA methyltransferase activity in a substrate- and methyl donor–dependent manner	Esophageal Squamous Cell Carcinoma cells Prostate Cancer cells	Promotes reversed DNA hypermethylation and reactivation of RARbeta, p16INK4a and MGMT trhough demethylation of promoter genes. Also inhibites cell growth	(172)

(Continued)

TABLE 4 | Continued

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
					Breast Cancer cells	Induces DNA hypomethylation and reactivation of RARbeta	
					Renal Cancer cells	Inhibits DNMT1 activity, thus induces demethylation of BTG3 promoter and has antiproliferative effects through cell cycle arrest	(173)
Peptide	**Beta amiloid peptide**	Component of Alzheimer's senile plaque	DNMT	Drecreases SAM/SAHlevels, promoting a global demethylation, through redox-dependent control over methionine synthase and methylation	Neuroblastoma cells	Soluble Aβ oligomers decreases intracellular glutathione levels by hampering cysteine uptake, followed by a global decrease in global DNA methylation	(174)
	BCM7	Natural Compound; food-derived peptide	DNMT	Decreases SAM/SAH levels, promoting decrease in cysteine levels, affecting redox status and methylation capacity of DNMTs	Neuroblastoma cells	Decreases cysteine absorption through opioid receptor activation. This reduction is followed by an increase of oxidized glutathione and an increase in DNA methylation	(175, 176)
	GM7	Natural Compound; food-derived peptide	DNMT		Neuroblastoma cells		
Section 2. DNMT and HDAC Dual Inhibitors							
Polyphenol	**Parthenolide**	Anti-inflammatory (not approved)	HDAC1 and DNMT1	Inhibits DNMT1possibly through alkylation of the proximal thiolate of Cys1226 of the catalytic domain by its -methylene lactone	Colon Cancer cells	Inhibits HDAC activity by molecular docking, downregulates HIF-1alfa and inhibits NF-kB pathway	(177, 178)
					Melanoma cells	Reduces MITF-M transcript level and HDAC1 and protein level.	(179)
					Breast Cancer cells	Induces proteasomal degradation of HDAC1, thus increasing global histone acetylation and p21/p53 expression and induces cell death.	(179–181)
					Thyroid Cancer cells	Down-regulates DNMT1 expression possibly associated with its SubG1 cell-cycle arrest. Promotes global DNA hypomethylation and reactivates HIN-1 gene trough demethylation of its promoter	(181)
					Myeloid Leukemia (AML) cells	Inhibits DNMT1 and decreases gene expression of DNMT1 and BF-kB pathway. Induces cell cycle arrest and interrupts the binding of Sp1 to DNMT1 promoter, thus reactivates tumor suppressor genes and inhibites HIF-α	(178, 181)
	Resveratrol (RVT/ RSV)	Natural Compound polyphenol (not approved);	HDAC1 and DNMT	Fits into the binding pocket of HDAC's though interaction with amino acids of the catalytic site and interacts with the zinc ion, disrupting HDAC-zinc dependent activity.	Hepatoblastoma cells	Antiproliferative effect on all cell lines; showed specific inhibition of HDACs and in turn a histone hyperacetylation in HepG2 cells.	(182)
					Breast Cancer cells	Decreases PRMT5 EZH2 ATP2A3 and HDAC2 expression, increasing H3ac and H3K27 marks; increases global level of H3K9ac and H3K27ac marks through increasing KAT2A/3B expression. Reduces the enrichment of H4R3me2s and H3K27me3; and increases activating histone marks (H3K9/27ac) within the proximal promoter region of BRCA1, p53, and p21 restoring its expression.	(183, 184)

(Continued)

TABLE 4 | Continued

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
					Thyroid Cancer cells	Decreases DNMT1 activity and demethylates CpG sites at promoters regions in CRABP2.	(185)
	EGCG	Natural Compound; thiol anti-inflammatory	DNMT and HDAC	Inhibitor of DNMT nuclear activity by direct interaction with a hydrophilic pocket of DNMT1 and catalytic binding site	Squamous Cell carcinoma cells	Induces reversal hypermethylation in RARβ, MGMT, p16INK4a, and hMLH1 promoter genes, promotes inhibition of cell growth.	(186, 187)
					Skin Cancer cells	Increases the levels of acetylation of histone H3 and histone H4 lysine residues through HDAC inhibition, leading to the upregulation of Cip1/p21 and p16INK4a.	(188)

host, (commonly *Poaceae* plants family). It was reported as a Maize Histone Deacetylase inhibitor (238) and proposed as an analog of **Apicidin** and **Artemisin**, a fungal metabolite (239), and antimalarial drug, respectively; with antiprotozoal HDACi activity proved for Malaria (*Plasmodium berghei*) in mice. However, recently HC-toxin has been rediscovered and identified as HDACi in different cancer cell lines (205). In breast cancer and neuroblastoma cell lines, HC toxin inhibited HDAC activity and promoted cell proliferation inhibition, cellular death, and induced H4 acetylation (205, 206). **Artemisin** has been repurposed as an HDAC1, HDAC2, and HDAC6 inhibitor in the breast cancer cell line MCF-7 (203) (**Table 5**, HDACi).

Psammaplin A (PsA) is a phenolic compound that derives from the marine sponge-association, *Poecillastra sp.* and *Jaspis sp.*, *(Pseudoceratina purpurea)* whose active substances are monomers of thiol groups with enzymatic inhibition activity (210, 240). These monomers play a key role for both HDACi and DNMTi activity (241). In endometrial cancer cells, PsA showed HDAC1 and HDAC6 inhibition, reduction of HDAC1 expression the elevation of histone H3 and H4 acetylation, induction of cell cycle arrest, and apoptosis (208, 209). **Burkholdacs A and B**, with a structure similar to **Thailandepsin A**, was identified as a novel HDACi through the systematic overexpression of transcription factors associated with *Burkholderia thilandensis* (227). They are bicyclic depsipeptide compounds, proposed as potent HDACi in brain cancer cells, but also in other cancer cell lines (226). Using a panel of 39 human cancer cell lines, burkholdacs have shown superior HDACi activity over Ramidopsine (approved HDACi) in at least six cancer cell lines (226). Burkholdacs' affinity for HDAC1 is greater than that for HDAC6. Structural changes in burkholdacs A and B structures may increase their activity and selectivity, giving rise to isoform selective inhibition of HDACs therapeutical potential (226) (**Table 5**, HDACi). Other depsipeptides have also been studied for repurposing. **Spiruchostatin A, and Plitidepsin (Aplidin)** are natural depsipeptides derived from *Pseudomonas sp.* (228) and *Aplidium albicans* (242), respectively. In cancer cell lines, reduced spiruchostatin A effectively inhibited HDAC1, an effect not observed when oxidized, and it showed an increase in the acetylation levels of specific lysine residues of histones H3 and H4 (228). Plitidepsin is currently in clinical trials to treat multiple myeloma (243, 244) but it has also displayed interesting properties against hematological malignancies (245). Some depsipeptides display a greater affinity for HDAC1 than HDAC6 and class II HDACs, but this does not appear to limit their activity as anti-cancer agents judging by *in vitro* effects in cancer cells (208, 226, 228). Structure-function studies on depsipeptides can lead to the generation of chemical analogs with enhanced selectivity as HDACi drugs (**Table 5**, HDACi).

HAT, HMT, HDM, and BET Inhibitors

Recently, HATi, HMTi, HDMi, and BETi have become of great interest for personalized cancer treatment. Multiple studies have consistently shown the enormous potential of known drugs and compounds for DR as epigenetic modulators (**Table 6**, HATi, HMTi, HDMi, and BETi).

TABLE 5 | Current HDACi repurposed drugs with applications in cancer therapy [*modified from Moreira-Silva et al. (9)].

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
HDAC inhibitors							
Acid	**Valproate (VPA)**	Antiepileptic	HDAC class I and HDAC2	Inhibits HDAC class I activity by binding to the catalytic site and promotes proteasomal degradation of HDAC2.	Melanoma treatment	Potentiates karenitecin-induced apoptosis in multiple melanoma cell lines and on xenografted mice, however, fails to enhance chemotherapy effects on dacarbazine plus interferon-α-treated melanoma patients.	(197, 198)
					Colon Cancer cells	Reduces relative HDAC2 mRNA expression, preventing cell colony formation and migration.	(199)
					Non-small Cell Lung Cancer cells	Increases major histocompatibility complex (MHC) class I chain-related protein A (MICA) expression and sensitizes cancer cells to γδ T-cell-mediated killing.	(200)
					Colon Cancer Tumor cells	Synergistically reduces viability of cancer cells in combination with mytomicin C.	(201)
					Ovarian Cancer cells	Upregulates WWOX and P27 genes and interferes with the cell cycle by promoting apoptosis and inhibiting cell proliferation, both *in vitro* and *in vivo*.	(202)
Phenolic	**Artemisin**	Antimalarial	HDAC1, HDAC2 and HDAC6	Not described	Breast Cancer cells	Inhibits cell proliferation, cell migration, invasion and induces apoptosis. Also inhibits HDAC 1, 2, 6 and up-regulates BRCA1, 2/Ras/ERα/ERβ/PR/Her expression	(203)
	Ginseng	Nutraceutical (not approved)	HDAC	Not described	Lung Carcinoma cells	Inhibits HDAC activity, increases p21 expression and induces apoptosis.	(204)
	HC toxin	Natural Compound antiprotozoal (not approved);	HDAC	Not described	Breast Cancer cells	Inhibits cell proliferation and induces cell cycle arrest at G2/M and apoptosis in a dose-dependent manner.	(205)
					Neuroblastoma Cells	Induces cell cycle arrest and apoptosis, induces neuronal differentiation and inhibits invasive growth. Increases p-RB, p15, p16, p21, p27 expression, and reactivates the RB tumor suppressor pathway. Also induces H4 acetylation while inhibits HDAC activity	(206)
	Psammaplin A (PsA)	Enzimatic inhibitor Bromotyrosine Natural Compound (not approved);	HDAC III (SIRT1)	Inhibits HDAC activity via the coordination of zinc ion in catalytic pocket of HDAC with sulfhydryl group activated by a reducing agent.	Ovarian Cancer cells, Colon Cancer cells and Cervical Cancer cells	Displays significant cytotoxic activity, inhibits cell proliferation and upregulates expression of tumor-suppressor gene gelsolin in a dose-dependent manner	(207, 208)
					Endometrial Cancer cells	Inhibits cell proliferation, significantly induces H3 and H4 acetylation, upregulates expression of cyclin-dependent kinase inhibitor, p21, and downregulates expression of pRb, cyclins, and CDKs, promoting cell cycle arrest.	(209)
					Breast Cancer cells	Inhibits proliferation induces cell cycle arrest at G2/M and reduces SIRT1 activity protein expression levels and reduces nuclear SIRT1 levels. Increases p53 acetylation (target of SIRT1) and increases DRAM expression	(208, 210)
Fatty acid	**Sodium Butyrate**	Anti-inflammatory	HDAC1	Not described	Gastric Cancer cells	Increases DAPK expression in human gastric cancer cells and this expression prompted apoptosis by decreasing FAK levels. Suggesting that DAPK expression prompts apoptosis by reducing the FAK protein level. Induce demethylation of the SFRP gene promoter	(211)
					Breast Cancer cells	Decreases cell proliferation induces cell cycle arrest at G1/G2 and decreases nuclear expression of DNA DSB repair proteins induced by etoposide (BRCA1 RAD51, ATM). Also increases H4 acetylation.	(212)

(Continued)

TABLE 5 | Continued

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
					Prostate Cancer cells	Inhibits HDAC1, 3 activity and induces H3, H4 acetylation, leading to hyperacetylation of H3 and H4 on the p21 promoter region, thus increasing p21 expression. Also induces cell cycle arrest, promoting apoptosis.	(213)
Hydroxamic acid	**Trichostatin A (TSA)**	Antifungal	HDAC class I, II and SIRT6	Hydroxamate. Pan-HDAC inhibitor, that obtain the binding energy associated with the strength of inhibition is derived from the bidentate chelation of hydroxamate	Breast Cancer cells	Decreases cell proliferation, inhibits HDAC activity, thus increases H4 hyperacetylation And increases ER acetylation and anti-tumor activity	(214)
					Myeloid Leukemia (AML) cells	Inhibits HDAC activity leds to histone hyperacetylation. Increases H4 acetylation and reduces Myc expression ZNF278 (Myc's coactivator), NM1, HOXB6 and MKRN3	(215)
					Esophageal Squamous Carcinoma cells	Decreases cell proliferation, induces cell cycle arrest at G1, down-regulates cell growth by inhibiting the activation of the PI3K/Akt and ERK1/2 pathways, and increases H4 acetylation levels	(216)
					Prostate Cancer cells	Increases apoptosis induces p21 expression and represses TMPRSS2-ERG expression AND affect acetylation status of p53 by inhibiting HDAC activity. Disrupts the epidermal growth factor receptor (EGFR),-STAT3 pathway, thus, inhibits proliferation in CRPC cells. Increases H4K16acetylation and promotes gene transcription, moreover decreases phospho-Akt pathway	(217, 218)
					Pancreatic Cancer cells	Restores cellular differentiation, reduces proliferation and restores p21 expression. Increases NDGR1 mRNA expression, also increases hypoxic responses	(219)
					Colon Cancer cells	Decreases cell growth and promotes apoptosis, down-regulates DNMT1 and HDAC1 expression, increasing p21, p27 and p57 expression	(220)
					Hepatocellular Carcinoma cells	Increases H3K9 and H3K27 acetylation and increases SERCA3 mRNA expression levels and promote ATP2A3 gene expression	(221)
	Vorinostat (SAHA)	Psoriasis disease treatmenr	HDAC class I, II and IV	inhibits HDAC activity by binding to the pocket of the catalytic site processes by removing acetyl groups from proteins	Advanced Prostate Cancer treatment	In a phase II trial, it was associated with significant toxicities limiting efficacy assessment in patients with disease progression on one prior chemotherapy	(222, 223)
					Follicular and Mantle Cell Lymphoma treatment	In a phase I trial in follicular and mantle cell lymphomaoral vorinostat was well tolerated up to 200mg bd for 14 consecutive days every 3 weeks in Japanese patients with NHL. Shown favorable results	(224)
	Panobinostat	HDACi multiple myeloma	HDAC	Pan-HDAC inhibitor, blocks the enzymatic activity of HDAC	Multiple myeloma treatment	It has improved progression-free survival when combined with bortezomib and dexamethasone in patients with relapsed multiple myeloma who previously received bortezomib and an immunomodulatory agent	(225)
Depsipeptide	**Burkholdacs A**	Pathogen bacteria (not approved)	HDAC1, HDAC6	Inhibits HDAC catalytic activity by reduction of the disulfide bond which generates a free thiol group that interacts with the catalytic site in HDACs.	Brain Cancer cells Colon Cancer cells Lung Cancer cells Ovary Cancer cells Stomach Cancer cells Prostate Cancer cells	In at least six cancer cell lines, it has shown superior HDACi activity over Ramidopsine (approved HDACi). Burkholdacs A presents more affinity for HDAC1 and was determined to be superior than B with respect to its HDAC1 inhibitory activity and isoform selectivity toward HDAC1 over HDAC6 and antiproliferative activity.	(226)

(Continued)

TABLE 5 | Continued

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
	Thailandepsin (Burkholdacs B)	Pathogen bacteria (not approved)	HDAC6		Cervical Cancer cells Breast Cancer cells		(226, 227) (227)
	Spiruchostatin A	Pathogen bacteria (not approved)	HDAC1 and HDAC6	Structural similarity with HDAC inhibitor FK228 (Romidepsin) which interacts with the active-site zinc in its reduced form, preventing it from interacting with substrate.	Breast Cancer cells Ovarian Cancer cells Brain Cancer cells Colon Cancer cells	Increases acetylation levels of specific lysine residues of histones H3 and H4.	(228, 229)
	Apicidin	Antiprotozoal for Malaria	HDAC3, HDAC4 and HDAC8	Cyclic tripeptide that chelate the active site zinc ion through the terminal carbonyl, hydroxy and/or amino functional groups	Promyelocytic Leukemia treatment	Inhibits cell proliferation an cycle arrest, promoting cell death. Increases H4 acetylation and inhibits HDAC activity, thus increases p21 expression.	(230)
					Lung, Colon and Pancreatic Cancer cells	Induces DNA demethylation via HMT suppression, reduces HP1 and DNMT1 recruitment to genes' promoter and induces p16, SALL3, and GATA4 expression. Also, decreases SUV39 and G9a expression in lung cancer cell lines.	(231)
					Cervical Cancer cells	Induces demethylation of CpG islands of the 1st exon of the PDH2 gene AND induces PHD2 and p21 gene expression and inhibits cell proliferation.	(232)
					Breast Cancer cells	Increases H3 and H4 acetylation and reduces ERalfa and ERb expression. Increases p21 and p27 expression and reduces cyclin D1 and cyclin E expression. Also reduces cell proliferation, thus promotig apoptosis.	(233)
					Endometrial Cancer cells	Increases H3 acetylation and reduces HDAC3, 4 expression, decreases cell proliferation and induces apoptosis.	(217)
					Ovarian Cancer cells	Decreases HDAC activity, reduces HDAC4 expression and blocks cell migration and invasion. Increases H3 and H4 acetylation and increases RECK expression through reducting the binding of HDAC4 to the Sp1 of its promoter, while reduces MMP-2 expression.	(234)
					Oral Squamous Cell Carcinoma cells	Inhibits cell growth, proliferation and reduces HDAC8 expression. Induces apoptosis and autophagy AND increases H4 acetylation.	(235)
	Platycodi	Nutraceutical (not approved)	HDAC	Not described	Lung Carcinoma cells	Inhibits HDAC enzymatic activity and induces the expression of p21. Stimulates cell death and inhibits cell proliferation.	(204)

HAT Inhibitors

Anacardic acid, a small molecule obtained from cashew nutshell liquid with known antitumor activity, inhibits the p300's and PCAF's HAT activity. Anacardic Acid is not specific to any particular HAT group, but it can be used to synthesize other specific HAT activity modulators based on this molecule (246). **Plumbagin** is an *in vivo*, potent acetyltransferase inhibitor, hydroxynaphthoquinone isolated from the roots of *Plumbago Rosea*. A single hydroxyl group in Plumbagin confers its HATi properties. Replacing this group with other chemical moieties results in complete loss of its inhibitory activity. Plumbagin has also been reported to suppress the activation of NFκ-B, leading to apoptosis potentiation. Plumbagin may be a potential anticancer agent, but its cell toxicity properties could be the main limitation of its use as a therapeutic molecule (253). **Garcinol** is a potent inhibitor of the p300 and PCAF HATs. It inhibits *in vivo* histone acetylation in HeLa cells but does not affect histone deacetylation. Garcinol suppresses chromatin transcription dependent on HAT p300 but does not affected transcription of DNA (249). **Lunasin** is a 43 amino acid peptide found in soybean, barley, wheat, and rye. Previous studies have shown that lunasin can suppress the proliferation and migration of cancer cells with no effect on wild-type cells. Lunasin is a competitive inhibitor of HATs. It inhibits histone acetylation and regulates the cell cycle. This binding is probably achieved through its helical structure, similar to chromatin-binding protein structures (267) (**Table 6**, HATi).

HMT Inhibitors

Allantodapsone was recovered from a virtual screening based on the PRMT1 structure. Allantodapsone inhibits H4R3 methylation in the hepatocellular carcinoma cell line HepG2 while leaving H3K4 methylation unaffected (255). **Ribavirin** is an antiviral drug that has become of interest as a therapeutic agent in cancer. Ribavirine selectively inhibits pediatric osteosarcoma and improves chemosensitivity (256). It also possesses *in vitro* growth inhibitory effects against various malignant cell lines at clinically reasonable concentrations; also, ribavirin treatment results in the reduction of EZH2 at RNA and protein levels, inhibition of EZH2 enzyme activity, and reduction of H3K27 methylation (257). The anti-malarial drug, **hydroxychloroquine**, has also been effective in treating rheumatoid lupus, arthritis, and *porphyria cutanea tarda*. Structural experiments have shown that hydroxychloroquine inhibits the allosteric binding of PRC2 to EED within the H3K27me3-binding region, thereby antagonizing the catalytic function of the PRC2. These findings suggest a new epigenetic function of hydroxychloroquine with possible therapeutic repositioning (258) (**Table 6**, HMTi).

HDM Inhibitors

Clorgyline is a selective MAO A inhibitor- used as an antidepressant until severe dietary adverse effects are commonly known as the "cheese effect" were reported for this drug (268). As a member of MAO inhibitors, clorgyline can also inhibit LSD1, and it has been demonstrated to have cell-type dependent synergic effects when combined with DNMTi (259). **Geranylgeranoic acid**, an acyclic diterpenoid present in medicinal plants, has recently been found to be a potent inhibitor of recombinant LSD1. Geranylgeranoic acid inhibits the proliferation and induces a neuronal phenotype through increasing the abundance of H3K4me2 of NTRK2 gene promoter in human SH-SY5Y-derived neuroblastoma cells (260). **Pargyline**, a MAO B selective inhibitor with antidepressant activity, affects the transition from androgen-dependent to androgen-independent in prostate cancer. Inhibition of LSD1 with a concomitant reduction of H3K4me2 and H3K9me2 levels have been reported for pargyline. Pargyline, in combination with androgen deprivation therapy, could be an effective adjunctive treatment for advanced prostate cancer (261). Unlike selective MAO inhibitors such as pargyline, non-selective MAO inhibitors strongly repress the nucleosomal demethylation of histone H3K4. **Tranylcypromine**, a drug used in treating severe depression, has demonstrated strong LSD1 inhibitory effects with an IC50 of less than 2 mM (262). Tranylcypromine contributes to GBM cell synergistic apoptosis in association with other HDAC inhibitors (263). Recently, molecular docking studies have highlighted the potential of approved drugs such as **decitabine**, **entecavir**, **abacavir**, **penciclovir,** and **DZNep** as KDM5B inhibitors. Their role as HDMi could be of great importance in lung cancer, melanoma, hepatocellular carcinoma, gastric cancer, and prostate cancer, among others. Decitabine is a DNMTi used in myelodysplastic syndrome (MDS), abacavir, entecavir, and penciclovir are antivirals used in the treatment of HIV, hepatitis B, and herpes infections, respectively. DZNep is a specific HMTi with promising results in cancer immunotherapy (269). Finally, **Polymyxin B and polymyxin E** are antibiotics used in multidrug resistant bacterial infections. These compounds were shown to inhibit LSD1 by competition with its substrate at the enzyme's cleft entry. Polymyxins have significant side effects that limit their application to untreated infections, but they could still be the target of drug repurposing for other diseases, such as leukemia (264) (**Table 6**, HDMi).

BET Inhibitors

Azelastine, a selective H1 antagonist, was found to be a promising BETi, displaying a stronger binding affinity than BETi control JQ1 for human BRD4 by docking-based methodologies. These findings highlight the importance of computational methods for molecular drug design and will uncover new BRD4 inhibition candidates (265). The antibiotic approved by FDA, **nitroxoline**, disrupts the association of BRD4 bromodomain with acetylated H4. Nitroxoline has shown strong selectivity at inhibiting all BET family members compared with non-BET proteins. By causing cell cycle arrest and apoptosis, nitroxoline successfully prevents the proliferation of MLL leukemia cells. The possible use of nitroxoline and its derivatives as BET inhibitors in BET related diseases is now under investigation (266) (**Table 6**, BETi).

CONCLUDING REMARKS

Drug repositioning has emerged as a viable strategy to increase drug discovery's overall productivity, resulting in a new and cheaper way to generate alternative therapies for various diseases, including cancer. The drug repositioning approach is

TABLE 6 | Current HAT HMT, HDM, and BET inhibitors repurposed with epigenetic applications in cancer therapy [*modified from Moreira-Silva et al., (9)].

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
HAT, HMT, HDM, and BET inhibitors							
HATi	**Anacardic acid**	Anti-inflammatory; food-derived (not approved)	HAT/Ep300 and Tip60	Not described	Cervical Tumor cells	Inhibits Tip60 HAT and ATM acetylation. Promotes resensitizing tumor cells to the cytotoxic effect of radiation.	(246)
					Myeloid Leukemia cells T-Cell Lymphoma cells Lung Cancer cells Prostate Cancer cells	Inhibits p300 HAT activity. Also, inhibits NF-kB activation, inhibits IkBalfa activation, p65 acetylation and nuclear translocation. It potentiates apoptosis via TNF-induced caspase activation and suppresses the expression of genes involved in invasion and angiogenesis.	(247, 248)
	Garcinol	Antioxidant benzophenone (not approved);	HAT2B/Ep300	Not described	Cervical Cancer cells	Inhibits p300 and KAT2B activity, HAT activity and induces apoptosis.	(249)
					Breast Cancer cells	Decreases H3K18 acetylation and increases DNA damage signaling markers. Inhibits HAT activity and induces cell proliferation arrest.	(250)
					Hepatocellular Carcinoma cells	Decreases HAT activity and inhibits STAT3 activation through acetylation. Decreases proliferation, tumor growth, survival and angiogenesis.	(251)
					Esophageal Carcinoma cells	Decreases p300/CBP levels, induces cell cycle arrest, thus induces apoptosis and inhibits migration and cell invasion and proliferation. Inhibits metastasis and inhibit HAT and its cofactors, decreasing TGF-beta pathway.	(252)
	Plumbagin	Nutraceutical quinone (not approved);	HAT3B/p300	Inhibits p300 HAT activity (non-competitive), through a single hydroxyl group of plumbagin that makes a hydrogen bond with the lysine 1358 residue of the p300 HAT domain.	Liver Carcinoma cells	Inhibits p300 HAT activity AND inhibits p300-mediated acetylation of p53 AND reduces H3 and H4 acetylation AND induces apoptosis AND modulates the enzymatic activity of p300. *in vivo*: reduces H3 acetylation.	(253)
	Lunasin	Natural Compound; food-derived peptide	HAT	Not described. Possibly a competitive inhibitor	Cancer preventive in mouse Fibroblasts	Suppresses foci formation in mice fibroblast cells induced by chemical carcinogens by the RGD motif and its chromatin-binding property, binding to deacetylated histones, and the reduction of histone acetylation.	(254)
HMTi	**Allantodapsone**	Antibiotic (Dapsone-derivated)	H4R3me	Inhibitory activity toward PRMT1	Hepatocellular Carcinoma cells	Inhibits cellular H4R3 methylation to the same level as AMI-1, while the H3K4 methylation level is barely impacted.	(255)
	Ribavirin	RSV infections and Hepatitis C	EZH2	Not described. Possibly a selective inhibitor of EZH2	Solid Tumors (Atypical teratoid/rhabdoid tumor)	Inhibits cell growth, induces cell cycle arrest and apoptosis. Also inhibits eIF4E and EZH2 activity decreasing its expression levels. Impairs cell migration, invasion and adhesion. In osteosarcoma enhances chemosensitivity.	(230, 256)
					Breast, Brain, Cervical, Colon and Prostate Cancer cells	Decreases EZH2 expression, inhibits HMT activity and decreases H3K27me3. Induces variable growth inhibition and downregulation of EZH2, eIF4E and IMPDH1.	(257)

(Continued)

TABLE 6 | Continued

Class	Compound	First indication	Epigenetic target	Drug-target interaction	Cancer model/New indication	Key features in mechanism	References
	Hydroxychloroquine (HCQ)	Antimalarial/Arthritis	PRC2	Disruption of PRC2-EED complex by allosteric PRC2-EED binding inhibition within the H3K27me3-binding pocket, thus antagonizing the PRC2 catalytic activity	Multiple Myeloma Cells	Decreases H3K27me3 levels in MM cells 3 by disrupting the H3K27me3- EED interaction within the PRC2 complex. Suggesting that its anti-tumor activity might rely on the reactivation of genes abnormally silenced via H3K27 hypermethylation.	(258)
HDMi	**Clorgyline**	MAO inhibitor	LSD1	Not described	Bladder Cancer cells Colon Cancer cells Promyelocytic Leukemia Cells	Induces DNA demethylation, inhibits LSD1, decreasing H3K4me2 and H3K4me, establishes an active chromatin state. Inhibits cell growth induces the expression of previously silenced genes by enriching H3K4me2 and H3K4me1 histone marks.	(259)
	Geranylgeranoic acid	Natural Compound (not approved)	LSD1	Not described	Neuroblastoma cells	Inhibits LSD1 activity, induces NTRK2 gene expression and increases H3K4me2. Moreover decreases cell proliferation.	(260)
	Pargyline	MAO-B inhibitor; antihypertensive	LSD1	Not described	Prostate Cancer cells	Inhibits cell migration and invasion AND inhibit EMT AND induces E-cadherin expression AND inhibits N-cadherin and Vimentin expression AND delayed PCa transition to CRPC AND decreases PSA expression AND decreases H3K4 and H3K9 di-methylation.	(261)
	Tranylcypromine	Severe depression	LSD1	Not described	Glioblastoma cells	Induces cell death AND inhibits LSD1 activity AND increases cell sensitivity to HDACi.	(262, 263)
	Polymyxin A/B	Antibiotic	LSD1	Inhibits LSD1 by competition with its substrate at the enzyme's cleft entry	Chemical inhibition of LSD1 assay	*In vitro* assays demonstrated that quinazoline core can represent a privileged scaffold for developing inhibitors that target epigenetic enzymes.	(264)
BETi	**Azelastine**	Anti-histaminic	BET-BRD4	Inhibits BRD4 through interactions with several key residues of the acetyl lysine binding pocket	Structural *in silico* assays by docking-based method	Docking-based database screening identified Azelastine drug as a promising novel template exhibiting binding affinity better than the control lead (+)-JQ1 for the human BRD4. Azelastine is having a low molecular weight, which gives a scope of further chemical modification to enrich its binding affinity for BRD4.	(265)
	Nitroxoline	Antibiotic	BET-BRD4	Occupies the acetylated lysine binding pocket of the first bromodomain of BRD4	MLL Leukemia cells	Prevents the binding of BRD4 to acetylated H4	(266)

growing due to a broad range of reposition candidate molecules that already have clinical and toxicity profiling developments. One factor that has strongly driven this approach is the increasing availability of biomedical data, including genomic data, which covers various aspects of cellular mechanisms, opening a search that is not restricted to biological factors involved in a disease. This omic perspective allows the deduction of complex interactions that can be inhibited or treated to cure or reverse a pathological condition. Advances in complementary bioinformatic analytical methods provide critical substrate candidates that enable their systematic evaluation. Therefore, a window of opportunity opens where the reuse of previously synthesized drugs can be investigated and given a new direction. Epi-DR has already shown a profit in epigenetics and cancer treatment, where it has proven its efficacy. Indeed, many epidrugs emerged this way, such as 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine) (146), Hydralazine (156), Vorinostat (SAHA), and Valproic acid (236).

Epigenetic alterations are considered to be among the earliest and most comprehensive genomic aberrations occurring during carcinogenesis, and therefore it has been classified as a hallmark of cancer (270). The impact of epigenetics in understanding cancer has been of great interest in recent years, and even more due to the advancement of the genomic era. Several works demonstrate the importance of epigenetic biomarkers that can predict the response or prognosis in various types of cancer. The promoter methylation of the MGMT gene in gliomas is a clear example, where it helps to indicate the use of precision medicine through the drug temozolomide (271). Another example is found in EHZ2 enzyme alterations, which indicate a poor prognosis in breast, prostate, and other types of cancers.

Epigenetic mechanisms have great flexibility to respond to environmental changes and modify gene expression. Consequently, search for artificial ways to induce epigenetic remodeling, which could improve therapy in the event of a disease as cancer. Therefore, the implementation of epigenetic therapies opens a new panorama for the fight against cancer. Epidrugs show enormous potential for clinical use, especially in cancer, because in these diseases, an epigenetic imbalance is a well-known characteristic that is both of origin, development, and severity of tumors.

Even though there are already some epidrugs approved by the FDA and the current knowledge about various mechanisms involved in gene regulation, promoted by the advancement of technologies that expand the information on specific epigenetic mechanisms, challenges remain in identifying epigenetic modifications of cancer and targeting them for therapeutic purposes. Among them stands out that epigenetic changes can be diverse in the types of cancer and between the different clinical phases and those that are dependent on environmental conditions. Therefore, we must distinguish between the dysregulation of driver genes and those whose changes are secondary to these. Also, the generation of epigenetic therapies as well as the molecular mechanisms that coordinate them is subject to understanding, and much research is still required of several of them to safely transport them to the clinic. However, identifying epigenetic alterations that affect the tumor's fate and behavior finding drugs that target them are some of the promises of epigenetic therapy in cancer.

In this sense, the concept of reusing a medicine offers a broad scope to investigate the hidden potential behind the medicine and to recycle it. The reincorporation of a drug with the potential to remodel epigenetic characteristics, which are beneficial for cancer management, is of great interest to the field. Offering great advantages in drug development times could lead to precision medicine therapy with new and clearly encouraging prospects for the future (**Figure 4**).

AUTHOR CONTRIBUTIONS

MM-C and MM-R wrote and designed the manuscript. RG-B coordinated, wrote, and designed the manuscript. RG-B and VJ-G revised the manuscript. VJ-G elaborated on the figures. CA-C wrote and revised the manuscript, and LH coordinated and directed the review development. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Glycosylated Nanoparticles for Cancer-Targeted Drug Delivery

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Nanoparticles (NPs) are novel platforms that can carry both cancer-targeting molecules and drugs to avoid severe side effects due to nonspecific drug delivery in standard chemotherapy treatments. Cancer cells are characterized by abnormal membranes, metabolic changes, the presence of lectin receptors, glucose transporters (GLUT) overexpression, and glycosylation of immune receptors of programmed death on cell surfaces. These characteristics have led to the development of several strategies for cancer therapy, including a large number of carbohydrate-modified NPs, which have become desirable for use in cell-selective drug delivery systems because they increase nanoparticle-cell interactions and uptake of carried drugs. Currently, the potential of NP glycosylation to enhance the safety and efficacy of carried therapeutic antitumor agents has been widely acknowledged, and much information is accumulating in this field. This review seeks to highlight recent advances in NP stabilization, toxicity reduction, and pharmacokinetic improvement and the promising potential of NP glycosylation from the perspective of molecular mechanisms described for drug delivery systems for cancer therapy. From preclinical proof-of-concept to demonstration of therapeutic value in the clinic, the challenges and opportunities presented by glycosylated NPs, with a focus on their applicability in the development of nanodrugs, are discussed in this review.

Keywords: drug delivery, glycoconjugates, glycosylated nanoparticles, glycodendrimers, cancer therapy, glycopolymers

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INTRODUCTION

Nanoparticles have long been known as the foremost systems to improve drug delivery for treatment of several diseases, especially cancer. However, development of effective, targeted, and safe drug delivery systems remains challenging in many cancer types due to limited target sites (1). Therefore, to develop strategies that facilitate specific delivery of therapeutic agents to the target site, reducing access to nontarget sites is urgently needed (2, 3). One strategy for applying targeted therapies is the use of carbohydrates and monosaccharides as ligands that represent crucial structures on tumor cell membranes and have been shown to be effective for cell-selective drug delivery (4).

Cancer metabolism is also a promising target for cancer therapy in the nanomedicine field. According to the classic theory known as "the Warburg effect," cancer cells require a much higher

glucose flux than normal cells because their phenotype is characterized by preferential dependence on glycolysis for energy production in an oxygen-independent manner (5). Hence, certain key proteins involved in this disruptive metabolism, such as GLUT, hexokinase-2 (HK2) and phosphoglycerate dehydrogenase (PHGDH), which are overexpressed in cancer, have been examined as possible targets (6). Additionally, energy source replacement with other monosaccharides, such as mannose, could retard tumor progression (7). Currently, repurposing of nanocarriers conjugated with glycan-based molecules is an interesting field of opportunity for cancer therapy and diagnosis. Hence, a wide range of functional nanocarriers, including polymeric, metallic, and metalorganic NPs, are being studied and developed in the biomedical field (8). NPs possess unique physical, optical, and electrical proprieties and can be conjugated with several therapeutic and target molecules that modify their interactions with cell membranes and biological systems, altering their toxicity and pharmacokinetic profiles (9). Furthermore, adsorption or conjugation of glycan structures can change the intrinsic properties and mobility of NPs in biological systems. Glycosylated nanomaterials interact differently with tumor-associated glycoprotein receptors, and generally, binding can be achieved through multivalent carbohydrates because both the membrane and microenvironment of cancer cells have been well studied (10–12). Therefore, this review aims to highlight the current novel strategies that have been developed for cancer therapy through the use of drug delivery systems that include carbohydrate-based NP systems as dendrimers, micelles, silica, and lipidic and metallic NPs, exploiting the modified metabolism of cancer cells as a therapeutic approach.

GLUCOSE METABOLISM AND TRANSPORTERS IN CANCER CELLS

The modified metabolism in cancer cells, which resorts to preferential use of glycolysis as the main energy source for ATP generation, promotes cancer cell growth, survival, proliferation, and long-term maintenance (13). The ATP production efficiency of glycolysis is much lower than that of oxidative phosphorylation, and cancer cells adapt to this disadvantage by increasing glucose uptake (**Figure 1A**) (5). Indeed, in the clinic, it has been reported that a high blood glucose level is associated with a poor prognosis in cancer patients (15, 16). Therefore, glucose plays an important role in cancer progression because it promotes cancer cell proliferation in a dose-dependent manner (17, 18).

Glucose is a hydrophilic molecule that must be transported and modified by specific proteins in the cell. Two classes of transporters are present in cells: the family of GLUT proteins and sodium-dependent glucose transporters (SGLTs) (19). These molecules are overexpressed in cancer cells; therefore, their inhibition can be a therapeutic strategy against cancer (20, 21). The use of compounds that suppress the growth of cancer cells through inhibition of glucose transporters has been widely explored in various types of cancer, including liver, colon, ovary, prostate, brain, and breast cancer (21–26). For example, in ovarian cancer cells, GLUT-1 and GLUT-3 protein levels are increased 6.5 and 4.1 times, respectively, and a GLUT-1/-3 inhibitor prevents cell growth, targets metabolic plasticity, and overcomes the cellular rescue mechanisms of cancer cells (22).

GLYCOSYLATION AFFECTS CANCER CELL MEMBRANES AND THE MICROENVIRONMENT

Cancer cells exhibit membranal structure changes *via* changes in external monosaccharide-related target molecules, such as proteins and lipids, that aid in tumorigenesis, malignant transformation, and tumor dissemination (27). For example, overexpression of sialic acid on the cell surface creates a negative charge on membranes and repulsion between cells, which helps cells enter the bloodstream (28). Changes in the intrinsic glycosylation of cell surface adhesion molecules, such as selectin ligands, integrins, and mucins, have been implicated in changes in the tumor microenvironment that can contribute to drug resistance and pH acidification (29), which lead to more aggressive cancer cell phenotypes; thus, their implications in the design of glycan-based therapies should be investigated (30). Therefore, glycans, glycoproteins, glycan-binding proteins, and proteoglycans are mechanistically implicated in cancer hallmarks (31, 32). For instance, lowered tumor extracellular pH (pHe) and upregulation of the membrane protein matrix metalloproteinase 2 (MMP2) in the tumor microenvironment has been exploited as a strategy to improve the selectivity of plasmid DNA release. Hence, DendriGraft poly-lysine, third-generation, (DGL-G3) conjugated with a cell-penetrating peptide (CPP), quenched by a pH-sensitive masking peptide, and linked by a metalloproteinase MMP2 substrate was a successful gene delivery system in a hepatoma cell line (32, 33).

Furthermore, tumor-associated macrophages (TAMs) can remodel the tumor microenvironment to reduce growth barriers, such as the dense extracellular matrix, and shift tumors towards an immunosuppressive microenvironment that protects cancer cells from targeted immune responses, making it difficult to deliver drugs with NPs larger than 100 nm (34). Glycoconjugates, such as mesoporous silica NPs (MSNs), can interrupt these biological interactions within tumors by altering TAM phenotypes through a process called polarization. By treating these MSNs with deglycosylases, the surface glycosylation of these NPs can be modulated without altering the protein coating. Reports indicate that increasing the size of silica particles can reduce their cellular uptake and minimize their M1-like macrophage polarizing capability, and surface modification of MSNs can further control their cellular uptake and modulate their polarization effects (28, 34, 35). Therefore, further investigation is required to determine the complete effects of carbohydrate changes in the external microenvironment and their role in inhibition of tumorigenesis.

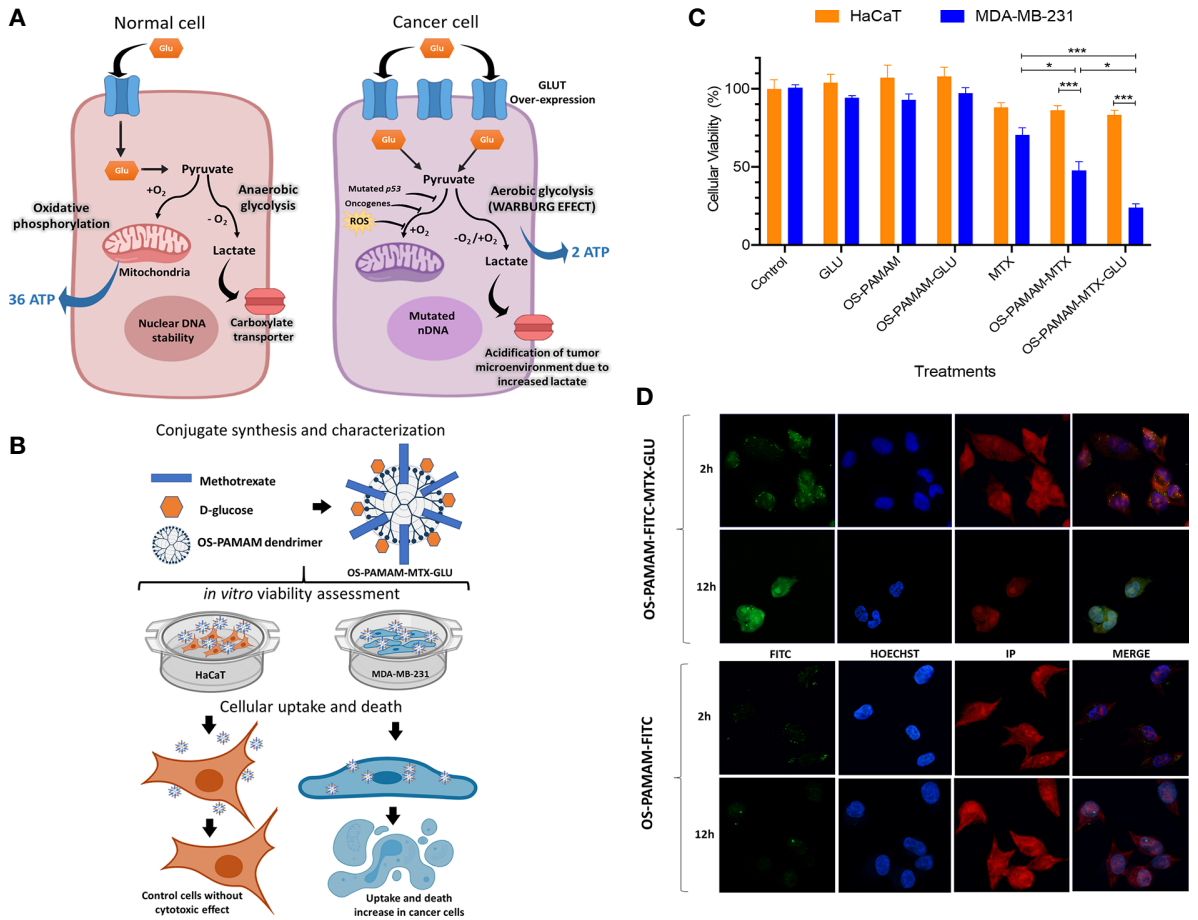

FIGURE 1 | A graphical representation of Warburg effect in cancer and experimental demonstrations of the improvement of glycosylated drug delivery systems for target cancer therapy **(A)** Metabolic differences between normal and cancer cells. In the presence of O_2, normal cells metabolize glucose in pyruvate followed by oxidative phosphorylation in the mitochondria generating 36 ATP per glucose molecule. In the deficiency of O_2, pyruvate is transformed to lactate *via* anaerobic glycolysis generating 2 ATP per glucose molecule. In cancer cell, mutations in mtDNA, nDNA or absence of *p53* gene, presence of oncogenes and ROS suppress oxidative phosphorylation and enhances lactate production *via* glycolysis even in the presence of O_2 (Warburg effect). **(B)** Glycosylated PAMAM dendrimers conjugated with methotrexate as a strategy for breast cancer target therapy. **(C)** Comparison of viability between MDA-MB-231 and HaCaT cell lines. Cells were exposed to OS-PAMAM-MTX-GLU and control treatments at the same concentration of free MTX and GLU was calculated in encapsulation assay for 4 h. Data represent mean ± SD (n = 16). Statistical analysis was performed by two-way ANOVA followed by *post hoc* Tukey's multiple comparisons test. ***P < 0.001, *<0.02. **(D)** Confocal images of MDA-MB-231 cells incubated for 2 and 12 h with OS-PAMAM-FITC and OS-PAMAM-FITC-MTX-GLU. For each group, the images from left to right showed the fluorescence of FITC (green), Hoechst 33342 (blue), and PI (red) stains. Images were acquired at 63×. Data has been contributed and modified from Torres-Pérez (14).

CARBOHYDRATE-BASED CARRIER MOLECULES FOR CANCER THERAPY

Specificity is a crucial aspect of drug administration in treatments against cancer because nonspecific agents can damage healthy tissues, causing adverse effects in patients (36). Carbohydrate changes in the external microenvironment of cancer cells also provide specific targets for carrier-based drug delivery. Hence, these carriers must be composed of biocompatible and biodegradable materials, which should be well characterized and conjugated (37). Among these, nanomaterials have been well accepted as nontoxic and nonimmunogenic agents (38).

NPs based on carbohydrates or conjugated to them have been explored as vehicles for drug administration in cancer (39, 40). Indeed, a wide variety of polysaccharides have been used, including chitosan (41), cellulose (42), glycogen (40), chitin (43), and dextran (44, 45), among others. There are two special cases. The first is hyaluronic acid (HA), a natural polysaccharide used in gene therapy and as a based-drug carrier. HA has shown a high molecular interaction with the CD44 receptor protein, a cell-surface glycoprotein involved in cell-cell interactions that is overexpressed in several types of cancer cells (46, 47). The second is the chitosan NPs, which are self-assembled, low-cost nanostructures with high positive charges that have the ability to encapsulate and deliver hydrophobic and negatively charged

drugs to cancer cells (48). Chitosan can be accumulated accurately by proving the interaction of charges and permeability with the cancer cell membrane. Also, it has shown a high biodegradability in sub-components of glutamic acid (49). This type of NP can be preferentially internalized *via* receptor-mediated endocytosis. Uptake studies have demonstrated an increase in the endocytic pathway, with both clathrin and caveolae activation, when receptors on the cellular membrane were blocked. Therefore, the intrinsic properties of NPs conjugated with ligand molecules, such as folic acid, can significantly improve drug delivery in chemotherapy strategies and reversion of multidrug resistance (50, 51).

CONJUGATION STRATEGIES FOR GLYCOSYLATED NANOPARTICLES USED IN CANCER THERAPY

Setting up a conjugation method requires several considerations, starting from an understanding of the chemical composition of both the cargo and the carrier molecule. The chemical composition of cargo molecules influences the physicochemical properties of nano systems including size, surface charge, and shape, but also, modifying biological effects. For therapeutic purposes, glycosylated nanoparticles (G-NPs) should be biocompatible, biodegradable, and soluble in biological fluids, and most importantly, they must have receptor-targeting properties (4, 8).

The most common monosaccharides, including glucose, mannose, fructose, and galactose, have usually been applied in the synthesis of glycoconjugates because of the ease of conjugation and their specific effect as a targeting ligand to some key receptors found in cancer cells (4). Monosaccharide molecules possess several groups, such as hydroxyl groups, which can be highly reactive to generate stable conjugation with carrier NPs through various linkage approaches, such as reductive amination (52, 53). Drug carriers usually have amino-terminal groups that allow hydroxyl groups to be linked directly to both NPs and/or drugs through the following strategies (54, 55):

1. Direct amide linkages with sugar-bearing carboxylated or activated ester derivatives. This is beneficial for conjugation of monosaccharides, for example, in surface-amino dendrimers modified with chemo drugs against breast cancer and glioma (14, 56), including antitumor immunotherapy using chitosan NPs and TCL vaccines coupled with mannose to target specific moieties in dendritic cells (DC) (57).
2. Introduction of thiourea linkages formed by treatment of NP-amino groups with isothiocyanate saccharide derivatives. This coupling is helpful for theragnostics when different linkage strategies must be employed for different cargo molecules or NP systems and has been used in dendrimers premodified with fluorescein isothiocyanate but also linked to gold NPs (58).
3. Monosaccharides can also be found in the derivate version containing amino groups, which are frequently used for carriers with peripheral carboxyl groups, for example, D-mannosamine conjugated to solid lipid nanoparticles (SLNs) through amidization. The resulting p-aminophenyl-a-D-mannopyranoside-modified SLNs (MAN-SLNs) effectively delivered docetaxel to the brain (59).

The advantages of these strategies include the following: i) the reactions are conducted at room temperature and are compatible with most drugs and degradable linkers; ii) the resulting products, such as poly(monochlorotriazine), can be conveniently derivatized (i.e. PEGylated). However, direct sacrifice of the reducing sugars, formers of extended linkers *via* amide-bond formation starting from sugar lactones described in the first syntheses, should be avoided, and the NP must have a spherical architecture to avoid a chelating effect (60).

PHYSICAL PROPERTIES OF GLYCOSYLATED NANOPARTICLES

The performance of drug delivery systems based on NPs in cancer therapy is affected by several physical properties, mainly size, shape, and surface electric charge, which modulate NP toxicity and stability. Also, these characteristics should be considered for glycoconjugates because most interactions with altered membrane molecules are closely related to the aforementioned parameters (61). In NPs, small changes in structure can lead to significant changes in properties and reactivity. Additionally, the directional organization of molecules on the nanoparticle periphery can help by increasing the electrophile affinity to target molecules due to the high surface area to volume ratio of NPs (62, 63). Therefore, the optimum drug dispersion and homogeneity in a nanoparticle system and the linkage to cargo molecules should be well controlled and reproducible to obtain the desired therapeutic effect (64).

Regarding size, reports on organic and inorganic NPs indicate that glycosylation increases the size and molecular weight of NPs (14, 64, 65). Additionally, glycoconjugates exhibit a neutralization of zeta potential without significant alterations in colloidal stability (34, 66). Furthermore, depending on the drug conjugation approach and the therapeutic strategy, cationic saccharide molecules, such as dextran spermine and aminated pullulan, or anionic molecules, such as pectin, heparin, and hyaluronic acid, can be modulated to obtain the desired therapeutic effect (67).

Regarding cancer therapy with drugs, it is crucial to avoid side effects due to the toxicity of NPs. Nonspecific toxicity is primarily influenced by surface chemistry, functionality, size, chemical composition, and zeta potential (65, 68). Organic glycoconjugates are natural products of living systems also upshot as multifaceted drug delivery vehicles that can reduce the toxicity associated with unmodified drug carriers and

therapeutic agents. An additional attribute of these carriers is their ability to positively alter the pharmacokinetic profile of drugs through stabilization (2, 38, 69). Furthermore, glycans and carbohydrates can neutralize the very positive or very negative charges of NPs, such as dendrimers or gold NPs, which can compromise the integrity of the plasma membrane, causing necrotic cell death (70, 71). Therefore, attached glycans play a critical role in maintaining NP stability and conformation and can define many of the physical properties of NP systems, which positively influences the safety of the proposed nanosystems through improvement of pharmacokinetic and biocompatibility (35, 72, 73).

APPLICATIONS OF GLYCAN-BASED NANOPARTICLES

Glycan changes in malignant cells, a hallmark of cancer, take a variety of forms: increase in incomplete or truncated glycan expression, loss of expression or excessive expression of certain glycans, and, less frequently, the appearance of novel glycans (26, 74). Furthermore, G-NPs have been studied to improve specific delivery of known and reassigned drugs as well as DNA, proteins, and peptides like vaccines. A database search was carried out with the words "glycoconjugates," "glycopolymers," "glycodendrimers," and "glycol AND drugs" "glycosylation AND nanoparticles AND cancer" in the Scopus server and Integrity (www.integrity.clarivate.com). The search revealed the increasing amount of research on G-NPs during the last 20 years (approximately 3,500 patents), especially because the number of technology patents around the world has doubled in the last 10 years. Therefore, these types of nanosystems have the potential to be used in cancer therapy and prevention, pathological imaging diagnosis, and theragnostics.

Glycosylated Nanoparticles as Carriers of Drugs and Small Molecules

The most common strategies for cancer therapy include the use of small molecular drugs, and NP systems improve the pharmacokinetic and pharmacodynamic profiles of these drugs due to the ability of NPs to remain in prolonged circulation in systemic models, increasing drug biodistribution and circulation, and reducing *in vivo* side effects (75, 76). For instance, overexpression of GLUT in breast cancer cells can enhance drug uptake (77). Moreover, our group performed a therapeutic strategy that included glycosylation of a one-step PAMAM dendrimer loaded with methotrexate (OS-PAMAM-MTX-GLU) (**Figure 1B**). This study showed that glucose conjugation led to a 150% increase in the internalization of OS-PAMAM conjugates in MDA-MB-231 breast cancer cells and reduced cell viability by up to 20%. Cancer cell death was significantly higher with the nanosystem than with free MTX, and the system displayed specificity because no effects were observed in noncancer cells (**Figures 1C, D**) (14).

Gold glyconanoparticles coupled to listeriolysin O 91–99 peptide (GNP-LLO_{91-99}) have been used as a novel adjuvant for cancer therapy. GNP-LLO_{91-99} exhibited antitumor activity by inhibiting tumor growth and migration in melanoma cells and generated an immune response by recruiting and activating DC (78). In addition, other strategies, including two glycosylated systems to deliver cisplatin (CDDP), mannose-decorated tobacco mosaic virus (CDDP@TMV-Man) and lactose-decorated tobacco mosaic virus (CDDP@TMV-Lac), have been reported. CDDP@TMV-Man induced enhanced endocytosis and apoptosis in galectin-rich MCF-7 cells, whereas CDDP@TMV-Lac showed superiority in endocytosis and apoptosis in HepG2 cells with overexpression of asialoglycoprotein receptors (ASGPR) (79). Currently, other strategies for cancer drug delivery using glycosylated carriers have shown a high antitumoral effect, reaching up to 95% cell death. In particular, the high affinity of galactose for the asialoglycoprotein receptor in cancer cells has provided outstanding therapeutic strategies, with special benefits in liver cancer (**Table 1**).

G-NP Carriers of Nucleic Acids

Due to recent developments in gene therapy, G-NPs have been employed for specific and higher nucleic acid (siRNA, DNA, and miRNA) transfection. A series of cationic block copolymers (PHML-*b*-PMAGal) and the statistical copolymers P(HML-*st*-MAGal) with pendant natural galactose and (L-)-lysine moieties were exposed to a human non-small cell lung carcinoma cell line. P(HML_{40}-*st*-$MAGal_4$) with 4.8% galactose content showed the highest gene transfection efficiency among the synthesized cationic polymers, 6.8-fold higher than the "gold standard" bPEI-25k (87). Combined treatments, such as using targeted NPs to deliver chemopeptides and gene therapeutics, have been delivered efficiently to cancer cells and tissues to avoid transfection cytotoxicity, overcome drug resistance, and stop tumor development. In one study, a novel mannosylated copolymer with a CPP grafted into Polyethylenimine (PEI) was prepared to target antigen-presenting cells (APCs) with mannose receptors. The gene transfection was significantly higher by the grafted CPP mannosylated than in control cells (88, 89).

G-NP Applications in Immunotherapy and Vaccines

The presence of altered glycans on cancer cells has been used as a diagnostic marker and tumor cell marker (90). Glycan aberrations have not only been used as markers but can also be linked to endogenous lectins, such as galectins, sialic acid-binding immunoglobulin type lectins, and selectins (91). For example, type C lectin receptors are widely expressed on myeloid cells, such as macrophages, neutrophils, and DC. Consequently, they can mediate specific interactions with tumor antigens and facilitate tumor rejection (92, 93).

Due to their relevance, incomplete or truncated glycan structures, often covered by sialic acid and commonly known as tumor-associated carbohydrate antigens (TACA), have been studied (94). These antigens have already been seen to be overexpressed in different cancer types, such as breast, pancreas, bladder, and colon cancer (95–98). For example, glycodendrimers were evaluated due to their dual properties as

TABLE 1 | Applications of the recent glycosylated nanoparticles for drug delivery in cancer cells.

Carrier	Average size ± SD (nm)	Ligand	Receptor	Applications	Cell line/cancer model	The decrease in tumor volume/cell viability (%)	Decrease in control cells (%)	Reference
Glycogen nanoparticles	175 ± 75	Galactose	Asialoglycoprotein receptor	The system has efficient accumulation and release of drugs at tumor sites, inhibiting tumor growth with only slight retention in normal liver tissues.	*In vivo* model HepG2/Liver epithelial cells	80	15	(40)
Solid-lipid nanoparticles	174,51 ± 5.1	Fucose	Lectin receptors	Efficient delivery of methotrexate mediated by fucose-decorated solid lipid nanocarriers in breast cancer therapy.	*In vivo* model MCF7/Breast epithelial cells	75	-*	(75)
Liposomes	81.9 ± 6.2	Mannose-6-phosphate	Type II insulin-like growth factor receptor	Selective induction of apoptosis in MCF7 cancer cells by specific liposomes functionalized with mannose-6-phosphate.	*In vitro* model MCF7/Breast epithelial cells	50	No significant differences with untreated cells	(80)
Polymer nanoparticles	54,84 ± 0.58	Galactose	Asialoglycoprotein receptor	Galactose-Containing Polymer-DOX Conjugates for Targeting Drug Delivery.	*In vitro* model HepG2/Liver epithelial cells	80	55	(81)
Polyethyleneimine-modified iron oxide nanoparticles	98.2 ± 2.3	Galactose	Asialoglycoprotein receptor	Targeted delivery and accumulation of siRNA in tumor cells for therapy of hepatocellular carcinoma.	*In vivo* model Hepa 1–6/Liver epithelial cells	70	–	(82)
Polymer nanoparticles	112 ± 5	Galactose	Asialoglycoprotein receptor	Polymeric NPs as potential carriers for hepatoma-targeted drug delivery and liver cancer therapy in clinical medicine.	*In vitro* model HepG2/Liver epithelial cells	95	No significant differences with untreated cells	(83)
Lipid nanoparticles	228,8 ± 5.42	Mannose	Mannose receptor	Increased supply of gemcitabine in lung cancer cells. The mannosylated formulation has higher cytotoxicity and can selectively kill cancer cells.	*In vitro* model A549/Lung epithelial cells	35	–	(84)
Lipid nanoparticles	239 ± 2,4	Galactose	Lectin receptors	Targeted delivery of doxorubicin to lung cells induces increased cytotoxicity related to that related to marked drug uptake and accumulation.	*In vitro* model A549/Lung epithelial cells	30	–	(85)
Mesoporous silica nanoparticles	180 ± 50	Mannose	Lectin receptors	Nanoparticles conjugated with D-mannose vehicles for controlled drug release in A549 cells.	*In vitro* model A549/Lung epithelial cells	45	10	(86)

**Not determined.*

targeting agents using a CD4- and CD8-directed melanoma antigen (gp100) and a glycan (LeY) recognized by the type C lectin receptors DC-SIGN and Langerin. Thus, the first glycovaccine with dual C-type lectin receptors (CLR) targeting properties was designed with glycosylated dendrimers, which reached multiple human skin DC and improved antitumor CD8+ T cell responses (99). These investigations demonstrate that glycans can be applied both in the construction of systems to detect biomarkers for tumor diagnosis and prognosis determination, as well as in the development of vaccines targeting carbohydrate antigens (91).

G-NPs Used in Theragnostics

The Warburg effect is a hallmark of cancer and serves as a target for both diagnosis and therapeutic strategies (100). Several glycoconjugates, such as 99mTc-labeled deoxyglucose derivates and glucosamine functionalized with multiwalled carbon nanotubes, have been employed as diagnostic agents for heart and brain cancer and showed superior accuracy over current diagnostic methods (101, 102). However, in recent years, theragnostic systems, such as silica and hyaluronic acid-based NPs that can be used to image cancer cells and at the same time can suppress tumor growth, have been designed by improving the solubility of hydrophobic drugs and glycosylation-mediated drugs and the tumor cell targeting efficiency, with minimum toxicity (103–105).

CONCLUSIONS AND PERSPECTIVES

Current evidence indicates that glycosylation strategies combined with drug delivery systems and immunological therapy present potential opportunities for cancer therapy and theragnostics. In particular, nanosystems proposed for lipidic NPs with galactose are the most well studied and promising strategy against several cancer types. However, targeted G-NPs for cancer treatment involving novel nanotechnologies and medical strategies have numerous challenges and issues. One of the challenges of targeted NPs is to induce a beneficial alteration in the solubility, stability, and pharmacokinetic features of the drug carried. Other challenges are related to control the diverse alterations in the tumoral microenvironment and the clinical safety and repeatability concerns.

Further nanomedicine innovations and basic research are crucial for the discovery of more specific cancer receptors and new glycan-based ligands or repurposed drugs against these receptors. Although the majority of carbohydrates and chemo drugs used in these experimental therapies are low-cost molecules, the sum of all the components and synthesis steps necessary to obtain the nanoconjugate can be expensive, and researchers have not fully examined the cost-effectiveness issues. Apart from accumulation of nonmetabolizable nanocomponents like gold, leakage of shelf life, toxicity of some substances employed for making NPs is another restriction. Therefore it is recommended to use organic NPs for therapeutic applications.

AUTHOR CONTRIBUTIONS

ST-P and ER-G designed this work of review. CT-P, ST-P and MP-E performed the literature search of the databases. ST-P and CT-P Writing—original draft preparation. ER-G, MP-E and SP-T Supervision, writing—reviewing and editing. All authors contributed to the article and approved the submitted version.

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Repurposing Individualized Nutritional Intervention as a Therapeutic Component to Prevent the Adverse Effects of Radiotherapy in Patients With Cervical Cancer

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Worldwide, cervical cancer was the fourth leading cause of cancer death among women, while in Mexico was the second cause (5.28%). Cancer patients receiving chemotherapy and radiotherapy have a high risk of malnutrition secondary to the disease and treatment, affects the patient's overall, with adverse effects on gastrointestinal symptoms. These use affects the medical therapy. The aim of the present study was to evaluate the benefits on individualized nutritional therapy on decrease weight loss and gastrointestinal adverse effects and to consider these outcomes in pharmacology research, especially in repurposing drugs. We conducted a longitudinal design with two comparation groups with medical diagnosis of cervical cancer and received radiotherapy weekly, 1) the intervention group (nutritional intervention and counseling -INC-) with 20 participants and 2) control group (retrospective cohort -CG-) with 9 participants. Weekly body composition, dietary intake, adverse effects (gastrointestinal symptoms), glucose, hemoglobin, and blood pressure were analyzed during 4 to 5 weeks. Both groups had weight loss weekly ($p = 0.013$ and $p = 0.043$ respectively) but the CG vs INC presented loss fat-free mass ≥500g in 67 and of 37% respectively. By the end of the intervention a 25% of the INC group had <10 g/dL of hemoglobin vs 60% for the CG. To compare the dietary intake of vitamins (A and folic acid), fiber ($p = 0.006$), iron ($p = 0.03$) and energy (mainly carbohydrates) ($p = 0.04$) were according to the recommendations in INC group ($p>0.05$). The number needed to treat was 4 (95% CI, 2 to 13). The nutritional intervention and counseling weekly during radiotherapy in cervical cancer to maintain/improve muscle mass, hemoglobin, and dietary intake above 70% of the recommendations for INC group compared to the evidence. Adequate nutritional status was maintained and decrease the rate of complications, mainly gastrointestinal symptoms, in INC group. The efficacy of drug repurposing can improve through individualized nutritional therapy for preventing adverse effects of radiotherapy in patients with cervical cancer.

Keywords: nutritional intervention, cervical cancer, body composition, radiotherapy, drug repurposing

INTRODUCTION

Cervical cancer (CC)was the fourth leading cause of cancer death (311,000 deaths) among women in worldwide while in Mexico was the second cause (5.28%) in 2018. The mortality was three times higher in Latin America and the Caribbean compared to North America (1). Women with ages 25 to 69 years and in lower socio-economic groups are more prevalent (2). Some health programs benefit the prevention of chronic degenerative diseases whose main risk factor is overweight and obesity (3). In Mexico vaginal cytology and human papiloma virus (HPV) vaccination are part of CC prevention as part of early detection programs. HPV is the main risk factor (96.6%) of CC (4, 5). There are more than 100 variants of HPV but only the 16 and 18 are associated to CC (70 to 76%) (6).The risk of having HPV increases from 2 to 10 times with the onset of sexual activity (It is exacerbated with greater number of sexual partners), an onset of sexual life before 18 years, adolescent pregnancy, multiparity and smoking (7, 8).

Nutritional intervention and individualized counseling (INC) are a nutritional therapy with dietary prescription based on the control of symptoms for avoiding the undernutrition, overnutrition or any deterioration of the patient. Unfortunately, there is not enough evidence on long term compliance and long term follow up. An INC has benefits in the treatment of many diseases and in this case, it will depend on the type of cancer or its stage (9). There is evidence the INC well implemented impacts and contributes to improve the prognosis of cancer treatment (chemotherapy and radiotherapy) but there is little evidence in cervical cancer (10).

It is known that healthy dietary habits can contribute to reduce CC risk trough maintenance immune system response due to antioxidant presence, avoiding susceptibility to infectious diseases such as HPV (11). The western diet (ultraprocessed foods and sugary drinks, low in fiber, high in saturated fat, sodium, additives) increases the risk of CC (OR = 3.26, 95% CI = 1.03, 10.3; p <0.05) (12). Some studies had associated deficiency of acid folic also other nutrients with a lack immune response (OR = 14.9, 95% CI = 2.65–84.38 and OR = 8.72, 95% CI = 1.55–48.82) (13), vitamin B12 (OR = 0.25, 95% CI, 0.10–0.58, p <0.01 and OR = 0.40, 95% CI, 0.17–0.88, p = 0.02) with an increased risk in prevalence of CC (14). The vitamin C intake has been associated with a decreased risk of cervical intraepithelial neoplasms (OR = 0.58, 95% CI = 0.38–0.89, p = 0.011; OR = 0.59, 95% CI: 0.39–0.89; OR = 0.59, 95% CI = 0.39–0.88 and OR = 0.62 95% CI = 0.40–0.95) (15), as well as the consumption of vegetables and fruits (OR = 0.50, 95% CI = 0.27–0.95). An inverse association between serum levels of carotenoids and tocopherol has high risk of cervical neoplasia (OR = 0.71, 95% CI = 0.56–0.92; p = 0.003 and OR = 0.75, 95% CI = 0.60–0.94; p = 0.008) (16). The Cervical Cancer Screening Study carried out in the United States found that a BMI greater than 29 increases the risk of HPV infection and its progressing to CC (17, 18). In Mexico is high the prevalence of obesity in women (30 to 40%) and a study found that a high energy intake and obesity were observed in women with HPV (19).

Radiotherapy (RT) in pelvic area (period of 6 weeks in average) generates adverse effects such as diarrhea (15% at onset and a 84.7% at finish RT), vomiting (19% at onset and 65% a at finish RT), nausea (39% at onset and a 45% at finish RT) enteritis, colitis and proctitis cause intestinal malabsorption, enterocolitis, ulcers, stenosis and suboclusive symptoms (20). 83% of patients with RT in the pelvic area in the past lost weight during treatment (21–23). According to the evidence, the individualized nutritional treatment must be part of cancer treatment especially in CC that helps to reduce adverse effects of RT. The nutritional objectives must focus on reducing fat-free mass loss and maintaining its functionality as well as reducing the adverse effects generated by the toxicity of RT and improving prognosis cancer (24). Ravasco et al., evaluated the RT toxicity in patients with colon cancer in the abdominal-pelvic area found that 65% of these patients (who received only standard recommendations) had radiotherapy-induced toxicity 90 days after treatment while that the group who received an individualized nutritional intervention only 9% of the patients presented it (25).

Due the benefits of nutritional intervention on the maintenance of body composition (preserving fat free mass) in cancer during RT and its association with reducing adverse effects (mainly gastrointestinal) (26, 27), the aim of the present study was to evaluate the benefits on individualized nutritional therapy with counseling on decrease weight loss and gastrointestinal symptoms, compared with historical cohort group on adverse effects. We hope that these outcomes could be considered in pharmacology research, especially in repurposing drugs.

METHODS

Study Population

Guanajuato is located in central Mexico, has 5,265,529 inhabitants (28), of which 51.7% are women (41% works at home). Guanajuato has a population of 657 513 emigrants (12.48% of the total population in the state) mainly to the United States. Migration is known to be a risk factor for increasing HPV exposure in women (29, 30). Since 2012 in Guanajuato has been implemented the program "Prevention and control of women's cancer" for addressing caused of mortality of CC (31). Last epidemiologic analysis showed a rate of 4.7 death per 100,000 habitants in Guanajuato state in 2018 and 4.2 per 100,000 habitants in 2019 (32).

Study Design

We conducted a longitudinal design with two comparation groups. The inclusion criteria were,for both groups, to have a medical diagnosis of CC with weekly radiotherapy in a public or private hospital, to have 18 years and over, to have been born in any city in the state of Guanajuato, medium to low socioeconomic and accept the informed consent. Participants who intake dietary supplements were not included and who did not have at least 80% weekly follow-up for cases or controls, were eliminated for the study. Non-probabilistic sample (consecutive cases by simple availability). A 100% of the cases and 70% of the controls (retrospective cohort) were selectec from the shelter "Jesús de Nazareth" located in Leon, Guanajuato.

Study Groups and Recruitment Phase

The study groups were: 1) the intervention group (nutritional intervention and counseling -INC-) with 20 participants and 2) control group (retrospective cohort -CG-) with 9 participants.

According to **Figure 1**, for INC group, 36 patients were assesed for elegibility in the recruitment phase; 7 were excluded (three participants did not meet the inclusion criteria and four did not accept to participate). Twenty participants were allocated to nutritional intervention and conseling group (INC). For retrospective cohort (CG) the sample size were nine. The historical cohort were study two years ago with the same characteristics. Both groups were followed up during RT of 3 to 5 weeks.

Intervention Group: Received a nutritional intervention (with individualized diet) and counseling during the radiotherapy treatment weekly (four to five weeks). The counseling was according to the gastrointesinal adverse effects and their tolerance. Before intervention, a complete nutritional evaluation was carried out body composition, dietary intake, adverse effects (gastrointestinal symptoms), glucose, hemoglobin, and blood pressure. The recommendations for energy and nutrient intake were based on ESPEN guidelines (33) for cancer patients. The dietary calculation was based with FAO/WHO/UNU, Harris-Bennedict formulas and ESPEN guidelines (suggest 25 to 30 kcal/kg/day with 1.2 to 1.5g protein/kg/day). Macronutrients were established of 20 to 30% for protein, 30 to 40% for lipids and 40 to 50% for carbohydrates and micronutrients were according to the *Recommended Daily Intake (RDI)* (34). The dietary recommendations were adjustment and individualized according to comorbidities presented in participants (diabetes, hypertension, hypothyroidism). The counseling was adjustmented according to the gastrointestinal adverse effects and food tolerance (for example, prescription of astringent diet when the diarrhea was presented or to increase energy density when anorexia appered), emphasizing the intake foods rich in carotenoids and antioxidants.

Control Group (Retrospective Cohort) (CG): *S*tandard counseling was prescribed, which included a list of foods allowed and to be avoided, as well as general recommendations for the control of adverse gastrointestinal symptoms. The CG data were draft from a study of 2016. The variables were the same for the INC group (body composition, nutritional status, adverse

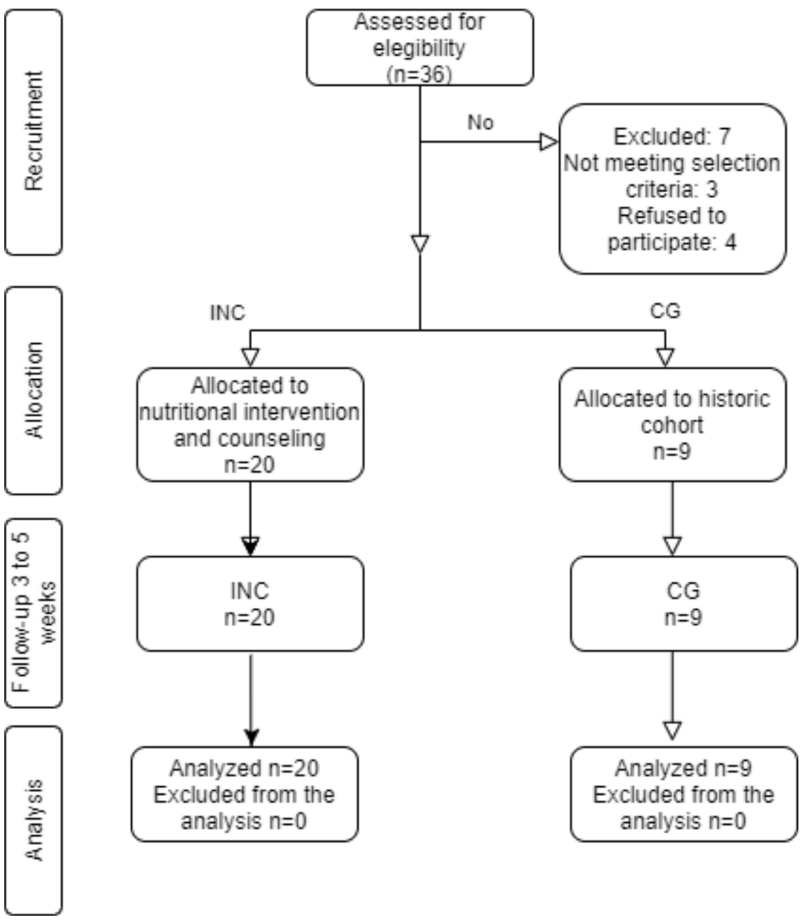

FIGURE 1 | CONSORT flow diagram. For INC group, 36 patients were assesed for elegibility in the recruitment phase; 7 were excluded (three participants did not meet the inclusion criteria and four did not accept to participate). Nine participants of historical cohort were the control group. Twenty participants were allocated to nutritional intervention and conseling group. Both groups were followed up during RT of 3 to 5 weeks.

effects, and dietetic intake). The sample size were 9 participants. They received only standard counseling (recommendations) weekly throughout the RT.

Nutritional Status

For the nutritional estatus, the anthropometric (body composition), dietary, biochemical, and clinical indicators were measured.

Anthropometric Variable (Body Composition)

Body composition was measured with a bioimpedance analyzer (OMRON® HBF-500INT). The definition for low fat mass was 9 to 23% percentage, acceptable value of 24 to 31% and unhealthy value for value ≥32%. Significant loss fat free mass was considered during radiotherapy treatment with ≥500 g. Body mass index (BMI) was considered malnutrition with <18.5 kg/m^2, adequate value with 18.5 to 24.9 kg/m^2, overweight with 25 to 29.9 kg/m2 and obesity grade 1 with 30 to 34.9 kg/m^2 and obesity grade 2 with 35 to 39.9 kg/m^2 (35). Arm, waist, and hip circumference (A value greater than 0.85 was considered cardiometabolic risk for Waist-to-hip ratio) was measured with a fiberglass tape (Vitamex®) according to the ISAK® technique (36). All anthropometric measurements were performed by a previously standardized nutritionist.

Diet

A 24-hour Recall was applied to assess the food and beverage intake (interview was in the last 24 h) with food replicas (NASCO®). The diet data was analyzed with Nutrikcal VO® Software. The energy, macronutrients (carbohydrates, proteins, and lipids), and micronutrients (vitamins and minerals) were calculated for one day and once weekly. The adequacy percentage was calculated for energy, macronutrients and micronutrients intake respect. An aceptable value for adequacy percentage was considered with 90 to 110%.

$$Percentage\ of\ adequacy = \frac{(Actual\ consumption \times 100)}{Required\ consumption}$$

A consumption frequency questionnaire was applied with 8 food groups according to the Mexican System of Equivalent Foods (37). Vegetables, fruits, cereals and tubers, legumes, animal foods, dairy products, oils and fats, and sugars with the following frequencies: once a week, two to four times a week and daily.

Biochemical Variables

Hemoglobin was measured from a capillary blood sample obtained with a sterile lancet with the Hemocue 201® kit (specificity greater than 90% and a sensitivity of 80%) (38). Capilar blood glucose was measured with an Accu-Chek® glucometer. The collection was carried out under postprandial conditions with a register of food intaked.

Clinical: Adverse Effects

The main adverse effects of radiotherapy, mainly the gastrointestinal symptoms, were reported by the participants weekly, considering previous studies and the experience with retrospective cohort (CG) (20, 25).

The VGS-GP (Subjective global assessment-generated by the patient) was applied, the score was interpreted, A: good nutritional status; B: moderate malnutrition or risk of malnutrition and C: severe malnutrition. This instrument was applied at the beginning (first week of radiotherapy) and at the end of RT.

Blood pressure: Blood pressure was measured with a digital wrist baunometer (Omron® R3), the participants were sitting and placing their wrist at heart level with the palm extended (39). This was measured at the beginning and at the end of the intervention.

Radiation Therapy Toxicity: The radiation toxicity was measured using the RTOG/EORTC acute toxicity scale for abdomen and pelvis (40). The acute toxicity scale was applied at the beginning (first week of radiotherapy) and at the end of radiotherapy.

Adherence to the intervention: Adherence to nutritional treatment was considered when the participants' attendance is at 80% of sessions (3 weeks in average).

Statistical Analysis

Descriptive analysis statistics were applied according to normal or non-normal distribution. For inferential analyzes, one-way Anova, Student's t, Chi^2 were applied. For the nonparametric variables, the Wilcoxon rank test, Student's t test, and the Friedman test were used. To measure the effect of the intervention, the number needed to treat (NNT) was calculated. 80% power was considered with an alpha of *0.05*. The association of risk factors with the main variables (weight loss, fat-free mass and adverse effects) was calculated con Odds Ratio (95% confidence intervale). Statistical analyzes were performed with SPSS® software V22 Free trial.

Ethical Considerations

Participants received written informed consent with detailed explanation of the intervention. The research was carried out considering the Declaration of Helsinki, Nuremberg Code. The study was approved by the Bioethical Committee of the University of Guanajuato (No. CIBIUG-P22-2017).

RESULTS

Their baseline characteristics showed that most participants were 51.5 years (rank 31 to 73 years) (p = 0.19). A 38.9% of participant were married for the INC group and 55.5% for the CG group (p = 0.18). A 50% of the participants in the INC group had primary complete. A 70% were housewives. The origin cities participants were originated are presented in **Figure 2**. A 44.5% of the participants in the INC group presented some comorbidities (diabetes mellitus, hypertension, hypothyroidism), while in the CG group only 22.2% presented some comorbidities (p = 0.259) (**Table 1**).

Anthropometric Variables (Body Composition)

Regarding anthropometric indicators, body weight and body mass index showed a significant decrease during the weeks for the INC group (*p = 0.013 and p = 0.043, respectively*). Likewise, there was a

tendency to decrease fat-free mass and body fat observed mainly in the CG group. At the beginning of RT the INC group had overweight in 25%, 35% were obese and 40% an adequate nutritional status; in the CG group 44% were overweight, 22% were obese and 33% an adequate nutritional status. There were no changes in nutritional status in the INC group, but in the CG group one of the participants developed malnutrition at the end of the RT (**Table 2** and **Figure 3**).

In the INC group the total weight loss during RT was of 1.1 kg (Rank 0.3 to 4.7 kg) while weekly weigh loss was 0.3 kg (rank 0.1 to 0.5 kg). For the CG group a statistically non-significant weigh loss of 2.7 kg (rank 0.9 to 6.2 kg) was observed throughout the five weeks while weekly was 0.9kg (rank 0.3 to 2kg). At comparing changes of weight weekly, mainly in the form of fat-free mass, for INC group the weight increased: last week (for weight $p = 0.044$ and fat-free mass p= <0.001), week two (p <0.001), week three (p = 0.14), week four (p = 0.048) and week five (p = 0.008). A 55% of the participants in the INC group lost body fat with a median of 2,100g (range from 1100 to 2,700g) while in the CG group 73.6% lost body fat with a median of 1,070g (range from 370 to 4,100g) *(p= 1.000)*.

Weight loss in fat free mass (FFM) had a median of 410g (110 to 2,500g) in the INC group and 1,060g (100 to 2,500g) in the CG group; the weekly loss was 240g (150 to 460g) and 320kg (200 to 730g), respectively. A 37% of the INC group presented a loss greater than 500 g of FFM, while in the CG group 67% presented it (*RR = 0.55; 95% CI: 0.26 to 1.17*) with a reduction in the relative risk of 0.45 (95% CI= −0.17–0.74), an absolute risk reduction of 0.30 (95% CI= −0.08–0.67). The NNT was 4 (95% CI= 2 to −13).

Diet

Energy intake decreased weekly in both groups (**Table 3** and **Figure 4**). According to adequacy percentage in the INC group during RT the energy intake was in 60 to 80% except in week five with 65%. For the CG group, in the first week a 44% had an energy intake greater than 60%, in the third week was a 50% and in the fourth week a 33%. Respect to protein intake in first week for INC group a 50% of the participants had more than 60% of requirement while a 33% for CG group. For second, third and fourth week the protein, lipids and carbohydrates intake were higher in the INC group compared to the control CG. There were no data for the fifth week in the CG group. The rest of the nutriments are presented in **Tables 3**, **4**.

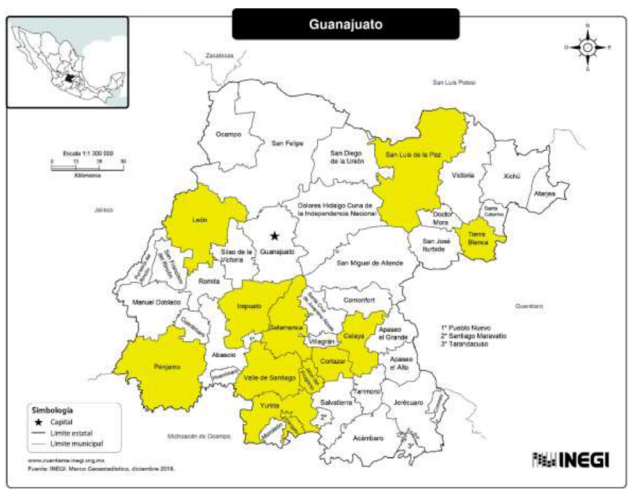

FIGURE 2 | Cities origin from Guanajuato State of the participants of this study (Leon, Celaya, Irapuato, Salamanca, Cortazar, PEnjamo, San Luis de la Paz, Tierra Blanca, Jaral del Progreso, Valle de Santiago, Yuriria and Uriangato).

TABLE 1 | Sociodemographic characteristics for both groups.

		INC n = 20	CG n = 9	P
	Age*	51.5(31–73)	51(35–83)	0.19*
Marital Status	Married	8(40)	5(55.5)	0.41**
	Single	5(25)	3(33.3)	
	Other	7(35)	1(11.1)	
Education level	Highschool	9(45)	–	
	Elementary	9(45)	–	
	None	2(10)	–	
Ocupation	Housewife	14(70)	4(44.4)	0.19**
	Employee	6(30)	5(55.5)	
Birthplace	Región del Bajío	13(65)	8(88.8)	0.18**
	Valles Abajeños	3(15)	1(11.1)	
	Sierra Gorda	4(20)	0(0)	
Comorbidity	Yes	9 (45)	2(22.2)	0.24**
	No	11 (55)	7(77.7)	
Stage of cancer	Stage I	2(10)	–	
	Stage II	12 (60)	–	
	Stage III	2(10)	3(33.3)	
	Stage IV	1(5)	–	
	No data	3(15)	6(66.6)	
Treatment	RT+CT	10(50)	3(33.3)	0.40**
	Previous Surgery	7(35)	2(22.2)	

*INC, Nutritional Intervention and Counseling; CG, Control Group. RT+CT, Radiotherapy and chemotherapy. Data is expressed in percentages and frequencys. *Median (Rank). Mann Withney U test. **Fisher's exact test.*

Lower intake for vitamin A was observed in the first week *(p = 0.04)*; fiber (*p = 0.006*) and iron (*p = 0.03*); for the second and fouth week the carbohydrates (p = 0.04) and folic acid (p = 0.04) intake were lower for the CG group in comparison with INC.

Biochemical Variables

There was a significant decrease in hemoglobin values, weekly in the INC group (*p = 0.009*); in second, third and fourh week the CG group had lower values (*p =0.016, p = 0.039*) (**Table 5**). At the end of treatment 21.3% of the INC group presented values less than 10 mg/dL. In the CG group, in the first week a 33% of the participants had hemoglobin values less than 10 mg/dL and at the end a 66%. maintaining blood sugar levels of 95–140 mg/dL. The glucose was maintained in normal values during RT.

Clinical Indicators: Adverse Symptoms

Regarding adverse symptoms for the first week, INC group had: nausea (58%), pain (53%), anorexia (32%) and dysgeusia (32%); in the last week, diarrhea (56%), fatigue (56%), anorexia (44%) and nausea (40%). For CG group the frequently adverse effect in the first week were nausea 33%, anorexia and a combination of diarrhea and constipation in one patient (**Table 6**). Dysgeusia

TABLE 2 | Anthropometric variables in Nutritional Intervention and Counseling group (NC) and Control Group (CG).

Indicator	Week 1		Week 2		Week 3		Week 4		Week 5		P value*	
	INC n = 20	CG n = 9	INC n = 19	CG n = 9	INC n = 17	CG n = 7	INC n = 17	CG n = 6	INC n = 15	CG n = 3	INC	CG
Weight (kg)	66.9 (42–114)	64.9 (49.1–104.3)	63.3 (41–114)	62 (48.7–100.1)	61.8 (41–96.3)	60.6 (47.6–90.2)	62.1 (40–111)	60.2 (43.1–98.2)	58.4 (41–109)	82.3** (75.4–98.1)	0.01	0.40
BMI (kg/m2)	27.7 (20–44)	26.9 (20.4–45.7)	27.6 (19–44)	26.8 (20.3–43.9)	26.8 (19–34.3)	25.9 (19.8–36.1)	26.6 (19–42.4)	26.1 (17.9–43.1)	25.7 (21–42.4)	28.3 (22.7–43)	0.04	0.40
Arm girth (cm)	28.4 (23–46.1)	30.3 (23–39.5)	28.7 (22–46)	29.5 (22.2–36.5)	29 (21–37.5)	27 (22.3–37.5)	28.5 (21–46)	28.3 (21.7–33.5)	28.5 (23–47.7)	31 (30–35.3)	0.47	0.15
Waist girth(cm)	89.3 (68–130)	88 (71–117)	88 (65–130)	84 (70–118)	88 (67–116)	82.5 (69.5–104)	85.5 (66–128)	89 (65.3–118)	85.5 (73–128)	91 (88.5–112)	0.18	0.06
Abdominal girth (cm)	96 (78–132)	98 (83–117)	96 (73–131)	95 (78–119)	95 (73–127)	92.2 (78–108)	94 (73–139)	94 (81.5–116)	97 (85–136)	99 (95.5–113)	0.66	0.25
Hips girth (cm)	102 (84–138)	106 (88–139)	100 (82–139)	97 (88.5–133)	100 (82–131)	96.5 (89–113)	99 79–138)	97 (85–135)	101 (85–139)	107 (104–130.5)	0.30	0.13
Fat free mass (kg)	16.7 (10–25.3)	21.7** (16.8–30.7)	16.9 (9.5–24.3)	21.5** (17.9–31.1)	16.5 (11–21.3)	20.8** (17.5–27.6)	16.2 (11–23.3)	21.2** (15.1–29.9)	15.9 (11–23.9)	25.8** (24.4–37)	0.57	0.40
Fat mass (kg)	29.1 (12–58.2)	25.5 (11.2–52.6)	28 (11–60.3)	25 (14.3–50.8)	27.4 (11–49.4)	22.3 (13.9–39.7)	22.8 (11–59.5)	23.4 (14.1–50.9)	24.2 (13–56.7)	46.9 (40.2–53.7)	0.30	0.40

*Friedman one-way repeated measure analysis of variance by ranks, **p < 0.05. Kruskall-Wallis test.*

was reported throughout the RT in the INC group and decreased it to 31% in the fifth week (**Figure 5**).

Other Variables

Regarding blood pressure, a significant decrease in diastolic pressure was observed in the INC group throughout RT ($p = 0.003$). At the beginning of nutritional intervention, the INC group 85.7% had a low nutritional risk (12 participants) and 14.3% (2 women) had a moderate risk. At the end of RT (n = 11), 90% (10 participants) remained at low risk.

The radiation toxicity was measured only in INC group (n = 14) where only one participant had grade three and the rest remained in grade 1 at the end of RT.

Association Analysis

An association was found between the presence of anorexia and a lower dietary intake in the first week ($p = 0.017$), but there was no association between this symptom and loss of body weight, fat mass or fat-free mass at the end of RT ($p= 0.082$). The presence of nausea, diarrhea, and dysgeusia were not associated with weight loss, fat mass, fat-free mass, and lower energy intake at the end RT ($p= 1.000$).

Nutrients and energy intake were not associated with weight loss, fat-free mass, and fat mass (p = 0.082). An intake less than 60% of the protein requirement was associated with a loss of fat-free mass greater than 500 g in the last week of radiotherapy in the INC group (p = 0.04), while an intake less than 60% for lipids requirement was associated with a loss of body weight greater than 500 g in the INC group in the last week of RT ($p= 0.028$).

A higher risk of had hemoglobin level less than 10mg/dL in the second week of RT in the CG group (*OR= 11.25; 95% CI= 1.57-80.3; p = 0.019). Other* risk factors for the CG group and INC as weight loss greater than 500 g and loss of fat-free mass greater than 500 g, energy intake less than 60%, serum hemoglobin at end of radiotherapy and the presence of symptoms such as anorexia are present in **Table 6**.

Adherence to the Intervention

About 27 participants who were initially considered for the INC group, 55% of them attended the 100% of nutritional interventional while a 45% attended 4 sessions.

DISCUSSION

Based on the immunological aspect associated with diet, there is evidence that an individualized nutritional intervention can be effective to improve nutritional intake, conserve nutritional status and quality of life and with a reduction in radiation toxicity (41). In the present study, individualized nutritional intervention did not decrease the adverse symptoms compared with retrospective cohort, but to maintain the body weight, fat-free mass, fat mass and diastolic blood pressure in the participants. Likewise, a higher energy and nutrient intake was observed in the intervention group.

It is known that malnutrition due to low body mass index (<18.5kg/m^2) and weight loss more than 5% are predictive indicators for developing radiation toxicity (42). In this study, 10% of INC group and 33% of CG group, presented loss weight greater than 5% after RT. It has been found that there is a positive association between body mass index and cervical cancer (*HR 1.10; CI 99%, 1.03–1.17*) (43). Furthermore, women whit overweight or obesity used not attend screening for CC (44). A 60% and 66% of the participants in both groups in this study had obesity or overweight, respectively. The obesity is known to promote inflammation through immune system dysfunction (45). Obesity is also associated with low functional level and a greater number of comorbidities in cancer patients (46). It is substancial to adrees both overweight and obesity as an especial issue that should be discussed and considered in the individualized nutrition therapy in cancer patients. The relationship between adipose tissue, muscle mass and other tissues in the body composition of the cancer patient

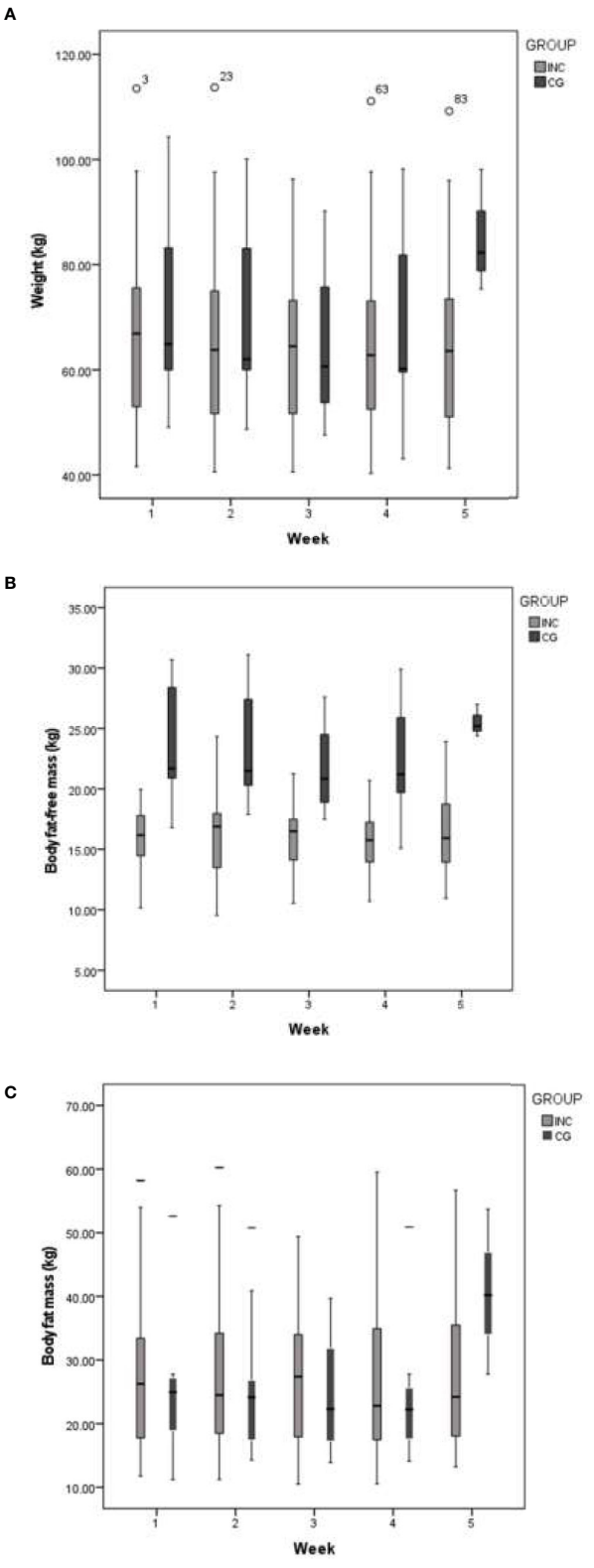

FIGURE 3 | Weekly changes in weight, body fat-free mass and body fat mass in both groups during RT. **(A)** Body weight. **(B)** Body fat-free mass and **(C)** Fat mass.

has important clinical implications. Bioimpedance analysis is accessible tecnique, portable and inexpensive method that give important data on body composition. Although few studies have analyzed the body composition of patients with cervical cancer, an association has been found between low values of phase angle with postoperative complications and hospital stay (47–49).

A study in 2004, carried out in patients with head and neck cancer, showed that an early and individualized nutritional intervention can decrease fat-free mass loss and considered such loss clinically significant when it was greater than 500 g, during radiotherapy, since this involves to an impact on physical functionality (50). In our study, body composition analysis demonstrated a trend toward greater fat-free mass loss in the CG group in comparison with INC group. The intention-to-treat analysis allowed to consider that receiving an individualized nutritional intervention could be a protective factor for a fat-free mass loss greater than 500g, which was 42% less likely that this occurs in the group with INC and that four is the number of patients that must be treated with an INC to avoid losing more than 500g in fat-free mass.

Respect to the dietary intake, it decreased during all treatment and weekly. A tendency to be lower was observed in the participants of the CG group, although it was not statistically significant. Interestingly, it has been observed in various studies that nutritional intervention can improve and increase dietary intake (20, 51).

In the aspect of dietary prescription to cancer patients, it is known that nutritional support could increase the speed of tumor growth however, when nutritional status is compromised, complications may be greater and have an impact on survival prognosis (52). Current recommendations encourage compliance with the energy requirement that covers from 20 to 30 kcal/kg/day, when an individualized calculation is not available, intake less of than 60% (individual requirement) is considered deficient (53), in this study the 65% of the participants in the INC group had a consumption greater than 60% and increased and maintained it at 75% the following weeks, compared to the control group, where initially 44% covered in the first week, in the third week a 50% and in the fourth week only 33% of the participants cover the requeriments.

The protein intake was in tendency to be higher (although not statistically significant) in the INC group compare with CG group. In both groups it was less than recommended in the intervention that was carried out individually, according to the needs of the participant (a contribution of 1 to 1.3 g/kg is recommended). This, together with the deficient consumption of micronutrients, which is also caused by the low dietary consumption, could constitute a significant risk to maintain an adequate weight and, therefore, avoid the problems caused by possible malnutrition. Even so, the consumption of folic acid, vitamin A and iron was significantly higher in the INC group, in the first, second and fourth weeks, respectively. This decrease in the consumption of micronutrients has also been found in other studies (54); however, it is still necessary to study the supplementation of some vitamins in these patients, so the recommendation is to follow the daily intake recommended by the national academy of sciences and for critically ill patients, evaluating each case individually (54, 55).

TABLE 3 | CompaNutritional intake in comparison between groups.

	Week 1		Week 2		Week 3		Week 4		Week 5		P value*	
	INC n = 18	CG n = 9	INC n = 18	CG n = 9	INCn = 15	CG n = 8	INC n = 15	CG n = 3	INC n = 15	CG n = 0	INC	CG
Energy (kcal)	1222.5 (258–2219)	945 (255–2401)	1141 (557–2254)	947 (477–1550)	1222 (454–2097)	1065 (407–2552)	1260 (609–2076)	422 (273–1642)	1056 (704–1401)	–	0.50	0.81
Proteins (g)	50 (8.1–77)	39.2 (9.1–149)	39.9 (19.3–83.4)	20.1 (13.8–82.1)	45.5 (19.8–68.7)	31.15 (10.2–168)	42.6 (11.3–93.6)	31.4 (23–47.7)	38.8 (13.4–87.4)	–	0.58	0.89
Lípids (g)	35.3 (3.6–102)	26.12 (5.2–85.8)	37.5 (2.2–104)	26.40 (14.9–77)	25.9 (2.1–88.4)	21.73 (2.70–99.8)	27.4 (11.5–72.5)	5.57 (4.6–86–3)	25.7 (10.5–55.7)	–	0.30	0.80
Carbohidrates (g)	166 (29.5–354)	131.7 (59–259.8)	199.3 (108–350)	164.9 (51.6–278)	198.6 (96.9–335)	179.4 (62.4–238)	204 (111–377.8)	62.4 (34–192)	169 (96–294.9)	–	0.60	0.61
Sugar (g)	27 (6.9–97.5)	35.2 (11.1–135)	33 (9.7–150.2)	21.90 (3.8–73.6)	40.7 (8.9–111)	29.90 (4.2–81.5)	27.7 (3.4–78.2)	15.4 (0–24.4)	29.2 (4.5–65.9)	–	0.15	0.18
Fiber (g)	14.9 (3.8–27.3)	14.3 (5.6–31.3)	18.7 (4.6–27.9)	7.90** (5.3–21.6)	17.4 (4–40.8)	12.75 (2.2–24.2)	18.8 (5.8–56.4)	2.20 (1.6–17.6)	13 (4.9–47.6)	–	0.10	0.80
Vitamin A (µg RAE/day)***	1165 (132–9688)	355** (99–1097)	1444 (34–9409)	475 (24–2293)	1155 (18–8486)	709 (45–1512)	651 (214–10612)	66 (21–1024)	540 (88–6192)	–	0.24	0.89
Vitamin B12 (mg)	1.29 (0.19–12.2)	0.45 (0–8.3)	0.95 (0–5.13)	1.2 (0–3.97)	1.1 (0–10.9)	0.43 (0–6.4)	1.04 (0–12.7)	1.2 (0.41–1.3)	1.1 (0–4.23)	–	0.89	0.71
Vitamin C (mg)	37.2 (12–199.7)	40.1 (9.5–599)	56.1 (7–126.9)	43 (12.2–145)	41.7 (13.6–201.8}	42.4 (27.1–222)	39 (1.7–131)	40.8 (0.6–76.7)	32.4 (4.3–177)	–	0.49	0.53
Folic Acid (mcg)	109.5 (29–392)	96.6 (27.3–389)	92.3 (14–379)	54.2 (7.7–554)	148 (28–922)	75.7 (18.9–224)	140 (22–1160)	18.9** (5–87)	72 (15.3–833)	–	0.57	0.80
Iron (mg)	9.01 (1.34–14.4)	5.30 (2.4–15.2)	9.15 (1.03–19)	4.4** (2.6–14)	10.2 (2.1–18.9)	6.3 (1.7–20.6)	9.8 (3.9–29.4)	3.3 (1.7–12.8)	6.9 (1.24–24.9)	–	0.52	0.80
Selenium (mg)	25 (2–57)	18 (1–51)	18 (0–46)	11 (2–52)	16 (2–74)	17.5 (4–118)	19 (1–110)	21 (13–24)	6.5 (1–105)	–	0.12	0.68
Zinc (mg)	3.3 (0–7.9)	2 (0.4–10.6)	2.6 (0–8.6)	1.7 (0–9.5)	3.1 (0–6.7)	2.1 (0.3–9.5)	2.8 (2–9.5)	2.8 (1.4–6.1)	1.4 (0–6.4)	–	0.51	0.45

*Friedman one-way repeated measure analysis of variance by ranks, **p < 0.05. Kruskall-Wallis test.***RE: µg retinol estimated per day.

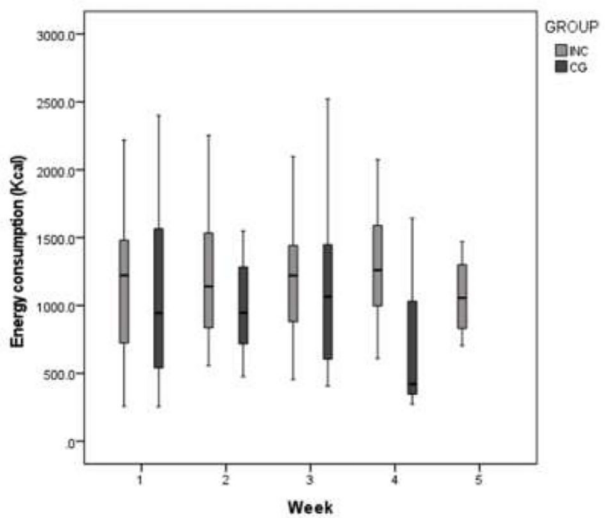

FIGURE 4 | Weekly energy intake in both groups (INC, Nutritional intervention with counseling group; CG, historical cohort (control group).

It is important to mention that adherence to nutritional treatment is an aspect that has a great impact on the results derived from the interventions; for example, one study showed that the risk of developing colorectal cancer can decrease up to 30% by having adequate adherence to the nutritional recommendations of the World Cancer Research Fund and the American Institute for Cancer Research (55). Multiple factors influence the adherence to nutritional treatment of patients; One of the main limitations for the participants to have an adequate fulfillment of their nutritional needs has been the attention to general recommendations that are given to them when they start their treatment. These recommendations generally restrict food that they consume daily and to which they have access. For example, the consumption of legumes (mainly beans and lentils) is maintained at least once a week in approximately 60% of the participants, despite the restriction.

Food availability and its relationship with the presence of cancer in a specific population, in 2018 an ecological study was conducted where found a correlation between with red meat intake, calories and animal fat with colorectal cancer ($r = 0.59$ *and* $r2$ *of* 0.29; $r = 0.56$ *and* r^2 *of* 0.16; $r = 6$, respectively) while a weak correlation was found with the availability of fruits and vegetables (56). These results should be analyzed, since it has not been considered individually, for example, individual high availability would not necessarily indicate a high consumption of food. Several factors influence this situation in the cancer patient for example gastrointestinal tolerance, sociodemographic variables. The anemia is an important variable that must be considered as a predictor factor in the prognosis of patients with chemotherapy and radiotherapy, mainly with values less than 10mg/dL in the last two weeks of treatment (57, 58).

TABLE 4 | Dietary intake and adequacy of energy and nutrients.

Intake adequacy percentage	Week 1		Week 2		Week 3		Week 4		Week 5		P value*	
	INC n = 18	CG n = 9	INC n = 18	CG n = 9	INC n = 15	CG n = 8	INC n = 15	CG n = 3	INC n = 15	CG n = 0	INC	CG
Energy (Kcal)	78 (15–147)	59 (14–150)	81 (33–125)	55 (25–103)	81 (27–137)	60 (25–148)	83 (37–153)	20 (18–102)	65 (45–113)	–	0.65	0.81
Proteins g	60 (9.8–102)	50 (10–187)	53 (23–95)	26 (14–102)	60 (24–92)	38 (12–197)	57 (14–138)	30 (29–59)	47 (19–134)	–	0.53	0.81
Lípids g	61 (7–198)	44 (8–160)	70 (4–178)	49 (24–154)	53 (4–189)	40 (5–176)	53 (21–155)	9 (7–161) **	52 (24–107)	–	0.41	0.89
Carbohidrates g	79 (14–202)	69.7 (26–143)	104 (59–158)	78 (25–139)	108 (46–148)	83 (33–119)	105 (57–223)	23 (18–96)	90 (54–163)	–	0.53	0.53

*Friedman one-way repeated measure analysis of variance by ranks, **p < 0.05. Mann-Whitney U test.*

TABLE 5 | Weekly changes in blood pressure, glucose and hemoglobin.

	Week 1		Week 2		Week 3		Week 4		Week 5		P value*	
	INC n = 20	CG n = 9	INC n = 19	CG n = 9	INC n = 16	CG n = 9	INC n = 12	CG n = 6	INC n = 15	CG n = 3	INC	CG
Systolic blood pressure mmHg	113 (90–140)	110 (94–151)	110.5 (90–131)	110 (96–133)	108 (87–161)	112 (87–126)	106 (90–117)	111 (60–124)	107 (90–130)	118 (100–127)	0.224	0.102
Diastolic blood pressure mmHg	70 (60–89)	66 (53–87)	69 (58–81)	70 (56–83)	62 (57–97)	61.5 (52–81)	60 (52–70)	78 (54–80)	61 (50–80)	75 (64–80)	0.003	0.209
Capiilar blood glucose mg/dL	114 (70–219)	110 (93–417)	126 (72–167)	137 (97–317)	123 (90–306)	130 (106–161)	128 (80–169)	102 (89–216)	136 (74–290)	138 (117–298)	0.363	0.171
Hemoglobin g/dL	12. 2(10–15.3)	11** (6.9–15)	12.1 (9.6–14.6)	10** (6.7–15.7)	12.2 (8.6–14.1)	9.4** (6.3–12.3)	11.2 (8.7–15)	9.5 (8.9–13.6)	10.9 (8.3–13.6)	9.5 (9–12.4)	0.009	0.736

*Friedman one-way repeated measure analysis of variance by ranks, **p < 0.05. Kruskall-Wallis test.*

TABLE 6 | Weekly frequency of adverse effects during RT in both groups.

Adverse effect	Week 1			Week 2			Week 3			Week 4			Week 5		
	INC n = 20	CG n = 9	P value*	INC n = 20	CG n = 9	P value*	INC n = 16	CG n = 9	P value*	INC n = 16	CG n = 6	P value*	INC n = 16	CG n = 3	P value*
Anorexy	6(32)	1(11)	0.27	7(35)	0(0)	–	8(50)	1(11)	0.05	6(37)	0(0)	–	7(44)	0(0)	–
Nausea	11(58)	3(33)	0.28	9(45)	4(44)	0.97	6(37)	3(33)	0.83	5(31)	4(66)	0.13	6(40)	1(33)	0.89
Vomit	1(5)	0(0)	–	2(10)	0(0)	–	1(6)	0(0)	–	2(12.5)	0(0)	–	1(6)	0(0)	–
Diarrhoea	2(10)	1(11)	1.00	7(35)	2(22)	0.49	5(31)	5(55)	0.23	8(50)	4(66)	0.48	9(56)	1(33)	0.46
Fatigue	10(53)	0(0)	–	9(45)	0(0)	–	6(37)	1(11)	0.15	9(56)	1(16)	0.09	9(56)	2(66)	0.73
Dysgeusia	6(32)	0(0)	–	9(45)	0(0)	–	6(37)	0(0)	–	6(37)	0(0)	–	5(31)	0(0)	–
Pain	10(53)	–	–	6(30)	–	–	9(56)	–	–	7(44)	–	–	7(44)	–	–
Constipation	4(21)	1(11)	0.34	5(25)	0(0)	–	4(25)	0(0)	–	4(25)	0(0)	–	3(19)	0(0)	–

*Data is expressed in frequencies and percentages. *Fisher´s exact test.*

Although glucose during treatment had not significant increases because its measurement was collected in post-prandial condition; even so, the maximum ranges present an increase higher than expected in a healthy person. A factor that may have had an influence was the presence of participants with comorbidities such as diabetes, as well as the association between elevated serum glucose levels with recurrence and mortality in patients who do not have diabetes (59). In the present study, only 25% of the patients in INC group, at the end, presented <10 mg/dL, compared to the CG group with values greater than 60%.

In the aspect of adverse effects, when comparing with a study carried out under the same methodology with the scale (RTOG/EORTC) with which the INC group was evaluated, in our study, in patients with chemo-radiotherapy, the frequency of participants with nausea, anemia and vomiting, it was lower (40, 50, and 6%, respectively) than that of the sample evaluated by said study (73.3, 69.2, and 20.9%). Frecuently diarrhea (56% in the INC group vs. 51.6%) was found by Izmajłowicz et al. Regarding lifestyle, risk factors such as smoking, should be addressed with re-relevance, since smoking has a RR of 2.4 (95% CI: 1.7, 3.4) and the risk remains

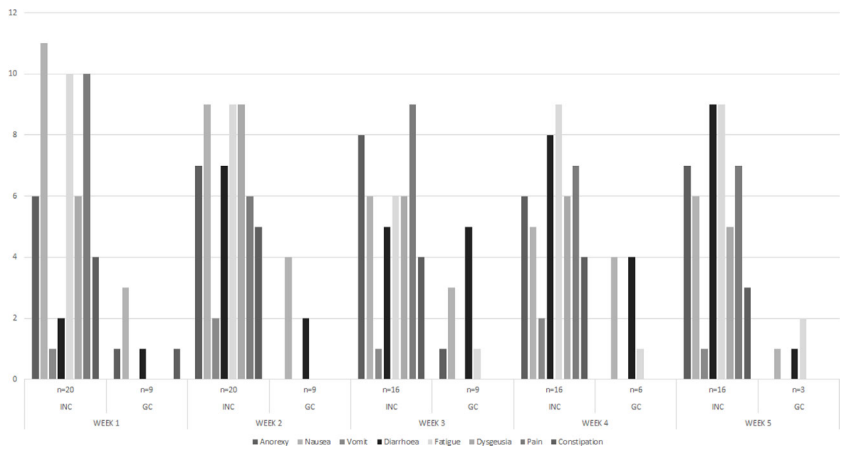

FIGURE 5 | Weekly frequency adverse effects presented during RT in both group.

TABLE 7 | Main risk factors associated with the nutritional variables in both groups.

Nutritional variables	INC n = 20	CG n = 9	OR (CI95%)	P value*
Weight loss greater than 500 gr.	15	7	1.167 (0.180–7.564)	0.631
Fat free mass greater than 500 gr.*	9	2	1.778 (0.134–23.52)	0.579
Serum hemoglobin <10mg/dl (second week)	2	5	11.25 (1.57–80.3)	0.019
Serum hemoglobin <10mlg/dl (final week)*	2	2	15.00 (0.896–251.06)	0.088
Energy consumption less than 60%	5	4	2.240 (0.424–11.837)	0.407
Anorexia	14	1	0.333 (0.33–3.335)	0.633

**Fisher's exact test.*

even with smoking cessation (*RR = 1.6, 95% CI: 1.0–2.7*) (60). The risk increases in women who smoked for a period of 16 years (OR: 3.23, 95% CI: 1.33, 7.69), and continue in recurrent smokers who consume more than 20 cigarettes a day (*OR: 2.57, 95% CI: 1.49 to 4.45*) (61).

It is important to mention that a limitation with comparison groups was the methodology to obtain the frequency of adverse effects that it was different in the CG group. In this sense, further research, and comparison of groups in which the variable of adverse effects has been measured with the same method is suggested. However, the outcomes in INC group could be used in pharmacological studies, with synergy of therapies and improve the prognosis of women with cervical cancer.

According to these findings, the implications and favorable effects of supervision, professional accompaniment in a nutritional intervention can be identified, which pays a methodology to be integrated for the research study of drug reuse and the nutritional intervention itself. The evidence shows an important synergy between some dietary components and drugs for the treatment of diseases associated with both lifestyle (hyperlipidemias, diabetes), and some, whose risk factors may be more complex, such as cancer (62, 63). A study by Kindelwal et al, in 2018, for example, showed that selenium-induced toxicity could be effective in treating breast cancer by considering an immunotherapeutic approach that can reduce the debilitating side effects that are associated with breast cancer drugs (64). The search for treatments that generate inhibition of cell proliferation mechanisms in Cancer, such as the inhibition of the ubiquitin system, proteasome, which is responsible for the degradation of proteins in the cell, in 80 to 90% is increasingly attractive through the reuse of drugs. In this sense, there is a growing interest in the use of some natural compounds such as flavonoids, polyphenols, isoflavones, curcumin and other compounds that are found intrinsically in food, the use of which could act in synergy with the anticancer drug, with a potential lower toxicity (65).

There are several factors that increase the cancer patient's susceptibility to malnutrition, this negatively impacts the prognosis, progression, and decrease in response to treatment (**Table 7**). Oncological treatments such as chemotherapy, radiotherapy, chemo-radiotherapy, or surgery can compromise food intake, nutrient absorption and affect the patient's nutritional status (66). INC is an adjunct to the treatment of various disorders, however, the evidence regarding cervical cancer is limited. The effect of various drugs already known on various mechanisms that can improve or complement the effect of basic therapies has been analyzed. For example, evidence shows that drugs such as emetine, fluorosalan, sunitinib malate, bithionol, narasin, tribromsalan, lestaurtinih can inhibit NF-kappaB (NFKB1) signaling, by inhibiting the phosphorylation of IkappaBelpha (NFKBIA), a transcription factor that plays an important role in

the growth of cells in CC (67). On the other hand, the role of zoledronic acid as a drug that could inhibit the proliferation of cervical cancer cell lines has also been studied, and in addition, in combination with paclitaxel or deoxorubin, it showed better inhibition of Ras oncogenes (68). the proposed mechanisms also include immunomodulation through PD-1/PD-L1 blockade, which has shown a response in up to 13 to 17% of gynecological cancers, probably due to the immunosuppressive effect that occurs in the microenvironment in tumors. gynecological and altered vasculature. It has been observed that the effect of this mechanism can improve benefits in conjunction with radiotherapy (69).

The search for new drugs has improve the quality life and saved lives although they are expensive and require many years of research and the effectiveness of these depend on pharmacokinetics and pharmacodynamics variables of each drug. The efficacy of a drug can be compromised by deterioration gastrointestinal absorption and therefore nutritional deterioration. The repurposing of drugs consists of finding new therapeutic indications for existing drugs, and therefore reducing the research times involved in the study of drugs with the advantage of knowing their risks already studied (70, 71). For cervical cancer, the treatment is chemotherapy and radiotherapy, but both have adverse effects towards the maintenance of nutritional status, which increases morbidity and mortality and therefore the prognosis of the disease. The nutritional intervention plus nutritional counseling should be reporpused as an essential part of the clinical trials for drug validation as it may improve benefits for patients with CC (71).

The design of drugs takes several years and very high costs, for which counting on the reuse of drugs and therapies as a nutritional intervention and counseling raises great hopes, since the effects of treatments (in this case, local radiation) affect all cells. The scheme for CC is to proceed with chemotherapy and radiotherapy, two systemic treatments with known adverse effects and high toxicity. On the other hand, the drugs that are developed to treat cancer and other diseases require high costs, due to the need to analyze the aspects of pharmacodynamics (absorption, distribution, elimination) where aspects such as nutritional status, gastrointestinal absorption and hemodynamic status stable. They are key to measure the effectiveness of a treatment, which is why this study supports a methodological proposal (72, 73).

It is urgently necessary to develop drugs and more effective, economic strategies that seek to decrease the resistance that has been generated to current drugs (some patients develop resistance to chemotherapy) or increase sensitivity to existing drugs or repurposing drugs.

Recommendations: The energy and nutritional intake was maintained with the intervention with adequate adherence to the intervention with, was better and remained constant weekly in the participants who received the intervention, although it remained below what was recommended. Food security could be an important factor to meet the requirements in this population; therefore, individualized nutritional intervention should consider this aspect, in the sense of food availability. Furthermore, it is recommended to conduct clinical trials with a greater sample size.

What this study provides: The effect of a nutritional intervention and individualized counseling vs. standard counseling from a historical cohort on adverse effects and body composition, and the findings are expected to contribute to the methodology in the study of cancer drug repurposing and improving the effectiveness of these.

Our study had several limitations. First, the sample size for the retrospective cohort group. Second, use the retrospective cohort as the control group. Third, the equipment used to measure blood pressure, the American Heart Association recommends using a home blood pressure monitor that measures upper arm blood pressure and not using wrist or finger blood pressure monitors; Fourth measure only one a week for food intake. On the other hand, many of the findings are consistent with other studies, but the retrospective cohort will observe differences despite these limitations. Nutritional treatment is known to help prevent nutritional deterioration and improve prognosis during radiotherapy and chemotherapy, but weekly monitoring is required, at least as in our study. This is important for drug studies, where these variables must be controlled in order to measure the effect of different drugs.

CONCLUSION

An individualized nutritional intervention and counseling with weekly monitoring and supervision throughout radiotherapy treatment demonstrated an impact on the maintenance of muscle mass, weight, hemoglobin, and a dietary intake above 70% of the recommendations for dietary intake and, a decrease in gastrointestinal adverse effects. Overweight and obesity found should be considered as part of the treatment for nutritional intervention and controlled counseling. These first findings reinforce the benefits of an individualized nutritional intervention to be implemented and reporpused in studies of drug reuse, achieving maintenance of nutritional status and a decrease in adverse effects, mainly anorexia and nausea as well as anemia. Improving the efficacy of pharmacological treatments and therefore improving their quality of life.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité Institucional de Bioética en la Investigación de la Universidad de Guanajuato (CIBIUG). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Repurposing Cationic Amphiphilic Drugs and Derivatives to Engage Lysosomal Cell Death in Cancer Treatment

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A major confounding issue in the successful treatment of cancer is the existence of tumor cell populations that resist therapeutic agents and regimens. While tremendous effort has gone into understanding the biochemical mechanisms underlying resistance to each traditional and targeted therapeutic, a broader approach to the problem may emerge from the recognition that existing anti-cancer agents elicit their cytotoxic effects almost exclusively through apoptosis. Considering the myriad mechanisms cancer cells employ to subvert apoptotic death, an attractive alternative approach would leverage programmed necrotic mechanisms to side-step therapeutic resistance to apoptosis-inducing agents. Lysosomal cell death (LCD) is a programmed necrotic cell death mechanism that is engaged upon the compromise of the limiting membrane of the lysosome, a process called lysosomal membrane permeabilization (LMP). The release of lysosomal components into the cytosol upon LMP triggers biochemical cascades that lead to plasma membrane rupture and necrotic cell death. Interestingly, the process of cellular transformation appears to render the limiting lysosomal membranes of tumor cells more fragile than non-transformed cells, offering a potential therapeutic window for drug development. Here we outline the concepts of LMP and LCD, and discuss strategies for the development of agents to engage these processes. Importantly, the potential exists for existing cationic amphiphilic drugs such as antidepressants, antibiotics, antiarrhythmics, and diuretics to be repurposed to engage LCD within therapy-resistant tumor cell populations.

Keywords: cancer treatment, therapeutic resistance, therapeutic targeting, therapeutic repurposing, necrosis, lysosomal cell death, lysosomal membrane permeabilization, cationic amphiphilic drugs

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INTRODUCTION

Despite decades of research into its underlying drivers and the development of corresponding therapeutic agents, cancer remains the second leading cause of death in the United States. Moreover, worldwide cancer incidence and death rates are predicted to increase by two-thirds over the next two decades as a result of an expanding and aging population (1). A potential barrier to therapeutic outcomes concerns the specific cytotoxic mechanism by which anti-cancer agents act. The overwhelming majority of conventional and targeted cancer therapeutics employed in the clinic today kill tumor cells *via*

caspase-dependent apoptosis, characterized by the breakdown of cellular components and their distribution into apoptotic bodies that are consumed by phagocytic cells (2). However, suppression of apoptosis is a hallmark of cancer (3); cancer cells engage a variety of strategies to subvert apoptotic mechanisms and engage anti-apoptotic pathways to promote their expansion, therapeutic resistance, and progression to malignancy. These general observations underscore the notion that engagement of non-apoptotic cell death pathways could offer an attractive alternative to the treatment of tumors that have proven refractory to currently employed therapeutic agents.

Necrotic cell death, characterized by plasma membrane rupture (2), has traditionally been considered a non-specific response to acute cellular stress. However, numerous observations over the last decade have revealed that cells can respond to stressful conditions by engaging a variety of pathways that trigger caspase-independent cell death. While these pathways appear distinct and their associated cell death mechanisms go by different names [e.g. necroptosis, ferroptosis, pyroptosis, parthanatos; (4)], their common underlying characteristic is plasma membrane rupture. Thus, our understanding of necrosis has expanded with the realization that it too is a programmed cell death mechanism (5–7). While the therapeutic potential of the various necrotic pathways for cancer remains to be fully explored, recent evidence suggests that engagement of lysosomal cell death (LCD) may offer a particularly attractive avenue.

Lysosomes canonically participate in the digestion of complex molecules such as glycoproteins and glycolipids, recycling basic building blocks such as amino acids and sugars for reuse (8). These organelles are comprised of a limiting lipid bilayer containing numerous structural proteins and channels, an internal glycocalyx lining protecting the limiting membrane from the acidic lysosomal lumen (9), and endosome-derived intraluminal vesicles (ILVs) that harbor enzymes, lipids, and cofactors involved in the highly regulated breakdown of delivered substrates (10, 11). Simultaneously, lysosomes serve as reservoirs for amino acids and Ca^{2+}, and engage in nutrient sensing and autophagy (12). However, one of the more underappreciated functions of lysosomes is their role in non-apoptotic cell death, where conditions that promote the breach of the limiting membrane (lysosomal membrane permeabilization, LMP) triggers cascades of events culminating in plasma membrane rupture (13, 14). In this mini-review we discuss LMP and LCD in detail, focusing on agents such as cationic amphiphilic drugs that promote these processes, and highlighting the potential for existing FDA-approved therapeutics to be repurposed for cancer.

LMP AND ITS ROLE IN CANCER

Release of cathepsins into the cytosol upon LMP results in the cleavage of multiple proteins, triggering a cascade of events culminating in plasma membrane rupture and LCD (15, 16). This process is akin to caspase-mediated apoptosis following compromise of the mitochondrial outer membrane (17). Interestingly, the degree of lysosomal compromise may dictate the mechanism of cell death; some evidence suggests that extensive LMP can initiate a largely necrotic outcome, while limited LMP can initiate an apoptotic fate (18, 19). As discussed below, a variety of external agents can induce LMP, including lysosomotropic detergents, v-ATPase inhibitors, and cationic amphiphilic drugs [CADs; (20)]. Moreover, LMP efficiency may be influenced by an array of internal factors, including reactive oxygen species (ROS) levels, cytosolic calcium concentration, and the lipid composition of the lysosomal limiting membrane (e.g. cholesterol levels), each of which is commonly dysregulated in cancer (21–23).

Transformation, the process that makes normal cells cancerous, confers marked behavioral changes to the cell, including altered metabolism, enhanced proliferation, increased invasiveness, and drug resistance. These changes are accompanied by dramatic alterations to cellular membranous components, including the cell surface and organelles (3). Increased lysosomal activity is essential to meeting the newly acquired growth demands, and tumor cells often exhibit alterations in lysosomal quantity, volume, membrane composition, hydrolase activity, and energy expended on pH maintenance (20, 21). Paradoxically, the transformation-associated changes critical to efficient tumor cell growth and invasiveness render the cancer cell limiting lysosome membrane more unstable, exposing a cancer-specific vulnerability that may be exploited therapeutically (24, 25). Upon LMP, cathepsins activate various pro-apoptotic proteins including p53, Bid, and TNF (26). However, LCD appears not to rely on p53 or caspases, but instead on ROS and Ca^{2+}-dependent calpain proteases (27, 28), providing support for the hypothesis that an LCD-based therapeutic strategy may be exploited in the treatment of tumors resistant to apoptosis-inducing agents.

LMP ASSAYS

Development of LCD-based therapeutic agents requires robust LMP assays that are sufficiently sensitive to detect low levels or early stages of lysosomal membrane compromise, and are readily adaptable to high throughput formats. Several assays are currently available, each with its strengths and drawbacks.

The LysoTracker probe, available in different colors, accumulates in acidic organelles such as lysosomes where its fluorescence is inversely correlated with pH (29). Thus, LysoTracker fluorescence is diminished as lysosomal pH increases from LMP induction (30), and quantification across dozens to hundreds of untreated versus treated cells can uncover the lysosomal impact of tested agents. However, loss of fluorescence can also reflect the accumulation of drug in lysosomes (31), making interpretations of untested compounds challenging.

A more direct method involves the quantification of fluorescently-tagged dextrans released from lysosomes into the cytosol upon LMP (32). Dextrans are hydrophilic polysaccharides that are endocytosed and delivered to lysosomes following their addition to media of cultured cells. Release of luminal dextrans through lysosomal pores alters fluorescence distribution from a highly punctate to a more diffuse pattern (32, 33). A strength of this method is a range of dextran sizes (10 to 250 kDa) may be employed to estimate the magnitude of drug-induced pores within

the membrane (33). A weakness is the dimming of puncta can be difficult to discern at low levels of LMP. However, it has been reported that loss of signal by flow cytometry allows quantification of LMP (34), useful for comparisons across drug candidates.

A similar approach involves the release of cathepsin proteases into the cytosol following LMP. Lysosomal resident cathepsins are canonically involved in the breakdown of proteins, however their cleavage of cytosolic proteins upon LMP is capable of initiating cell death pathways (35). Microscopic analysis of fixed cells with cathepsin antibodies reveals that staining evolves from a highly punctate pattern to a more diffuse pattern with increasing LMP (15). A notable strength of this method is that it can be applied to formalin-fixed, paraffin-embedded tissue samples to assess LMP patient samples and animal models. A variation on this theme assesses cytosolic cathepsin enzyme activity of lysed cells to quantify LMP (32).

Finally, the galectin assay sidesteps issues surrounding the subtle dimming of lysosomal puncta upon LMP, characteristic of the dextran and cathepsin assays, by inverting the strategy to assess increased lysosomal puncta in response to LMP. Galectins are a family of cytosolic and secreted lectins that bind to β-galactoside sugars. Upon LMP, cytosolic lectins diffuse through lysosomal pores and bind to the glycocalyx lining of the inner leaflet of the limiting lysosomal membrane (36); thus, staining of fixed cells with galectin antibodies reveals a more robust punctate pattern after cellular exposure to LMP-inducing agents (32). Galectin abundance in most cells and its immediate translocation to lysosomes make this the most sensitive of LMP assays (37). Moreover, this approach may be coupled with dextran or other lysosomal markers to facilitate high-throughput screening (33).

LMP-INDUCING AGENTS

In general, three classes of drugs have been demonstrated to induce LMP to provoke lysosomal cell death. The physicochemical properties of lysosomotropic detergents, consisting of a weak base moiety attached to a lipophilic tail, allow these agents to partially permeabilize the limiting lysosomal membrane (38). Their selective accumulation in acidic compartments coupled with the elevated fragility of cancer cell lysosomes relative to non-transformed cells make this class of molecules attractive candidates for the development of novel anti-cancer therapeutics (39). *O*-methyl-serine dodecylamine hydrochloride (MSDH), a synthetic detergent under analysis as a potential anticancer therapy, appears to be endocytosed in an inert vesicular form by cells at neutral pH and reconfigures to a toxic micellar form at lysosomal pH (40), suggesting a mechanism by which MSDH may specifically act toward lysosomal membranes and not other membranous structures such as the plasma membrane. L-leucyl-leucine methyl ester (LLOMe), a lysosomotropic agent that provokes the death of cancer cells, also exhibits considerable toxicity toward primary cells (41), highlighting the need for further research into the mechanisms and cell type selectivities of these agents as cancer treatments. Additionally, lysosomotropic detergents have been explored as potential vehicles for directly delivering drugs to lysosomes to induce a more targeted effect (42).

v-ATPase inhibitors block the ATP-dependent proton pump involved in maintaining the cellular pH of lysosomes (20). While inhibition of proton transporters, such as Na^+/H^+ exchanger isoform 1 (NHE1), monocarboxylate transporters (MCTs), and carbonic anhydrases (CAs), have been explored in the context of cancer because of dysregulated cytosolic and extracellular hydrogen ion concentrations associated with tumor metabolism (43), a v-ATPase inhibition anti-cancer strategy specifically focuses on disrupting lysosomal pH to provoke LMP. For instance, Bafilomycin A1, a macrolide antibiotic that targets v-ATPase, has exhibited some promise as an antitumor agent (44). Bafilomycin A1 mechanism of action involves the elevation of lysosomal pH and release of cathepsins into the cytoplasm, and has the potential to subvert therapeutic resistance (45). Interestingly, omeprazole, a gastritis and duodenal ulcer treatment targeting the H^+/K^+-ATPase of parietal cells through pH modification, has exhibited effectiveness in pancreatic cancer cells by eliciting alterations in lysosomal lipid metabolism to trigger cell death (46). Together, these observations underscore the potential of pH manipulation in the development of novel lysosome-acting therapeutics.

The chemical structure characteristic of CADs, a weak base moiety attached to a hydrophobic region (47), ensure that these molecules accumulate in lysosomes. At neutral pH, the hydrophobic portion permits diffusion across membranes, while at lower pH the base becomes protonated and the charged molecule becomes trapped within the lysosomal lumen (31). CADs are found among a wide variety of drug classes, including antidepressants, neuroleptics, cardiac antiarrhythmics, and tranquilizers (48). Mechanistically, lysosomally trapped CADs are thought to inhibit ILV-localized hydrolytic enzymes to suppress the breakdown of complex lipids (49), which in turn accumulate to levels that compromise lysosomal limiting membrane integrity. For example, siramesine, originally developed as a potential antidepressant, selectively kills cultured cancer cells by inhibiting the lysosomal sphingolipid catabolic enzyme acid sphingomyelinase (ASM) (50–52). Likewise, antihistamines such as loratadine and ebastine exhibit similar efficacy in killing cancer cells through lysosomal membrane destabilization, and epidemiologic evidence points to their effectiveness in reduced cancer mortalities when delivered in conjunction with chemotherapy (53). Accumulating observations suggest that CAD mechanism of action may be particularly well suited to inducing the non-apoptotic death of cancer cells.

DRUG-INDUCED PHOSPHOLIPIDOSIS AS AN ANTICANCER STRATEGY

Phospholipidosis results from the excessive accumulation of phospholipids within cells upon CAD treatment (47). As CAD action inhibits breakdown of complex lipids delivered to the lysosome (31, 49), lipid substrates accumulate to form multilamellar bodies reminiscent of membranous cytoplasmic bodies found in lysosomal storage diseases (LSDs) such as Tay-Sachs disease and GM2 gangliosidoses (54). LSDs encompass over 70 very rare genetic diseases that arise from deleterious mutations in genes responsible for lysosomal function and homeostasis (55). While these diseases have dire human health consequences (55),

often leading to death within the second or third decade, harnessing the ability of drugs to induce a transient but acutely lipidotic state to specifically kill cancer cells offers tremendous therapeutic potential for tumors refractory to other treatment options. It is important to note that clinical studies suggest CAD-induced phospholipidosis in normal tissues is reversible with drug withdrawal (54), so adverse side effects may be rapidly ameliorated.

The primary consideration in cancer therapeutic development is the ability of drug to reach its tumor tissue and molecular target at sufficient concentrations to elicit a pharmacologic effect, while minimizing the impact at normal tissues. Certainly, the intrinsic ability of CADs to concentrate in lysosomes, the site of their molecular target, facilitates efficacy, and the abundance and lability of transformed cell lysosomes relative to those of normal cells minimizes off-target concerns. At the same time, CADs tend to accumulate within tumors relative to normal tissue because of their lower cytosolic pH and the lower difference between cytosolic and lysosomal pH (56, 57). On the other hand, CADs also distribute efficiently to tissue types rich in lysosomes, including lung, liver, and kidney (56), suggesting these sites may be most susceptible to CAD-induced side effects.

Siramesine and hexamethylene amiloride (HMA) are examples of CADs that illustrate the potential of the phospholipidosis induction strategy in cancer therapy. As mentioned above, siramesine specifically targets a variety of cancer cell lines through ASM inhibition (50, 51), and its mechanism of action appears to involve inhibition of ASM binding to and activation by the acidic lysosome-specific lipid bis(monoacylglycero)phosphate (BMP) within ILVs (51). Lysosomal accumulation of ASM substrates upon drug treatment may contribute to membrane destabilization and LMP. Interestingly, altered sphingomyelin metabolism common to tumors may further sensitize cells to siramesine, and evidence has been presented that siramesine can reverse drug resistance to confer tumor cell sensitivity to conventional chemotherapeutics (51), underscoring the potential of this drug as a repurposed anticancer therapeutic. HMA, a derivative of the diuretic amiloride that has been used clinically for over 50 years, is cytotoxic toward breast cancer cells independent of tumor subtype or species, while exhibiting marginal impact on non-transformed cells from a variety of tissues (58). Notably, HMA cytotoxicity contrasts with that of conventional chemotherapeutics in that it kills tumor cells trapped in G1 phase of the cell cycle, suggesting that poorly proliferative tumor cell populations resistant to traditional chemotherapeutics are susceptible to CADs. Mechanistically, HMA induces the formation of multilamellar bodies in lysosomes of treated tumor cells upon very short (1–3 h) exposure, and triggers a caspase-independent and cathepsin-, Ca^{2+}- and ROS-dependent cell death mechanism within 24 hours (58). Further studies assessing HMA-induced lysosomal lipid metabolism are warranted.

NON-CAD INDUCTION OF LCD

Though CADs offer a straightforward approach to engaging LCD to combat cancer, other agents and strategies also show significant promise. In addition to the membrane-permeabilizing lysosomotropic detergents and pH modifying v-ATPase inhibitors discussed above, agents that interfere with lysosomal iron disposition are also attractive candidate LCD inducers. As a primary store of cellular iron, acute dysregulation of lysosomal iron homeostasis triggers the ferroptosis cell death mechanism (4, 59, 60). Salinomycin, an antimicrobial used to treat coccidiosis, and its derivative ironomycin have exhibited anti-cancer effects, notably toward cancer stem cells (61, 62). Their mechanism of action appears to involve the sequestration of iron within the lysosome, leading to the production of high levels of ROS that destabilize the limiting membrane, induce LMP, and ultimately necrotic cell death (63).

CONCLUSIONS

Lysosomes are powerful organelles that maintain steady-state levels of a variety of cellular metabolites by mediating their breakdown upon delivery. Acute disruption of these homeostatic processes can lead to LMP, which ultimately engages LCD programmed necrotic cell death. **Figure 1** summarizes the biological and chemical factors implicated in triggering LMP, highlighting the therapeutic potential of repurposed CADs, v-ATPase inhibitors, lysosomotropic detergents, and ferroptosis inducers. Although underappreciated in the cancer therapeutics field, accumulating studies point to exploitation of this mechanism for tremendous potential in targeting apoptosis-resistant tumor cell subpopulations. Advantages include the selective action of LCD inducers toward tumor versus non-transformed cells, minimizing side effects, and the ability of LCD inducers to trigger death in quiescent cell populations resistant to conventional chemotherapeutics.

A significant consideration in cancer therapeutic development concerns the effective integration of a novel drug with existing treatment paradigms. A popular approach is to incorporate the newly developed drug into current standard-of-care treatment protocols, with the hope of realizing synergistic efficacies with added agents. Such an approach could prove particularly effective with LMP-inducing agents. It has been suggested that lysosomes contribute to drug resistance by sequestering chemotherapeutics (doxorubicin, mitoxantrone, sunitinib, etc.), decreasing their availability and effective concentrations at target sites (64); thus, the abrupt release of stored chemotherapeutics into the cytosol after CAD treatment could allow for a potent one-two punch (65). On the other hand, doubling up on therapeutic agents runs the risk of developing synergistic toxicities and unwanted off-target effects. Given that LCD-inducing agents uniquely target quiescent and apoptosis- and therapy-resistant cell populations, a more fruitful strategy may involve delivery of such agents following standard-of-care treatment to eradicate remaining tumor cells and minimize chances for recurrence.

Going forward, LMP screens will identify novel agents that might be developed into more effective anti-cancer therapeutics. This is somewhat ironic in that the phospholipidosis side effect of CADs has been known for decades, and many investigators omit compounds from screens for drugs for other disease states whose structures might provoke such a phenotype. Deeper analysis will

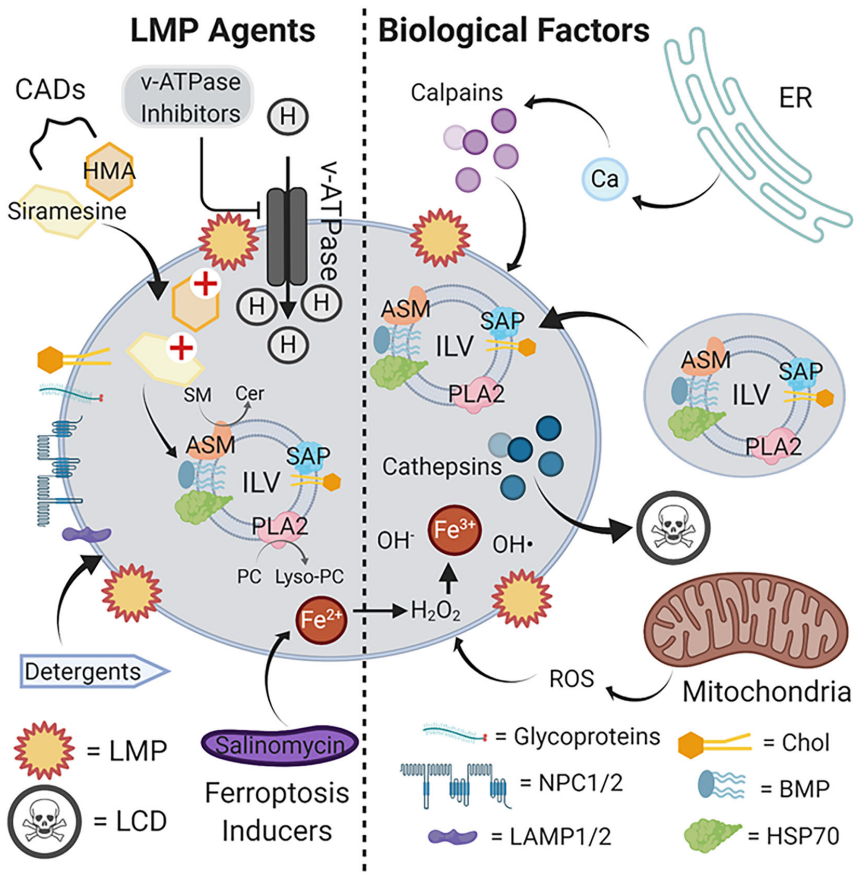

FIGURE 1 | LMP agents and biological factors that induce lysosomal rupture and cell death. The limiting lysosomal membrane employs various glycoproteins, NPC1/2 and LAMP1/2, and a characteristic lipid composition to maintain its functional integrity as a barrier with the cytosol. Internally, ILVs delivered from endosomes *via* vesicles bind to and activate lysosomal hydrolases responsible for the metabolic breakdown of delivered glycolipids to maintain cellular steady-state levels. CADs such as siramesine and HMA compromise this metabolic pathway, likely by interfering with the activation of enzymes such as ASM by ILV-localized acidic lipids such as BMP. The resulting accumulation of glycolipid substrates such as SM leads to limiting membrane destabilization, LMP and cathepsin release, and ultimately cell death. By suppressing proton import, v-ATPase inhibitors similarly suppress lysosomal hydrolase function by elevating luminal pH beyond the optimum for catalytic activity. Lysosomotropic detergents directly disrupt the limiting membrane by partially solubilizing lipid components, and ferroptosis inducers such as salinomycin sequester iron in lysosomes to promote ROS accumulation and limiting membrane destabilization through lipid oxidation. Internally-produced ROS from mitochondria can also contribute to limiting membrane destabilization, as can the release of Ca^{2+} from intracellular stores such as the ER to activate calpains. Abbreviations: ASM, acid sphingomyelin; BMP, bis(monoacylglycero)phosphate; CADs, cationic amphiphilic drugs; Cer, ceramides; Chol, cholesterol; ER, endoplasmic reticulum; HSP70, heat shock protein 70; HMA, hexamethylene amiloride; ILV, intraluminal vesicle; LAMP1/2, lysosomal-associated membrane protein 1/2; LCD, lysosomal cell death; LMP, lysosomal membrane permeabilization; lyso-PC, lysophosphatidylcholine; NPC1/2, Niemann-Pick disease 1/2; PC, phosphatidylcholine; PLA2, phospholipase 2; ROS, reactive oxygen species; SAP, saposins; SM, sphingomyelins. Illustration created using Biorender.

uncover the specific mechanisms by which CADs act. In this regard, it is important to note that different CADs exhibit somewhat different phenotypes and cytotoxic parameters, not surprising given that the phenotypes of lysosomal storage diseases can differ substantially. It is likely that different CADs preferentially target different enzymes of lysosome metabolism. As the molecular targets of CADs are uncovered and their structures elucidated, rational drug design approaches may be utilized to develop inhibitors; conferring CAD characteristics to lysosomal enzyme inhibitors of moderate efficiency could markedly enhance their potency in cells by promoting their lysosomal accumulation. These approaches will likely take years to decades to fully unfold. In the meantime, efforts to repurpose roughly six dozen existing clinically-employed CAD molecules to anti-cancer purposes could give us a substantial leg up on the LCD approach.

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MH and KC contributed to the research for and the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Strategies for Targeting Gene Therapy in Cancer Cells With Tumor-Specific Promoters

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Cancer is the second cause of death worldwide, surpassed only by cardiovascular diseases, due to the lack of early diagnosis, and high relapse rate after conventional therapies. Chemotherapy inhibits the rapid growth of cancer cells, but it also affects normal cells with fast proliferation rate. Therefore, it is imperative to develop other safe and more effective treatment strategies, such as gene therapy, in order to significantly improve the survival rate and life expectancy of patients with cancer. The aim of gene therapy is to transfect a therapeutic gene into the host cells to express itself and cause a beneficial biological effect. However, the efficacy of the proposed strategies has been insufficient for delivering the full potential of gene therapy in the clinic. The type of delivery vehicle (viral or non viral) chosen depends on the desired specificity of the gene therapy. The first gene therapy trials were performed with therapeutic genes driven by viral promoters such as the CMV promoter, which induces non-specific toxicity in normal cells and tissues, in addition to cancer cells. The use of tumor-specific promoters over-expressed in the tumor, induces specific expression of therapeutic genes in a given tumor, increasing their localized activity. Several cancer- and/or tumor-specific promoters systems have been developed to target cancer cells. This review aims to provide up-to-date information concerning targeting gene therapy with cancer- and/or tumor-specific promoters including cancer suppressor genes, suicide genes, anti-tumor angiogenesis, gene silencing, and gene-editing technology, as well as the type of delivery vehicle employed. Gene therapy can be used to complement traditional therapies to provide more effective treatments.

Keywords: cancer, gene therapy, targeted treatment, specific promoters, non-viral vectors

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INTRODUCTION

Cancer Basics and Available Treatments. Why Use Gene Therapy?

Nowadays, cancer is one of the main causes of death worldwide. According to the World Health Organization (WHO), cardiovascular disease related deaths are the main cause of death worldwide, being cancer in the second place, responsible for one in six deaths. Nevertheless, it is believed that in the future it could become the first cause of death (1, 2). Cancer is not exclusive of high economy

level countries; developing countries contribute with a little more than a half (56%) of new cancer diagnosis per year and with 64% of deaths due to cancer worldwide. Hence, it is considered as an important obstacle to the economic and social development among all countries. WHO estimates that by the year 2030 cancer cases could surpass 20 million cases per year worldwide, as a consequence of the current demographic exPLoSion and the increase of elderly people. Nonetheless, there is a possibility of diminishing these figures given that more than 30% of all types of cancer are preventable by avoiding the main risk factors such as smoking, alcoholism, unhealthy diets, and sedentary lifestyle plus some chronic infectious diseases, especially those of viral nature (2).

Vaccination and early diagnosis along with proper therapies are important aspects to be taken into consideration to significantly reduce cancer related deaths. All the same, these strategies have not been successful given tumor variability and complexity. Regardless of the neoplasms origins, the main features common to all tumor cells include: continuous proliferative signaling, tumor suppressors evasion, apoptosis resistance, replicative immortality, cell-energetics deregulation, metastasis, and angiogenesis activation and immune system evasion (3). Surgery and radiotherapy are the treatments used to treat local non-metastatic tumors whereas antineoplastic medicine such as chemotherapy, hormones, and biological therapies are preferably used to handle metastatic tumors. Toxicity rendered by chemotherapeutic drugs, which induce undesirable massive destruction of normal cells, next to the upgrowing knowledge of molecular biology of tumor cells and the exclusive tumor features, have arisen the need of searching alternative targeted and efficient treatments against cancer, being gene therapy one of the most promising procedures for accomplishing such a purpose. Gene therapy consists in the introduction of therapeutic nucleic acids (TNAs) into target cells in order to achieve a beneficial molecular effect for patients. TNAs' delivery into cancer cells, such as genes, oligonucleotides, or interference RNAs, has enabled cancer battling by means of gene substitution, or genetic expression regulation either over-expression or repression (4–8). Notwithstanding, TNAs effect is transient and most of the time the desired effect has not been achieved. Because of this, expression plasmid vectors have been designed and constructed with the purpose of avoiding transient effects of TNAs. At first, therapeutic genes were under transcriptional control of ubiquitous eukaryotic viral promoters such as those from cytomegalovirus (CMV), Rous sarcoma virus (long terminal repeat, LTR), simian virus 40 (SV-40), and Epstein–Barr virus (EB) (9), which mediate non-specific expression of therapeutic genes in neoplastic cells and normal cells likewise. For this reason, the need of designing new expression systems with cancer/tissue specific promoters in order to drive gene expression of therapeutic genes towards target cells has arisen.

Cancer/tumor-specific promoters have been used to perform gene therapy in many types of neoplasia; within the most studied ones we have hepatocellular carcinoma, breast, lung, colorectal, pancreas and prostate cancer (8, 10–13). Gene therapy success in cancer treatment relies not only on a good molecular strategy, which consists of the design of specific genetic material being exclusively expressed within tumor cells, but also on the need of a safe, efficient and specific gene delivery system. Accordingly, a wide variety of genetic vectors have been developed for TNAs delivery, within them, viral vectors have shown the highest efficiencies but their greatest disadvantage is their immunogenicity. In this sense, non-viral vectors have proven to be safer when it comes to *in vivo* TNAs delivery, even when they are less efficient. So, the search of a safer, more efficient and specific genetic vector is still going on.

The aim of the present review is to provide a general view of the most recent molecular strategies in which cancer/tumor-specific promoters have been used in the design and construction of appropriate genetic vectors so targeted gene therapy can be performed on specific neoplasia.

CURRENT MOLECULAR STRATEGIES IN CANCER GENE THERAPY

Gene transferring technologies allow a wide variety of treatment possibilities which can be used in complementing conventional therapies as well as providing new treatment strategies. New delivery systems as well as more sophisticated gene expression systems are being studied with the aim of achieving cancer treatment and removal. That is why the use of nucleic acids such as recombinant DNA, interfering RNA (iRNA), microRNAs (miRNAs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats/CRIPR-associated protein 9 (CRISPR/Cas), and suicide genes have aroused great interest among the scientific community (14–16).

Antisense Oligonucleotides Technology in Cancer

Antisense oligonucleotides (ASOs) are defined as single-stranded, highly modified, synthetic RNA or DNA oligonucleotides designed to selectively bind to target RNA molecules, which are encoded by the gene of interest, through Watson–Crick base-pairing with the purpose of modulating its function (17, 18). Binding of ASO to its complementary target can trigger different action mechanisms (16). These mechanisms can be classified as those that bind to RNA and interfere with its function without promoting RNA degradation and those that promote RNA degradation (19, 20). Even when several ASO-based treatment candidates have gone into different clinical trial stages, none has been approved for cancer treatments yet (18). Despite this, ASO-mediated intervention is a potential therapeutic approach for targeted manipulation of gene expression for cancer treatment.

Interfering RNAs

RNA interference technology has made considerable progress, especially when it comes to cancer treatment (21), since first described in *Caenorhabditis elegans* in 1998 (22). RNAi is a

double-stranded RNA (dsRNA)-based gene silencing technology that evolved as a natural cell defense mechanism against RNA viruses. This mechanism identifies pathogenic dsRNA molecules and targets them for cleavage. Up to now, three classes of small RNAs have been described in animals: microRNAs (miRNAs), small interfering RNAs (siRNAs), and PIWI-interacting RNAs (piRNAs) (23). Usually, these RNAs guide Argonaute proteins to target RNAs *via* Watson–Crick base-pairing, usually resulting in gene silencing (24).

The miRNAs are single-stranded, non-coding RNA molecules, of 21–22 nucleotides in length, which main function is silencing gene expression at a post-transcriptional level by the imperfect binding to the target mRNA, specifically in nucleotides 2–8 of the miRNA, known as the seed region (25, 26). In cancer, some miRNAs are over-expressed, inducing tumor development (oncomirs) and others are downregulated, blocking inhibitory control over some oncogenes, or cell differentiation, and apoptosis control (tumor suppressor miRNAs) (27–29).

The siRNAs are synthetic double-stranded RNA molecules of 21–23 nucleotides in length which induce gene silencing at post-transcriptional levels by binding to the target mRNA in specific binding sites which leads it to its degradation and thus to translation inhibition (30–33). It is worth mentioning that siRNA union with its target mRNA is highly selective and when compared against miRNAs, this union is 100% complementary, discriminating sequences even with one different nucleotide (34).

The piRNAs are a kind of small non-coding RNA of 24–32 nucleotides in length, named this way because of their interactions with the PIWI subfamily of Argonaut proteins which exerts a transposon gene silencing effect besides other kinds of regulation such as epigenetics, gene and protein regulation, genome rearrangement, spermatogenesis, and germ stem cells maintenance (35–37). The piRNAs can be mainly involved in epigenetic regulation rather than post-transcriptional regulation of many biological phenomena such as cancer (38, 39).

RNA interference therapies also include small hairpin RNAs (shRNAs) (40), which are RNA molecules that can be synthesized from expression vectors within cell nucleus, to be then transported to the cytoplasm and processed by endogenous machinery to give siRNAs. Each shRNA can encode more than two siRNAs to silence the target mRNA (41, 42). The shRNAs can be transcribed by type II or type III RNA polymerase throughout type II RNA polymerase depending promoters or type III RNA polymerase depending promoters in the expression cassette design to carry on RNA interference (40, 41).

Gene Editing Techniques in Cancer

Development of gene editing techniques has enabled the possibility of directly targeting and modifying specific gene sequences in almost every eukaryotic cell, displaying an enormous potential for its use in many fields which range from basic research to applied biomedicine and biotechnology (43). The latest advances in the development of programmed nucleases such as ZFNs, TALENs, and CRISPR/Cas have enormously accelerated transition from basic research to the advances in gene edition within clinical practice (15).

ZFNs are proteins which arise from the specific union of a Cys_2-His_2 protein and the Fok1 restriction endonuclease cleavage domain resulting in DNA target sequence cleavage (44–46). On the other hand, there are transcription activator-like effector nucleases (TALENs); these ones arise from the binding of a DNA binding domain and a nuclease catalytic domain of Fok1 (47). Using gene editing techniques such as ZFNs and specific TALENs, has been specifically inhibiting cervix cancer cell growth (48–50) and acute lymphoblastic leukemia (45, 51). As well, ZFNs have been used for fighting the resistance to therapeutic agents in breast cancer cells (52).

The most recent gene editing technology developed is CRISPR/Cas. It is originally present in bacteria and archaea as an "immune system" to protect these organisms against phage and other viral infections. One interesting feature of CRISPR systems is that they use a guide RNA (gRNA) that binds to the DNA target site while a nuclease known as CRISPR-associated caspase protein (Cas) cleaves specific DNA strands which are complementary to the RNAg of the CRISPR system (53). This gene editing system can be used for raising adaptive immunity, fighting carcinomas and specific mutation editing (54). It has been shown that using this gene editing technique, the reduction of tumor size, migration capacity, and drug resistance can be achieved in pancreatic (55), prostate (56–58), colon (59), and breast cancers (60, 61).

The potential of gene editing techniques for its use in basic research as well as in clinical cancer treatment has begun to develop strongly. In the future, grouped gRNA will provide a complete set of susceptible genes which could be modified in most cancer cell lines (62). This resource, along with the available information about cancer cell line genetics and epigenetics will allow us to achieve new safer, more efficient cancer gene therapies.

Suicide Gene Therapy

Suicide gene therapy is based on introducing suicide genes to express enzymes or proteins that trigger the death of tumor cells (63–65), directly or indirectly. Direct suicide gene therapy consists of a gene that encodes for a protein that is cytotoxic and when expressed within the tumor cell induces its death. This has been achieved with the diphtheria toxin A complete gene or with segments of it, whose expression can change cell membrane stability and reduce tumor cell viability (14, 66–68). Indirect suicide gene therapy uses genes that express enzymes such as the Herpes Simplex Virus Thymidine Kinase (HSV-tk) accompanied by the administration of ganciclovir (GCV) (65, 69, 70). Another gene that has been used is the *Escherichia coli* cytosine deaminase gene; it encodes for an enzyme that catalyzes conversion of 5-fluorocytosine (5-FC) into 5-fluoruracile (5-FU), a drug used in conventional chemotherapy of HCC, prostate, colon, and breast cancers because it causes cell proliferation inhibition and induces cell death (14, 67, 71). Furthermore, this approach has been carried out by using a suicide gene which encodes for a modified human Caspase 9 (iCas9), which is strongly recognized by the bioinert synthetic molecule (B/B Homodimer, AP20187), alone or delivered by mesenchymal stromal cells (MSCs), inducing a dimer formation and activation apoptosis pathway in breast and lung cancer cells (72–74).

DIRECTING GENE THERAPY IN CANCER

In recent years, gene therapy has had considerable progress. However, the big challenge still remains: for gene therapy to be successful, the TNAs must be delivered and expressed within the target cells (75). In order to direct gene expression of TNAs on the desired cells or tissues, a specific carrier of TNAs must be developed, that is, a genetic vehicle must be designed in a fashion that it can target specific cells, such as cancer cells, and deliver TNAs in a localized manner (76–79). The second approach has to do with the presence of regulatory sequences which direct gene expression only within the target cells, even if the recombinant DNA molecule can enter cells whether they are cancer cells or not.

An important feature that determines cancer-focused gene therapy success is the regulatory sequence which controls gene expression. Because of this, expression of therapeutic genes must be controlled by cell or tissue specific promoters (80, 81). Promoters are *cis*-acting regulatory regions which direct transcription of mRNAs that, in turn, are translated into proteins. Functionally, a promoter is a DNA sequence located upstream the 5′ end of the coding region of a gene that includes the binding regions for transcription factors (82). Currently, they are being highly studied in biotechnological processes, since they cannot only increase transcriptional activity but also provide additional levels of control, *e.g.*, at the expression or stage-specific level of a gene in a particular organ or tissue. Ideally, such a promoter should provide the maximum specific expression of the therapeutic gene in the target tissue and must be strong enough to ensure the safety and efficiency of the system.

Some promoters show a specific activity only in certain cell types, making them potential candidates for transcriptional targeting. Promoters which can be used to transcriptionally target cancer can be classified into three different categories: tissue-specific, cancer-specific, and tumor-specific (76, 82).

Tissue-Specific Promoters

Transcriptional targeting by using tissue-specific promoters resorts to promoters of genes which are specifically active in certain tissues. Although they have been widely studied for its use in cancer gene therapy (83, 84), one of the main limitations of this type of promoter is that gene expression may lead to cytotoxic effects in normal as well as in the tumor tissue derived from the same cell type. Therefore, the use of such promoters must be restricted to tissues in which damage is not critical for the survival of the host, for example, prostate, melanocytes, or thyroid. If the tissue/organ is critical, then therapeutic genes must be delivered directly to the tumor site to prevent normal tissue to be affected (85). Nonetheless, the use of cancer- and/or tumor-specific promoters rather than tissue-specific promoters could be the best option to avoid adverse effects in normal cells (86).

Cancer-Specific Promoters

One of the main obstacles to current cancer therapies is the lack of tumor specificity. So, specifically targeting gene expression to tumor cells is one of the most important goals of cancer gene therapy (82, 85). Cancer-specific promoters are those that are functional for various types of cancers without any particular tissue/tumor specificity. However, their main feature is that they are functional within cancer cells but have no activity in normal cells. Telomerase was the first gene to be classified as cancer specific and whose promoter (hTERT) has been used to drive the expression of genes selectively in a wide variety of tumor cells (85, 87, 88). It has been observed that ~90% of human cancers express high levels of telomerase, while its activity is generally absent in normal somatic cells (89, 90). Whereby, this promoter clearly has a real potential in targeting a wide range of different tumor types.

At present, other cancer specific promoters have been studied, among them is epidermal growth factor receptor (EGFR), human epidermal growth factor receptor/neu (HER2/NEU), vascular endothelial growth factor receptor (VEGFR), folate receptor (FR), transferrin receptor (CD71), mucines, tumor resistance antigen 1-60 (TRA-1-60), cyclooxygenase (COX), cytokeratin 18, cytokeratin 19, survivin and chimeric antigen receptors (CAR) (82, 91–93). Most of the genes controlled by these promoters are over-expressed in cancer cells. That is why genetic constructions have been designed using these promoters to direct gene expression of suicide genes only in tumor cells for cancer gene therapy strategies (14).

Tumor-Specific Promoters

Tumor-specific promoters are those which are active in a limited type of cancer cells and their activity varies widely in different tumors. Nevertheless, their main feature is that they are little or non-active in normal cells (**Figure 1**) (13). So it can be ensured that tumor-specific promoters are specific for a malignant process but show no specificity for a certain type of tissue given that they respond and are activated by the tumor microenvironment. Within this group are alpha-fetoprotein promoter (AFP), thyroid transcription factor 1 (TTF-1), glypican-3 protein (GPC3), human secretory leukocyte protease inhibitor (hSLPI), ERBB2, Mucin 1 (MUC1), L-plastin, α lactalbumin (LALBA), cyclooxygenase 2 (COX2), epithelial glycoprotein (EPG2), A33, uPAR, carcinoembryonic antigen (CEA), breast cancer 1 (BRCA1) and BRCA2 (85, 94).

Genetic Vectors

With the development of a wide variety of recombinant DNA technologies, as well as a better understanding of genetics and molecular biology, the promise of treating genetic diseases in order to cure them or to improve quality of life of the patients seems to be a closer reality. Nevertheless, there is still a long way to go. For gene therapy to be successful, one of the most important things to take into consideration is the use of appropriate gene delivery systems. One of those issues related with gene therapy is the need of delivering therapeutic nucleic acids into the target cells, tissues and/or organs in a safe and efficient way. To do so, it is imperative to develop strategies which allow us to specifically target the delivery of the therapeutic nucleic acids and in doing so, maximizing cell transfection. As of 2017, around 70% of all gene therapy clinical trials were carried out by viral vectors. However, there are several significant concerns regarding the application of viruses as a carrier, including immunogenicity, insertion mutagenesis, as well as reports of deaths following

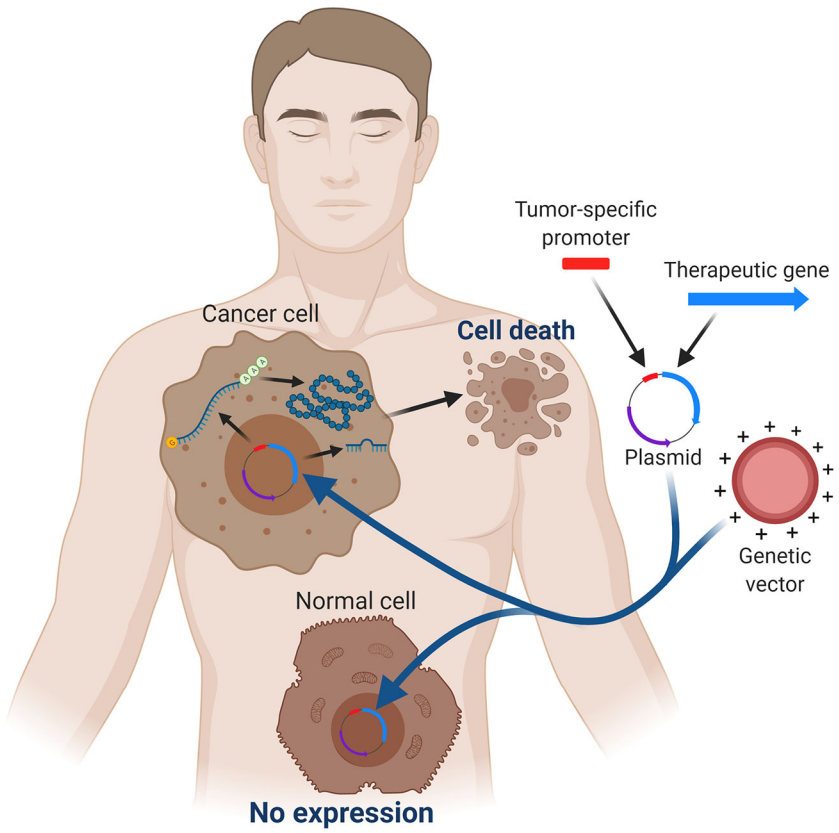

FIGURE 1 | The principle of targeted gene expression controlled by a tumor-specific promoter. Specific transcription factors in cancer cells are able to induce therapeutic gene expression. On the other hand, expression of the therapeutic gene is not obtained in normal cells because they lack the specific transcription factors. Created with BioRender.com.

administration of viral vectors for gene administration (95). Therefore, considerable attention has been paid to the application of non-viral vectors with the ability to specifically direct therapeutic nucleic acids to the target cells, promoting the entry and release of these within cells, to obtain the desired biological effect (96–102).

MOST COMMON CANCERS TREATED WITH GENE THERAPY: THE POTENTIAL OF SPECIFIC PROMOTERS AND GENETIC VECTORS

As a new promising strategy in gene therapy, the use of cancer/tumor-specific promoters for targeting TNAs has grown in recent years. Several studies have proven that, by using these types of promoters, along with different molecular strategies and delivery vectors, TNAs can be delivered safely and efficiently within the desired cells, allowing the treatment of cancer without harming healthy tissues (**Table 1**).

Hepatocellular Carcinoma

Hepatocellular carcinoma is one of the four types of cancer with the highest death rate worldwide causing 781,631 deaths per year (1); this is because of a late diagnosis and ineffective treatments as well as those which give rise to adverse effects. Target gene therapy seems to be a promising approach (171).

Cancer/tumor-specific promoters impede gene expression of the therapeutic gene within normal cells, reducing toxicity and all the same sustaining anti-cancer efficacy. AFP promoter is the most common tumor-specific promoter used in HCC gene therapy due to the high level of activity in this cancer. AFP promoter is usually active in the fetal stage and then suffers inactivation 6 months after birth. Notwithstanding, it can be reactivated in abnormal conditions like cirrhosis and certain types of cancer such as HCC or, less importantly, pancreatic cancer and lung cancer (11, 87, 103, 104, 172). For that reason, it has been widely used in gene therapy for HCC in order to direct the expression of genes such as sodium/iodide symporter (NIS) with the purpose of improving radiotherapy efficiency (105, 106) and HSV1-tk gene to increase tumor-sensitivity facing chemotherapy (103, 107).

TABLE 1 | Features of tumor-specific promoters and type of gene used in cancer gene therapy.

Cancer type	Promoter	Type of gene used	Specificity	Advantages	Disadvantages	References
Hepatocellular carcinoma	AFP	Increase sensitivity (NIS); Suicide gene HSV1-tk; shRNA against Beclin1	High activity in liver cancer	Tumor specificity	Weak promoter	(11, 87, 103–108)
	EA4D (enhanced AFP)	Apoptotic protein tBid	High activity in liver cancer	Stronger than wild type AFP promoter and the same tumor specificity	Useless in other types of tumors	(109)
	a2bm (variant AFP)	Oncolytic adenovirus E1A	High activity in liver cancer	Stronger than wild type AFP promoter and the same tumor specificity	Useless in other types of tumors	(11, 106, 110–112)
	hTERT	Cytotoxic gene ADI	High activity in a wide variety of tumor cells	Widely used for other tumors	Tumor non-specificity, basal expression in normal cells	(88, 112, 113)
	GPC3		High activity in liver cancer	Tumor specificity, stronger than AFP	Useless in other types of tumors	(86, 114–116)
Breast Cancer	ErbB2	Suicide gene HSV1-tk	High activity in breast and prostate cancer	Tumor specificity	Useful in a limited number of tumors	(117–120)
	MUC1	Suicide gene HSV1-tk	High activity in breast cancer, pancreatic cancer and cholangiocarcinoma	Higher activity in tumor cells	Basal expression in some normal cells	(121, 122)
	LALBA	Adenoviral genes E1A and E1B	High activity in breast cancer	Tumor specificity	Useless in other types of tumors	(123, 124)
Lung cancer	hTERT	Pro-apoptotic protein MP-VSV	High activity in a wide variety of tumor cells	Widely used for other tumors	Tumor non-specificity, basal expression in normal cells	(80)
	TTF-1	Tumor suppressor miR-7	High activity in lung cancer	Tumor specificity	Useless in other types of tumors	(125–128)
	hSLPI	Combinations: suicide gene HSV1-tk and IL-12 gene; miRNA targeting EGFR and CASP3	High activity in lung, breast and ovary cancer	Tumor specificity	Useful in a limited number of tumors	(8, 77, 129–131)
	CEA	Suicide gene E and drug PTX	High activity in lung, gastrointestinal, colorectal and breast cancers	Tumor specificity	Useful in a limited number of tumors	(13, 32, 132–134)
Colorectal cancer	CEA	Suicide gene E	High activity in colorectal, gastrointestinal, lung and breast cancers	Tumor specificity	Useful in a limited number of tumors	(135–138)
	COX-2	Tumor suppressor 15-PGDH	High activity in colorectal cancer	Tumor specificity	Useless in other types of tumors	(12, 91, 139–142)
	A33	Adenoviral gene E1A	High activity in colorectal, intestinal-type gastric and pancreas cancer	Higher activity in tumor cells	Tumor non-specificity, basal expression in normal cells	(138, 143, 144)
	hTERT	Adenoviral gene E1A; combination of suicide gene HSV1-tk and IL-18 gene	High activity in a wide variety of tumor cells	Widely used for other tumors	Tumor non-specificity, basal expression in normal cells	(69, 71, 145, 146)
	uPAR	Suicide gene HSV1-tk	High activity in a wide variety of tumor cells	Widely used for other tumors	Specificity in the invasive edge of a tumor	(147–150)
	FGF18	Suicide gene HSV1-tk	High activity in a wide variety of tumor cells	Widely used for other tumors	Tumor non-specificity, basal expression in normal cells	(150–152)
	KDR	Combination of suicide genes HSV1-tk and CD	High activity in a wide variety of tumor cells	Widely used for other tumors	Tumor non-specificity, basal expression in normal cells	(138, 153)
Pancreatic Cancer	CCKAR	Pro-apoptotic gene BikDD	High activity in pancreatic cancer	Tumor specificity	Useless in other types of tumors	(154, 155)
	MUC1	DTA toxin	High activity in pancreatic cancer, breast cancer and cholangiocarcinoma	Higher activity in tumor cells	Basal expression in some normal cells	(156–158)
	hTERT	Oncolytic adenovirus Telomelysin	High activity in a wide variety of tumor cells	Widely used for other tumors	Tumor non-specificity, basal expression in normal cells	(89, 90, 159, 160)
	SHIP1		High activity in pancreatic adenocarcinoma that overexpresses PDX-1	Stronger than wild type insulin promoters	Tumor non-specificity, basal expression in normal cells	(10, 161)

(Continued)

TABLE 1 | Continued

Cancer type	Promoter	Type of gene used	Specificity	Advantages	Disadvantages	References
Prostate cancer	GRP78	Suicide gene HSV1-tk	High activity in prostate, gastric, breast, pancreatic, lung and colon cancers	Tumor specificity	Useful in a limited number of tumors	(162–164)
	hON-522E	Suicide gene HSV1-tk	High activity in prostate cancer and in a wide variety of other tumor cells	Higher activity in tumor cells	Tumor non-specificity, basal expression in normal cells	(165, 166)
	PSA	Suicide gene thymidine kinase; apoptotic protein Apoptin	High activity in prostate cancer	Higher activity in tumor cells	Tumor non-specificity, reported expression in normal cells	(167–170)
	PSMA	Apoptotic protein Apoptin	High activity in prostate cancer	Higher activity in tumor cells	Tumor non-specificity, reported expression in normal cells	(167, 168, 170)

AFP promoter has also been used in RNAi strategies (AFP-Cre/LoxP-shRNA), obtaining a specific shRNA against the cell death and autophagy regulatory protein (Beclin1) gene mRNA, inhibiting translation and HCC growth (108). Furthermore, a more transcriptionally active variant of AFP promoter (EA4D) was found and by means of the genetic construction pGL3-EA4D-tBid/H, the growth of HCC AFP+ tumors was specifically and remarkably inhibited. Nonetheless, there was no effect on AFP– tumors (109).

The main drawback of AFP promoter is that it is a weak promoter when compared to strong promoters such as CMV or CAG promoters (106, 110–112). Due to this disadvantage, its usefulness in gene therapy assays was limited. As a result, chimeric variants of AFP promoter bearing enhancers have been developed, deriving in the modified AFP promoter known as a2bm. This variant promoter bears two enhancer A and one enhancer B regions and as a result a 500-fold increase in transcription rate was obtained, besides being HCC specific. Later, AFP-a2bm was further modified using hypoxia-response elements (HRE), increasing transcriptional activity under hypoxic conditions. This allowed to overcome the hypoxic tumor environment and to target HCC with high specificity, proving it as a promising candidate for HCC treatment based on gene therapy (11).

Human telomerase reverse transcriptase (hTERT) promoter is a type of cancer-specific promoter; this can be used to drive the expression of genes in a wide variety of tumor cells without any particular tumor-specificity and has also been used to target therapeutic genes towards HCC along with arginine deaminase (ADI) gene, which encodes for an arginine-degrader enzyme (88), a potential agent against arginine-auxotroph tumors such as HCC and melanomas (113). This was done by substituting CMV promoter with hTERT promoter in an adenoviral vector aiming to direct ADI expression within the cancer cells, resulting in cytotoxicity on cancer cell lines and even eliminating tumors after two weeks of treatment in a mouse model (88). This gene therapy procedure with ADI-PEG20 (ADI PEGylated with PEG 20,000) has already passed phase I/II clinical trials but due to its low efficacy and specificity has been used only as an adjuvant therapy (112, 113).

Another tumor-specific promoter for HCC is the GPC3, an oncofetal protein belonging to the proteoglycan family only expressed in fetal development. However, the expression of this protein is reactivated mostly in HCC but also has been observed in malignant melanoma, neuroblastoma, and colon cancer (114–116). GPC3 promoter activation in HCC was demonstrated using luciferase and enhanced yellow fluorescent protein (eYFP) reporter genes in HCC cell lines and compared to normal hepatocytes (86).

Prostate and breast cancer over-expressed 1 (PBOV 1) encodes for a protein which is quite over-expressed in many types of cancer but not in normal tissues (173). Nevertheless, its role in the initiation and progression of hepatocellular carcinoma (HCC), was unknown (174). In order to reveal the role of this gene in HCC, a study was carried out in which a PBOV-1 plasmid and a PBOV-1 siRNA plasmid were delivered into HCC cells so that its expression levels and its effects on growth and metastasis could be investigated. Then, the need for an efficient and safe genetic vehicle arises. Epidermal growth factor receptor (EGFR) is a cell transmembrane protein which is known to be over-expressed in many epithelial tumors (175). So, anti-EGFR monoclonal antibodies could be potent ligands directing therapeutic nucleic acids towards epithelial tumors such as HCC. An EGFR single-chain antibody-modified graft copolymer of polyethylene glycol (PEG) and polyethylenimine (PEI) complexed with superparamagnetic iron oxide nanocrystals (SPION) was developed. The use of EGFR single-chain antibody improves tumor-targeted gene delivery, and the use of polymers is useful for nucleic acids protection against the nuclease activity *in vivo*. Nevertheless, the cationic and non-biodegradable characteristics of PEG and PEI remain to be an obstacle to overcome when it comes to its use in clinical trials (176, 177).

Breast Cancer

Breast cancer is the result of an abnormal and disordered growth of epithelial cells of mammary ducts or lobules and is characterized by metastasis capability, being mainly a hormone-depending disease (65% of all breast cancer cases) (1, 2). Due to lack of early diagnosis and timely treatments, it is the fifth cause of cancer death worldwide. Therefore, different

directed gene therapy strategies have been developed using tumor-specific promoters, achieving encouraging results in breast cancer treatment.

ERBB2 protein is an oncoprotein that belongs to the EGFR family (117). It is over-expressed in about 20% of invasive breast cancers. Particularly, it has been shown that ERBB2 over-expression boosts invasion and metastasis of breast cancer and is correlated with poor survival of patients (118). Identification of the deregulated ERBB2 pathway in breast cancer pathogenesis has led to the development of ERBB2 targeted therapies. In a study, HSV1-tk gene under ERBB2 251 bp promoter (p256-TK) transcriptional control was transfected in breast cancer cells, resulting in a higher ganciclovir sensibility without affecting normal cells (119, 120).

The MUC1 gene encodes a mucin-like high molecular weight glycoprotein and is over-expressed in breast cancer and cholangiocarcinoma (121). It has a 114 bp enhancer region capable of modulating heterologous promoter transcription. It has been shown that positive DF3 breast cancer cell lines are more susceptible to cell death by GCV when HSV1-tk is delivered and driven by this enhancer. Afterwards, a replica of the expression vector was constructed and introduced in an adenovirus vector to be delivered to breast cancer cells, inhibiting tumor growth and intraperitoneal metastasis in a breast cancer mouse model (122).

LALBA is a protein that regulates lactose production in the milk of most mammals. It constitutes the regulatory subunit of the lactose synthase heterodimer (LS), whereas β-1, 4-galactosyltransferase constitutes the catalytic domain. The dimer allows LS to synthesize lactose by transferring galactose residues to glucose (123). LALBA is breast specific and expresses in more than 60% of breast cancer tissues. LALBA promoter showed a significantly higher activity in MDA-MB-435S and T47D breast cancer cell lines when compared against normal breast cell lines or other tumor cell lines. Furthermore, the replication efficiency of the vector and as a consequence its tumor cell destroying capability were increased as shown versus normal cell lines (negative LALBA promoter cells) (124).

Cationic porphyrin microbubbles (CpMBs) have been synthesized from a porphyrin grafted lipid which has two cationic amino groups (PGL-NH2) and the fluorocarbon inert gas C3F8. This design has two purposes: first of all, the porphyrin group can be used as a photosensitizer in order to carry on photodynamic therapy (PDT); secondly, the amino groups provide positive charges which can interact with a siRNA that can be used for FOXA1 knockdown (FOXA1 KD) in estrogen receptor positive breast cancer cells (ER + BC cells) (178, 179). *In vivo* experiments were carried on in which female Balb/c nude mice were injected with cells from the MCF7 cancer cell line and then subjected to treatment with CpMBs/siRNA followed by ultrasound targeted microbubble destruction (UTMD) being guided by contrast enhanced ultrasound (CEUS) (180). Promising results were obtained with this novel CpMBs in combination with ultrasound technology, which lead to a more efficient accumulation of porphyrin and siRNA into tumor cells (181, 182).

New approaches aim to treat breast cancer using co-delivery systems which can transport drugs and therapeutic genes within breast cancer cells. Among the many gene delivery systems developed, inorganic materials are highly promising. Hydroxyapatite nanoparticles have shown many advantages such as low cytotoxicity, wider surface areas and they are easy to fabricate and modify (183). Amine-functionalized hydroxyapatite nanoparticles (NHAP) were synthesized from 3-aminopropyl-triethoxysilane (APS) and HAP nanoparticles. Then, candesartan (CD) and p53 plasmid were added to give a drug–gene co-delivery vehicle. These nanoparticles showed the desirable characteristics of surface charge and particle size good enough to provide pDNA condensation and protection. After 72 h post-incubation in *in vitro* assays, cells treated with these nanoparticles showed viability above 90%. Transfection efficiency of these nanoparticles was about 26%. Finally, the design of this co-delivery system showed a strong inhibitory effect on angiogenesis *in vitro*, and *in vivo* analysis demonstrated a superior anti-tumor effect in a mouse model (181, 184, 185).

Lung Cancer

Lung cancer is the main cause of cancer deaths worldwide, accounting for 1,761,007 deaths from the 2,093,876 new reported cases in 2018 (1) despite advances in chemotherapy, surgery, and radiotherapy.

The matrix protein (MP) of the vesicular stomatitis virus (VSV) induces apoptosis in tumor cells in the absence of other viral components. Wild-MP gene was used to construct pVAX-M recombinant plasmid, which showed an efficient suppression of malignant tumors growth by inducing apoptosis in *in vivo* and *in vitro* assays. Afterwards, phTERTM plasmid encoding VSV MP under transcriptional control of hTERT promoter was constructed, displaying the same anti-tumor effect but specifically directed against lung adenocarcinoma (80).

On the other hand, thyroid transcription factor 1 (TTF-1) is a member of the Nkx2 transcription factors family, classified as a tissue-specific oncogene given that it is expressed mainly in lung cancer cells but not in other types of cancer and whose expression levels are tightly linked with patient prognosis (125). Based on the above, a miR-7 expression vector under TTF-1 promoter transcriptional control was constructed (p-T-miR-7), this expression vector displayed a reduction of tumor growth rate, migration and metastasis of lung cancer cells *in vivo* and *in vitro* suggesting the usefulness of miR-7 to develop new gene therapy strategies selectively against lung cancer (126–128).

As discussed previously, suicide gene therapy is another interesting approach in cancer gene therapy. From this point of view, an expression vector was constructed from HSV1-tk and human interleukin-12 genes under transcriptional regulation of tumor-specific hSLPI (human secretory leukocyte protease inhibitor) promoter, which is known to be active in lung, breast, and ovary cancers (8, 77, 129). This vector displayed a more specific anti-tumor effect because of hSLPI promoter transcriptional regulation, besides demonstrating that suicide gene therapy combined with immune gene therapy provides a stronger anti-tumor effect than gene therapy using a single gene

(77). Otherwise, a recombinant adenovirus (Ad-SLPI-EGFRamiR-SLPI-revCASP3) expressing an artificial miRNA targeting EGFR and recombinant caspase-3 (CASP3) under transcriptional regulation from SLPI promoter was constructed. This displayed a specific novel anti-cancer gene therapy strategy which combines EGFR inhibition as well as CASP3 induced apoptosis (130, 131). The inhibitory effect caused by this adenovirus was commensurable to the therapeutic effects of cis-platinum and cetuximab (8).

CEA belongs to a cell-surface glycoproteins family and is the most used tumor marker in clinical diagnosis of colorectal, gastrointestinal, lung and breast cancers (32). It is normally expressed in epithelial cells of the fetal gastrointestinal tract (132, 133); however, non-small-cell lung carcinoma (NSCLC) patients have elevated CEA serum levels, something that has been correlated with low survival rates (13, 134) so that CEA promoter has been used in directing E gene (pCEA-E) along with (PTX) in order to specifically target lung cancer cells, improving anti-tumor effects of PTX. *In vivo* assays corroborated this combined therapy effectiveness and demonstrated that CEA is an excellent tumor-specific promoter for targeting therapeutic genes expression within lung cancer cells inducing apoptosis and with no harm to normal cells (13).

Human Wnt inhibitory factor-1 (hWIF-1) has been described as an effective anti-oncogene useful for NSCLC gene therapy. The use of viral vectors to deliver these therapeutic genes into NSCLC cells has failed, given the inherent disadvantages of these genetic vehicles such as immunogenicity and insertional mutagenesis. So, a novel genetic vehicle based on PEI and branched PEI1800 coated with SP5-2 peptide, which specifically targets NSCLC cells, was developed. When proved on A549 cells, this vehicle provided a 50% transfection efficiency, showing, this way, that this is a promising genetic vehicle which can be useful for delivery of therapeutic nucleic acids on cancer cells (98).

Nanocarriers, such as nanostructured lipid carriers (NLC), have proven to be potential candidates to work as non-viral genetic vehicles due to important features such as increased chemical stability, higher loading capacity of nucleic acids, lower cytotoxicity and controlled release (186). Another strategy used in the design of safer and more efficient genetic vehicles is the construction of dual ligand-decorated lipid carriers. Transferrin (Tf) is a protein which has been used for targeted gene therapy, given that most cancer cells of lung carcinoma overexpress transferrin receptors (187). In a similar way, hyaluronic acid (HA) has also been used for a similar purpose for most of the non-small cell lung cancer (NSCLC). Then, for the sake of finding a more promising genetic vehicle, transferrin and hyaluronic acid containing polyethylene glycol-distearoyl phosphoethanolamine (PEG-DSPE) ligands were synthesized. The systemic delivery efficiency of nucleic acids using this novel genetic vehicle was evaluated *in vivo* in a human lung adenocarcinoma A549 cell-bearing mouse model. These nanocarriers showed a sustained release of pDNA, which can lead to the persistence of the therapeutic effect when used for *in vivo* purposes. Even more, the presence of the Tf and HA ligands on the surface of NLC granted them lower cytotoxicity when compared with uncoated NLC (100, 101).

Colorectal Cancer

Colorectal cancer (CRC) originates in colon and rectum, usually starting with the forming of a polyp because of an epithelial proliferation from colon and rectal mucosa. The probability for a polyp of turning into a malignant neoplasia depends on the type of polyp according to its histology. Adenomatous polyps are likely to turn into cancerous neoplasia because of their pre-cancer nature for being a type of adenoma. Meanwhile, inflammatory, and hyperplasic polyps are not considered as pre-malignant lesions (188). CRC is the third type of cancer with the highest incidence worldwide and the second one on the list of cancers with higher death rate (1, 2).

As in lung cancer, CEA is an oncofetal tumor-marker over-expressed in more than 90% of CRC cells. High CEA levels have been found in serum as well as high levels of its mRNA in end-stage CRC patients (135). CEA levels have been used in predicting and keeping track of recurrence and metastasis of CRC in stage-II patients (136, 137). CEA promoter has been used to direct therapeutic gene expression in CRC cells, such as E gene against colon cancer, triggering a high inhibition of cell growth compared to normal human colon cells. Moreover, it has been shown in mice carrying subcutaneous MC-38 colon cancer cells that there is a significant decrease in tumor size and low Ki-67 levels compared against untreated tumors (138).

COX-2 is an enzyme that catalyzes initial oxidation of arachidonic acid for prostaglandins synthesis, an essential factor in carcinogenesis and tumor evolution. COX-2 is over-expressed in 93% of colon cancers and in 87% of rectum cancers (139). Over-expression levels of COX-2 have been shown to be related to cancer progression and death rate in patients with CRC (140, 141). COX-2 gene promoter has been found to be active in CRC cell lines but not in normal human intestinal epithelial cell lines, via analysis of its transcriptional activity using luciferase reporter gene (91). This promoter has also been used to control 15-hydroxyprostaglandin dehydrogenase (15-PGDH) gene expression, a repressed gene in most cancers. By doing so in colon cancer cells, growth and migration of CRC cells was inhibited (12, 142).

A33 protein is a member of the transmembrane protein family of the immunoglobulin superfamily, only found in the small intestine and colon, associated with gut immune response, cell adhesion processes and cell traffic. A33 protein over-expression is correlated with many cancers such as primary and metastatic CRC (95%), diffuse gastric cancers (63%), intestinal-type gastric cancers (83%) and pancreas cancer (50%), nonetheless it has not been found in normal colon epithelium (138, 143). The A33 promoter has been used to specifically drive the expression of the E1A anti-cancer protein gene to decrease tumorigenic potential, inhibit cell growth and activate apoptosis in cancer cells. Production of favorable levels of E1A mRNA has been demonstrated in different CRC cell lines, but not in normal colon cells, with a slight activity in HCC and melanoma cell lines, so that the A33 promoter can be used as tumor-specific promoter (144).

The hTERT is synthesized in cells with high levels of enzymatic activity (*e.g.* tumor cells) but not in normal tissues (145). Telomerase is highly active in malignant tumors and high levels of hTERT mRNA have been correlated with poor prognosis for CRC patients versus patients with low telomerase levels (146). hTERT promoter has been used in directing therapeutic gene expression such as E1A, showing a high specificity towards CRC, inhibiting 75% of cancer cell growth, obtaining apoptosis and necrosis levels of 32.3 and 31.5% respectively (71). Furthermore, combined gene therapy with interleukin 18 (IL-18) gene and HSV1-tk under hTERT promoter transcriptional control has been carried out, this strategy confers a specific anti-tumor immunity, partially or completely eliminating tumors (69).

The uPAR gene encodes for a serine protease that catalyzes inert zymogenic plasminogen into plasmin. It has been observed that the uPAR gene is positively regulated by activated RAS signaling pathway, the main signaling pathway in CRC (147). This gene is over-expressed in many tumors such as those of pancreas, liver, breast and especially gastrointestinal (148). The specific tumor union of activator protein (AP-1) to uPAR promoter has been found in approximately 40% of CRC patients and 38.9% of them showed this specific tumor union in resected tumors in contrast to low or absent attachment in the corresponding normal mucosa, demonstrating the specific tumor activity of uPAR in CRC and not in normal tissues (149). uPAR promoter specific activity has been demonstrated in colon cancer cell lines (SW480) and CRC (HTC116) by means of lacZ reporter gene. By administering HSV1-tk under uPAR promoter transcriptional regulation within SW480 and HCT116 cell lines cell growth rate decreased significantly by ganciclovir administration (150).

Fibroblast growth factor 18 (FGF18) is a crucial mitogen in the embryonic stage taking part in bone and cartilage development (151). Its over-expression has been linked with different types of cancer, mainly in CRC, promoting the transition of colon carcinogenesis from adenoma to carcinoma (151, 152). Activity of FGF18 promoter has been tested with *lacZ* reporter gene in SW480 and HCT116 cell lines versus normal human umbilical cord colon cells, and it was found that galactosidase activity was much higher in cancer cells than in normal cells. Moreover, specific tumor activity of FGF18 promoter was demonstrated by expressing HSV1-tk gene within CRC cells, significatively inhibiting its growth after treatment with ganciclovir (150).

The receptor that contains the endothelial cell type specific tyrosine kinase domain (KDR) is the receptor of the vascular endothelial growth factor (VEGF), which plays a vital role in the growth and development of endothelial cells. KDR expression has been detected in a wide variety of cancer cells and vascular endothelial cells but not in normal cells (153). By screening the expression of CD and HSV1-tk genes (KDR/CD-TK) in colon cancer cells, KDR promoter expression has been proven to be specific for this type of cells, finding high levels of CD/TK mRNA in SW480 and SW620 (KDR positive human colon adenocarcinoma) which were found to be highly susceptible to 5-FC and ganciclovir prodrugs and with no effect on LS174T cells (KDR negative human colon adenocarcinoma) (138).

Copolymers are becoming more attractive to scientists due to the features and advantages that emerge when using new combinations of monomers. In colorectal cancer gene therapy, a novel nanocarrier was developed using a copolymer of poly-(ethylene glycol)–poly-(caprolactone) which were used to co-loading 5-Fluorouracil and the enhanced green fluorescent protein coding gene. Transfection efficiency was tested on colorectal cancer-bearing mice, showing 70–90% of transfection percentage 24 to 72 h post-transfection, respectively (99, 189).

An interesting approach for colorectal cancer (CRC) gene therapy and, specifically, in the development of novel genetic vehicles is the construction of nanoparticles (NPs) made of mesoporous silica nanoparticles (MSNs) which can be modified using polymerized dopamine and AS1411aptamer (190). The use of the AS1411aptamer confers these nanoparticles high tumor specificity, given that this aptamer binds specifically to nucleolin, which is a protein over-expressed on the cell surface of many types of tumor, including CRC (191). *In vivo* and *in vitro* assays demonstrated that these NPs can effectively target CRC cells (98).

Pancreas Cancer

Pancreas cancer is one of the most aggressive malignant human neoplasia and the seventh cause of death due to cancer worldwide accounting for 458,918 of new cases and 432,242 deaths in 2018 (1), given the lack of proper therapies (82).

Cholecystokinin type A receptor (CCKAR) promoter has relatively high activity in pancreatic cancer cells when compared with normal cells, this tumor-specific promoter was modified for enhancing its activity to be used within pancreas cancer cells for directing BikDD expression, a powerful pro-apoptotic gene, demonstrating its effective and specific anticancer effect (154). Furthermore, the versatile expression vector "VISA" (VP16-GAL4-WPRE integrated systemic amplifier) which contained the same tumor-specific CCKAR promoter was constructed to direct the expression of BikDD in pancreas cancer *in vivo*. The targeted expression of BikDD by the CCKAR-VISA vector showed a significant antitumor effect in pancreatic cancer and prolonged survival in the mouse model used (155).

A strategy with Diphtheria toxin A (DTA) against pancreatic cancer has also been developed, using the tumor-specific promoter MUC1, due to its over-expression in pancreas ductal adenocarcinoma (PDA) and its association with tumor aggressiveness (156). This strategy has been used to direct the expression of DTA only within tumor cells, since this toxin inhibits protein synthesis and is lethal for cells (157). However, some normal cell types, such as gastrointestinal and breast epithelial cells, express MUC1 (158), so this pMUC1/DTA construct may cause gastrointestinal side effects in treated patients.

Telomelysin (OBP-401) is an oncolytic modified adenovirus in which the hTERT promoter controls viral replication, therefore it only replicates within cells that overexpress hTERT such as pancreatic cancer cells (89, 90, 159). Telomelysin has been shown to effectively lysate pancreas cancer cells and reduce

xenograft tumors in murine models by itself as well as in combination with docetaxel (160).

With the purpose of developing a more efficient pancreatic cancer gene therapy, a synthetic human insulin super-promoter (SHIP1) was designed to improve the activity and specificity of the human insulin promoter. SHIP1 has been shown to be a promoter with higher activity than that of endogenous human insulin promoters and rat insulin promoters (RIP), which are used to direct expression in pancreatic adenocarcinoma that overexpresses pancreas and duodenal Homeobox gene 1 (PDX-1) (10, 161). This new gene therapy strategy using synthetic super-promoters could be used to more efficiently direct therapeutic genes expression in various types of cancers.

An interesting approach in the development of genetic vehicles for treatment of cancer is the use of exosomes to enhance targeting of oncogenic Kras in pancreatic ductal adenocarcinoma (PDAC). Exosomes, which are vesicles of nanometric dimensions and are produced by all cells, were obtained from cell cultures of human foreskin fibroblasts (BJ) (192). Then, these exosomes were electroporated with a siRNA or a plasmid for shRNA silencing of KRAS mutations, which are the key driver of pancreatic cancer (193). After that, their biological effect was evaluated in *in vitro* and *in vivo* essays. Exosomes are characterized by the presence of transmembrane and membrane anchored proteins, one of these is CD47, a protein that signals for the inhibition of phagocytosis via the interaction with the ligand for signal regulatory protein alpha (SIRPα), allowing evasion of phagocytosis by circulating monocytes and increasing half-life of exosomes in circulation. Kras mutant cancer cells are known to have an enhanced micropinocytosis activity, so that exosomes uptake is favored, allowing the targeted delivery of the therapeutic nucleic acid (96, 194–196).

One of the most recent approaches to treat cancer is the use of engineered cells by a non-viral vector which carries a therapeutic protein which induces apoptosis in cancer cells. In this new approach, human mesenchymal stem cells (hMSCs) are genetically modified with complexes of branched polyethyleneimine (bPEI) and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) gene (197). To overcome the low transfection efficiency of the TRAIL-gene vector within hMSCs, photochemical internalization (PCI) was the method utilized to carry on genetic modification. After genetically modifying hMSCs, the transfection and secretion of TRAIL protein into culture supernatants was evaluated as well as the evaluation of *in vivo* therapeutic effect in tumor-bearing mice and histologic analysis. TRAIL is a member of the tumor necrosis factor (TNF) superfamily which is able to form homotrimer with death receptors (DRs) on the cell membrane; when it does, it triggers apoptosis pathway in cancer cells and has a negligible effect on normal cells (198). To overcome the limitations of *in vivo* application of DNA complexes with polymers, hMSCs were applied for direct secretion of TRAIL protein; these cells were used given their ability for homing to tumor sites and their immunity privileges which prevent them from being rejected *in vivo*. Finally, the use of PCI is justified because it maximizes cellular internalization of DNA-bPEI complexes; this technique uses near infrared light (NIR) along with a photosensitizer, which enhances the cell membrane permeability and allows photo-induced endosomal escape efficiency for enhanced gene transfection (97, 199–201).

Prostate Cancer

Prostate cancer is the second type of cancer with the highest incidence and the fifth cause of death among men worldwide, taking 358,989 lives per year (1) and representing a high risk for elder men.

One promoter used in the development of gene therapies against tumor cells of prostate cancer is the glucose-regulated protein 78 (GRP78) promoter. This promoter is inactive in healthy adult tissues but it has been proven to be highly active in a wide range of cancer cells like prostate, gastric, breast, pancreatic, lung and colon (162–164). One strategy developed consisted in using GRP78 promoter as the regulatory sequence in the HSV1-TK suicide gene. Under the enzymatic action of the protein encoded by this gene, GCV is transformed into GCV-monophosphate and then into GCV-diphosphate and GCV-triphosphate. This last product showed cytotoxic effects specifically on prostate cancer cells (164).

Another promoter receiving attention is the human osteonectin promoter (hON-522E). This promoter regulates transcription of osteonectin, a protein known to play roles in cell adhesion, proliferation and migration. Osteonectin is over-expressed in many types of cancer such as prostate cancer, where it is involved in metastasis (165, 166). A vector was constructed with hON-522E promoter regulating transcription of a HSV1-TK suicide gene, demonstrating induction of cell death *in vitro* (PC3M) and slowing the growth of prostate tumors in an xenograft model, without other organ toxicity (166).

Prostate-specific antigen (PSA) is a cytoplasmic protein present in prostate gland cells and prostatic duct epithelial cells. Its expression has been documented in normal prostate tissues but is known to be over-expressed in prostate cancer cells. On the other hand, prostate-specific membrane antigen (PSMA) is an intrinsic protein on membranes of prostatic epithelial cells and has been found to be over-expressed in prostate cancer, especially in metastasis (167, 168). Therefore, promoters of these two proteins seem to be suitable candidates for directing gene therapy in prostate cancer. One approach was constructing a plasmid containing the thymidine kinase suicide gene under transcriptional control of the human PSA enhancer/promoter fragment. Further directionality was achieved by using JC polyomavirus virus-like particles, which show tropism towards androgen receptor positive prostate cancer cells, as the genetic vehicle carrying the recombinant plasmid. The constructed plasmid could kill 22Rv1prostate cancer cells *in vitro* by inhibiting growth of these cells in a xenograft mouse model (169). In a similar way, a recombinant plasmid was constructed using PSA and PSMA regulatory elements ruling transcription of apoptin. When transfecting human prostatic adenocarcinoma cell line LNCaP, viability was significantly decreased (170).

Cationic polymers are known to interact with the negatively charged therapeutic nucleic acids, forming stable nanocomplexes. One of the most used cationic polymers is polyethylenimine, given that it binds DNA with high efficiency and has a proton sponge

effect which is useful in endosomal escape of nucleic acids. Besides, it is a polymer that can be modified with targeting agents. Chlorotoxin is a peptide which binds in a specific fashion with matrix metalloproteinase-2 (MMP-2), which is over-expressed in certain types of cancer such as brain, prostate, skin, sarcoma, among others and plays a role in cancer metastasis (202). So, a novel genetic vehicle was constructed by conjugating chlorotoxin with PEI and forming nanocomplexes using a plasmid which contains a gene which encodes for melittin, a peptide present in bee venom and that has some anti-cancer activity. The transfection experiments showed that this genetic vehicle can reach transfection efficiencies of 49% with no cytotoxic effect (203–206).

Sometimes, it is necessary to use more than one genetic delivery strategy in order to archive the requirements of a safe and efficient transfection of mammalian cells. In an attempt to fulfill these needs, a complex consisting of 11-mercaptoundecanoic acid modified nanocages (AuNCs)/polyethylenimine (PEI)/miRNA/hyaluronic acid (HA), abbreviated as AuNCs/PEI/miRNA/HA was designed and constructed. Due to the presence of HA, these complexes can be specifically targeted for intracellular delivery of miRNA via HA receptor mediated endocytosis, using a layer-by-layer method. The use of HA as a mediator of endocytosis has additional advantages such as its unimportant nonspecific interactions with serum components, improving its *in vivo* availability. Attachment of PEI onto AuNPs surface making use of 11-mercaptoundecanoic acid provides the surface for the interaction with the negatively charged miRNA, besides PEI property of strong endosomal escape. Finally, the use of AuNPs has proven to be quite dynamic due to their physicochemical properties as well as the ease with which AuNPs surface can be functionally modified. Ultimately, the use of photothermal therapy, which makes use of NIR light, enhances the antitumor effect of these new genetic vehicles (98, 102, 207).

PERSPECTIVES

As recombinant DNA technologies have arisen, the need of finding new treatments for diseases has become an important topic within the scientific community. Cancer is one of the most aggressive and deadliest diseases among humans and given its genetic basis and the lack of effective conventional treatments, it is a suitable candidate for using gene therapy. As we have reviewed in this work, there are different genetic engineering techniques which can be used for controlling or restoring normal gene expression within cancer cells. Nevertheless, that approach requires further research to be delivered safely and efficiently within cancer cells as well as being expressed just inside the tumor cells of interest rather than in normal tissues. Cancer and tumor-specific promoters have been shown to be highly effective when it comes to targeted gene expression, mainly because they direct gene expression only within a specific type of tumor cells or cancer cells and not within normal tissues, which results in targeted therapies involving cell death just for malignant tissues while keeping normal tissues safe and intact. Along with these cancer and tumor-specific promoters, the right delivery system, that is, a safe, efficient, and specific genetic vector, must be available in order to render a highly promising therapy, with specificity and efficacy enough to ensure cancer elimination and normal tissue preservation.

So, it is of pivotal importance to continue in the search of new promoters which can direct gene expression only within the desired cells and new genetic vectors that can deliver recombinant DNA molecules also in a specific fashion with no cytotoxicity and high transfection efficiencies so that gene expression is assured to take part only where needed. To do so, cancer genetics must be further studied so that the principles controlling gene expression in cancer can be fully understood and manipulated in a beneficial way via gene therapy. Simultaneously new genetic vectors must be developed and, in doing so, new materials must be studied in order to fulfil the needs of biocompatibility, no cytotoxicity, high transfection rates, tissue specificity and high loading capacity of genetic material, with the purpose of providing a new tool in cancer treatment that can (surpass the shortcomings of the conventional therapies) complement or reduce toxicity of conventional treatments available nowadays, increasing survival rates and improving life quality of patients suffering cancer.

CONCLUSIONS

Cancer and tumor-specific promoters have proven to be an important feature in the construction of recombinant DNA molecules for cancer gene therapy. Yet, a more advanced knowledge of cancer genetics is required in order to find effective and safe elements to control gene expression. New molecules and materials for the development of genetic vectors have opened the possibilities to deliver nucleic acids into cells, nevertheless, the challenge of finding a genetic vector that is safe and efficient remains.

AUTHOR CONTRIBUTIONS

MM-S: Development of most topics, figure creation, and table creation. DB-E: Participation in the development of the entire gene therapy topic of the most common cancers. OM-G: Development of the perspectives chapter and participation in the drafting of the entire article. EA-H: Participation in the organization and review of the topics. MI-H: General idea, coordination and advice of the whole article. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Repurposing of Drug Candidates for Treatment of Skin Cancer

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Skin cancers are highly prevalent malignancies that affect millions of people worldwide. These include melanomas and nonmelanoma skin cancers. Melanomas are among the most dangerous cancers, while nonmelanoma skin cancers generally exhibit a more benign clinical pattern; however, they may sometimes be aggressive and metastatic. Melanomas typically appear in body regions exposed to the sun, although they may also appear in areas that do not usually get sun exposure. Thus, their development is multifactorial, comprising endogenous and exogenous risk factors. The management of skin cancer depends on the type; it is usually based on surgery, chemotherapy, immunotherapy, and targeted therapy. In this respect, oncological treatments have demonstrated some progress in the last years; however, current therapies still present various disadvantages such as little cell specificity, recurrent relapses, high toxicity, and increased costs. Furthermore, the pursuit of novel medications is expensive, and the authorization for their clinical utilization may take 10–15 years. Thus, repositioning of drugs previously approved and utilized for other diseases has emerged as an excellent alternative. In this mini-review, we aimed to provide an updated overview of drugs' repurposing to treat skin cancer and discuss future perspectives.

Keywords: skin cancer, drug repurposing, melanoma, nanocarriers, drug delivery systems

INTRODUCTION

Skin cancers are highly prevalent malignancies worldwide, ranked at the twentieth place of incidence (1, 2). There were an estimated 100,000 new melanoma cases in the United States during 2020, with the approximate death of 6,850 people. The prevalence is higher in men, and the incidence varies according to the geographic region and by country (3). Skin cancers include melanomas and nonmelanoma skin cancers (NMSC). Melanomas are tumors that develop in

melanocytes, and these may appear in diverse body regions. Specialists consider melanoma one of the most dangerous cancers (4). Patients in advanced stages commonly have a discouraging prognosis, and the five-year survival in those patients is <5%. Remarkably, patients without treatment exhibit a median survival between six and nine months (5). The main types of NMSC include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). NMSCs have a higher occurrence than melanoma, but they are less lethal, especially if diagnosed early (6). BBCs are skin tumors produced by the abnormal growth of basal cells. It is the most frequent type of skin cancer (7), and they are curable in many cases when detected timely. On the other hand, SCC is the second most frequent skin cancer type; it develops in the squamous cells located in the epidermis (8). SCC generally exhibits a benign clinical pattern; notwithstanding, it may sometimes be aggressive and metastatic (9).

Skin cancers develop more frequently in body regions exposed to the sun; however, they may also appear in areas that do not usually get sun exposure. Thus, their development is multifactorial, comprising endogenous (skin type and genetic factors) and exogenous (degree of sun exposure and sun protection conduct) risk factors (10). Among exogenous aspects, ultraviolet radiation (UVR) is the most notable risk factor. UVR can produce DNA damage, mutations, inflammatory responses, and oxidative stress, leading to skin cancer development (11). Among the UVR types, ultraviolet A (UVA) penetrates deeper into the skin, producing more considerable skin damage than the ultraviolet B (UVB). Nevertheless, UVB is mostly related to inflammatory responses and DNA damage as a critical tumor-promoting event (12).

Skin cancer management depends on the type; it is usually based on surgery, chemotherapy, immunotherapy, and targeted therapy (7, 9, 13, 14). In this respect, oncological treatments have demonstrated some progress in the last years; however, current therapies still present various disadvantages such as little cell specificity, recurrent relapses, high toxicity, and increased costs (14). Furthermore, the pursuit of novel medications is expensive, and the authorization for their clinical utilization may take 10–15 years (15). Thus, repositioning drugs previously approved and utilized for other diseases has emerged as an excellent alternative (16). In this mini-review, we aimed to provide an updated overview of drugs' repurposing to treat skin cancer and discuss future perspectives.

DRUG REPURPOSING FOR SKIN CANCER

Drug repurposing is the process of giving new applications for existing drugs; it may considerably diminish development costs (and times) to search for effective strategies to treat skin cancer (17). Repurposing drugs possess various advantages, including data availability about clinical tests, chemical composition, and possible toxicity, which can accelerate their application in clinical trials (18). Although various drugs have been proposed for their repurposing in skin cancer (**Table 1**), most of them have only been evaluated in preclinical studies, and extensive clinical trials are needed before their approval for skin cancer treatment. Nonetheless, these drugs represent a promising alternative because almost all are cheap and without significant adverse effects on therapeutic doses. Drugs that have been suggested for repositioning in skin cancer are discussed in the next sections in alphabetical order as an example of the most prominent proposals to date.

Digoxin

Digoxin is a compound utilized to treat arrhythmia and heart failure symptoms. Its mechanism of action includes inhibition of the hypoxia-inducible factor-1α (HIF-1α) (42), which contributes to angiogenesis, metastasis, and tumor resistance in many cancers (43). Concerning this, Eskiocak et al. (21) suggested a therapeutic effect of digoxin against melanoma. The authors reported that digoxin exhibited low cytotoxic activity in mice xenografted with metastatic melanomas derived from patients. However, the authors found a synergistic beneficial effect when simultaneously administered with a MEK inhibitor, extending experimental mice's survival. The possible mechanism of action included acidification of cytoplasm, rises in mitochondrial Ca^{2+} levels, depletion of ATP, and mitochondrial function reduction. Likewise, the combination of digoxin and DMXAA (an anti-vascular agent) inhibited tumors' regrowth in mice harboring B16F10 melanoma tumors (44). The enhancement in the efficacy may be explained by the inhibition of HIF-1α and stimulation of the immune function. Concerning human studies, a recent clinical trial explored the effects of parallel administration of digoxin and trametinib on BRAF wild-type metastatic melanoma patients (45). The results exposed a reasonable rate of control of disease in those patients for up ten months. Thus, this approach could be useful in metastatic melanoma patients refractory or intolerant of immunotherapy; nonetheless, additional clinical trials with a higher number of patients will be crucial.

Doxycycline

Doxycycline is a broad-spectrum tetracycline antibiotic (46). Some studies reported that doxycycline might inhibit several matrix metalloproteinases that participate in diverse cancers' metastasis (47). Thus, it has been suggested that this drug could be repositioned as an anti-cancer treatment (48). An interesting study demonstrated that doxycycline inhibited the growth of melanoma cells (49). The anti-tumor effects might be mediated by various mechanisms, including inhibition of the NF-κB pathway, decrease of antiapoptotic proteins, cytochrome C release, and activation of caspase-8 (50). Likewise, doxycycline inhibited the adhesion and migration of a melanoma cell line, with subsequent apoptosis induction (51). This activity appeared to be mediated by inhibition of focal adhesion kinase, which participates in migration and cell adhesion regulation. Likewise, a very recent study showed that doxycycline diminished the viability and proliferation of a melanoma cell line (COLO829 cells) by decreasing intracellular levels of reduced thiols and impairing the homeostasis of the cells (22). Finally, a recently finished clinical trial found that the concomitant administration of doxycycline, temozolomide, and ipilimumab produced no

TABLE 1 | Drugs proposed for chemoprevention and treatment of skin cancer.

Drug	Other uses	Study model	Mechanism of action	Reference
Albendazole	Useful for giardiasis, trichuriasis, filariasis, neurocysticercosis, among other diseases	A375 and A2058 metastatic melanoma cells lines	Induction of DNA damage and cells arrest in the G2/M phase of the cell cycle, sensitizes them to radiation therapy	(19)
Desmopressin	Synthetic hormone commonly used for nocturia and enuresis	Mice xenografted with B16-F0 melanoma cells	Modulation of proteolysis and coagulation	(20)
Digoxin	Antiarrhythmic agent used in heart failure, and other heart disorders such as atrial fibrillation	Primary melanocytes (hMEL1) or melanoma cells derived from patients	Inhibits the ATP1A1 Na þ/K þ pump, which is highly expressed by melanoma	(21)
Doxycycline	Antibiotic used to treat infections such as skin infections and rosacea. It can also be used to prevent malaria	Human (A2058 and A375) and mouse (B16F10) melanoma cells	Inhibition of the MMP-2 and MMP-9 metalloproteinases activity, activation of apoptosis signal-regulated kinase 1, c-Jun N-terminal kinase, and caspases, which induces apoptosis	(22, 23)
Fenofibrate	Used with other medications to reduce fatty substances such as cholesterol and triglycerides	Human (SkMell88) and mouse (B16F10) melanoma cells	Anti-metastatic activity involving down-regulation of Akt phosphorylation	(24)
Flubendazole	Anthelmintic drug used in parasitic infestations	A375, BOWES, and RPMI-7951 cells	Anti-melanoma activity related to enhanced transcription of p53 and NF-κB, as well as phosphorylation of JNK	(25)
Haloprogin	Antifungal drug used to treat skin infections such as athlete's foot	Mouse B16F10 skin melanoma tumor model	In combination with RAPTA-T, shown to be a profitable candidate for its use as a melanoma growth inhibitor through cancer cell death induction	(26)
Itraconazole	Used to treat fungal infections as aspergillosis, blastomycosis, and coccidioidomycosis	SK-MEL-28 and A375 human melanoma cells	Inhibits the proliferation and colony formation through the Hh, Wnt, and PI3K/mTOR signaling pathways blockade	(27)
Leflunomide	Used in active moderate-to-severe rheumatoid arthritis and psoriatic arthritis	Human melanoma cell lines	A selective inhibitor of *de novo* pyrimidine synthesis, blocking the synthesis of DNA and RNA; reduces cell proliferation and causes cells to arrest in G1 of the cell cycle.	(28)
Lidocaine	Local anesthetic and antiarrhythmic	Human keratinocytes	It induces membrane permeability and excessive production of reactive oxygen species (ROS).	(29)
Mebendazole	Used to treat parasitic worm infestations as ascariasis, worm infections, and giardia, among others	Human melanoma cell lines	Bcl-2 phosphorylation in melanoma cells, avoiding its interaction with pro-apoptotic Bax, through apoptosis induction	(30)
Metformin	Commonly used to treatment of type 2 diabetes, also used in the treatment of polycystic ovary syndrome	Human melanoma cell lines	Induces cell cycle arrest in the G0-G1 phase, and it´s responsible for autophagy and apoptosis induction	(31, 32)
Naproxen	Used to treat pain, menstrual cramps, inflammatory diseases such as rheumatoid arthritis, and fever	Mice irradiated with UVB	Reduction in the incidence of tumor lesions by naproxen may be due to its ability to increase TNF-a levels and decrease PGE2.	(33)
Niclosamide	Anti-helminthic drug, has been used to treat tapeworm infection	In vitro: human and mouse melanoma cell lines. In vivo: a mouse xenograft model of A375 cell line	Induces cell apoptosis *via* the mitochondrial-mediated apoptotic pathway, also inhibits tumor growth by decreasing the expression of p-STAT3, MMP-2, and MMP-9	(34)
Nicotinamide	Treatment and prevention of niacin deficiency and certain conditions related such as pellagra	Human keratinocytes	Chemopreventive effects, replenishes cellular ATP after UV irradiation, and enhancement of DNA repair in UV-irradiated human skin	(35)
Pimozide	Decreasing the activity of a natural substance (dopamine) in the brain, Tourette syndrome patients	B16 cell-bearing mice	Antitumor activity *via* the regulation of proliferation, apoptosis, and migration	(36)
Piroxicam	Used to the treatment of rheumatoid arthritis and osteoarthritis, acute musculoskeletal disorders, and dysmenorrhea	Patients affected by Actinic keratoses	A non-selective NSAID* that inhibits the activity of COX-1 and COX-2, inducing apoptosis and inhibit recruitment and production of growth factors and other carcinogenetic mediators	(37, 38)
Propranolol	β-blocker commonly used for high blood pressure	Patients with melanoma	Inhibition of angiogenesis and migration of cancer cells	(39)
Rafoxanide	Anthelmictic used mainly for the treatment of *fasciola hepatica*	A375 and A431 cells and mice xenografted with those cells	Inhibition of CDK4/6, increase of cell apoptosis, and arrest of cell cycle	(40)
Telmisartan	Angiotensin receptor 1 inhibitor widely used as an antihypertensive drug	Human melanoma cells A375, 518a2, and HTB140	Induction of apoptosis, generation of reactive oxygen species, and alteration of cell bioenergetics	(41)

**NSAID, Non-Steroidal Anti-Inflammatory Drugs.*

significant clinical improvement in patients with melanoma (NCT01590082). Although this finding could appear disappointing, preclinical studies suggest a therapeutic usefulness of doxycycline and warrant further clinical trials.

Fenofibrate

Fenofibrate is an agonist of the peroxisome proliferator-activated receptor-α; it is indicated for managing mixed dyslipidemia and hypertriglyceridemia (52). A variety of studies have reported that fenofibrate exerts anti-tumor activities in several cancers (53, 54), including melanoma (55, 56). Panigraphy et al. (56) exposed that fenofibrate significantly inhibited the proliferation of melanoma cells (B16-F10 cells) and suppressed primary tumors' growth *in vivo* in a murine model. Those effects were mediated by the inhibition of inflammation and angiogenesis in the surrounding host tissue. Additionally, fenofibrate significantly decreased melanoma metastases when administered *via* oral in mice, suggesting that this compound possesses chemopreventive activity (55). Interestingly, a down-regulation in the phosphorylation of Akt might explain this anti-metastatic effect (24). Finally, a very recent study proposed that the effects of fenofibrate on growth and metastases of melanoma could be produced by inhibiting the TLR4-dependent signaling pathway (57). Despite the studies suggesting beneficial effects of fenofibrate in melanoma, currently, there are no ongoing clinical trials; thus, this drug perhaps requires additional studies in animal models before its evaluation in patients.

Flubendazole

Flubendazole is an anthelmintic compound (58); its mechanism of action depends on the disruption of microtubules' structure and function. This activity attracted considerable interest in the drug as a possible anti-cancer treatment (59); thus, recent studies explored the therapeutic potential of flubendazole against skin cancer (25, 60). For example, a pioneering study conducted by Čáňová et al. (25) demonstrated inhibition of cell growth and proliferation in three distinct types of melanoma cell lines (A375, BOWES, and RPMI-7951), finally leading to caspase-dependent apoptosis. A subsequent report demonstrated that these effects were related to enhanced transcription of p53 and NF-κB and phosphorylation of JNK, eventually producing cell cycle arrest and disturbances of the microtubules network (61). Likewise, another study reported that flubendazole suppressed tumor growth and prevented metastasis in mice with xenografts of human melanoma cells (60). According to the authors, those anti-tumor activities were produced by a decrease in STAT3 and PD-1 levels. This drug is not being evaluated in any clinical trial, so its application may need further evaluations in cellular and animal models.

Itraconazole

Itraconazole is an antimycotic drug commonly utilized worldwide, which has demonstrated the therapeutic potential for skin cancer treatment. In this regard, Kim et al. (62) revealed that itraconazole suppressed the growth of BCC in mice by inhibiting the Hedgehog signaling pathway. This exciting finding provided the foundation for a subsequent Phase II clinical trial in BCC patients (63). The research revealed that the administration of itraconazole *via* oral reduced cell proliferation and tumor area; thus, the authors concluded that itraconazole possesses beneficial effects against BCC in humans. Also, Liang et al. (27) reported that itraconazole inhibited the proliferation of human melanoma cells (A735 and SK-MEL-28 cells) *in vitro*. Interestingly, the drug also suppressed the melanoma growth *in vivo* in a xenograft mice model. Further experiments revealed that the effects were mediated by suppressing Wnt, Hedgehog, and PI3K/mTOR signaling pathways. All these studies provided the basis for clinical trials. In this regard, three clinical trials are studying the effects of itraconazole in patients with skin cancer. Two of them are focused on the molecular effects of locally applied itraconazole on the growth of BCC (NCT02120677 and NCT02735356), whereas the other one is assessing the efficacy and safety of orally administered itraconazole in patients with BBC (NCT02354261).

Leflunomide

Leflunomide is a compound utilized for the management of rheumatoid arthritis (64). This drug inhibits the enzyme dihydroorotate dehydrogenase (DHODH), which is pivotal in pyrimidine synthesis (65). Since leflunomide impedes the replication of dividing cells, it provided a rationale to propose its use in preclinical cancer studies (66). For example, White et al. (67) explored the possible benefits of utilizing leflunomide to treat skin cancer. They found that leflunomide produced a substantial reduction in melanoma development *in vitro* (RPM17951, A375, and Hs.294T cell lines) e *in vivo* (xenograft in mice). According to the authors, the inhibition of DHODH repressed transcription elongation of genes necessary for melanoma growth such as *myc* and *mitf*. More recent studies have provided more information about molecular targets for leflunomide. For example, O'Donnell et al. (68) stated that the anti-proliferative effects of leflunomide on A375 melanoma cells are dependent on the Aryl Hydrocarbon Receptor. A similar study found that leflunomide caused cell cycle arrest and autophagy through the phosphorylation of Ulk1 and AMP-activated protein kinase (AMPK) in A375 melanoma cells (69). Finally, another study demonstrated that the combination of leflunomide and selumetinib (an inhibitor of MEK) had a synergic effect in reducing BRAFwt and mutant melanoma cells' proliferation and growth of melanoma tumors in xenografted mice (28). Interestingly, a clinical trial intended to explore the efficacy and safety of the combination of leflunomide and vemurafenib in patients with V600 mutant metastatic melanoma was prematurely terminated due to adverse effects (NCT01611675). Therefore, despite available information about approved drugs, their possible toxicity can be a critical concern in drug repurposing when combined with other substances.

Mebendazole

Mebendazole is a drug employed to helminths infestation (70), which has also been proposed for drug repurposing in skin cancer (71). A pioneering study exposed that mebendazole produced apoptosis in melanoma cells (30). The apoptotic response was promoted by the phosphorylation of B-cell

lymphoma 2 (Bcl-2) and the decrease in X-linked inhibitor of apoptosis (30, 72). Interestingly, the combination of mebendazole, temozolomide, and Bcl-2 antisense had a synergistic effect in inhibiting the growth of two melanoma cell lines (73). Likewise, the combination of mebendazole and trametinib effectively inhibited the proliferation of melanoma cells derived from patients carrying $NRAS^{mut}/BRAF^{WT}$ and reduced their growth in xenografted mice (74). Therefore, the concomitant administration of mebendazole with other medications could be an alternative for melanoma treatment. However, this drug has not been assessed in any clinical trial with patients with skin cancer. Thus, its clinical evaluation could require further evidence from preclinical studies

Metformin

Metformin is a drug commonly used in type 2 diabetes mellitus; it reduces serum glucose levels through diverse mechanisms (75). Notably, melanoma is strongly dependent on glucose metabolism (76), and several epidemiological studies presented a relationship between the use of metformin and lower skin cancer risk (77). Concerning this, an investigation revealed that metformin inhibited tumor growth in mice xenografted with SCC cells (A431 cell line); the effect appeared to be caused by the inhibition of the mTOR and NF-κB signaling pathways (78). Similarly, Tomic et al. showed that metformin decreases the proliferation of melanoma cells *in vitro* and reduces tumor growth *in vivo*; those effects were mediated by a cell cycle arrest (31). In comparison, other studies suggested a variety of molecular mechanisms to explain the anti-melanoma properties of metformin, including the decrease of protein TRIB3 expression (79), upregulation of miRNAs expression (80), and induction of immune response in the tumor microenvironment (81). Furthermore, metformin prevented the development of metastasis *in vitro* e *in vivo* by activating the p53 tumor suppressor protein and AMPK (82). Besides, metformin enhanced the anti-proliferative effects of binimetinib (an inhibitor of MEK) in a model of metastatic melanoma cells (83). The molecular mechanism involves P-ERK downregulation and AMPK upregulation. Due to these preclinical pieces of evidence, various clinical studies have been undertaken. Remarkably, at least five clinical trials are ongoing exploring the therapeutic effects of metformin in skin cancer (NCT01638676, NCT01840007, NCT02143050, NCT03311308, and NCT04114136). Although metformin is being studied only as an adjuvant in all the studies.

Pimozide

Pimozide is an antagonist for dopamine receptors; it is employed to treat Gilles de la Tourette syndrome and schizophrenia (84). Additionally, pimozide has shown promising results for managing several cancers, including skin cancer (36, 85–87). An early clinical trial showed that pimozide might have beneficial effects in patients with formerly medicated metastatic melanoma (86). The possible molecular mechanism for this anti-metastatic effect could be mediated by inhibition of ARPC2, a subunit of the Arp2/3 complex involved in migration and invasion (85). Moreover, preclinical studies demonstrated that the combination of pimozide with other drugs might enhance its anti-melanoma activity. For example, pimozide's simultaneous use and an inhibitor of indoleamine 2,3-dioxygenase (an enzyme that participates in melanoma tolerance) had a synergistic effect against melanoma in a mouse xenograft model (36). The authors indicated that pimozide inhibited STAT3 and STAT5, regulating tumor immunity. Likewise, Zhao et al. (87) co-administered pimozide and siRNA targeting PD-1 to mice xenografted with melanoma cells. Their results revealed an increase in the anti-tumor effects by inducing apoptosis and enhancing immune function. Lastly, a cutting edge study explored the anti-cancer effects of pimozide and a CpG oligodeoxynucleotide (CpG ODN) on mice xenografted with B16 cells (88). Their results revealed that the combination of those compounds suppressed the melanoma growth and extended experimental subjects' survival. Those findings were due to the induction of apoptosis, repression of invasion, and enhancement of immune response. Despite all these findings, there are no clinical trials with this drug to date. Those studies shall be necessary to support its repurposing for skin cancer.

Piroxicam

Piroxicam is a non-steroidal anti-inflammatory compound that blocks the cyclooxygenases-1 and -2 (COX-1 and COX-2) enzymes (89). Several studies have shown that those enzymes participate in the development of actinic keratoses and SCC (90, 91); thus, piroxicam could help their prevention and treatment. In support of this hypothesis, Campione et al. (38) demonstrated that piroxicam's topical application (1%) had beneficial effects in patients with actinic keratoses. Numerous studies combining piroxicam (0.8%) and sunscreens (SPF 50+) have found very similar results (92–97), which suggests that piroxicam might serve as a chemopreventive agent for SCC. On the other hand, a recent study reported that piroxicam exhibited cytotoxic activity on SCC cells (A431 cell line), highlighting the drug's therapeutic potential (98). Interestingly, piroxicam had no effects on the proliferation of melanoma A375 cells (99), suggesting that its anti-cancer activity is specific for SCC. Nevertheless, this drug has not been assessed in any clinical trial with patients with SCC; thus, its clinical efficacy has not been proven yet.

CONCLUSION AND PERSPECTIVES

The development of efficacious treatments for skin cancer is costly and time-consuming; hence, old drugs' repositioning has arisen as an affordable approach. This procedure requires a thorough search through multiple dataset analyses and structure-based virtual screening to select a suitable compound for repurposing (13, 100, 101). Moreover, extensive *in vitro* e *in vivo* analyses are necessary before undertaking clinical trials. In this respect, advances in knowledge of skin cancer cellular and molecular mechanisms have provided essential information for drug repurposing. Likewise, although clinical trial execution usually requires a long time to evidence security and efficacy, the repositioning of drugs for skin cancer will consume less time than the development of novel medications.

Interestingly, even with the evidence for repositioning old drugs for skin cancer, to our knowledge, there is limited evidence from ongoing clinical trials. Possibly, the degree of improvement, and therefore of clinical relevance, does not support the commercial profitability of the discoveries, except for metformin with at least five clinical trial registries, one of them in phase 2. It is noteworthy that metformin, itraconazole, leflunomide, and doxycycline have been proposed as adjuvants, so possibly they would not be one of the primary and first-choice drugs. Nevertheless, the concurrent use of drugs targeting different signaling pathways may enhance their anti-cancer effectiveness, therefore extending the patients' survival and reducing the relapse risk. Also, this clinical strategy would allow lowering costs related to expensive current anti-cancer medications.

Finally, as in other drug strategies for treating cancers, pharmaceutical technology tools are necessary for adequate administration and effect at skin cancer's cellular level. In this respect, several nanoformulations can enhance the efficacy of drugs to treat cancer; thus, this approach will allow well-known drugs to be used to treat skin cancer. Although nanosystems for skin cancer are not commercially available, several formulations have been proposed as nanocarriers to effectively deliver known antineoplastic therapeutic agents for skin cancers (102, 103).

AUTHOR CONTRIBUTIONS

HC and GL-G conceived the article. HC, OR-H, SA-A, SB-C, GF-G, and GL-G wrote the first draft of the manuscript. MG-T, MG-C, IC-F, and JS-R contributed to the discussion and the search for information. HC, MG-T, MG-C, JS-R, and GL-G critically reviewed the manuscript and edited the final version. IC-F elaborated the **Table 1**. All authors contributed to the article and approved the submitted version.

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REVIEW
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Pathway-Based Drug-Repurposing Schemes in Cancer: The Role of Translational Bioinformatics

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Cancer is a set of complex pathologies that has been recognized as a major public health problem worldwide for decades. A myriad of therapeutic strategies is indeed available. However, the wide variability in tumor physiology, response to therapy, added to multi-drug resistance poses enormous challenges in clinical oncology. The last years have witnessed a fast-paced development of novel experimental and translational approaches to therapeutics, that supplemented with computational and theoretical advances are opening promising avenues to cope with cancer defiances. At the core of these advances, there is a strong conceptual shift from gene-centric emphasis on driver mutations in specific oncogenes and tumor suppressors—let us call that the silver bullet approach to cancer therapeutics—to a systemic, semi-mechanistic approach based on pathway perturbations and global molecular and physiological regulatory patterns—we will call this the shrapnel approach. The silver bullet approach is still the best one to follow when clonal mutations in driver genes are present in the patient, and when there are targeted therapies to tackle those. Unfortunately, due to the heterogeneous nature of tumors this is not the common case. The wide molecular variability in the mutational level often is reduced to a much smaller set of pathway-based dysfunctions as evidenced by the well-known hallmarks of cancer. In such cases "shrapnel gunshots" may become more effective than "silver bullets". Here, we will briefly present both approaches and will abound on the discussion on the state of the art of pathway-based therapeutic designs from a translational bioinformatics and computational oncology perspective. Further development of these approaches depends on building collaborative, multidisciplinary teams to resort to the expertise of clinical oncologists, oncological surgeons, and molecular oncologists, but also of cancer cell biologists and pharmacologists, as well as bioinformaticians, computational biologists and data scientists. These teams will be capable of engaging on a cycle of analyzing high-throughput experiments, mining databases, researching on clinical data, validating the findings, and improving clinical outcomes for the benefits of the oncological patients.

Keywords: pathway-based methods, drug repurposing, translational bioinformatics, computational oncology, PharmaOncology

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INTRODUCTION

Drug development is perhaps one of the most complex and challenging endeavors in biomedical science. Aside from the already daunting complexities behind pharmacological drug designs, there are also enormous difficulties derived from clinical, regulatory, intellectual property and commercial issues. Such a challenging environment has caused drug development to be a really slow and uncertain process. In the search for alternatives to treat the patients suffering from diseases such as cancer, researchers and clinicians have turned the attention to drug repurposing strategies. There are several advantages in the use of repositioning schemes for already existing validated, toxicologically safe and—no less-important—regulated pharmaceuticals to treat neoplasms. This is, however, a route not devoid of its own challenges and caveats. To cope with molecular heterogeneity (in particular mutational variances) a shift has recently made to resort to pathway-centered strategies that are aimed to approach the endeavor of drug repurposing armed with semi-mechanistic understanding of the mechanisms of action of the repurposed drugs on its new applications.

A number of successful approaches in this regard rely on the integration of methods from translational bioinformatics to face cancer data analysis with a clinician's perspective in mind; computational intelligence to diminish biases both individual and methodological and systems biology to think in terms of processes and organisms aside from molecular cues. Only by effectively combining such theoretical approaches with improved clinical diagnostics and out of the box thinking, we will be able to live up to the promise of personalized oncology. Such endeavors will be particularly relevant for the treatment of tumors with scarce therapeutic options and those prone to develop resistance to therapy.

The rest of this work will be organized as follows: the following section will discuss the essentials of pathway-based drug repurposing methods. In particular, we will elaborate on how these methods are situated in relation to *de novo* drug designs, and what is the role played by advances in pharmaceutical informatics and personalized medicine. We will further describe the commonalities and differences of pathway-based repurposing and mutation centered approaches, by contrasting the strengths and limitations of both strategies. The following section is a discussion of recent advances in the field, including novel computational tools, a growing emphasis on the impact of these strategies in the clinical outcomes and the role of artificial intelligence and machine learning in drug repurposing approaches in cancer. We will also discuss on the development of novel omic approaches to probe tumors, the important role of drug delivery and precision drug targeting for repurposing, and recent advances in functional proteomics relevant to drug repositioning. Finally, some brief concluding remarks are outlined.

We will now pay attention to the importance of drug repurposing schemes as compared to *de novo* drug design, as this will guide the rest of our discussion of pathway-based approaches to anti-cancer therapy.

PATHWAY-BASED DRUG REPURPOSING

Drug Repurposing Versus *De Novo* Design

Developing new anti-cancer drugs is of course a very important endeavor in itself. However, its timeline and route-maps are often very slow and costly. It is thus desirable that, in parallel with the synthesis and design of new anti-cancer compounds and their therapeutic combinations, we also consider strategies for the repurposing of the large number of already approved drugs (both anti-cancer and non-anti-cancer labelled) that may target known or soon-to-be-discover cancer players. Drug-repurposing has been considered as a good cost-effective strategy in order to widen-out the catalog of therapeutic options in oncology. A strategy that, in addition to be better suited to tackle better with molecular heterogeneity, is cheaper and faster to escalate to preclinical, clinical and tier studies stages, even up to clinical trials (1). In the case of approved drugs with known pharmacological interactions this may even pave the way to the development of tailor-made drug cocktails based on pathway-founded personalized medicine studies.

The latter point gains relevance in the light of a large body of evidence on the fact that combination therapies may lead to more powerful and effective results. In particular for the treatment of late-stage neoplastic tumors than single or sequential drugs combinations, given the large inter and intratumoral population heterogeneity (2, 3).

Of course, this is not to say that individualized, tailor-made polypharmacy therapy is free of caveats. Of notable relevance is the obvious fact that repurposing schemes did not follow the development and testing procedures that the pharmaceutical industry often impose on their new products, regarding dosage, tissue specificity and so on, and the fact that repurposed drugs were not designed with multi-therapy in mind (4).

Aside from these fundamental limitations there are other challenges to systematic approaches to drug repurposing for anti-cancer therapy. There are also defiances of a methodological and multi-disciplinary nature: the rational design of multi-drug repurposing schemes is a daunting task requiring the collaboration of clinical oncologists and cancer biologists with computational biologists, bioinformaticians and even experts in artificial intelligence, to name but a few disciplines. In this regard, oncologist and pharmaceutical officers need to adapt current practices to benefit from the input of professionals trained to manage the enormous wealth of information on chemical, pharmacological and genomic databases. Also, the use of biomedical informatics specialists to analyze electronic health records of the patients subjected to certain treatments. Let us consider some of these instances in more detail.

Pharmaceutical Chemo-Informatics in Cancer Therapy

One relevant application of high level computational analysis is the use of data mining and computational intelligence for drug chemo-informatics, or pharmo-informatics. Particularly relevant for repurposing schemes is *off-target analysis*. The vast majority of drugs and other compounds used in pharmacological therapy have a large number of off-target effects (OTEs), i.e., additional targets or

mechanisms aside from the main (intended) therapeutic mechanism of action (MoA). OTEs are often the actual basis of a large number of drug repurposing strategies. Due to combinatorially large "search spaces", consequence of the systemic nature of MoAs, looking at OTEs is an endeavor that is difficult (and extremely slow) to perform by humans alone. Computationally assisted interrogations of the very large datasets currently available on drugs, its targets and its MoAs, allow for a sped-up process—often by narrowing down the available options—allowing the clinician to select from a handful alternatives and not from among thousands of them (5, 6).

Additional computer-aided methods of drug-repurposing include the hybrid use of knowledge discovery in databases (KDD) and molecular profiling/modelization to search for novel drug-target interactions. The use of machine learning and other computational and statistical intelligence techniques to screen the huge molecular catalogues, searching for drug-target interactions is gaining a lot of attention. By combining KDD and machine learning with high-throughput *in vitro* assay screening (HTS) it has been possible to devise efficient therapeutic strategies to treat multifactorial diseases such as cancer, largely outperforming single-drug approaches (7).

Interestingly, not only mono-therapeutic drug target interactions need to be considered in these designs. The relevant issues of molecular and phenotypic heterogeneity in cancer tumors need to be taken into account to reach clinically-worthy anti-cancer therapeutic interventions, such as the case of targeted immunotherapy (8). Immunotherapy has gained a lot of attention recently. However, although a number of patients respond quite successfully, a large fraction does not share such benefits. This is very likely associated with the fact that there are important effects linked to the immunosuppressive nature of the particular tumor microenvironments. In such situations, it may be advisable to resort to personalized designs centered on the individually-perturbed metabolic and signaling pathways. The recent work by Li and collaborators considered how metabolic circuits are able to regulate intrinsic tumor-suppressing immunity pathways. A relevant number of these interactions have made their way onto the clinical trial stage (see, Table 1 in 8). Systematic repurposing of immunomodulatory drugs like thalidomide, lenalidomide and pomalidomide has been validated and supported by comprehensive assessment studies (e.g., QSAR) of computationally predicted biomarkers in patient-diverse cohorts (9).

The clinical oncology community remains skeptical, since the pharmacological efficacy of such treatments is still quite heterogeneous (10). One avenue to overcome skepticism (and to level up such variability) is the inclusion of immunotherapeutic drugs in polypharmacological designs. This strategy has been deeply discussed by Shen and collaborators, regarding the use of thalidomide as a drug to increase delivery and therapeutic efficacy of cis-platin (11). Thalidomide and its derivative compounds, however, are still subject of scrutiny (both as mono-drugs and in combination therapy) due to a series of reports of adverse side effects, including neurotoxicity (12) and teratogenic events (13).

Patient-Centric Drug Repurposing

Aside from molecular mechanisms and off-target effects, drug-repurposing schemes face additional demands related to individual heterogeneity. These challenges start with the availability of optimal diagnostic tools that consider factors helping to stratify such heterogeneous response to therapy. This is yet another instance in which computationally-assisted methodologies (CAMs) and AI may prove useful (14–17). Aside from CAMs/AI, modelization approaches based on systems biology frameworks would permit improved phenotyping and prognostics, leading to better-suited drug repurposing strategies (18, 19). Computational studies, relying on patient-wise genomic information, are becoming an invaluable tool to study the influence of genetic alterations in tumor progression and cell survival. This information, in turn, is fundamental to unveil tumor-specific weaknesses pointing out to clues for the development of optimal constrained sets of targeted therapeutic interventions, including drug repurposing designs (20–22).

Drug repurposing schemes extend far beyond designing drug lists or drug-cocktails. Additional consideration has to be given to making proper regimes available to the patient (1, 23). The first one of such considerations deals with the establishment of appropriate dosage to achieve anti-cancer pharmacological activity, which in general may be quite different from the dosage intended for the original use of the repurposed drugs. Computational tools have been actually developed to solve this issue (24–26). There are other non-technical (or better, not biological) issues to take into account. One of them is related to intellectual property, in particular on how to deal with patent and licensing issues, both in the case of generic and proprietary treatments. There are also economic challenges to be overcome, taking into account that cancer-related clinical trials are often more expensive, need longer follow-ups and are very prone to failure than those of non-cancer drugs. Pharmaceutical companies may find the endeavor of conducting repurposing trials to be financially unworthy. Those latter issues, although relevant, are out of the scope of the present work and hence will not be further discussed, the interested reader may refer, for instance to (5) and references therein. Coming back to the drug-repurposing molecular studies issue, we will further discuss some aspects of translational bioinformatics strategies to improve the design of personalized, pathway-based anticancer drug repurposing schemes. We will start by considering mutation-targeted therapy as this was the beginning of anti-cancer treatments beyond the use of broad cytotoxic agents.

Mutation-Specific Therapies as an Approach to Personalized Medicine in Cancer: Pros and Cons of Silver Bullets

Since the discovery of the first cancer-associated mutations and oncogenes, one central goal of anti-cancer therapy was that of looking for *cancer-causing mutations* (in particular tumor-drivers), to later resort to a tailor-armed approach to the molecular structure of *silver-bullets*, i.e., drugs able target tumors on an extremely specific fashion, while having no significant effects on non-tumor cells, often by targeting tumor-specific mutations.

In **Figure 1A**, we present a schematic workflow for mutation profiling design of personalized anti-cancer drug repurposing. High-precision DNA sequencing is used to find a tumor specific mutation in a patient's genome. If this mutation is annotated in a "cancer-panel", the clinician will gain knowledge that may allow (specially if such a mutation is absent in the germline genome or in the healthy tissue) the search of a targeted therapy. Therapeutic

alternatives may include monoclonal antibodies able to recognize the effect of the mutation at the protein level (27–29), composing an antibody-drug conjugate complex (30–33) or synthesizing a small molecule drug (34, 35). Armed with this knowledge, it is possible to look up into pharmacological databases, finding related drugs, along with off-targets and side effects (36–43). Those drugs are the long-sought *silver bullets*.

Unfortunately, with a few exceptional cases of highly penetrant mutations; most cancer patients have not benefited from these approaches (44, 45). Due to tumor mutational heterogeneity, most cancer mutations are rare, subclonal, often not causal and hence poorly annotated. The sequencing of more and more tumors, in combination with strong efforts to annotate the new variants may change this over time. However, things are not changing fast. A large scale study on the benefits of genome-driven oncology, the MOSCATO study (46, 47) concluded that purely genomic searches for cancer therapy are able to improve clinical outcomes in the minority of patients who undergo molecular screening. These results have diminished the emphasis on mutation-centered drug designs (48, 49). Mutational heterogeneity is fundamental to understand the challenges of mutation-centric studies. In recent times, mutational tumor variability has been unveiled at an unprecedented scale (50). Furthermore, pharmacologically-induced mutation is known to increase the malignancy and therapeutic-resistance (51).

The mutation frequency of well-known driver genes in metastatic breast cancer, for instance, has increased as a consequence of previous pharmacological treatment (52, 53). In this regard, the APOBEC family of APO enzymes, for instance, is known to be relevant for mutational heterogeneity (54, 55). These facts have led the pharmaco-oncology and clinical oncology experts to look up for alternative ways to face cancer

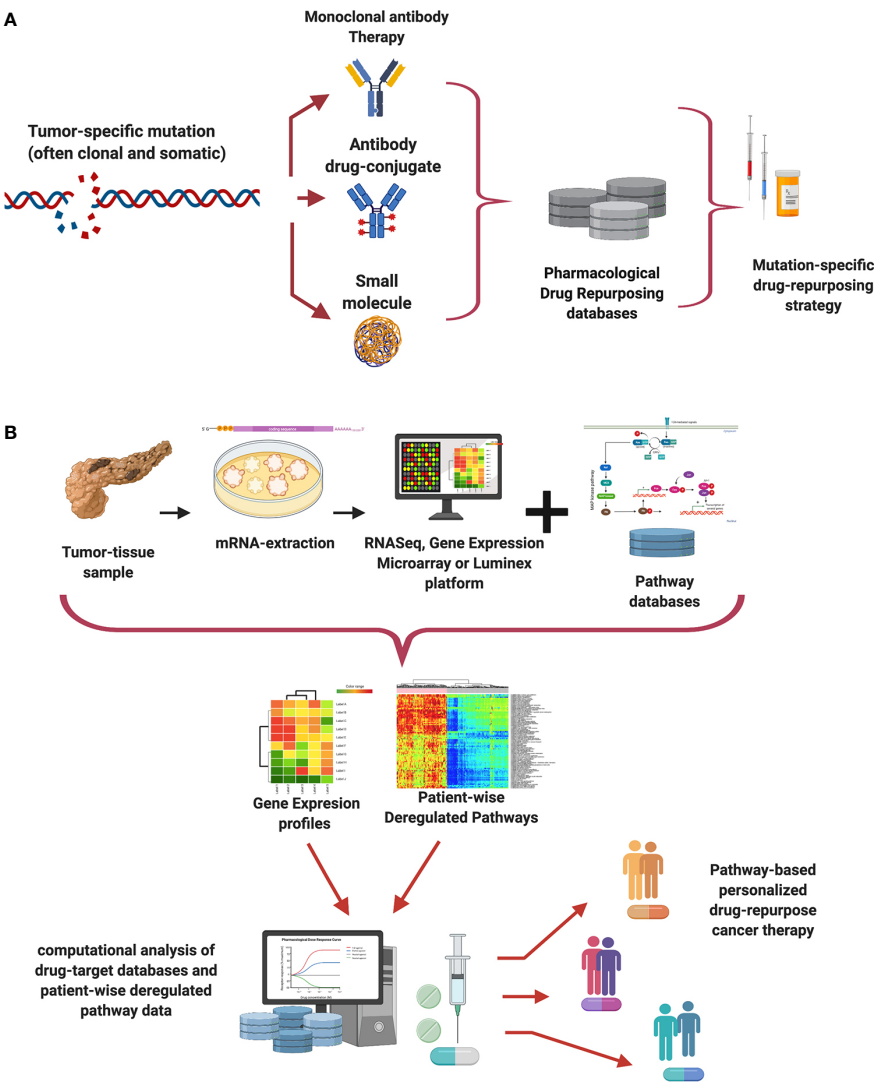

FIGURE 1 | Mutation-specific and pathway-centric approaches to drug re-purposing. Panel **(A)** exemplifies a simplified workflow for drug repurposing based on tumor mutation profiling, Panel **(B)** shows one possible pipeline for drug repurposing based on pathway activities as proxied by gene expression analysis.

therapeutics and drug repurposing. One of these avenues that is gaining a lot of momentum recently is that of pathway-based designs.

Combining Pathway Analysis, Network Approaches, and Data Mining: the Shrapnel Approach

Alternatives to mutation-based therapeutic design exist and are becoming relevant. This is the case of studies based on functional pathway analyses based on gene expression profiling. One of these approaches combines pathway enrichment (56), pathway crosstalk (57) with the so-called *pathway deregulation analysis* (58) and network strategies (59) including probabilistic modeling and knowledge discovery in databases (60).

Figure 1B presents a simplified view of a pathway-based drug-repurposing workflow. Since it is known that gene expression, although quite heterogeneous, is better aimed at capturing functional similarities at the pathway level than mutational profiling. Such methods are transcriptome-based designs instead of a genome-based. The workflow starts by taking a tumor-biopsy sample from one patient. mRNA is extracted and purified from the sample. Then gene expression levels of the sample are measured either by RNA-Sequencing or by other technologies such as expression arrays, or a Luminex panel (7).

The rationale behind such pathway based methods has to do with a systems biology view on how to cope with the emergence of complex phenotypes (say tumors and tumor responses to therapy) from a myriad of (sometimes unknown) biomolecular interactions, metabolic reactions and signaling events. In the cases when the emergence of the phenotype is largely determined by one (or a handful) mutation events, genomic-variant centered approaches have proven quite efficient. However, more often than not, the emergence of the tumorigenic and tumor response to drug phenotypes is due to the interplay of a number (perhaps large) of mutually intertwined biological processes. Pathway based approaches to drug repurposing are intended to deal with such cases.

The gene expression sample profile is analyzed in the context of this large data corpus (sometimes by clustering or subtyping it), the next step consists in database mining from pathway databases such as KEGG (61), Reactome (62, 63), and Pathway Commons (64). One may either look up for a specific set of pathways (metabolic or immune system, for instance) or consider all currently annotated pathways. Once the set of pathways has been selected, it is possible to interrogate the databases looking for pathway-targeting drugs, this is molecules targeting key genes in the deregulated pathways.

Pathway deregulation metrics will allow for further filtering *via* joint analysis of pathway deregulation, differential gene expression, drug-target interactions, off-target, and side effects databases such as PharmGKB (65, 66), DrugBank (67), the Therapeutic Target Database, TTD (68) and others. Once these steps have been followed, we end up with a list of suggested therapies mapping the abnormal pathways linked to cancer in the different patients. These prioritized lists are the starting point of the work of the clinical oncologists and pharmaco-oncologists, as such, they are intended as mere tools, which, however useful, complement but do not replace the expertise of the clinical oncologist.

This workflow belongs to a more general family of pathway-based methods for individualized anticancer drug repurposing. As is known, biological functions are often represented as an interaction network of molecules within the cells. Such interactions are often captured in semi-mechanistic terms as pathways to try to capture the plethora of higher order biological functions (61). As we have said, often pathway-based strategies are founded on gene expression and other molecular profiling studies. Let us review some general ideas in this regard.

Gene Expression and Other Means of Molecular Profiling

One important challenge for the development of personalized drug repurposing approaches of anticancer therapy is molecular and phenotypic heterogeneity of the tumors. To tackle such variability, large scale databases like The Cancer Genome Atlas—Genomic Data Commons— (69–72) and others (73, 74), allow for analyses helpful to discern the commonalities and differences in gene expression features and associate them with the phenotypes and survival in thousands of cancer patients. Such systematic, data-driven studies, in turn, opened-up the possibility to create dynamic maps of tumor features and vulnerabilities by classes. Using these maps such as the CMAP led to the discovery of vulnerability biomarkers to guide clinical interventions (75).

Computational biology and AI studies of these huge omic databases along with clinical, data driven translational applications, are significantly improving patient-specific diagnostics and prognostics (76, 77). These, in turn, paved the way to enhanced designs to cancer therapeutics (78). Such large computational endeavors have also increased the success of targeted assays to determine the efficacy of competing therapies such as chemotherapy and hormone-guided designs (79) or the effects of combinatorial immune therapies (8).

Pathway Activity Profiling

Moving on from gene expression profiling to actual biological function is a daunting, unfinished task. However, a common approximation is given by analyzing which molecular pathways are deregulated, i.e., their activity functions in abnormal ways. Perhaps, the optimal experimental way to do this would be by resorting to massive phospho-proteomic and metabolomic experiments. However, technical and logistic challenges for accuracy and reproducibility of current proteomic technologies have discouraged further studies along these lines for the present moment. Hence, gene expression profiling has become the standard proxy used in large cohort studies of oncogenic pathway activity.

From Deregulated Pathways to Repurposed Drugs

After analyzing the individual repertoire of dysfunctional pathways (as proxied by the expression of key genes within them), it has been possible to devise pathway-centric approximations to drug repurposing. Let us discuss some remarkable cases. The case of breast neoplasms with challenging phenotypes is quite illustrative. A recent study led to the identification of nine breast tumor subtypes (instead of the usual 4 or 5 considered in the PAM50 classification). One of these subtypes, that went unobserved until this study, comprising about 7% of the cases (on a cohort of around

2000 tumors and 144 controls) resulted deregulated for 38 PKA pathways (80).

The importance of this finding for the therapeutic options to treat these tumors lies in the fact that despite being many protein kinase-driven pathways of great phenotypic impact, most of these pathways are all inducible by a single molecule: PRKACB which is a druggable gene. PRKACB is a target for Staurosporine, a p-glycoprotein/abcb1 inhibitor. Staurosporine induces cell death in (Luminal A-associated) MCF7 human breast cancer cells (81), and is known to also disrupt HUNK, a cell cycle-associated kinase in Her2+ tumors (82). In this way, Staurosporine is able to treat two different breast cancer subtypes (luminal and Her2+) by disparate yet related mechanisms that inhibit proliferation *via* PKA pathways. The same large scale study identified 9 EGFR-related pathways which can be targeted by FDA-approved drugs such as Anlotinib (83, 84). Anlotinib main use in cancer was already established to treat aggressively, drug-resistant tumors such as glioblastoma (85); Poziotinib (86–88). Other available EGFR-targeting molecules include Dacomitinib (89) and cationic polyamidoamine dendrimers (90).

Due to the binding nature of EGFR control, EGFR-modulation can also be attained by using glucocorticoids (91). However, hormone-mediated mechanisms of action are often less specific than other EGFR modulators mentioned, so caution must be taken (92, 93). We must notice that EGFR-centered therapies have resulted to be less effective than initially expected due to kinase repertoire heterogeneity. However, EGFR-targeting may result useful in combination therapy, for instance, to increase chemosensitivity in triple negative breast tumors. The mechanism proposed for this enhanced chemoselectivity is *via* reprogramming apoptotic signaling networks (94). The variability in response to EGFR-targeting is useful to introduce additional issues to be considered in the design of repurposing strategies. Two quite relevant among these issues are the effects of active pathway crosstalk and the role of secondary targets, in particular in relation to pharmacological resistance.

Coping With Pharmacological Resistance: The Role of Pathway Crosstalk and Secondary Targets

A final, yet extremely relevant, issue to be considered in the design of pathway-based, individualized cancer therapy is the fact that the clinical efficacy of a drug goes well beyond the (static) molecular portrait given by the action of the drug on the pathway or pathways under consideration. The dynamic nature of drug activity depends on its effect, at the level of systemic, even organismal perturbations. Such phenomena occur within a densely interconnected signal transduction and metabolic network (95, 96). Given this, one must consider the MoA not only within the single instance of the prioritized pathways, but also in the context of all other biological phenomena occurring on their close surroundings (i.e., in the pathways' network neighborhood). The phenomenon of pathway crosstalk, for instance, it is known to exert important effects on the onset and progression of pharmacological resistance (57, 97). Of course, pathway crosstalk has gone beyond network connectivity since, as stated, it is a highly dynamic process. For the cases in which one is able to anticipate crosstalk phenomena that may result relevant to pharmacological efficacy this must be considered in the initial design. At least dosage and coadjuvant therapies to prevent or diminish its effects must be analyzed in advance (98–100).

To date, a number of bioinformatic and computational biology resources have been developed to cope with the issue of pathway crosstalk in the context of drug repurposing (101–103). A recently proposed strategy is the use of crosstalk inhibition studies (104–107). However, other approaches include the evaluation of drug synergism (108–111), as well as cohort studies to evaluate and categorize crosstalk induced resistance (57, 112–114).

Aside from pathway crosstalk phenomena, in which the activity of several interconnected pathways is cross-regulated, there is also the issue of secondary molecular (and/or functional) targets. A secondary target of a drug has been defined as any target (a gene, protein, metabolite, etc.) whose associated MoA or downstream effects are not in line with the intended therapeutic mechanisms (115–117). Secondary target studies have been carried out for a long time. However, the availability of comprehensive database resources for high throughput assessment of secondary targets is relatively recent (118, 119).

Among the more relevant resources in the context of anti-cancer therapeutics, we can mention, for instance, the one maintained by the COSMIC consortium drug resistance database (CCDRD) (https://cancer.sanger.ac.uk/cosmic/drug_resistance (120, 121). CCDRD is indeed a quite comprehensive catalog of drug resistance events in cancer that is, however, limited in that it only considers somatic mutations. As we have already discussed, somatic-mutation therapy provides only a narrow window for therapeutic advances limited by the mutational heterogeneity of the tumors. Other approaches, although based on less comprehensive resources are also being considered (122). An outstanding example of its applications is the case of pembrolizumab (Keytruda) which is an immune checkpoint inhibitor drug. After looking up for secondary targets of pembrolizumab, Dang and coworkers found that some of them actually provide synergistic therapeutic effects (123).

DISCUSSION

Recent Advances

Aside from the established computational frameworks for oncological drug repurposing already discussed, there is also a series of nascent, promising strategies that may complement them. Machine learning (ML) studies, for instance, are providing means of discovery relying more on the increasing abundance of omic and clinical data than on a deep knowledge of cancer biology (which is the case for most of the approaches already presented). The recent work of Issa and collaborators (124) summarizes well recent ML applications. Of noteworthy attention is the fact that some computational learning algorithms are already being applied beyond genomic and transcriptomic data. The role machine learning (Random forests, support vector machines, LASSO optimization) for ligand-based and docking studies (125, 126) for instance, has already resulted in therapeutic advances for the patients (127, 128). Feature selection techniques applied to the characteristics of the targets and the drugs, have allowed advances in

the so-called *proteochemometrics*, which aims to optimize the metabolic efficacy of drugs, something that must not be overlooked, in particular when facing polypharmaceutical designs (129).

Machine learning algorithms in cell phenotyping are also starting to gain attention as a route to the design of anti-cancer drugs (130) and repurposing strategies (131). Machine learning in transcriptomic data has been extensively used in recent years, as already discussed. An application that stands out, having revealed the efficacy of a very common over the counter drug (cimetidine, an already off-patent approved anti-ulcer drug with favorable safety profile) to be repurposed to treat lung adenocarcinoma was presented and validated years ago by Sirota and coworkers (132) and its results have been successfully replicated by an independent group (133). The work by Sirota and collaborators exemplifies well one way in which the translational bioinformatics approach should proceed. Starting with high throughput, highly curated information from the CMAP (6), they applied machine learning tools (at that time in the state of the art), discovered novel dysregulated pathways, in lung adenocarcinoma, find key genes involved, look up for FDA approved targets. Validated their findings in cell lines and mouse xenografts and make their data and codes available to allow for replication studies. After this, they started clinical trials to make the treatment available to the patients. If one were to summarize the 'ideal' workflow of translational bioinformatics, the work by Sirota and collaborators may be a very good example (132).

Two nascent applications of ML to drug repurposing in cancer are the use of computational learning in electronic health records (EHR) databases (134, 135) and in immune profiles (17, 136). Both are promising for different reasons: On the one hand, EHR databases may provide massive access to data at a relatively low cost, enabling hypothesis generation to be tested in molecular/omic studies. On the other hand, immune 'fingerprinting' has shown to be somehow less heterogeneous at the individual level than genomic/transcriptomic profiling while at the same time being highly individual-specific.

The emergence of database resources for repurposing such as RepurposeDB (137) is also worth noticing. Particularly relevant is the fact that computational learning approaches and KDD over such databases have revealed that, aside from purely pharmacological and biochemical features, there are also epidemiological factors influencing the effectiveness of a repurposed drug. Scanning the feature selection spaces allows for innovative treatments within the spectrum of repurposed drugs. Such is the case of the DrugPredict algorithm (138) which, based on molecular and epidemiological data, have been used to repurpose the non-steroidal anti-inflammatory drug Indomethacin for the treatment of chemo-resistant ovarian cancer. Since it has been demonstrated that induced robust cell death in primary patient-derived platinum-sensitive and platinum-resistant ovarian cancer cells.

Computational intelligence techniques combined with systems (particularly network) biology studies constitute relevant lines of research to comprehensively map the interactions pertinent to drug repurposing. The work of the group of Dragici in this regard is worth mentioning (139). This group developed an open source bioinformatic drug repurposing tool called DrugDiseaseNet (https://github.com/azampvd/DrugDiseaseNet). With this tool, the team has managed to reproduce the results of several noteworthy repurposing studies, most notable, the one by (132).

Also, using machine learning combined with network approaches, Tan and collaborators were able to analyze the comprehensive Library of Integrated Network Cell Signatures (LINCS) database (140) to uncover specific druggable targets in glioblastoma (48).

The Impact on Clinical Outcomes

Ultimately, the success or not of drug repurposing schemes—as in every other therapeutic intervention—must be measured in relation to their impact on clinical outcomes. Of course, there are numerous reports, including data from pre-clinical assays, clinical trials, and observational studies supporting the anti-cancer efficacy of a wide range of repurposed drugs (141). Indeed, one main advantage of repositioned drugs is the fact that, often there are extensive data on pharmacokinetic properties and toxicity available.

However, drug repositioning may require further validation on novel side effects—due, for instance, to different dosage—and other considerations for which clinical trials must be run. The outcome of such studies varies widely. For instance, repurposing of raloxifen (a mineral density enhancer), was validated as an anti-breast cancer therapy in a multicentric study in 180 hospitals in 25 countries and become ultimately FDA-approved as a coadjuvant in breast cancer therapy. Digoxin (a cardiac glycoside) on the other hand, even if quite promising in the experimental stage, bring no survival benefit when compared to conventional platinum-based therapy, and also had significant toxicity and pharmacological interactions (141). That was also the case for the repurposing of Latrepirdine, Ceftriaxone and Topiramate (142). All three drugs were extremely promissory on experimental pre-clinical tests and were relatively well evaluated regarding toxicity and side effects, even at anti-tumor doses, but fail to deliver at the clinical outcome test.

Interestingly, translational bioinformatic approaches have been advanced for the evaluation of clinical outcomes in relation to drug repurposing (143). By performing computational literature mining in databases such as ClinicalTrials.gov and others, it has been posible to pre-evaluate clinical outcomes and focusing repurposing trials on possible *red alerts*. Aside from *positive* clinical outcomes, data mining for adverse events, side effects, and drug-drug interactions, is making possible to sped-up clinical trials for repurposing drugs by standardizing, cataloguing, and processing annotated vocabularies (143, 144). However, standardize, large scale clinical outcome data is not easily available. One alternative that has been proposed (142) is that of online, self-reported patient data (145). This approach has several advantages such as faster data collection, reduced costs, and enhanced patient-engagement, but is still facing challenges related to privacy and systematic curation.

Aside from database reporting and archiving, recent efforts have been made in the use of artificial intelligence (AI) and machine learning for the large scale analysis of clinical outcomes (146). An interesting resource in this regard is the *Clinical Outcome Search Space* (COSS), an AI platform for drug repurposing (147). In spite of these advances, not all the

experts agree on the actual efficacy of drug repurposing regarding clinical outcomes.

Tran and Prasad (148), for instance, recall that observational studies alone, may be extremely biased by selection and that this may affect some of the drug repurposing strategies, hence many of the repurposing clinical trials are doomed by design. In order to prevent such biases, randomized controlled trials in large, heterogeneous populations, evaluating *oncological outcomes*, even at the adjuvant level are needed. Such was the case, for instance for the repurposing of metformin as a neo-adjuvant therapy. As of 2020, there are 132 completed, 85 under recruitment, and 32 finishing clinical trials for metformin as an anticancer drug as reported in the ClinicalTrials.gov website (149). In spite of the large samplesize of studies such as the TAXOMET, the STAMPEDE, and the METEOR, and the fact that the drug has been discussed for oncological use for some years, there is no consensus on the real significance regarding clinical outcomes. A striking contrast with this has been the relative success of statins as antineoplastic agents to treat lung cancers (150). However, the very fact that we face such enormous differences in clinical outcomes for repurposed drugs call for optimized means to evaluate *a priori* when a repurposing candidate drug is worth to enter clinical trial stages.

The Role of Artificial Intelligence and Machine Learning in Drug Repurposing: Challenges and Opportunities

As we have already mentioned, one possible avenue of improvement of drug-repurposing analytics is the use of computational intelligence and machine learning approaches. Such views and methods are particularly relevant to try to cope with the enormous challenges in *interpreting* the vast amounts of heterogeneous experimental and clinical data often present in drug repurposing studies in cancer. The challenge to *make sense* of the data has been approached in several ways. One of such methodologies is *baseline regularization* (BR). Kuang and collaborators introduce BR (151) to analyze EHR data, including drug-prescriptions, physical, and biochemical measurements (lab tests, anthropometrics, etc.). BR make use of statistical relationships to account for changes in the patient's indicators correlating with the use and dosage of certain drugs of interest. These relationships are then used to identify, assess, or validate drug repurposing candidates.

Deep learning methods such as Deep Neural Networks (DNN), Convolutional Neural Networks (CNN), Support Vector Machines (SVM), and Naive Bayesian analysis (NB), as well as Natural Language Processing (NLP), have also been used to find patterns, useful to predict pharmacological effects, from transcriptomic, genomic, EHR, and bibliographic data (124). A DNN method, for instance, was introduced in a study analyzing perturbation experiments from 678 drugs across several cell lines from the LINCS project (152). ML and DNN have also been used for rational drug discovery, moving on from classic measures such as Quantitative Structure-Activity Relationships (QSAR) to high-throughput, event-based studies for the identification of novel and repurposed drugs (153, 154).

Aside from trying to tackle the complexities of data interpretation in experimental and pre-clinical data, ML approaches have been also developed for the inference and prediction of drug response patterns (155). To do so, MoA data, as well as genomic and transcriptomic databases are being complemented with novel experimental techniques such as those based on single cell assays (these techniques and their use in drug repurposing may be discussed in the next subsection). Computational intelligence techniques are being applied *on tandem*, all along the drug repurposing and development strategies, in the so-called end-to-end (E2E) applications (156). However, powerful these approaches are, we have good reasons to be cautious, even skeptical of them, and as is the case with all clinically-inclined interventions, wait until their effectiveness is proven in controlled, randomized clinical trials.

Novel Omic Approaches: Single-Cell Sequencing, Structural Genomics, Epigenomics

Technical advances in relation to drug repurposing tools not only consist in the development of computational and bioinfomatic tools to analyze existing experimental data types. Some functional features of biological relevance for drug repurposing are indeed being able to probe only be the use of novel experimental ways to measure biological activity (157). We can mention, for instance, the rapidly developing field of single cell sequencing. Single cell biology has been envisioned as a means to comprehend intra-tumor heterogeneity with greater precision, and with this gained knowledge being able to overcome the diagnostic and therapeutic challenges often posed by such enormous cell-to-cell tumor variability (158). One outstanding example of such tumor heterogeneity is glioblastoma multiforme (GBM). One important component of the essential intractability of advanced stage glioblastoma multiforme is precisely cell-to-cell variability, even within the so-called glioma-imitating cell population. To analyze therapeutic challenges in glioblastoma, Niklasson and coworkers analyzed single cell sorted RNASeq libraries derived from biopsy-captured GBM samples (159) to evaluate mesenchymal states connected to therapy resistance *via* immunomodulatory mechanisms.

To study c-MET inhibitors and their potential role in overcoming drug resistance, Firuzi and collaborators studied spheroid models of pancreatic and stellate cells (160). Single cell proteomic assays confirmed previous sequencing findings regarding the relative effects of repurposed drugs tivantinib, PHA-665752 and crizontinib. Single cell RNASEq and single cell shotgun proteomics have also been used in combination to discern the role of cancer associated fibroblasts in chemoresistance inesophageal adenocarcinoma (161). This study shows that phosphodiesterase 5 inhibitors are able to regulate the activated fibroblasts phenotypes in the benign disease and are promising drugs to enhance response to chemotherapy. Multiscale modeling, including the role that single cell models of ErbB receptor-mediated Ras-MAPK and PI3K/AKT signaling, has been used to study the response to a drug-reposition treatment in prostate adenocarcinoma (162). There single cell sequencing assay data was used to account for subclonal heterogeneity. To evaluate ATRi/BD98 inhibition in cell cycle defects induced by ATR inhibitors in cancer cells, single cell sequencing and single cell gel electrophoresis (COMET) were used by Chory and coworkers (163). These single cell assays allowed the researchers to characterize the MoA of the ATR inhibitors

via inhibition of ATP-dependent chromatin remodeling complexes SWI/SNF.

Aside from single-cell assays, advances in techniques to probe on structural abnormalities in the genome such as microsatellite instabilities, gene fusions and chromotripsis have revealed clues to the design and repurposing of anticancer drugs. In a recent analysis on the use of gene variants and networks for drug repurposing in colorectal cancer, Irhan and collaborators (164) discussed how to use colorectal cancer biomarkers, such as microsatellite instabilities (MSI), for the repurposing of PIK3CA modulators. Finding molecules such as copanlisib, either alone or in combination with nivolumab as promissory drugs. On a similar line of thought, Fong and To (165) presented the use of immune checkpoint inhibitors as effective therapies for colorectal cancer patients with MSI or mismatch repair variants. This has led to the FDA approval of pembrolizumab (Keytruda) combined with nivolumab as PD-1 inhibitors and of ipilimumab as a CLT4-inhibitors in those tumors. In connection to anti-breast cancer therapies, pembrolizumab has also been approved for metastatic tumors with marked MSI. Such is also the case of coadjuvant theory with aspirin and Celecoxib as (anti-PD-1 antibody) for advanced stage breast cancer (166, 167). MSI has also been a factor to consider for the repurposing of co-adjuvant drugs to treat advanced stage melanoma (Indoximod), metastatic non-small cell lung cancer (Metformin), in both cases to enhance pembrolizumab activity.

Another set of structural variants of interest for anti-cancer drug repurposing is that of gene fusions. Perhaps the paradigmatic case is that of acute myeloid leukemia (AML) (168). The case of niclosamide is relevant since it targets some relatively common gene fusions (or their associated chimeric proteins), aside from targeting relevant transcription factors such as CREB, STAT3 and NF-κB. Chromosomal aberrations and gene fusions in intimal sarcoma have also helped to identify potential therapeutic targets (169). In particular, the PDE4NIP/NOTCH2 and the MRPS30/ARD2 fusion positive tumors have been identified as druggable targets. In colon cancer, KCTD12/CDK1 fusion positive tumors have been shown to become vulnerable to vemurafenib *via* a coadjuvant treatment with adefovir dipivoxil (170). This allows the repurposing of the BRAF V600E inhibitor vemurafenib from melanoma to colon cancer therapy.

Epigenomic markers—most notably methylation patterns—have also unveiled avenues for drug repurposing (171). Some of these were found *via* KsRepo a methylation-based drug repurposing method for acute myeloid leukemia (172) that has allowed to reposition four drugs: alitretinoin, cytarabine, panabinostat, and progesterone for AML. Methylation profiles (in particular, m^6A DNA/RNA methylation) have been proven to be relevant to the action of repurposed drugs such as afatinib in non-small cell lung cancer (173). DNA methylation profiles also have been useful as a tool to find out novel and repurposed therapeutic targets in bladder cancer (174).

In particular, a novel useof 5-azacytidine a nucleoside analogue and decitabine that may function as a DNA methyltransferase inhibitor, have been found to re-activate tumor suppressor genes, inhibiting tumor cell growth and increasing apoptosis in bladder cancer cells. These results remain consistent from *in vitro* assays all along to clinical trials.

Drug Delivery Mechanisms and Chemo-Resistance

A relevant and often overlooked challenge in drug repurposing—in particular when the repositioned drug was originally a non-oncological one—is the issue of drug delivery efficacy and its relationship with proper drug targeting and chemo-resistance. One example of how to overcome these challenges is the reduction of chemo-resistance *via* coadjuvant therapy with mebendazole (175). Aside from coadjuvant therapy, perhaps the best solution to optimize drug delivery to the tumors is *via* advancing delivery technologies (176, 177). Lei and coworkers, for instance, discussed the use of nanomedicine such as nanoparticle albumin-bound paclitaxel (nab-PTX), abraxane, or a liposomal formulation of irinotecan as effective improvements of anti-cancer drug delivery for pancreatic ductal adenocarcinoma (177).

The use of exosomes has been extensively studied recently, in particular since they may play a role, not only in drug delivery, but also in regulating autocrine and paracrine signaling pathways which may regulate drug responses (178). Extracellular vesicles have also been used to navigate through the tumor microenvironment in glioblastoma. Such vesicles have resulted useful to deliver drugs, even through the blood-brain barrier (179). Caution, however, mut be taken since these vesicles have also biological roles such as the promotion of angiogenesis, immune suppression and facilitating recurrence, all of them pro-tumor effects. Hence, a lot of research efforts must be devoted to develop effective drug delivery mechanisms that enhance drug-targeting and reduce chemo-resistance in relation to anti-cancer repurposed therapy.

Emerging Proteome-Based Studies

We have mentioned that most high-throughput pathway activity and drug MoA studies are based on either sequencing known genomic targets or novel mutations or measuring gene expression by RNASeq, microarrays, or Luminex-type assays. However, quite recently proteomic-wise techniques are enhancing our capacities to probe cellular activity at the (functional) proteome and phospho-proteome level. One of such techniques is isobaric labeling mass spectrometry (8). This technique has allowed to identify and dose-stratify the binding of the drug staurosphorine to 228 cellular kinases on a single experiment. Proteomic and phosphoproteomic analyses have also allowed to reveal mechanisms of activation of NEK2 and AURKA kinases in cancer (180), thus allowing the use of drugs targeting such kinases in six different cancer types within the Clinical Proteomic Tumor Analysis Consortium (CPTAC): Breast cancer, clear cell renal carcinoma, colon cancer, lung adenocarcinoma, ovarian cancer, and uterine corpus endometrial carcinoma.

Advances in the experimental tools to study cancer proteome-wise, have also called for the development of new methodological, computational, and analytical techniques, useful in drug repurposing strategies. As an example, Saei and collaborators developed a comprehensive chemical proteomics profiling approach for target deconvolution of a redox active drug auranofin (originally and anti-rheumatic called Ridaura) as an anti-cancer drug auranofin was

found to target genes such as TXNRD1, NFKB2, and CHORDC1, all of them known to be involved in the perturbation of oxidoreductase pathways in cancer (181). Bioinformatic platforms for the predictive analytics of drug-protein-disease data are in turn, being developed. Such is the case of rb"cando.py", a bioinformatic platform to analyze changes in proteome profiles related to drug perturbation. This method has been applied successfully to analyze repurposing of ribavririn and a novel compound LMK-235 in breast cancer and AML. The results have been validated in *in vivo* experiments and are being considered to enter a clinical phase in the near future (182). These are but a handful of examples that, however, make us anticipate further, near-future developments, in the high-throughput study of phenomena of interest for systematic drug repositioning strategies to treat cancer.

Concluding Remarks

Drug repurposing in cancer is a quite complex endeavor. In order to cope with all the complexities and subtleties involved, there is a need for collaborative, multidisciplinary teams, including clinical oncologists and oncological surgeons, molecular oncologists, cancer cell biologists and pharmacologists but also bioinformaticians, computational biologists and data scientists. One emergent and quite successful avenue of research and intervention, is that of basing repurposing strategies on functional, semi-mechanistic basis as the one supplied by pathway-based analysis. This comes as no surprise, since the ultimate goal of pharmacological interventions is the modulation of functional traits and processes both at the functional and physiological levels. Hence pathway-based studies provide a close proxy as to these functional processes that make us hypothesize that findings based on these may prove to be more effective in terms of providing effective anticancer therapy.

The present review discusses recent advances in the application of computational molecular biology and bioinformatic approaches using high throughput omic data, mining of extensive, well-annotated databases and a cycle of experimental and clinical validation, to face some of the more evident challenges for anti-cancer drug repurposing. The field is flourishing so this review is not meant to be comprehensive but rather to serve as an introductory journey into a wide and fascinating research topic.

AUTHOR CONTRIBUTIONS

EH-L devised the project, established the outline, and made the figures. EH-L and MM-G performed literature surveys and revisions. EH-L and MM-G wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CRISPR-dCas9-Based Artificial Transcription Factors to Improve Efficacy of Cancer Treatment With Drug Repurposing: Proposal for Future Research

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Due to the high resistance that cancer has shown to conventional therapies, it is difficult to treat this disease, particularly in advanced stages. In recent decades, treatments have been improved, being more specific according to the characteristics of the tumor, becoming more effective, less toxic, and invasive. Cancer can be treated by the combination of surgery, radiation therapy, and/or drug administration, but therapies based on anticancer drugs are the main cancer treatment. Cancer drug development requires long-time preclinical and clinical studies and is not cost-effective. Drug repurposing is an alternative for cancer therapies development since it is faster, safer, easier, cheaper, and repurposed drugs do not have serious side effects. However, cancer is a complex, heterogeneous, and highly dynamic disease with multiple evolving molecular constituents. This tumor heterogeneity causes several resistance mechanisms in cancer therapies, mainly the target mutation. The CRISPR-dCas9-based artificial transcription factors (ATFs) could be used in cancer therapy due to their possibility to manipulate DNA to modify target genes, activate tumor suppressor genes, silence oncogenes, and tumor resistance mechanisms for targeted therapy. In addition, drug repurposing combined with the use of CRISPR-dCas9-based ATFs could be an alternative cancer treatment to reduce cancer mortality. The aim of this review is to describe the potential of the repurposed drugs combined with CRISPR-dCas9-based ATFs to improve the efficacy of cancer treatment, discussing the possible advantages and disadvantages.

Keywords: cancer treatment, drug repurposing, CRISPR-Cas9, artificial transcription factors, CRISPR-dCas9-based ATFs

INTRODUCTION

Cancer represents one of the most important health challenges in the world. It can be treated by the combination of surgery, radiation therapy, and/or drug administration. Surgery and radiation are used to treat cancer that is confined locally and drug therapy is used to kill metastasized cancer cells (1, 2). Cancer therapies provide different efficiency degrees depending on the tumor type and

therapy applied; however, anticancer drug-based therapies are the main treatment used in different tumor types (2). Anticancer drugs are classified as pregenomic and genomic era drugs. Pregenomic era drugs are targeted against a tumor phenotype, whereas genomic era drugs are developed after the target is identified by using molecular techniques that consider intratumoral genetic heterogeneity (1, 3, 4). Furthermore, cancer drug development takes an average of 11.4–13.5 years and an investment from 161 to 1,800 million dollars per drug (5–7). An alternative solution to this problem is drug repurposing which is the application of a drug for another indication than it was originally approved. It helps to reduce development costs and gets a more rapid return on investment in the development of repurposed drugs (7–11). Some repurposed drugs have demonstrated antitumor efficacy by inducing cancer cell death or suppressing various genes related to cancer (12). There are different mechanisms by which the repurposed drugs cause antitumor effects, however, it is important to study mechanisms that regulate gene expression related to proliferation and cell death to improve the cancer treatment efficacy and avoid drug resistance. The possibility to combine pregenomic era drugs and molecular tools could increase tumor cell killing and reduce the likelihood of drug resistance (1).

The artificial transcription factors (ATFs) are molecular tools that can manage the gene expression to induce changes in different cell stages (13, 14). Within the different types of ATFs, the emerging CRISPR-dCas9-based ATFs have been used to precisely regulate gene expression in different *in vitro* and *in vivo* studies. Aforementioned, these molecular tools are a promising strategy for cancer treatment at the transcriptional level (15, 16). In addition, it is important in cancer research to identify new drug combinations that generate synergistic effects and thereby achieve more efficient therapies (4, 17).

For this reason, in this review, we described the possibility to implement a cancer therapy with CRISPR-dCas9-based ATFs combined with repurposed drugs, to regulate gene expression related to pharmacodynamics of the repurposed drug and/or MDR genes of the cancer cells.

DRUG REPURPOSING USED IN CANCER THERAPIES

Cancer drug development requires preclinical and clinical studies to extensively test and characterize their pharmacological properties, efficacy, antineoplastic effects, and toxicity (5, 12). The time to develop and license new drugs are often longer than the identification of new targets for chemotherapeutic intervention (18). The pharmacodynamics of the cancer drug has to be identified and validated to proceed to clinical trials. For that reason, drug repurposing is a great opportunity for alternative cancer therapy development, since it is faster, safer, easier, and cheaper (3) and because most of the non-cancer drugs have little or tolerable adverse effects for human health, contrary to chemotherapeutic agents that have relevant side effects that significantly reduce life quality (19).

Despite drug repurposing in cancer advantages, drugs are affected by multidrug-resistant (MDR) mechanisms that decrease their pharmacodynamic, enhance degradation of the drug, and reduce uptake. In this way, it is important to tackle genetic heterogeneity and drug resistance in cancer through the drug combination with molecular tools. One possible solution for this situation is to use CRISPR technology to silence MDR genes and increase cancer treatment effectiveness (1).

CRISPR THERAPY IN CHEMOSENSITIVITY

Multiple drug resistance is caused by the differential expression of genes in tumor cells, commonly called multidrug resistance genes (MDR). This resistance is responsible for unsuccessful chemotherapies and causing high mortality in a short time. An alternative to overcome this challenge is to silence or inactivate these MDR genes (20–22). In recent years, the clustered, regularly interspaced short palindromic repeats (CRISPR) in combination with a CRISPR-associated nuclease 9 (Cas9) have been used for this purpose due to its practical use, versatility, and its cleavage efficiency in almost any target sequence (20, 23). The CRISPR-Cas9 system is formed of an RNA-guided endonuclease (Cas9/sgRNA complex) which consists of the single guided RNA (sgRNA) fuses with Cas9. The sgRNA is formed by a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA) (21, 22).

For cancer therapy, each CRISPR therapeutic target is selected by the tumor type. For example, CRISPR-Cas9 targeting the CXC chemokine receptor 4 (CXCR4) was evaluated *in vitro* and *in vivo* studies on hepatocarcinoma, which significantly decreased its expression and inhibited cell proliferation and migration leading to less invasiveness and also significantly increased the chemosensitivity to cisplatin (24). In another study, the CRISPR-Cas9 system was used to deactivate the Nuclear Erythroid 2-Related Factor (NRF2) gene in lung cancer cells. It showed an increase in the sensitivity to chemotherapeutic agents such as cisplatin and carboplatin (25).

Similarly, CRISPR has been evaluated to increase chemosensitivity in breast cancer by inactivating or down-regulating the MDR1 gene (also known as ABCB1) that significantly increased the doxorubicin cytotoxicity in resistant chemotherapy breast cancer cells. These data suggested that the mutation of the MDR1 gene by intracellular administration of the CRISPR-Cas9 complex recovered the drug susceptibility and avoided multidrug resistance in breast cancer cells (26).

In ovarian cancer, chemosensitivity with CRISPR-Cas9 has also been increased from the inactivation of the MDR1 gene that encodes the P-gp protein. This decrease in expression was associated with a greater sensitivity to doxorubicin (27). Likewise, the PARP-1 gene has been suppressed by CRISPR-Cas9 in ovarian cancer and caused a greater sensitivity to cisplatin in cancer cells (28). In osteosarcoma, P-gp expression can be effectively blocked by CRISPR-Cas9, and P-gp inhibition was associated with reversal of doxorubicin resistance in MDR osteosarcoma cell lines (KHOSR2 and U-2OSR2). For that

reason, the CRISPR-Cas9 system increased the long-term chemotherapy efficacy by overcoming P-gp-mediated MDR in the clinical setting (29).

Although sometimes it is enough to inactivate a gene to reverse chemotherapy resistance, the tumor types can have several target genes that can lead to the same goal of making it chemosensitive. For example, p53 was overexpressed to make cells more sensitive to doxorubicin chemotherapy and a greater effect on chemosensitization of resistant osteosarcoma cells was obtained (30).

Due to the above, the use of the CRISPR-Cas9 tool combined with chemotherapy can enhance the efficacy of the elimination of various tumor cell types. However, the specificity of Cas9/sgRNA needs to be carefully evaluated since Cas9/sgRNA can have undesired off-target targets and cut essential genes for the patient (20). For this reason, the CRISPR-dCas9 system has been used due to its deactivated nickase activity that does not make cuts in the genetic sequence and it does not permanently inactivate genes and thereby reduces desire off-target effects (31, 32).

CRISPR-dCas9-BASED ARTIFICIAL TRANSCRIPTION FACTORS

ATFs are used to express and/or suppress target genes. They consist of molecular domains such as DNA-binding domains (DBD) that confer sequence specificity and may target similar sites in the genome with different affinity degrees. Several DBDs are used for the design of ATFs, including zinc fingers (ZF) (**Figure 1A**), transcription activator-like effectors (TALEs) (**Figure 1B**), and the CRISPR-dCas9 system (**Figure 1C**) (14, 32, 33).

ATFs also have an effector domain (ED) that interact with DBD to activate or repress transcription of target genes by blocking the transcription process (14, 34, 35).

The CRISPR-dCas9 system nuclease activity is deactivated by mutations (Cas9 mutated is called dCas9) and an ED can be incorporated to allow its function as ATFs. The dCas9 retained the DNA-binding specificity of wild-type Cas9, without causing a DNA double-strand cleavage altering the host DNA sequence (26). Furthermore, the dCas9 protein requires sgRNA for its specificity. Multiple sgRNAs can be easily designed and synthesized, making the CRISPR-dCas9 system suitable for testing more than one target simultaneously (14, 35–37).

The combinatorial effect of the use of CRISPR-dCas9-based ATFs with certain chemotherapeutics makes it possible to completely eradicate tumor cells. It has been seen that a long non-coding RNA (lncRNA) KCNQ1OT1 was overexpressed in squamous cell carcinoma tissues and lung cancer which were resistant to cisplatin (38, 39). By using CRISPR-dCas9-based ATFs (**Figure 1C**) with interfering function in expression called CRISPR interference (CRISPRi), KCNQ1OT1 expression was inhibited in CAL27-res and SCC9-res cells that improved the

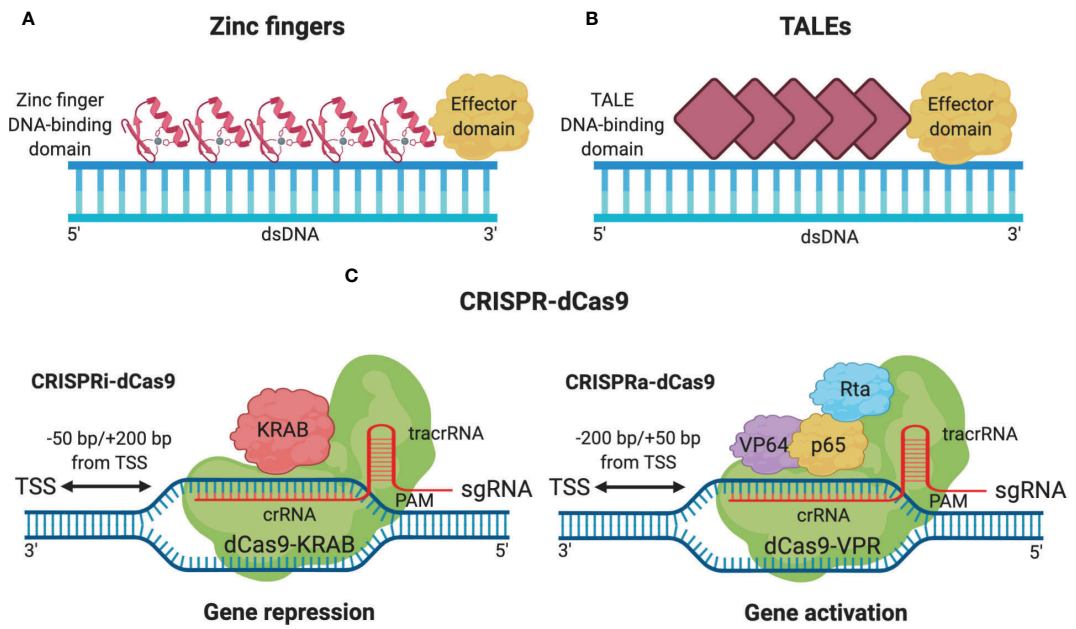

FIGURE 1 | ATFs types used in the repression or activation of transcription. **(A)** Zinc finger-based ATF is composed of an effector domain and a DNA-binding domain like the Cys2-His2 (C2H2) domain which contains multiple cysteine and histidine residues which are ligands for the zinc ion. **(B)** TALEs-based ATF also consists of an effector domain and a DNA-binding domain (diamond red) composed of 33–35 amino acid repeat arrays (each repeat domain specifies a single DNA base). **(C)** CRISPR-dCas9-based ATFs comprise a dCas9 protein and a single guide RNA (sgRNA). Interference of transcription mediated by dCas9 associated with KRAB domain (CRISPRi) and activation of transcription associated with VP64, p65, and Rta domains that conglomerate ribonucleic complexes (RNAP) to activate the transcription process (CRISPRa). Created with BioRender.com.

chemosensitivity to cisplatin. While using CRISPR-dCas9-based ATFs with activating function in expression called CRISPR activator (CRISPRa), expression levels of KCNQ1OT1 increased by promoting cell growth and returning chemoresistance in the cells. The CRISPR-dCas9-based ATFs are useful tools in gene overexpression and underexpression, which improves chemosensitivity (38).

Currently, CRISPR-dCas9-based ATFs have been applied in drug resistance, epigenetic regulation, and immune regulation in various cell lines such as squamous cell carcinoma (CAL27-res and SCC9-res), breast cancer (E0771), pancreatic adenocarcinoma (Pan02), melanoma cells (B16F10), hepatoma (Hep3B), lung (H157), etc (22, 38, 40–42).

CRISPRa has been used to express the target antigenic peptide (SIINFEKL) in breast cancer (E0771), pancreatic adenocarcinoma (Pan02), and melanoma (B16F10) cells, in an orthotopic model in mice to enhance the elimination of tumor cells through the immune response generated by the peptide (40). Similarly, in lung cancer cells (H157), CRISPRa activated the expression of MASPIN (mammary serine protease inhibitor) that led to a concomitant cell proliferation inhibition and apoptosis induction (42).

Several *in vivo* studies with mice models have validated the use of CRISPR-dCas9-based ATFs as regulators of the gene expression related to cancer development. However, more studies on the off-target effects of this tool are still lacking before moving to clinical phases (36). In addition, CRISPR technologies *in vivo* transfection efficiencies are still relatively low; hence for the implementation of this technology in the clinic for cancer treatment, it is necessary to continue with scientific research on the most plausible *in vivo* administration of these ATFs to target tissues (43). The ideal *in vivo* delivery system should cause low immunogenicity and direct the dCas9/sgRNA to the interested organ or cell type (44). There is a variety of *in vivo* delivery systems like viral vectors (adenovirus and lentivirus) that are very efficient (36), but they could have side effects due to their potential carcinogenesis and immunogenicity (15, 45, 46). Another delivery system is the DNA plasmid. However, since the size of CRISPR/Cas9 plasmids is larger than other plasmids, they exhibit a higher charge density, and more polycations are required to condense them (46, 47). Currently, with the help of nanotechnology, different administration methods of the system based on metal, polymeric, or lipid nanoparticles have emerged (44, 48, 49). The use of these nanoparticles can improve transfection efficiency, reduce off-target effects, decrease systemic toxicity, and immune risks associated with transfection (45).

Since cancer is involved in multiple and complex cellular pathways that affect the efficacy of the therapies, drug combination therapies might be an alternative strategy to have a higher success rate in the clinical application (17). For this reason, in this review, it is proposed to use CRISPR-dCas9-based ATFs for cancer therapies in combination with repurposed drugs whose action mechanisms are to regulate the expression of oncogenes and tumor suppressor genes or to inactivate MDR genes. **Table 1** summarizes the repurposed drugs analyzed in this study from several pharmacological classes. As selection criteria, all drugs are currently being evaluated in clinical trials for cancer therapy, and they have been observed in *in vitro* studies on various cancer types,

TABLE 1 | Repurposed drugs proposed for cancer therapy combination with CRISPR-dCas9-based ATFs.

Drug	Pharmacological class	Original indication	Effect of the drug on gene regulation related to cancer	Complementary gene regulation with CRISPR-dCas9	Cancer types	Refs.
Digitoxin	Cardiac glycosides	Cardiac conditions	Expression of p21	Suppression of HIF-1 and HIF-2	Prostate, lung, breast	(12, 19, 50)
Chlorpromazine	Antipsychotic drugs	Psychosis, schi-zophrenia, bipolar disorder	Expression of p21 Suppression of oncogene K-Ras	Expression of p53	Colorectal, glioma, leukemia	(12, 51, 52)
Mebendazole	Microbiological agents	Parasitic worm infection	Expression of pro-apoptotic Bcl-2	Suppression of ABL and BRAF oncogenes	Colorectal, melanoma, glioblastoma	(12, 53–55)
Ritonavir	Antiviral	HIV treatment	Expression of p53 Suppression of pRb	Expression of p21	Ovary, breast pancreatic	(12, 56)
Nelfinavir	Antiviral	HIV treatment	Expression of DR5 and inhibition of AKT	Expression of SREBP-1 and ATF6	Lung, ovary, breast	(12, 57)
Naproxen	NSAIDs	Antiinflammatory	Suppression of genes for COX-enzymes	Expression of PI3K	Leukemia, breast, colorectal, osteosarcoma	(12, 58)
Ibuprofen	NSAIDs	Pain, fever, antiinflammatory	Expression of Akt, p53, Bcl-2 and Bax	Suppression of genes for COX-enzymes	Colorectal, melanoma	(12, 59, 60)
Aspirine	Salicylate	Pain, fever	Suppression of Sp transcription factors family	Suppression of genes for COX-enzymes	Colorectal	(4, 6, 19, 53–55)
Metformine	Oral antidiabetic	Diabetes II	Inhibition of mTORC1 and Activation of AMPK	Expression of AMPK	Hepatocarcinoma, breast, colorectal, prostate	(12, 56)
Artesunate	Microbiological agents	Malaria	Expression of pro-apoptotic proteins such as caspase-3	Suppression of antiapoptotic proteins and MYC oncogenes.	Lymphoma, myeloma, hepatocarcinoma	(12, 61, 62)
Itraconazole	Microbiological agents	Fungal infections	Inhibition of 14-alfa-lanosterol demethylase	Decreased AKT1 activity	Lung, prostate	(12, 63)
Doxycycline	Antibiotic	Bacterial infections	Suppression of MMP-2 and MMP-9	Expression of TIMP-2	Hepatocarcinoma, lung, prostate, colorectal	(12, 64, 65)
Lithium	Antidepressant	Depressant	Inhibition of glycogen synthase kinase 3	Suppression Smad3 and TGFBIp	Prostate, colorectal	(12, 66, 67)

which have an action mechanism with antitumor effect due to their ability to regulate gene expression involved in cell proliferation and death (5, 12, 19). It is proposed to combine the gene regulation effects of repurposed drugs and the CRISPR-dCas9-based ATFs to obtain a cancer therapy with a higher success rate. With this combination, target genes can be synergistically regulated from CRISPR-dCas9-based ATFs to enhance the effect of repurposed drugs. Additionally, genes involved in the signaling pathway of processes related to cancer development can be complementarily regulated, as well as, MDR genes can be silenced to have a higher success rate in the treatment (4–6, 17, 19).

DISCUSSION

Cancer is a complex, heterogeneous, and highly dynamic disease with multiple evolving molecular constituents. Due to the genomic instability of cancer cells, every individual cancer cell has a set of mutations. This tumor heterogeneity causes several resistance mechanisms in cancer therapy, mainly the target mutation (3, 4, 22). CRISPR-dCas9-based ATFs can be used in transcriptional therapeutics to optimize gene expression and design a more controllable system, for example, repurposed drug inducible system, improving the potency of gene manipulation, multiplexing and resource limitation and dosage and gene expression pattern (68). For cancer therapy, CRISPR-dCas9-based ATFs had been developed to activate tumor suppressor genes and silence oncogenes and the tumor resistance mechanisms for targeted therapy (22). Some potential strategies for CRISPR/Cas9 interventions targeting cellular genes in cancer have proposed downregulation of oncogenes (ErbB, *src, abl, fps, yes, ras, raf*, and *myc*) and genes related to chemoresistance (MDR-1, MRP, GST-p, UGT1A1 and Cytokine P450) and for upregulation of tumor suppressor genes (pRb, p53, APC, SMAD4, PTEN, BRCA1/2, and ATM) (22, 69).

For that reason, it is proposed to use CRISPR-dCas9-based ATFs for cancer therapies in combination with repurposed drugs whose action mechanisms are to regulate the expression of oncogenes and tumor suppressor genes or to inactivate MDR genes. This could allow for synergy or complementarity between CRISPR technology and repurposed drugs in cancer therapy since both strategies express or repress certain genes involved in cancer, and its combined use could generate a synergistic effect that enhances therapy when the repurposed drug regulates the expression of the same gene that will be the target for CRISPR-dCas9-based ATFs.

For example, in **Table 1**, the digitoxin causes cell cycle arrest in the G2/M-phase since it induces the expression of p21, an inhibitor of cyclin-dependent kinases and they suppress HIF-1 and HIF-2 expression which are transcription factors often increased in tumors that regulate essential genes related to hypoxic environments for tumor adaptation (12, 19, 50, 51). For this reason, the CRISPR-dCas9-based ATFs can be combined with these repurposed drugs to equally activate p21 expression and generate a synergistic effect or to suppress HIF family expression and generate a complementary effect to make chemotherapy more effective.

Cancer treatments are handled by multiple therapeutic tools. These can be used depending on the patient types and their diagnosis. In this sense, the repurposed drugs in combination with CRISPR-dCas9-based ATFs may be an innovative alternative that promises to be able to cover certain tumor types more efficiently. Drug repurposing and CRISPR-dCas9-based ATFs have been used for cancer therapy, and they have received increasing attention from biotechnology research due to the economic advantages they represent for the pharmaceutical industry, as well as the molecular advantages they confer on the patient during the cancer treatment. The use of drug repurposing alternatives for cancer treatment represents fewer side effects for patients and a wider range of applications as molecular advantages. Nevertheless, cancer efficacy of drug repurposing is still affected by MDR genes (1, 19). This challenge may be solved with CRISPR-Cas9 technology or CRISPR-dCas9-based ATFs in combination with drug repurposing by the inactivation of MDR genes. Despite CRISPR-Cas9 technology providing an effective inactivation of any gene, it cleaves one target at a time and in a non-specific way, which represents other disadvantages (25, 27, 29). For this reason, CRISPR-dCas9-based ATFs is the best option for cancer therapy combination since it not only has a majority of therapeutic targets but also the DNA double-strand is not broken, and the host DNA sequence is not altered. CRISPR-dCas9-based ATFs are even relatively cost-effective in comparison to the *de novo* construction of protein-based ZF and TALEs DNA-binding domains. Other advantages are that CRISPR-dCas9-based ATFs are more specific compared to TALEs and ZINC fingers and may have several genetic targets to regulate at the same time (36, 70). However, the CRISPR-dCas9 specificity can decrease depending on the complexity of the DNA due to the inaccessibility to the therapeutic target (43, 71).

Regarding CRISPR-dCas9-based ATFs limitations, the dCas9/sgRNA complex is bigger than other ATFs as TALEs or Zinc Fingers, and cell delivery may be difficult (14, 22, 35, 37).

Other limitations of the CRISPR-dCas9-based ATFs are the off-target effects due to the possibility of dCas9 binding to nucleotide sequences similar to the target PAM sequence. However, the optimization of the length of the sgRNA allows reducing the off-target effects without sacrificing efficiency in the objective (43, 72). Despite the above, more information is needed to corroborate the real negative impact of the off-target effects generated by dCas9 since it only performs partial and temporary binding with the off-target sequences without damaging them (36, 70).

Finally, cancer therapy with drug repurposing combined with CRISPR-dCas9-based ATFs has not yet been carried out on an experimental basis; however, it is important to explore in future research the possibility to combine these methods for cancer therapy due to the potential advantages to reduce cancer mortality in a cost-effective manner and with more efficient results.

AUTHOR CONTRIBUTIONS

AM-E and BL-C: Conceptualization, methodology, writing—original draft preparation. ER-G: Supervision, writing—

reviewing, and editing. All authors contributed to the article and approved the submitted version.

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Mifepristone Repurposing in Treatment of High-Grade Gliomas

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Glioma is the most common and aggressive primary tumor of the central nervous system. The standard treatment for malignant gliomas is surgery followed by chemoradiotherapy. Unfortunately, this treatment has not produced an adequate patient response, resulting in a median survival time of 12–15 months and a 5-year overall survival of <5%. Although new strategies have been sought to enhance patient response, no significant increase in the global survival of glioma patients has been achieved. The option of developing new drugs implies a long and costly process, making drug repurposing a more practical alternative for improving glioma treatment. In the last few years, researchers seeking more effective cancer therapy have pursued the possibility of using anti-hormonal agents, such as mifepristone. The latter drug, an antagonist for progesterone and glucocorticoid receptors, has several attractive features: anti-tumor activity, low cytotoxicity to healthy cells, and modulation of the chemosensitivity of several cancer cell lines *in vitro*. Hence, the addition of mifepristone to temozolomide-based glioblastoma chemotherapy may lead to a better patient response. The mechanisms by which mifepristone enhances glioma treatment are not yet known. The current review aims to discuss the potential role of mifepristone as an adjuvant drug for the treatment of high-grade gliomas.

Keywords: brain cancer, glioma, mifepristone, repurposing, repurposed drug, resistance

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INTRODUCTION

Glioma is the most common primary neoplasm in the central nervous system, making up approximately 30–45% of tumors in the central nervous system (CNS). These tumors are very invasive, making a complete surgical resection impossible (1).

The World Health Organization (WHO) has classified gliomas into four grades, based on degree of malignancy. Grade 1 tumors, most frequently found in children, are considered gliomas with a low risk of malignancy and a reduced potential for proliferation. Grade 2 tumors, appearing mainly in young adults 20–30 years of age, are slow growing and in some cases have a tendency to progress to a more malignant tumor. Grade 3 tumors present a high rate of mitotic activity and are very invasive, giving a median survival time of 1–3 years. Grade 4 tumors, also called glioblastoma multiforme (GBM), correspond to the maximum degree of malignancy, being characterized by rapid growth, necrotic zones, and an accelerated rate of progression. Grade 3 and 4 are malignant or high grade gliomas, mainly characterized by a poor prognosis, resistance to chemoradiotherapy, and rapid tumor recurrence (2).

Additionally, in 2016, the WHO reclassified the gliomas by molecular profiling. This classification includes mutation in isocitrate dehydrogenase 1 or 2 (IDH1 or IDH2), wild type IDH, or not otherwise specified (NOS). The IDH proteins are involved in the conversion of isocitrate into alpha-ketoglutarate in the tricarboxylic acid cycle. In low-grade gliomas, isocitrate dehydrogenase (IDH) mutations were found with higher frequencies (83%), compared to grade III astrocytoma (70%) or primary and recurrent GBM (5%) (3). These mutations have been correlated with better prognosis, leading to a higher median survival in patients with IDH mutations in all gliomas.

Malignant gliomas are rare tumors in epidemiology. They constitute 2% of the total cases of cancer in women and 2.8% for men (4). In spite of the low prevalence of gliomas, it is urgent to find an effective medical treatment because the average survival rate is under 2 years.

Physiopathology of Glioma Development

Diverse reports in "The Cancer Genome Atlas" (TCGA) describe three main signaling pathways involved in the pathogenesis of gliomas. This include: RTK/RAS/PI3K (receptor tyrosine kinase, RAS, phosphatidylinositol 3-kinase), p53, and retinoblastoma (5). Another important pathway involved in glioma pathology is that related to angiogenesis (the formation of new blood vessels from a pre-existing vascular network). One of the main stimulants for the expression of angiogenic factors is hypoxia. This factor induces the synthesis of vascular endothelial growth factor (VEGF), which is considered the most important signal protein mediating angiogenesis.

Various strategies have been utilized to inhibit the expression of VEGF, such as the bevacizumab, a humanized monoclonal antibody. According to two prospective phase-III trials, the addition of bevacizumad to the first-line treatment (temozolomide and radiotherapy) did not improve overall survival in patients with newly diagnosed glioblastoma. Progression-free survival was prolonged but did not reach the pre-specified improvement target (6, 7).

Yang et al. evaluated the transcriptome of patients with glioblastoma, observing differences between men and women in the gene pathways associated with survival. In men, the pathways most commonly linked to overall survival participate in cell division. Thus, the pharmacological blockage of the progression of the cell cycle may be more effective in men. In women, the genes most closely related to overall survival are involved in the regulation of invasiveness; therefore, drugs targeting integrins could function better in women (8).

Another common focus of research on glioma tumors is the PI3K/Akt signaling pathway (9). The *PTEN* gene, encoding for a protein that inhibits the PI3K/Akt pathway, is inactivated in 40 to 50% of the cases of glioblastoma. This pathway is closely related to resistance to treatment because its activation promotes proliferation, invasion, and angiogenesis, and has been related to the conversion of anaplastic astrocytoma (grade 3) into GBM (grade 4) (9, 10).

Resistance to Treatment

Treatment of malignant gliomas begins with surgical resection, which is often incomplete, leaving residual cells that are capable of migrating and proliferating. Therefore, surgery is accompanied by 60 Grays (Gy) of radiotherapy (2 Gy/daily) and chemotherapy with the alkylating agent temozolomide (75 mg/m^2, daily by 6 weeks), followed by a dose of maintenance of 150–200 mg/m^2 for 5 days every 28-day cycle for six cycles. However, it has not given the desired response, resulting in a median survival time of 15 months (11, 12).

Among the most important challenges in the treatment of gliomas are the restrictions on access to the brain imposed by the blood-brain barrier (BBB), the limited response to therapy, and neurotoxicity stemming from the treatments (13). The vast majority of chemotherapy treatments for glioma tumors have failed due to the reduced concentrations of the drug that reach the CNS (14, 15).

The mechanism of action of temozolomide consists of inhibiting the replication of DNA. This prodrug is spontaneously transformed into monomethyl triazeno imidazole carboxamide (MTIC) upon entering the organism. The cytotoxicity of MTIC is reportedly caused by the alkylation of guanine at positions O6 and N7 of DNA (16). Unfortunately, the adducts generated are removed by the repair enzyme 06-methylguanine-methyltransferase (MGMT), leading to resistance and recurrence. The silencing of the MGMT gene seems to improve the response to treatment in patients receiving alkalizing agents (17). The methylation of the CpG island of the MGMT enzyme promoter is associated with better survival of patients with high grade gliomas that are given alkylating agents as chemotherapy.

Besides the repair mechanisms for damaged DNA, another mechanism involved in resistance to treatment is an insufficient accumulation of the drug at the target site stemming from alterations in transporters dependent on ATP (**Figure 1**) (18). Among such transporters overexpressed in the BBB and glioma tumor cells are the P-glycoprotein multidrug resistance protein 1 (P-gp/MDR1/ABCB1) and the multidrug resistance-associated protein (MRP/ABCC1) (19). Several drugs showing affinity for P-gp include actinomycin D, taxanes, anthracyclines, and temozolomide (20, 21).

The blockage of the ATP-dependent transporters could lead to a considerable enhancement of glioma treatment. Temozolomide is reported to compete with substrates of P-gp, thus blocking their activity (20), and to diminish the activity of P-gp by inhibiting its ATPase (22). However, there is controversy about whether temozolomide actually increases or decreases the expression of this protein. According to one study, temozolomide boosts the expression of P-gp through the EGFR pathway (23), while another found a temozolomide-induced reduction in the expression of P-gp in BBB cells caused by the methylation of the WNt3a gene promoter (24). A reduced expression of P-gp would sensitize glioma cells to treatment.

Drug Repurposing for Combination With Temozolomide in the Treatment of Gliomas

Despite extensive research on the design and development of new molecules to achieve a better response to glioma treatment, patient overall survival, and the median survival time have not

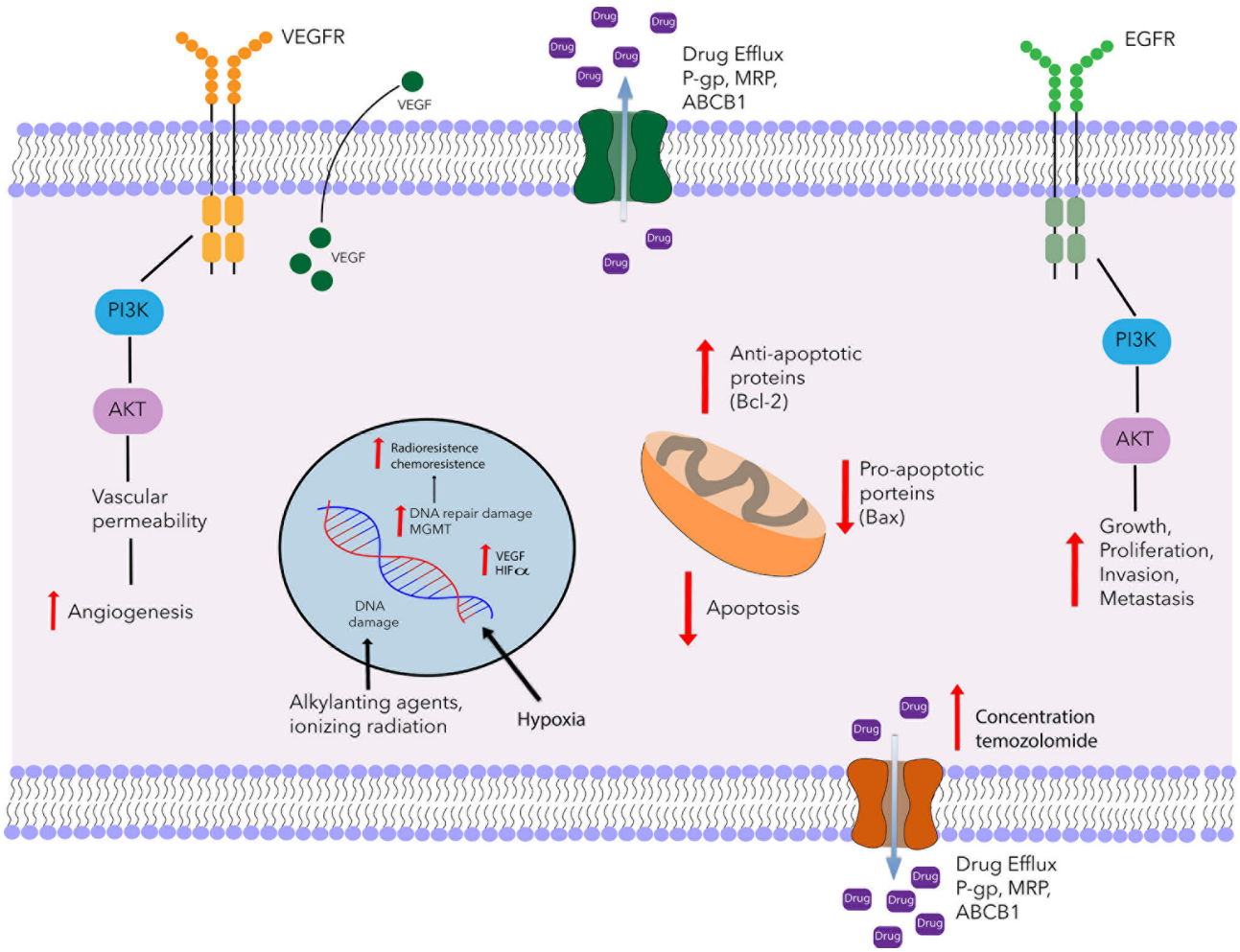

FIGURE 1 | Schematic portrayal of resistance mechanisms of glioblastoma. Glioblastoma multiforme is characterized by an angiogenic tumor and resistant to chemotherapy. One of the mechanisms of temozolomide resistance is mediated by MGMT, which removes methyl group O6-MeG lesión and restores the cellular replication of glioblastoma cells. Another mechanism of resistance is the ATP-dependent transporters family (P-gp, MRP, BCRP). These proteins are involved in the uptake and efflux of several drugs including temozolomide. Also, in glioblastoma tumors, there is an increase of VEGF expression and over-expression of VEGFR promoting the formation of tumor blood vessels. Another tyrosine kinase receptor that is involved in chemoresistance is the epidermal growth factor receptor (EGFR), activates MAPK, and PI3K signaling promoting growth, proliferation, invasion, and metastasis. On the other hand, it has been reported that radioresistance is partly due to the presence of hypoxic regions, hypoxia is associated with tumor angiogenesis and invasiveness, therapeutic resistance, and poor prognosis. In addition to the resistance mechanisms described above, there is dysregulation of the apoptosis genes, such as the up-regulation of Bcl-2 and the down-regulation of Bax. The combination of these mechanisms contributes to a chemo-radioresistance.

yet improved. One strategy employed in recent years is the repositioning of drugs, which consists of finding new applications for approved drugs. An advantage of drug repurposing is that is known a profile of safety and efficiency, making it a candidate for rapid incorporation into other clinical treatments, implying less risk and greater cost-benefit.

Cost is an important factor in the development of new compounds for the treatment of diseases. The cost of developing new drugs is generally in the range of a billion dollars (25, 26). The US Food and Drug Administration (FDA) considers glioblastoma as a rare disease, which limits the initiative of the pharmaceutical industry to invest in the development of new drugs for this neoplasm. Hence, drug repurposing may be the most suitable strategy under these circumstances. The administration of multiple therapeutic agents with various targets in malignant gliomas likely provides more benefits than standard chemotherapy based on a single agent.

Although intense research efforts have been made to improve the current treatment of glioblastoma, there are scant reports on the repositioning of drugs for use in combination with temozolomide. An experimental and clinical study on human glioma cells, on an animal model, and in patients with recurrent GBM demonstrated that the combination of temozolomide with

inhibitors of the tumor promoter gene *GSK3β* (glycogen synthase kinase-3 β) reduces in the progression of the disease and protects against the neurodegenerative effects provoked by radiotherapy (27). Such inhibitors include cimetidine, valproate, olanzapine, and lithium carbonate (commonly prescribed to treat gastro-duodenal ulcer, epilepsy, schizophrenia, and bipolar disorder).

Temozolomide was tested in combination with hydroxyurea on an orthotopic experimental model of glioma, finding an increase in the percent survival of the animals (28). Roix *et al.* (2014) evaluated 182 compounds *in vivo*, identifying three (candesartan, risedronate, terbinafine) that lead to a better response of animals when given in combination with temozolomide. Preclinical trials are still necessary to explore their efficacy (29).

In the last few years, our group has investigated mifepristone (an abortifacient drug) as a plausible repurposing candidate for treating a wide range of cancers. A synergistic action has been demonstrated when it is combined with cisplatin or temozolomide plus radiation (30–34).

The Repositioning of Mifepristone for Cancer Treatment

Mifepristone (RU486) was the first antiprogestogen developed. In 1981, it was described as an antagonist of glucocorticoid, progesterone, and androgen receptors. Its first use was as an emergency contraceptive pill to induce abortion (35). In the year 2000, mifepristona was approved by the FDA as an abortive agent. In practice, this drug has been utilized for a great variety of diseases and clinical conditions, including the termination of pregnancy, endometriosis, Cushing's syndrome, and metabolic syndrome (36).

Due to its antiproliferative and antimetastatic activity, mifepristone has been evaluated individually as a potent agent in metastatic ovarian cancer with positive progesterone receptor (PR) (37). However, the loss of PR may represent a more aggressive panorama in the development and progression of cancer. Mifepristone is also known to produce effects not mediated by hormonal receptors, acting on hormone receptor negative cells. It was found to inhibit cell growth in ten different PR negative cancer cell lines. The mechanism described was a decrease in the activity of checkpoint Cdk2 of the cell cycle, generating cell cycle arrest in phase G1 (38). Hence, the effect of mifepristone *does not require* of the presence of the PR. Additional studies carried out on PR negative cancer cells support the finding of mifepristone-induced antiproliferative effects on breast (39), cervical (40), endometrial (41), ovarian (42) and prostate cancer (43).

Nowadays, there are several clinical trials in which mifepristone is used alone or in combination with another drug to treat different types of cancer. In the case of prostate cancer, a phase II clinical trial has been conducted. In this study, 200 mg of mifepristone was administered daily, which was well tolerated, with no incidence of clinical adrenal insufficiency (44).

Another clinical trial (phase I/II) is currently recruiting patients with metastatic, castration-resistant prostate cancer. The first goal is to establish the safe and pharmacologically active dose of mifepristone and enzalutamide (androgen receptor antagonist). The second goal is to determine if mifepristone in combination with enzalutamide delays time to prostate-specific antigen (PSA) progression compared to enzalutamide alone [ClinicalTrials.gov identifier (NCT number): NCT02012296].

On breast cancer patients, the combination of paclitaxel-charged nanoparticles plus mifepristone (300 mg) was evaluated in a phase I trial. This combination was found to be well tolerable by patients (45).

Mifepristone administration has been documented to improve both length and quality of life in patients with advanced non-small cell lung cancer (46, 47) and renal cancer (48). In advanced pancreatic cancer this drug had palliative benefits (49). Mifepristone seems to be well tolerated at a dose of 200–300 mg.

Mifepristone as a Sensitizing Agent for Malignant Gliomas

Epidemiological studies show that the incidence of glioblastoma is approximately 40% higher in men than in woman. Furthermore, the woman/men ratio is lower in pre-menopausal women, and increases in parallel with the decrease in female hormone levels, which suggests that these hormones have preventive effects on gliomagenesis (50). These results correlate with a lower incidence and better overall survival for women with brain tumors (50, 51). Estrogens also improved the percentage of animal survival in an orthotopic model of experimental glioma (52). These data suggested that estrogen might be responsible for better overall survival.

On the other hand, it has been found that the expression of glucocorticoid and progesterone receptors is elevated in patients with high-grade glioma. It is known that this type of receptors play an important role in cell proliferation. In this way progesterone and glucocorticoids could promote the development of gliomas.

Experimental studies have showed that progesterone is capable of stimulating the infiltration and migration of astrocyte in the rat cortex (53). Mifepristone, due to its anti-progestational and anti-glucocorticoid action, blocks the capacity of progesterone to stimulate the growth, migration and invasion of human astrocytoma cells lines (54, 55). Nowadays, several clinical and preclinical studies are being carried out to understand and confirm the impact of steroid hormones on gliomatosis.

Recently it was demonstrated the participation of progesterone in the growth of glioblastoma stem cells (56). Several studies have suggested that stem cells may be responsible for resistance and recurrence in glioblastoma. It is still unknown whether mifepristone could inhibit the growth of glioma stem cells; however, some authors have shown that mifepristone reduces the breast cancer stem cells population (39, 57). Therefore, future research is required to determined whether mifepristone could regulated glioma stem cells.

The methylation of the MGMT promoter is a prognostic factor associated with increased temozolomide response and overall

survival in glioblastoma. Schiffgens *et al.* (2016), suggested that the silencing of the MGMT gene may be influenced by the sex. In this study a greater proportion of females presented promoter methylation in comparison with males. However, the authors mentioned that it is necessary to corroborate these results due to the low sample size in its study (50, 58).

Glucocorticoid receptor is expressed in neurons, oligodendrocytes, astrocytes, and microglia of the brain (59). Glucocorticoids as dexamethasone are frequently used in patients with high-grade glioma as a therapy to address edema and increased intracranial pressure. However, its use is controversial on glioblastoma progression (60). An increase in proliferation, angiogenesis, invasion and anti-apoptotic effects has been described in preclinical studies. In addition, dexamethasone seems to decrease the efficacy of temozolamide (61, 62). Therefore, the addition of mifepristone, as GR antagonist, could increase the effect of temozolomide and decrease cellular proliferation (60).

According to some authors, the glucocorticoids are involved in eliciting the expression of the MGMT gene, which means that these drugs could contribute to an elevated MGMT protein level. Biswas *et al.* and Ueda *et al.* detected an upregulation of MGMT in glioblastoma cell lines during glucocorticoid treatment (63, 64).

Mifepristone seems to have great potential for glioma treatment as a glucocorticoid receptor antagonist. In C6 glioma cells implanted intracranially in rats, our group recently described the capacity of mifepristone to reduce the expression of the MGMT protein. This led to a higher rate of apoptosis and consequently to diminish tumor proliferation in rats treated with the mifepristone/temozolomide combination (33, 34). Consequently, one of the mechanisms possibly involved in the mifepristone-induced sensitization to temozolomide is the modulation of the expression of the MGMT enzyme.

It is still unknown whether mifepristone epigenetically inhibits MGMT. However, different nuclear transcription factors as SP1, AP-1, NF-kappa B, and HIF-1alpha, can activate the transcription of MGMT gene in glioblastoma (65). Some of the above-mentioned transcription factors may participate in MGMT gene regulation by mifepristone.

Mifepristone has also been found to inhibit the expression of the VEGF, which is overexpressed in glioblastoma. We find that there was an additive effect by temozolamide and mifepristone in the inhibition of VEGF levels. Mifepristone also blocks the function of drug efflux proteins such as P-gp. This protein is highly expressed by endothelial cells in gliomas, and a key role has been attributed to P-gp in the chemoresistance of gliomas. In our work we show the participation of mifepristone in the inhibition of P-gp expression, and on the increased intracerebral concentration of temozolomide. The tumor growth rate was slower than that found with temozolomide alone, indicating a chemo-sensitizing effect. According to the current results, mifepristone could contribute to the modulation of tumor relapse in glioblastoma by decreasing the levels of VEGF, MGMT, and P-gp (34). Further research is needed to explore other mechanisms of drug resistance of glioblastoma tumors.

Several clinical studies show that mifepristone is capable of crossing the BBB and has demonstrated palliative effects on brain tumors, such as meningiomas (66, 67) and glioblastoma (68). It has also been found to improve the quality of life of patients with glioma. These characteristics make mifepristone an attractive repurposing candidate for the treatment of malignant gliomas. It is considered a safe drug, which even with prolonged use has relatively mild adverse effects, such as fatigue, nausea, vomiting and diarrhea. The administration of mifepristone monotherapy was reported for two clinical cases of cancer: a patient with non-small-cell lung carcinoma (NSCLC) and brain metastasis, and another with bilateral kidney cancer and metastasis. In both patients, the result was a better quality of life.

Based on the experimental data, as well as in clinical trials, mifepristone appears to be a promising approach against glioblastoma. The addition of mifepristone to glioblastoma treatment could improve the quality of life of patients, and has the potential to control the progression of tumors.

Future Directions

The continuous effort to identify new treatments for glioblastoma is yet to lead to significant improvements in the survival rate. The molecular complexity of glioblastoma has forced the scientific community to pay attention in new strategies, such as drug repurposing.

Recently, new evidence has emerged about the role of stem cells in the development of cancer and resistance to therapy. Several studies have suggested that cancer stem cells could be responsible not only for reduced treatment efficacy but also their recurrence (69). One of the most challenging aspects when treating gliomas is the complete elimination of cancer stem cells. Glioblastoma stem cells are reported to abundantly express the MGMT and P-gp proteins, leading to greater resistance to treatment, a higher level of hypoxia, and more frequent tumor recurrence (70, 71). As a result, these proteins have become an important therapeutic target to improve patient response to glioblastoma treatment. As we have mentioned, several studies reported that mifepristone decreased MGMT and P-gp expression (33, 34) (**Figure 2**). Future research is required to determine whether mifepristone can regulate glioma stem cells, offer greater benefits during tumor recurrence and improve the prognosis of patients with glioma.

CONCLUSION

A great variety of strategies exist for the development of new glioblastoma treatments. Yet in the vast majority of cases, none of them has yet been able to control the progression of tumors or recurrence. Mifepristone has been found to improve the quality of life of patients with glioma. It is considered a safe drug that, even with prolonged use, has relatively mild adverse effects. Considering the chemoresistance mechanisms reviewed, mifepristone could possibly be a sensitizing agent in therapy against malignant gliomas and we recommend it for clinical trials on a combined mifepristone/temozolomide plus radiation

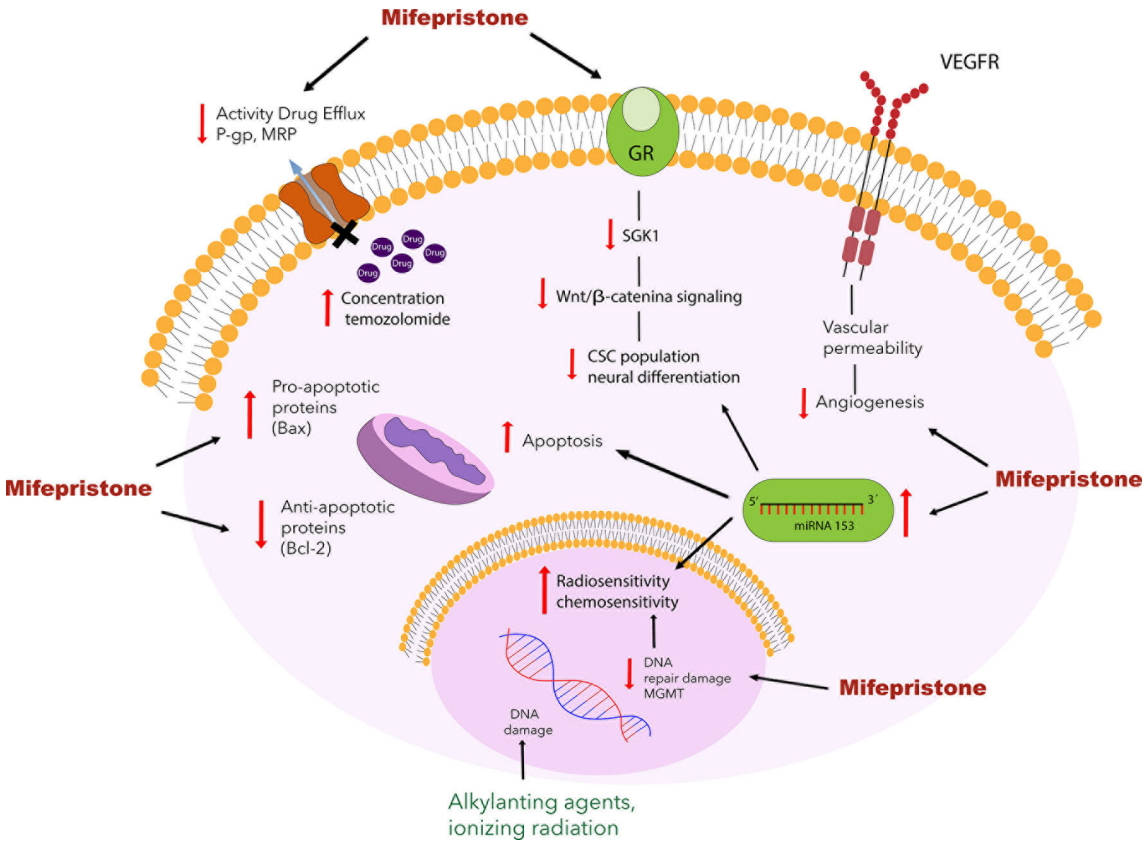

FIGURE 2 | Schematic portrayal of the possible mechanisms of mifepristone as a chemo-sensitizing agent for glioblastoma. Mifepristone decrease the level of anti-apoptotic protein Bcl-2 and increase the levels of pro-apoptotic protein Bax leading to apoptosis. Mifepristone has also been found to inhibit the expression of VEGF and block the function of drug transporting proteins such as P-gp and MRP. Another mechanism involved in the mifepristone-induced sensitization to temozolomide is the modulation of the expression of the MGMT enzyme, it has been reported that mifepristone reduces the expression of the MGMT protein in combination with temozolomide, leading to apoptosis. As described elsewhere, mifepristone decreases the growth of stem cells by inhibiting the Wnt/β-catenin signaling pathway, which involves glucocorticoid receptors and increasing the miRNA-153.

treatment for glioblastoma, which holds promise for improved therapeutic efficiency and patient overall survival.

AUTHOR CONTRIBUTIONS

ML-M and PG-L organized topics and contributed in manuscript drafting. MV-L and RJ collected the literature. All authors contributed to the article and approved the submitted version.

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REVIEW
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Overcoming Glucocorticoid Resistance in Acute Lymphoblastic Leukemia: Repurposed Drugs Can Improve the Protocol

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Glucocorticoids (GCs) are a central component of multi-drug treatment protocols against T and B acute lymphoblastic leukemia (ALL), which are used intensively during the remission induction to rapidly eliminate the leukemic blasts. The primary response to GCs predicts the overall response to treatment and clinical outcome. In this review, we have critically analyzed the available data on the effects of GCs on sensitive and resistant leukemic cells, in order to reveal the mechanisms of GC resistance and how these mechanisms may determine a poor outcome in ALL. Apart of the GC resistance, associated with a decreased expression of receptors to GCs, there are several additional mechanisms, triggered by alterations of different signaling pathways, which cause the metabolic reprogramming, with an enhanced level of glycolysis and oxidative phosphorylation, apoptosis resistance, and multidrug resistance. Due to all this, the GC-resistant ALL show a poor sensitivity to conventional chemotherapeutic protocols. We propose pharmacological strategies that can trigger alternative intracellular pathways to revert or overcome GC resistance. Specifically, we focused our search on drugs, which are already approved for treatment of other diseases and demonstrated anti-ALL effects in experimental pre-clinical models. Among them are some "truly" re-purposed drugs, which have different targets in ALL as compared to other diseases: cannabidiol, which targets mitochondria and causes the mitochondrial permeability transition-driven necrosis, tamoxifen, which induces autophagy and cell death, and reverts GC resistance through the mechanisms independent of nuclear estrogen receptors ("off-target effects"), antibiotic tigecycline, which inhibits mitochondrial respiration, causing energy crisis and cell death, and some anthelmintic drugs. Additionally, we have listed compounds that show a classical mechanism of action in ALL but are not used still in treatment protocols: the BH3 mimetic venetoclax, which inhibits the anti-apoptotic protein Bcl-2, the hypomethylating agent 5-azacytidine, which restores the expression of the pro-apoptotic BIM, and compounds targeting the PI3K-Akt-mTOR axis. Accordingly, these drugs may be considered for the inclusion into chemotherapeutic protocols for GC-resistant ALL treatments.

Keywords: acute lymphoblastic leukemia, glucocorticoid-resistance, drug repositioning, signaling pathways, tamoxifen, cannabidiol, BH3 mimetics, tigecycline

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) represents a heterogeneous group of hematological malignancies, originated from T (T-ALL) or B (B-ALL) cells progenitors. They suffered genetic alterations that preclude their further maturation and cause an unlimited self-renewal. Initially, malignant lymphocytes accumulate within the bone marrow (BM), where they constantly proliferate, displace healthy lymphoid precursors, devastate hematopoietic niches, and compromise the hematopoiesis. Later, a part of malignant cells leaves the BM and invades extramedullary sites, such as lymph nodes, spleen, liver, mediastinal space, and central nervous system (CNS). A proper treatment should begin immediately, otherwise clinical complications become incompatible with life (1, 2).

The established therapy consists of high-dose multi-agent protocols, which combine genotoxic drugs, antimetabolites, spindle inhibitors, and glucocorticoids (GCs). Albeit more than 80% of patients go to the remission after the induction therapy, there are also groups that are refractory to it. Many patients, who have reached the remission, will relapse later. A poor response to the initial GCs administration has been identified as a prognostic factor of unfavorable outcome (3, 4).

Over decades, synthetic GCs prednisolone (PRD), prednisone (PRED), and dexamethasone (DEX) were used widely as anti-inflammatory and immunosuppressive agents due to their lympholytic properties. They were among the first drugs, which were used for ALL treatment and remain as essential components of the antileukemic chemotherapy. The recent standard protocol, adopted by the Berlin-Frankfurt-Münster group, starts with 1 week of the GCs monotherapy, which serves as a prediction test and determines the future treatment strategy. Response to GCs varies among ALL patients, and GC resistance has been associated with an elevated risk of a minimal residual disease and poor survival (5–7). A more aggressive chemotherapy with toxic adverse effects is usually prescribed for these patients (3, 7–10). The understanding of underlying mechanisms could trigger the development of novel strategies that help to overcome steroid resistance in ALL.

Nowadays, much attention is paid not only to the development of new compounds, but also to a deeper understanding of the mechanisms of action of approved drugs, which may lead to their expanded or alternative use. Drug repurposing (or repositioning) is a very rational approach, since it implies the use of already approved drugs with identified mechanisms of toxicity and known side effects, thereby reducing the cost and time of the entire "from bench to bedside" process (11).

In the present review we have critically analyzed the available data regarding GC effects on leukemic cells, seeking the way to overcome the GC resistance by usage of certain repurposed drugs. The manuscript is divided into three parts. The first chapter describes the mechanisms of GC toxicity in sensitive cells. In the second chapter we discuss the mechanisms of GC resistance in ALL, with a focus on where the involved signaling pathways converge. In the third chapter we propose some drugs, already approved for treatments of other diseases, which can affect these converging points, thus overcoming/reverting GC resistance in ALL.

FACTORS DETERMINING GCs EFFECTS IN LYMPHOID CELLS

Endogenous and Synthetic GCs

Primary endogenous GCs (cortisol in humans) are steroid hormones, generally produced by adrenal cortex in a response to physiological and/or emotional stress. GC synthesis is under the regulation of the hypothalamus-pituitary-adrenal axis. The duration of GC secretion is rather short, the clearance rate is rapid, and elevated GC levels, achieved during acute stress response, quickly return to their basal values. Because most cellular types in mammals express receptors for GCs (GRs), GCs display systemic effects, including a potent immunosuppression [reviewed in (12)]. According to early observations, adrenocorticotropic hormone administration leads to a decrease in mass of lymphoid organs in rats (13). Numerous subsequent studies demonstrated that GCs change the production of some interleukins, cytokines, and adhesion molecules, and cause cell death in lymphocytes (12).

Inverse relationship between the size of adrenal gland and thymus, the primary lymphoid organ, where T lymphocytes maturate, was also observed (14). At the same time, local GCs are naturally produced by stromal cells in the thymic cortex, providing the GC-rich microenvironment required for the T cells selection (15, 16). A crosstalk between the T cell receptor (TCR)- and GR- triggered pathways determines pro-survival or pro-apoptotic fates of thymocytes (17, 18).

Pharmacological effects of endogenous and synthetic GCs are similar. But synthetic GCs possess a greater relative potency and are significantly more stable [reviewed in (12)].

Structural and Functional Diversity of GRs

GCs, being small lipophilic molecules, diffuse freely across the plasma membrane into target cells. Classically, they exert their

Abbreviations: AML, Acute Myeloid Leukemia; ALL, Acute Lymphoblastic Leukemia; ATG, Autophagy Related Gene; AURKB, Aurora Kinase B; BCR, B Cell Receptor; B-ALL, B cell Acute Lymphoblastic Leukemia; BM, Bone Marrow; CA4, Carbon Anhydrase 4; CB, Cannabinoid Receptor; CBD, Cannabidiol; CLL, Chronic Lymphocytic Leukemia; CNS, Central Nervous System; CRAC, Calcium Release Calcium Activated Channel; CREB, Cyclic-AMP Responsive Element Binding Protein; DEX, Dexamethasone; ER, Estrogen Receptors; ETP, Early T cell Precursor; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; GC, Glucocorticoids; GLUT, Glucose Transporter; GR, Glucocorticoid Receptors; GRE, Glucocorticoid Response Element; GSI, Gamma Secretase Inhibitor; HIF-1α, Hypoxia-inducible Factor-1 alpha; HMA, Hypomethylating Agents; HTS, High-Throughput Screening; IL, Interleukin; KD, Knockdown; LSO, Lymphocyte-Specific Open Chromatin; LSC, Lymphocyte-Specific Closed Chromatin; MAPK, Mitogen Activated Protein Kinases; MDM2, Murine Double Minute 2; MDR, Multidrug Resistance pump; mPTP, Mitochondrial Permeability Transition Pore; mTOR, Mammalian Target of Rapamycin; OXPHOS, Oxidative Phosphorylation; P-gp, P-glycoprotein; PI3K, Phosphatidylinositol 3-Kinase; PRD, Prednisolone; PRED, Prednisone; PTEN, Phosphatase and Tensin Homolog; SGK1, Serum and Glucocorticoid inducible Kinase 1; TAM, Tamoxifen; TCA, Tricarboxylic Acid; TCR, T Cell Receptor; TGC, Tigecycline; TRAIL, TNF-Related Apoptosis Inducing Ligand; T-ALL, T cell Acute Lymphoblastic Leukemia; VDAC, Voltage Dependent Anion Channel; $\Delta\Psi m$, Mitochondrial Membrane Potential.

effects by binding to their specific intracellular GRs, which are ligand-inducible transcriptional factors, belonging to the nuclear receptor superfamily. In the absence of a specific ligand, GRs are retained in the cytoplasm by their association with chaperone proteins. Ligand binding causes a formation of the GC-GR complex, its conformational change, and translocation to the nucleus, where it exerts genomic effects either through the direct binding to the specific DNA binding motif (the GC response element, GRE) or through the interaction with other transcriptional factors. GRE is an enhancer element, capable to modulate the activity of associated gene promoters, causing activation (transactivation) or inhibition (transrepression) of target genes expression [reviewed in (19, 20)]. Another, less appreciated regulatory function of GRs is related to the ability of GC-GR complexes to bind mRNA, triggering its rapid degradation (21, 22).

Although all GRs are encoded by unique *NR3C1* gene, their structure, stability, and functional characteristics are diverse. This diversity is generated by multilevel mechanisms at the transcriptional, post-transcriptional, translational, and post-translational levels [reviewed in (23–26)]. Based on these comprehensive reviews, here we briefly describe the mechanisms, relevant for GC resistance in ALL.

At the transcriptional level, there are several promoters that have alternative binding sites for various transcriptional factors that can increase or alternatively suppress the expression of the *NR3C1* gene (23). Among activators there are AP-1/AP-2, NF-κB, estrogen receptor (ER), cyclic-AMP responsive element binding protein (CREB), whereas GC responsive factor-1 and c-Ets-1/2 are reported as repressors. Interestingly, NF-κB also controls expression of anti-apoptotic and proliferative genes and it is frequently constitutively upregulated in ALL and may be related to drug resistance (27–29). AP-1 is involved in the GC response in ALL patients (30) and high CREB expression was correlated with a poor outcome (31).

Remarkably, *NR3C1* possesses binding sites for GRs themselves, providing an autoregulatory loop (23). Interactions of GRs with other relevant transcriptional factors can upregulate (interaction with c-Myb) or downregulate (interaction with c-Ets) the *NR3C1* expression (23). c-Myb was shown to interact with GR and enhances its expression level in pre-B-ALL (32, 33). Accordingly, a different tissue microenvironment and cellular context may contribute to the control of the *NR3C1* expression through upregulation of different transcriptional factors.

A different translation initiation of the GR transcript and an alternative RNA splicing result in a formation of several receptor isoforms, which possess different functional features (23–26).

Classical GRα protein is the most abundant isoform, accounting for about 90% of GR transcripts in all tissues (23). It efficiently binds GCs, possesses the nucleus-targeted sequence and DNA binding domain. Remarkably, there are eight alternative translation initiation sites in exon 2, resulting in eight GRα translational isoforms, named GRα-A to D, which are characterized by a different length of the N-terminal and by unique transcriptional target genes (34, 35).

Alternative splicing of the 9β instead of the 9α exon results in the GRβ isoform, which is unable to bind GCs, but is transcriptionally active (36). It resides constitutively in the nucleus and can alternatively regulate many genes, controlled by the GRα (37, 38).

GRγ isoform is less studied, but intriguing data evidencing unique GRγ properties were reported (39). GRγ is identical to GRα but contains an insertion of a single arginine near the nuclear localization signal, which slows down the nucleus-cytosol shuttling upon ligand binding when compared to GRα. GC and DNA binding capacities are similar to those of GRα, but their target genes are distinct. In particular, it was shown that GRγ controls nuclear genes, encoding mitochondrial proteins. GRγ is predominantly localized in the cytoplasm and in its unbound state targets mitochondria. The authors suggest unique functional profile of GRγ, which includes the regulation of mitochondrial function and ATP production.

Thus, distinct GR isoforms demonstrate non-redundant properties. Importantly, more than one isoform is usually found in the same cell, forming the cell-specific pattern. Consequently, cellular response to the GC application is the result of their complex crosstalk.

Stability of the GR mRNA is another factor, which may determine the GR expression level. mRNA stability is controlled by various mechanisms, including microRNAs (miRNAs) (23–26) and a previously mentioned GC-dependent mRNA decay (21, 22).

Further on, numerous GR mutations and polymorphisms may be related to either GC hypersensitivity or resistance [complete lists of GR mutations and polymorphisms known up to 2018 can be seen in (26)]. Finally, post-translational modifications, occurred at different physiological and pathological conditions, such as phosphorylation, ubiquitination, acetylation, nitrosylation or oxidation, are all capable to change the GR functional activity (23).

Effects of GCs on Sensitive Lymphocytes

Effects of GCs on lymphoid cells include G1-phase cell cycle arrest and cell death, predominantly via the intrinsic (mitochondrial) apoptotic pathway (17, 20, 40–43).

To understand the early response of leukemic cells to GCs, parallel time-course metabolomics, proteomics and isotope-tracing studies were performed recently, using the B-ALL—derived cell line RS4;11 (44). The earliest genomic effect (4 h after the GC exposure) is a downregulation of the proto-oncogenic transcription factor *MYC*. CDK4, responsible for cell cycle progression, is decreased, whereas apoptotic markers *BCL2L11* (encoded BIM protein) and *CD93* are increased over time.

Puffal's group reported that DEX repressed the expression of genes, coding for key regulators of the early B cell development (*ITGA4, IL7R, BCL6*) as well as various genes related to the B cell receptor (BCR) signaling (*CD79B, CSK, FYN, BTK, PIK3CD, PIK3C2B, PIK3R2*). Pro-survival *BCL2* and *MYC* as well as *CXCR4* coding for the BM homing receptor were reported among the repressed, whereas pro-apoptotic *BCL2L11* and the major regulator of cellular redox signaling *TXNIP* among the activated genes. Remarkably, the mechanisms of cell death are most likely redundant, because no one among pro-apoptotic genes was determined as absolutely required (45).

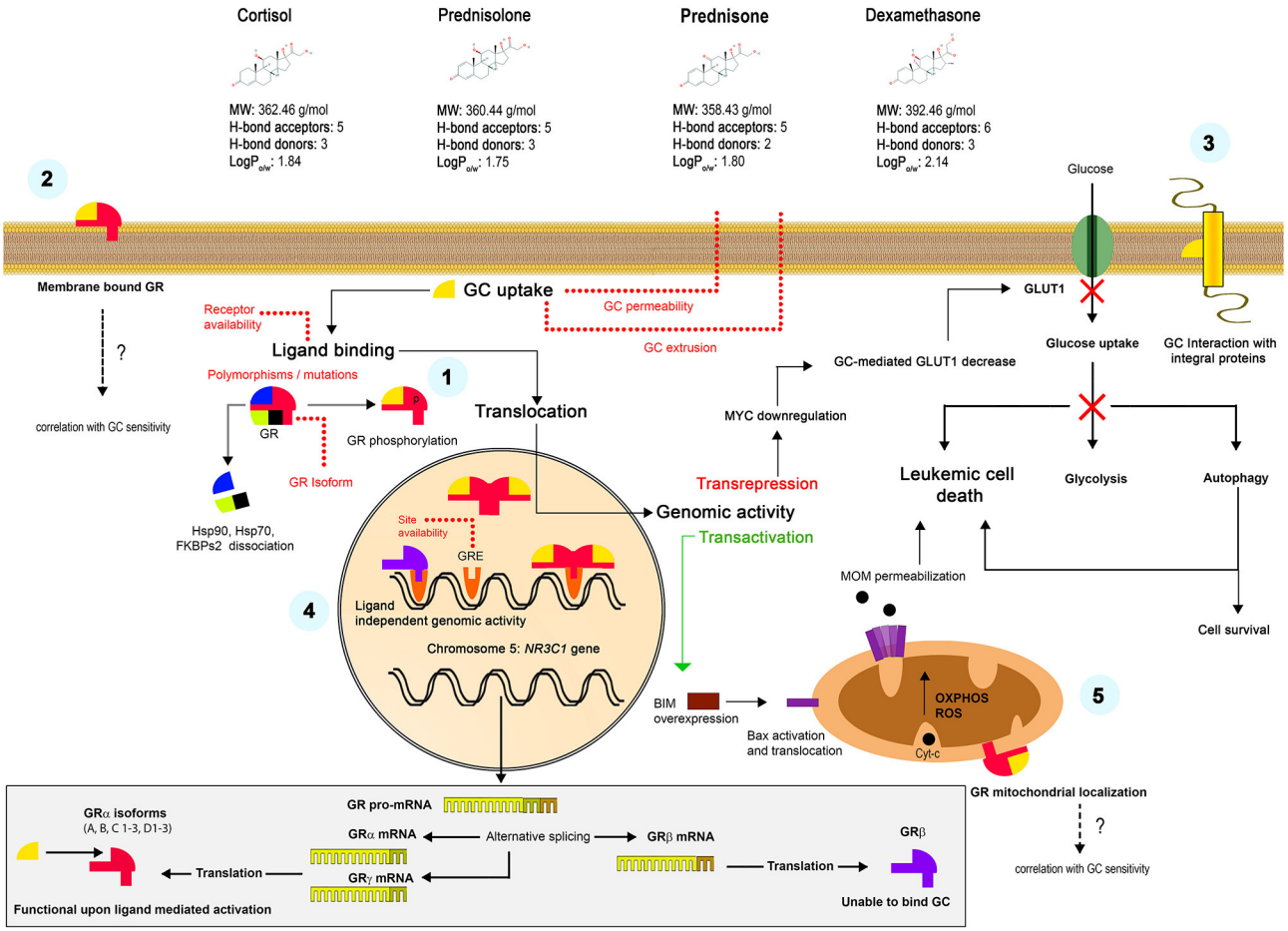

FIGURE 1 | Mechanisms of the GC action. Liposoluble GCs freely diffuse through the plasma membrane. Classically, they bind to specific intracellular GRs (α or γ isoforms) with the formation of GC-GR complexes, their posterior translocation to the nucleus and interaction with the GRE, which results in a transactivation or transrepression genomic activity (1). Alternative non-genomic mechanisms were also proposed, including the interaction with surface receptors (2) or the GCs retention in the plasma membrane and the interaction with integral proteins (3). Unable to bind GC, but transcriptionally active β isoform constitutively resides in the nucleus and can alternatively regulate many genes (4). GC-GR complexes translocate to mitochondria and interact with the OMM proteins, causing non-genomic effects (5). The lower panel shows the formation of alternative GR isoforms. See Chapter 1 for more details.

IL7R and BCR pathways, in turn, work through the PI3Kδ stimulation, leading to the activation of ERK/MAPK and Akt/mTOR axes, involved in growth and survival (45, 46). Accordingly, PI3Kδ inhibition enhances the GC-regulated cell death even in resistant B-ALL (45). Different ALL were shown to be heterogeneous in the strength of the PI3K signaling [(47), and references therein].

Cell and tissue specificities of GC effects also depend on a specific pattern of the chromatin accessibility. Although the GR-associated transcriptome of lymphoid cells has not yet been decoded, lymphocyte-specific open (LSOs) and closed (LSCs) chromatin domains, characterized by different methylation degree, were described. The Bcl-2 family member *BCL2L11* was recently identified in highly accessible chromatin regions, critical for the GC-induced cell death in lymphocytes (48). BIM protein, which precludes the anti-apoptotic activity of Bcl-2, Bcl-XL, and Mcl-1, was demonstrated to trigger apoptosis in GC-sensitive cells (48, 49).

It is well-known that endogenous GCs are essential for regulation of energy metabolism in different human tissues under physiological and stress conditions (50). Expression of metabolism-related genes changed considerably in a response to GC treatments in ALL, causing strong alterations in cell metabolism (51–54). DEX treatment reduces the surface expression of the glucose transporter GLUT1, resulting in a decreased glucose uptake and a profound inhibition of glycolysis, both in cell lines and primary ALL (55). A consequent apoptotic cell death was correlated with the inhibition of glucose uptake (55). GLUT1 gene is not a direct target for GRs. The mechanism, underlying the inhibition of glycolysis, seems to be related to *MYC,* which is known to induce the expression of glucose transporters and some glycolytic enzymes in leukemic cells, and it is downregulated rapidly during GC treatments (44).

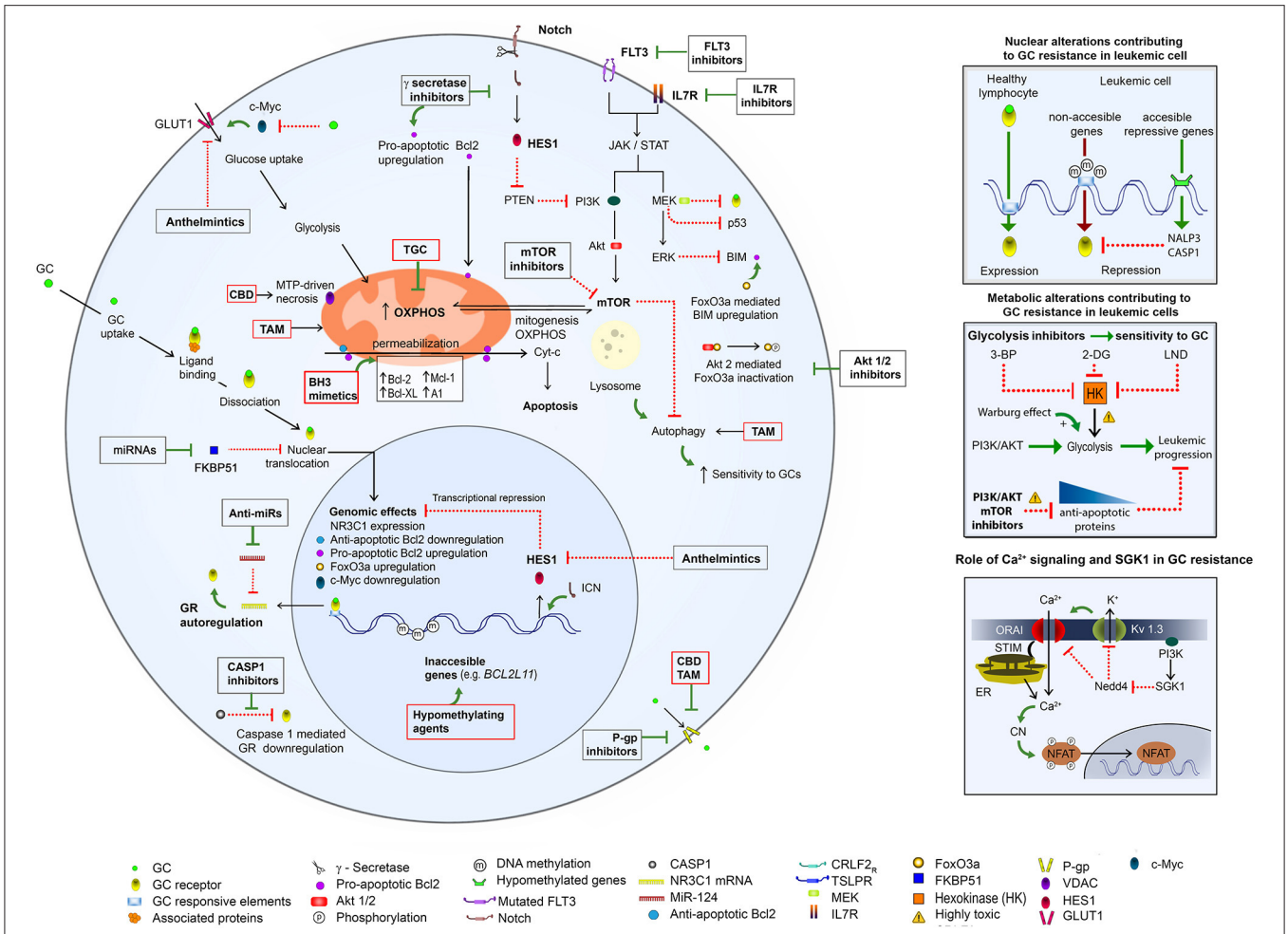

FIGURE 2 | An overview of mechanisms of GC resistance in ALL and pharmacological strategies to overcome it. (**Left**) GC resistance in ALL is related to different genetic aberrations (see references in the text), which cause (1) upregulation of Notch, IL7R, Flt3, and MEK/ERK pathways, with a consequent upregulation of PI3K/Akt/mTOR and Glut1 and acceleration of cellular growth and metabolism; (2) downregulation of the proapoptotic proteins (BIM) and upregulation of the antiapoptotic proteins (Bcl-2, Bcl-XL, Mcl-1, and A1), with a consequent apoptosis inhibition; (3) overexpression of MDR proteins. A hypermethylation of *BCL2L11* results in its inaccessibility to the transcriptional upregulation by a GR (**left and upper right**). The mTOR activation causes upregulation of glycolysis (**middle right**) and OXPHOS, and inhibition of autophagy. Upregulation of glycolysis can be opposed by the inhibition of hexokinase (HK), the first glycolytic enzyme. Ca^{2+} signaling is involved in the NFAT activation via the Ca^{2+}-dependent dephosphorylation by calcineurin (CN). A sustained Ca^{2+} signal is achieved due to a repression of the recycling of ORAI (main Ca^{2+} influx component) and Kv1.3 (mediating K^+ efflux, which supports Ca^{2+} influx) proteins via the PI3K/SGK1 pathway (**lower right**). The above mechanisms can be opposed by inhibitors of FLT3, IL7R, γ secretase, Akt1/2, P-gp, glycolysis, PI3K/AKT, SGK, and mTOR as well as by BH-3 mimetics and hypomethylating agents. A possible toxicity of PI3R/AKT/mTOR inhibitors as well as of glycolysis/ hexokinase (HK) inhibitors 3-bromopyruvate (3-BP), 2-deoxy-D-glucose (2-DG), 1-(2,4-dichlorobenzyl)-1H-indazol-3 carboxylic acid) (lonidamine, LND) needs to be considered. Some re-purposed drugs may improve antileukemic protocols: (1) TGC, which targets mitochondria and OXPHOS; (2) CBD, which targets mitochondria, causes the MTP-driven necrosis and inhibits P-gp; (3) TAM, which targets mitochondria, induces autophagy, inhibits P-gp and enhances the sensitivity to GCs; (4) anthelmintics, which inhibit GLUT1 and Hes1. For more details please consult the text.

A switch to the mitochondrial oxidative phosphorylation (OXPHOS) for ATP production is a rescue strategy in GC-treated leukemic cells, in case of suppressed glycolysis (56, 57). When glycolysis is inhibited, mitochondrial activity appears to rely on autophagy (56, 58). There are various reports on a massive accumulation of autophagosomes in GC-treated ALL cells (44, 59, 60). Originally, autophagy was evolved as a pro-survival mechanism under starvation, but when the threshold level is exceeded, it can eventually lead to cell death (61). A non-protective autophagy was suggested to be an important process, preceding cell death in GC-treated leukemic cells (44, 59, 60).

Some rapid effects of GCs could be explained by non-genomic mechanisms. In particular, the translocation of the GC-GR complex to mitochondria instead of nucleus, with a subsequent direct interaction with the Bcl-2 superfamily proteins and triggering on the intrinsic apoptotic pathway was evidenced in experiments on mouse thymocytes (16, 62, 63). Another mechanism proposed the interaction with surface GRs and triggering of alternative signaling pathways (64).

TABLE 1 | GC sensitivity and GR expression in T- and B-ALL.

ALL phenotype	Leukemic cells	Status/modifications	GC sensitivity	GR expression	Notch dependence	Components of resistance mechanism	References
T-ALL cell lines							
CD3+ CD4+ CD8-	Jurkat parental (wt)	Relapse CD4+	Resistant	*	+	GRs↓ Notch↑ PTEN↓ Akt↑ nuclear GR translocation↓	(66–69)
	Jurkat /GR-A	Stable expression of GR translational isoforms in Jurkat (wt)	Sensitive	****	ND	Reverted sensitivity to GCs	(70)
	Jurkat /GR-B		Sensitive	****		Reverted sensitivity to GCs	
	Jurkat /GR-C		Sensitive	****		Reverted sensitivity to GCs	
	Jurkat /GR-D		Resistant	****		Resistant, like Jurkat (wt)	
CD3+ CD4+ CD8-	CCRF-CEM parental (wt)	Relapse	Sensitive	***	+	-	(68, 71–73)
	CEM-C7-14	Sub-clone of CCRF-CEM	Sensitive	***as parental	ND	-	(74, 75)
	CEM-C1-15	Sub-clone of CCRF-CEM, isolated without selective GC pressure	Resistant	***as parental	+	ND	(7, 73–75)
	CEM-C7//HDR	Prolong culturing CEM-C7–14 under hypoxia +single Dex treatment	Resistant	*/–	ND	GRs↓	(75)
	CEM-C7//H	Prolong culturing CEM-C7–14 under hypoxia (no Dex)	Sensitive	****	ND	-	(75)
	6T-CEM	HPRT-deficient	Sensitive	***	ND	-	(66)
	CCRF-CEM/MTX R3	Selected for resistance to MTX	Resistant	**	ND	GRs↓, nuclear GR translocation↓	(68, 72)
cyCD3+ CD4+ CD8+	MOLT-3	Relapse	Resistant	*	+	Notch↑ PTEN↓ Akt↑ GRs↓ nuclear GR translocation↓	(67, 76)
CD3+ CD4+ CD8+	CUTLL1	Relapse	Resistant	**	+	Notch/HES↑	(77, 78)
CD3+ CD4+ CD8+	UP-A t(8:14)(q24;q11) translocation LL13	Diagnosis	Sensitive	ND	-	-	(79)
CD3+ CD4+ CD8+	HBP-ALL	Diagnosis	Resistant	***	+	Notch↑	(66, 80), (81)
CD3+ CD4+ CD8+	T-ALL1	Relapse	Resistant	ND	+	Notch↑	(66, 77, 80)
CD3- CD4+ CD8+	ALL-SIL	Relapse	Sensitive	**	+	-	(7, 66, 80)
Precursor T lymphoblast	DND-41	ND	Sensitive	**	+	-	(76, 80)
	KOPTK-1	ND	Resistant	*	-	Akt↑ Notch↑	(76, 80)
ALL lymphoblasts	KE-37	Diagnosis	Sensitive	****	+	-	(68)
B-ALL cell lines							
Pro-B	HAL-01 CD3-	t (17, 19)(q22;p13) TCF3-HLF (E2A-HLF) fusion gene	Resistant	*	ND	Apoptosis resistance nuclear GR translocation↓	(68)
Pro-B	UOC-B1	Relapse TBL1XR1 delitions	Resistant	****	ND	Decreased GR recruitment at gene regulatory regions nuclear GR translocation↓	(68, 82)
Pre-B	Reh parental (wt)	Relapse	Resistant	*/–	ND	GRs↓ BIM↓ p53↓ apoptosis resistance	(66, 68, 82)

(Continued)

TABLE 1 | Continued

ALL phenotype	Leukemic cells	Status/modifications	GC sensitivity	GR expression	Notch dependence	Components of resistance mechanism	References
	Reh/MEK4-KD	Reh wt MEK4 shRNA	Sensitive	**	ND	Reverted sensitivity to GCs: GR↑ + BIM↑	(82)
	Reh/MEK2-KD	Reh wt MEK2 shRNA	Sensitive	*/– as parental	ND	Reverted sensitivity to chemotherapy in general: pERK↓ + p53↑	(82)
Pre-B	NALM6 parental (wt)	Relapse	Sensitive	****	+	-	(66, 68, 83)
	NALM6/ DEX	NALM6 wt prolong exposure to DEX	Resistant	*	ND	GRs↓+ FLT3 point mutations	(84)
	NALM6/ HDR	NALM6 wt Prolong culturing under hypoxia + single Dex treatment	Resistant	ND	ND	Similar to CEM-C7//HDR?	(75)
	NALM6/ CELCR2-KO	NALM6 wt shCELSR2	Resistant	Lower than NALM6 (wt)	ND	Low ratio of BIM:BCL2 after PRED treatment	(85)
Pre-B	(697) parental (wt)	Relapse t(l;19) translocation	Sensitive	***	ND	-	(86)
	(697)/Bcl-2	Infected with recombinant Bcl-2 retrovirus	Resistant	*	ND	BCL-2↑ + GRs↓ + GSH↑	(87)
	(697)/DEX	(697): prolong exposure to DEX	Resistant	*	ND	GRs↓ + GSH↑	(84, 87)
Pre-B	RS4;11 Parental (wt)	Relapse	Sensitive	*****	ND	-	(84)
	RS4;11/DEX	RS4;11: prolong exposure to DEX	Resistant	*/–	ND	GRs↓+ FLT3↑ point mutations	(84)
Pre-B	(380) parental	Relapse Translocations: t (8, 14); t (14, 18)	Resistant	ND	ND	*IGH-BCL2* fusion *MYC-IGH* fusion Bcl-2↑ + Myc↑	(86, 88)
Primary samples							
Pediatric ALL patients (no phenotypes reported)	Xenografts	ND	Variable sensitivity depending on NR3C1 polymorphism	**	ND	BIM↓ in resistant samples	(68)
Pediatric ALL patients (no phenotypes reported)	*In vitro* primary samples	Relapse GR Somatic mutations not found in 49/50 patients	Variable sensitivity	Positive variable	ND		(89)
Pediatric ALL patients (no phenotypes reported)	Primary culture (BM/PB)	Diagnosis (43) Relapse (11)	Variable sensitivity	***	ND	Higher relation GRγ/GRα in resistant samples?	(90)
B-ALL	Freshly isolated (BM) primary cultures	Diagnosis	Resistant Sensitive	Variable	ND	Lower CELSR2 and higher BCL2 in GC resistant samples	(85)
Ph+-ALL	Xenograft derived strain (ALL-4CL)	Relapse	Resistant	**	ND	BIM ↓ Apoptosis resistance	(68)
CD11a+, CD19+;CD20+ CD38-; CD49e+	IM-9	ND	Sensitive	*	ND	-	(91)
Biphenotypic leukemias	Xenograft derived strain (ALL-7CL)	Diagnosis	Resistant	****	ND	BIM↓ Apoptosis resistance	(68)

(Continued)

TABLE 1 | Continued

ALL phenotype	Leukemic cells	Status/modifications	GC sensitivity	GR expression	Notch dependence	Components of resistance mechanism	References
9 ALL lymphoblasts	Primary cultures; patients exposed to PRD (n=49)	Relapse	Sensitive Resistant	*** *	ND	Positive correlation between endogenous expression of NR3C1 in ALL cells and sensitivity to GCs and clinical outcomes	(66)
ALL lymphoblasts	Primary culture of patients exposed to PRD (n=9)	ND	Sensitive Resistant	*** *	ND		(92)
T-ALL (incl. ETP) lymphoblasts	Primary culture from BM / Peripheral blood	Diagnosis	Resistant Variable depending on mutation	/– *** (mutated)	ND	GRs↓ (deletion) Truncated GR, unable to bind to DNA [G (371)X mutation]	(93)
ETV6/RUNX1- ALL	BM	Relapse	Resistant	*/–	ND	Patients with *NR3C1* aberrations display a poor prognosis	(94)

*Very low expression; **Moderate expression; ***Intermediate expression; ****Increased expression; *****Very high expression.
ND, Not determined.
HPRT, hypoxanthine phosphoribosyl transferase.
GSH, glutation.
MTX, methotrexate.

Finally, the accumulation of highly lipophilic GC molecules in plasma membrane was postulated, which alters the function of membrane integral proteins such as ion channels or receptors (65). Proposed mechanisms of the GC action in sensitive cells are summarized in the **Figure 1**.

MECHANISMS OF GC RESISTANCE IN ALL

Here we present evidence for multiple mechanisms of GCs resistance (summarized graphically in the **Figure 2**). Most likely, different mechanisms may be responsible for GC resistance in the same leukemic clone.

GC Resistance May Be Caused by an Altered GRs Expression

GRs Expression Is Heterogeneous in ALL

The level of the GRs expression among ALL patients and derived cell lines appears to be highly heterogeneous (**Table 1**). Sensitivity to GCs in hematological malignances was initially thought to be directly dependent on the number of functional GRs. This assumption seems to be logical and various reports confirmed it (66, 94–98), although conflicting results were also reported (7, 51, 71, 89). Regarding lineage differences, the levels of GRs were reported to be lower in T-ALL (90, 99–102), which is in line with the fact that T-ALL display GC resistance more frequently than B-ALL (6, 7). Additionally, a reduced binding affinity to DEX was revealed in T-ALL clinical samples as compared to B-ALL (102).

Importantly, GCs by itself may cause an acute decrease in GR expression. This effect varied significantly, depending on the leukemic phenotype and on the chosen therapeutic protocol. The receptor re-establishment was observed predominantly during the first 15 days after the last DEX administration (90, 103). Remarkably, GC-resistant clones isolated from relapsed ALL patients usually express lower GRs levels due to alterations in *NR3C1* expression (66, 92, 94, 103–106). These data suggest that a chronic exposition to GCs in newly diagnosed ALL patients can promote the appearance and selection of ALL clones with a low GR expression, their evasion and re-appearance during the relapse.

However, high GR expression was found also in GC-resistant cases (**Table 1**). The opposite situation, when a high GC sensitivity is paralleled with a low level of GRs, is rare. Some early studies reported GC resistant clones, derived from sensitive cell lines, with apparently unaltered functional cytosolic GRs (74). These data argue for alternative mechanisms of the GC resistance, rather than simply to be caused by a decreased GR expression.

Somatic Loss-of-Function Mutations and Polymorphisms in the NR3C1 Gene May Alter GC Sensitivity

Somatic loss-of-function mutations in the *NR3C1* gene may change a proper functionality of GRs. Recurrent *NR3C1* inactivating aberrations, including deletions, missense, and nonsense mutations, which can be detected already at the first diagnosis, were reported to be responsible for GC resistance in pediatric T-ALL patients (93). Xiao et al. (98) reported

frequent relapse-specific genetic alterations in adult patients with B-ALL, revealed by the longitudinal whole-exome sequencing analysis on diagnosis/ relapse pairs. In particular, recurrent truncated mutations were detected in the *NR3C1* gene (98). *NR3C1* deletions were also reported in ETV6/RUNX1-positive relapsed patients (94). Ectopic expression of the *NR3C1* reverses GC resistance, while the *NR3C1* deletion, in contrast, confers a resistance to GCs in ALL cell lines and xenograft models (66). Polymorphisms were found in healthy individuals and ALL patients. Moreover, it has been observed that GR polymorphisms conferred an increased or decreased GC sensitivity (107).

Epigenetic Regulation of *NR3C1* Expression May Be Altered in ALL

Alterations in the GR protein expression, associated with the methylation status of the *NR3C1* gene, have been described in some human pathologies [reviewed in (108)], but not in ALL. However, there are other epigenetic mechanisms regulating the *NR3C1* expression, such as silencing or repressive RNA. ALL patients exhibit high levels of miRNAs. In particular, miR-124 is overexpressed in GC resistant leukemic cell lines and poor PRD responders. *NR3C1* was found to be a target for miR-124, which acts as a GR suppressor, inhibiting the apoptosis induced by DEX (109). Conversely, *FKBP51,* a GR repressor that decreases GR autoregulation and activity, was shown to be a target for miR-100 and miR-99a. miRNA expression, which limits the *FKBP51*, reestablishes the *NR3C1* autoregulation and activity and confers GC sensitivity (110).

Differential Expression of GR Isoforms in ALL

Since GR isoforms are functionally non-redundant (see section Structural and Functional Diversity of GRs), their pattern in leukemic cells may be associated with a different GC sensitivity. In particular, GC resistance was associated with a high GR β/α ratio (111–113) that may be explained by the fact that GRβ alternatively regulates GRα-dependent genes (discussed in Structural and Functional Diversity of GRs). Pro-inflammatory cytokines TNFα and IL-1 can selectively upregulate the GRβ expression, as it was demonstrated for leukemic cell lines (112). Remarkably, leukemic niches in B-ALL are characterized by a proinflammatory microenvironment, producing enhanced levels TNFα, IL-1 and IL-12 (114) that may support the GC resistant phenotype.

GRγ is an important positive regulator of mitochondrial function (see section Structural and Functional Diversity of GRs). GRγ up-regulation is related to an increase in the mitochondrial mass, oxygen consumption, and ATP production (39). Accordingly, an enhanced GRγ expression is associated with some GC resistant cases (90, 115).

As far as different GR translational isoforms can mediate differential regulatory patterns of GC-induced genes, the question about their capacity to induce apoptosis in ALL was addressed. With genetically modified Jurkat cells, expressing individual GR isoforms GRα-A-D, it was demonstrated that DEX efficiently decreased Myc expression and induced apoptosis in GRα-A-B but not in GRα-D expressing cells (70).

GR May Be Cleaved by Upregulated Caspase 1

A low somatic methylation of the *CASP1* gene and its activator *NLRP3* was observed in ALL patients, with a resulting upregulation of caspase 1. It was revealed that GR may serve as a target for inflammasome and may be cleaved by caspase 1, resulting in a decreased receptors' number (116).

Alterations in Signaling Pathways in ALL May Be Involved in GC Resistance

Genetic alterations, which cause ALL, occur in two steps. Chromosomal rearrangements, which result in upregulation of oncogenic proteins and maturation arrest, are considered as driving leukemogenic events and are associated with unique expression profile. Gene rearrangements in ALL often place the oncogenic transcriptional factors under the control of promoters or enhancers of the BCR/TCR or *BCL11B* genes, among others. During the pre-leukemic phase additional mutations occur and give rise to ALL. These secondary mutations alter basic cellular processes, including survival, cell cycle progression, proliferation, and apoptosis. They are related to a variety of signaling pathways, including Notch, Il7R/JAK/STAT, RAS/MEK/ERK, and PTEN/PI3K/AKT/mTOR ones. Several comprehensive reviews, which describe in detail sequential genetic rearrangements and mutations in leukemogenesis were published recently (117–120). Targeting mutated genes and pathways was proposed as a basis for the "precision medicine" (121, 122). However, this strategy requires further studies, concerning a complex crosstalk between altered signaling pathways, to reveal the most promising therapeutic targets, in particular when patients present multiple genomic lesions (120). In addition, whereas genetic biomarkers are widely used for risk predictions in B-ALL, few genetic abnormalities were reported to show a prognostic significance in T-ALL (119, 123). Accordingly, functional studies should be of a primary importance. In a continuation we will discuss those signaling pathways, which are upregulated in GC resistant phenotypes, and intend to determine the most frequent abnormalities and convergent points.

Notch Activation

More than 50% of human T-ALL are known to exhibit Notch activating mutations (80, 119, 124). An enhanced expression of Notch receptors was also reported in B-ALL primary samples and cell lines (125). Remarkably, an aberrant Notch upregulation is associated not only with an increased proliferation but also with chemoresistance. Notch inhibition by a highly potent γ secretase inhibitor (GSI) reversed chemo- and GC- resistance in both B- and T-ALL [(77, 125); **Table 1**].

Notch is involved in the regulation of the *NR3C1* expression and GR protein levels. The underlying mechanism was shown to involve *HES1*, a transcriptional repressor, which is upregulated by Notch signaling and binds to *NR3C1* promoters, responsible for the GR autoregulation (67, 77, 126). Notch-dependent positive regulation of mTOR pathway in ALL is also related to *HES1* (127). *HES1* inhibits the tumor suppressor phosphatase and tensin homolog (PTEN), which is a negative regulator of the phosphatidylinositol 3-kinase (PI3K), whereas the activation of PI3K is the primary step in the PI3K–AKT–mTOR1 axis (128).

Notch acts as a positive modulator of the interleukin 7 (IL-7) receptor, IL7R (129).

Upregulation of Cytokines' Receptors in GC Resistance

IL-7 is a cytokine, produced by thymic and BM stroma, which supports survival and proliferation of both healthy and leukemic lymphocytes. Activating mutations in the *IL7R*α gene was reported in 6% of pediatric ALL, with a higher prevalence in T-ALL (130, 131). Primary T-ALL samples developed GC resistance, when cultured with IL-7 (123). GCs induce their own resistance by activating the IL7R (123). IL7R mediates its downstream effects through the JAK/STAT and PI3K/Akt/mTOR pathways. Deprivation of IL-7 or blockade of downstream effectors enhances the efficiency of DEX in T-ALL cells (132, 133). Whole genome and targeted exome sequencing, undertaken recently in T-ALL patients, revealed frequent (32%) IL7R mutations among the abnormalities, identified for 151 genes (134). Specific IL7R mutation, when expressed in steroid-sensitive cell lines, induces GC resistance through an upregulation of MEK-ERK and PI3K/Akt/mTOR. Accordingly, IL7R inhibitors revert the apoptosis development in response to GCs (134). Inhibitors of MEK and PI3K/Akt efficiently block the IL7R signaling (135).

The cytokine fms-like tyrosine kinase 3 ligand (FL) and its receptor FLT3 form an important axis in the hematopoiesis regulation. An aberrant up-regulation of FLT3 is commonly found in ALL, including a high intrinsic FLT3 level or gain-of-function mutations that promote constitutive FLT3 activity (136). Similar to IL-7, the FLT3 signaling converges with the PI3K/Akt/mTOR pathway. Some GC resistant ALL cells are characterized by a constitutive activation of the FLT3 signaling. They also exhibit a suppressed GR activity due to the Akt-mediated phosphorylation (76, 84).

MAPK Axis

Genome-scale short hairpin RNA screening was used to identify the mediators of GC resistance in B-ALL cell lines (137). Two different mitogen-activated protein kinases (MAPK), MEK2 and MEK4, were shown to be important for GC resistance but act through distinct mechanisms. MEK4 knockdown (KD) significantly increases both GR expression and transcriptional activity. The latter phenomenon seems to be related to the phosphorylation of GR on Ser226, which may cause its nuclear export and degradation. Accordantly, PRD-induced expression levels of *GILZ* and *BIM*, related to apoptosis, are higher in MEK4 KD samples. In contrast, MEK2 KD does not affect the GR expression but increases the sensitivity to various cytotoxic agents. Underlying mechanism involve the MEK2-dependent ERK suppression, which in turn causes an upregulation of the p53 and sensitizes leukemic cells to a drug-induced apoptosis (137).

Metabolic Re-programming and Upregulation of the PI3K/Akt/mTOR Pathway Is Related to GC Resistance in ALL

As for other cancer types, a re-programmed energy metabolism is typical for ALL. It includes the upregulation of both glycolysis and OXPHOS. ATP production predominantly via glycolysis (Warburg effect) gives the advantage to use the truncated tricarboxylic acid (TCA) cycle for biosynthesis of lipids, proteins, and nucleic acids (57, 138). The expression pattern of genes, associated with the glucose metabolism, is different in GC-sensitive and GC-resistant B-ALL. In particular, expression levels of hypoxia-inducible factor-1 alpha (HIF-1α), glucose transporters, carbonic anhydrase 4 (CA4), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are significantly higher in GC-resistant ALL (52, 139). An enhanced glucose consumption and glycolytic rate are correlated with GC resistance in ALL. Inhibition of glycolysis, either by RNA of interference or by synthetic compounds, reverts GC resistance in cell lines and primary samples of both B- and T-ALL (52, 54). Inhibitors of glycolysis and OXPHOS pathways were shown to enhance the sensitivity to GCs in T-ALL *in vitro* (54). A synergy between GCs and metabolic inhibitors was suggested as a valuable strategy for ALL treatments (56).

Under conditions of an increased rate of glycolysis and, as a consequence, limited availability of pyruvate, the TCA cycle is replenished with glutamine, which also leads to an increase in the rate of glutaminolysis in most types of cancer (57, 138, 140). Leukemic cells require a rapid source of ATP and, at the same time, enough biosynthetic precursors, for their accelerated proliferation. Consequently, ALL, especially GC-resistant ones, predominantly make ATP via glycolysis (54), whereas glutaminolysis serves as an extra source of biosynthetic precursors (57, 140). In Notch1-induced T-ALL, glutaminolysis represents a key carbon source and is critically dependent on the up-regulation of mTOR pathway (127, 141, 142). This metabolic reprogramming was shown to induce resistance to anti-Notch1 therapy (141). Consequently, the inhibition of glutaminolysis and mTOR was proposed as a potential strategy against Notch1-driven and, even, against anti-Notch1 therapy resistant ALL (141, 142). GCs not only suppress glycolysis, but also prevent the entry of glutamine into TCA cycle (44). It remains to be elucidated, whether the suppression of glutaminolysis by GCs in GC-resistant ALL is insufficient to minimize its metabolic contribution.

A balance between OXPHOS and glycolysis is under the control of the outer mitochondrial voltage-dependent anion channel, VDAC1, which mediates ion and metabolite exchange between mitochondria and cytosol (143). A comparison of GC-resistant and GC-sensitive B-ALL lines revealed that an enhanced VDAC1 expression is a crucial biomarker for GC-resistance (144). In addition to its role in the metabolic reprogramming, the VDAC1 closed or open conformation favors ALL death or survival, respectively (57).

PI3K-Akt pathway is constitutively hyperactivated in more than 80% of primary T- and B-ALL (45, 47, 145–149). Mutations in *PIC3CA* and *PIKRA*, encoding catalytic and regulatory PI3K subunits, are observed frequently in different ALL subtypes (150). Akt up-regulation is required for an increased glucose metabolism, which underlies a sustained cell growth (126, 151). Akt up-regulation is characteristic for GC-resistant phenotypes (152). Akt phosphorylates GR at Ser134, which prevents its translocation from the cytosol to the nucleus (76). In T-ALL,

the inhibition of Akt2 enhances the sensitivity to GCs more efficiently than the inhibition of Akt1 (152).

Critical downstream effector of the Akt is the mammalian target of rapamycin (mTOR), which is upregulated in many cancers (153, 154). mTOR contributes to leukemogenesis and GC resistance in ALL (155). PI3K/Akt/mTOR pathway appears to be critical for a proliferative response of leukemic cells to CXCL12, IL-7 and different stroma-derived mediators (156). Notch1 and Akt pathways interplay in ALL through *HES1*, which negatively controls *PTEN*, the main negative regulator of Akt signaling [(126, 151), see also section Notch Activation]. Overall, T-ALL patients often display an increased PI3K/Akt/mTOR pathway activation (145, 157). mTOR is known as an important regulator of a balance between survival, autophagy, and cell death (153, 154). The underlying mechanism to a large extent is related to mTOR involvement into the regulation of mitochondrial function and biogenesis [(158) and references therein].

Autophagy May Be Involved in GC Response

Autophagy is an essential recycling process, which is responsible for degradation of unnecessary, dysfunctional or damaged organelles and proteins in living cells. mTOR is a central checkpoint that negatively regulates autophagy. Metabolic stress is known to cause autophagy (61). In the context of anti-cancer treatments, autophagy may allow cells to survive during chemotherapy but may also act as a pro-death mechanism. This dual outcome is reported for various types of cancer (159, 160), including acute leukemias of myeloid and lymphoid lineages (161).

During unfavorable metabolic circumstances, caused by chemotherapy, autophagy may provide energy and macromolecules, required for survival and proliferation of cancer cells. Autophagy is an important mechanism, which maintains OXPHOS in leukemic cells, when glycolysis is inhibited by GCs (see section Effects of GCs on Sensitive Lymphocytes). As a result, GC-treated cells may be more sensitive to mitochondria-targeted compounds. A combination of these two classes of drugs was shown to cause a synergistic effect (56).

The expression of autophagy-associated genes was studied in samples, derived from B-ALL pediatric patients, where a differential expression was demonstrated for the GC-sensitive group as compared to the GC-resistant one (162). In general, key autophagy inducer genes are downregulated, while the inhibitors of autophagy are upregulated in GC-resistant cells (162). Activation of *BECN1*, a key autophagy inducer, is required for the DEX-dependent cell death in ALL (42) and for a sensitization of DEX-resistant ALL cells to obatoclax (163, 164) and MEK1/2 inhibitor (165).

In GC-resistant, in contrast to GC-sensitive ALL cell lines, autophagy is not induced by DEX (60, 164). Interestingly, a sensitization to GCs is achieved in GC-resistant Jurkat cells by a co-treatment with the autophagy-inducing drug tamoxifen (TAM) (166). Obatoclax reverts the GC resistance through the autophagy-dependent necroptosis, while knock-down of the autophagy-related gene 7 (*ATG7*) and *BECN1* completely prevents the re-sensitization to DEX (163, 164). These data indicate that autophagy can contribute to death of GC-treated cells.

Hypoxic Conditions Favor the GC Resistant Phenotype

BM leukemic niches represent a sanctuary for blasts, which therefore evade chemotherapy and are responsible later for a relapse. Like hematopoietic niches, they possess a hypoxic microenvironment (167). Leukemic cells, cultured under hypoxic conditions *in vitro*, were shown to develop the GC resistance (75, 168). Hypoxia is a signal, regulated mainly by the HIF-1α. Under hypoxic conditions, T-ALL cells up-regulate the HIF-1α expression, which activates Notch1 signaling, favoring cell cycle progression and limiting GC sensitivity [(67, 77, 169); discussed in Notch Activation]. Under hypoxic conditions, HIF-1α is overexpressed and ALL cells response to PRED is impaired, as evidenced by lower levels of BIM and higher levels of antiapoptotic proteins Mcl-1 and Bcl-2 (168). Therefore, hypoxia, together with a high production of IL-7 and Notch ligands (see Notch Activation and Upregulation of Cytokines' Receptors in GC Resistance), form a complex microenviromental network in leukemic niches, favorable for the maintenance of GC resistant clones. GC-resistant ALL cell lines, derived from relapsed cases, show mostly a low GR level (**Table 1**).

Resistance to GCs Can Be Mediated by Ion Channels and Ca^{2+} Signaling: The Role of SGK1

Ca^{2+} signaling is a principal component in the activation of healthy lymphocytes via TCR/BCR and the expression of about ¾ of genes, involved in the activation, is Ca^{2+}-dependent (170). ALL cells proliferation does not depend on the antigen binding to TCR or BCR, but still relies on the otherwise altered Ca^{2+} signaling. There is also an invariant signaling axis for the proliferation of both healthy lymphocytes and ALL cells, including a sustained Ca^{2+} influx via the plasma membrane Ca^{2+} channel, CRAC, Ca^{2+}-binding protein calmodulin, which activates calcineurin; the latter dephosphorylates the NFAT, allowing its import by nucleus and a consequent initiation of genes transcription [**Figure 2**; for a review see (170, 171)].

GCs acutely induce the expression of serum-and-glucocorticoid-inducible kinase-1 (SGK1), which is involved in a variety of pathologies, including tumor growth and resistance to GC-chemotherapy [for a review see (172, 173)]. In particular, SGK1 was found among key upregulated genes in GC-resistant B-ALL (162). Among multiple SKG1 targets is the Orai-1, the main channel-forming subunit of CRAC. SGK1 phosphorylates the Nedd4-2 protein, which binds then the 14-3-3 protein. The resulting protein complex is unable to ubiquinate the Orai-1 protein, thus precluding its degradation (174). An enhanced CRAC activity underlies pro-survival scenarios in tumor cells (174, 175). Activation of CRAC by thapsigargin suppresses, whereas chelation of intracellular Ca^{2+} potentiates, the sensitivity of ALL to the GC treatment (176).

CRAC is not unique route for Ca^{2+} entry. TRPV5 and TRPV6 channels, which display a high Ca^{2+}/Na^{+} selectivity, are scarcely expressed in quiescent healthy T cells, but robustly in T-ALL (177, 178). SGK1 activity increases the membrane surface

expression of TRPV5 and TRPV6 channels (174, 179). Another important member of the TRP channels family, TRPC3, is less selective albeit permeable for Ca^{2+}. In T-ALL upon the mitogenic stimulation it can mediate an extra Ca^{2+} signal, additional to the CRAC-generated one (180). Notably, TPC3 gene expression is strongly upregulated upon T-cells activation (177). A specific block of the TRPC3 by Pyr3 suppresses the GC-induced Ca^{2+} signal in ALL and synergistically enhances the DEX-mediated cell death (176). K^+ efflux via K^+-selective channels causes membrane repolarization, which underlies a sustained Ca^{2+} entry via CRAC [**Figure 2**; (170, 171)]. Voltage-dependent K^+ channels in B and T cells are functionally represented by the single member, Kv1.3 (181, 182). The Kv1.3 current is robustly presented in T-ALL, albeit it is lacking in B-ALL (183, 184). The surface expression of Kv1.3 channels is downregulated by Nedd4-2 and upregulated by different SGK isoforms (185). Therefore, it may be hypothesized that an increase in the Kv1.3-mediated current by SGK1 may contribute to the GC-resistance in T-ALL but not in B-ALL, via a promotion of Ca^{2+} entry. In several malignant tumors, including ALL, there is an aberrant expression of the cardiac K^+ channel hERG, which in its non-conducting (closed) state forms the signaling complex with β-integrin and CRC4. This aberrant signaling complex mediates both ERK1/2 and PI3K/Akt pro-survival pathways, which cause the SKG1 induction. Consequently, hERG1 contributes to the GC resistance in B-ALL, whereas a pharmacological block of hERG1 sensitizes B-ALL to GC treatments (186). Development of low molecular weight inhibitors with a high (100-fold) preference for SGK1 as compared to the generically similar kinase Akt and preclinical tests on colorectal cancer supports a synergistic effect of the SGK1 inhibitors with radio- and chemotherapy (173). At the same time, SGK1 can increase the degradation and ubiquitylation of Notch protein (187) and Notch pathway is up-regulated in most patients with T-ALL [reviewed in (171)].

Alterations in the Regulation of Apoptosis Are Related to GC Resistance

Bcl-2 Superfamily

The GC-induced cell death in sensitive leukemic cells is executed mainly through the intrinsic apoptotic pathway (discussed in section Effects of GCs on Sensitive Lymphocytes). Specific pattern and interactions of pro- and anti-apoptotic proteins of the Bcl-2 family determines the sensitivity to the apoptosis in leukemias (57).

The pro-apoptotic BIM, belonging to the BH3-only group, is the most studied in ALL. As it was mentioned previously, GC administration causes BIM overexpression in sensitive cells (68, 188), since GCs bind and stimulate the promoter, situated in lymphocyte-specific open chromatin domains (48, 49). In contrast, BIM enhancer was found to be highly methylated and therefore inaccessible for transcription in the GC resistant ALL (48).

Transcription factor FoxO3a is a well-known BIM regulator, which binds to BIM promoter and enhances BIM expression in a sensitive phenotype (152, 189). Akt2 kinase, up-regulated in the GC-resistant ALL, is responsible for Fox3a phosphorylation at Ser253. Resulting p-FoxO3a (Ser253) form is unable to translocate to the nucleus. Akt2 possesses a stronger binding capacity to FoxO3a than Akt1. Akt2 silencing significantly decreases FoxO3a phosphorylation at Ser253 and Akt2 inhibitors efficiently restore the GC resistance in ALL. DEX administration can upregulate the FoxO3a expression and decrease the p-FoxO3a, as a result favoring BIM expression and apoptosis (152).

In T-ALL, the inactivation of Notch signaling as well as limitation of the PI3K/mTOR pathway leads to a decreased Akt expression and activity, thus, promoting FoxO3a nuclear translocation and upregulation of BIM expression (77, 190).

Mutations of genes, which activate the extracellular signal-regulated kinase (ERK) pathway, are recurrently found in ALL. It has been also observed that BIM protein can be phosphorylated by ERK at Ser55, Ser65, and Ser100, preventing its efficient interaction with BAX, and, consequently, impeding apoptosis (191, 192).

ALL cells usually display high levels of anti-apoptotic proteins [reviewed in (35)]. In particular, a high level of Mcl-1 expression was associated with the resistance to PRED in MLL-rearranged infantile ALL clinical samples (193). Downregulation of Mcl-1 by RNA of interference induces PRED sensitivity in an ALL cell line (193). A comparative analysis of gene expression in clinical samples, obtained from children diagnosed with B-ALL, reveales an upregulation of the pro-survival Bcl-2 family members Mcl-1 and Bcl-2A1 (A1) in GC-resistant samples (162). Overexpression of Bcl-2 as well as of Mcl-1 tends to protect against the GC-induced apoptosis *in vitro* (139, 194). It was suggested that upregulation of Mcl-1 is related to upregulation of Akt/mTOR pathway (127). In this context, mTOR inhibitor rapamycin causes Mcl-1 downregulation and sensitizes ALL cells to GCs (188). Similarly, PI3K/mTOR inhibitor BEZ235 decreases the levels of the pro-survival Bcl-2 members but increases that of BIM (190).

p53 and MDM2

Mutations that inactivate p53, a genome-guardian protein, responsible for genetic stability and DNA repair, are frequently observed in several cancer types. The murine double minute 2 (MDM2) protein represents the main negative regulator of the p53 activity. MDM2 overexpression has been found in BM samples from ALL patients. Interestingly, p53 expression in MDM2 overexpressing patients is poorly detected, which correlates with an unfavorable outcome (195). In 11 different T- and B-ALL cell lines high levels of MDM2 are detected. Also, the analysis of 42 B-ALL relapsed patients demonstrated that most of them possess MDM2 alterations. Those, who failed to re-induce a remission after the chemotherapy with the use of PRED, were characterized with a high level of MDM2 expression (196, 197). MDM2 contribution to GC resistance was also evidenced in a preclinical ALL model. Mixed lineage leukemic xenografts in deficient mice were treated with DEX and, additionally, with RG7112, a MDM2 inhibitor. Mice, treated with RG7112, display p53 overexpression and cell cycle arrest, while DEX efficiency to induce apoptosis is increased (198). It has been reported that GR can interact with p53 and, upon GC administration, MDM2 was recruited, promoting a degradation of both GR and p53 (199).

Multidrug Resistance Contributes to GC Resistance

Other aspect that might explain the lack of sensitivity to GCs is a higher expression of drug-efflux pumps or transporters such as P-glycoprotein (P-gp), multidrug resistance 1 (MDR1) pump, and multidrug resistance-associated protein, MRP1 (200). Although P-gp is overexpressed both in GC- resistant and sensitive pre-B ALL cells, its activity does not correlate with GC sensitivity (87, 201). As it was demonstrated recently, inhibition of an upregulated MDR1 in a GC-resistant B-ALL sensitized cells to DEX (202).

Integrative Genomic Analysis as a Tool to Reveal Key Elements in GC Resistance

Many pathways are involved in GC resistance in ALL (see sections GC Resistance May Be Caused by an Altered GRs Expression, Alterations in Signaling Pathways in ALL May Be Involved in GC Resistance, Alterations in the Regulation of Apoptosis Are Related to GC Resistance, and Multidrug Resistance Contributes to GC Resistance). The challenge is to reveal how these different pathways interact, to determine the exact position of each component and key elements in a complex signaling.

Functional genomics studies and a genome-wide shRNA screen, performed by Pufall's group, have identified two classes of GC-regulated genes, which contribute to GC sensitivity in B-ALL: (a) effector genes, which contribute to cell death and (b) buffering genes, which decrease GC efficacy. Aurora kinase B (AURKB) is overexpressed in resistant ALL in the relapse and is involved in the GC signaling by phosphorylation and suppression of the GR coregulator complex EHMT1/2. AURKB inhibitors potentiate GC sensitivity in B-ALL cell lines and relapsed clinical samples by enhancing GC regulation of effector genes (203).

Pharmacogenetic complex approach, based on three novel methods, was recently suggested by Evans group (85). They combine the polygenomic analysis of primary B- and T-ALL cells with an advanced biostatistical method, in order to identify genes, associated with GC resistance. Further on, they undertook a genomewide CRISP-knockout screening in human ALL cell lines, to prioritize genes, which determine GC resistance. This integrated approach corroborated a polygenomic character of GC resistance. Numerous previously known genes and pathways were confirmed, namely, those involved in B cell development, BCR and IL7R signaling, apoptosis, drugs transport, and inflammation. But, in addition, 14 previously not tagged genes, underlying GC resistance, were identified. Among these is *CELSR2,* which is suppressed in GC resistant samples, possessing also a lower *NR3C1* and a higher *BCL2* expression. A novel resistance mechanism was suggested, where the CELSR2 protein, as a mediator of a non-canonical Wnt signaling (204), positively controls the *NR3C1* and negatively the *BCL2.* Based on these findings, a combined treatment with PRD and Bcl-2 antagonist venetoclax was proposed and successfully validated on *CELSR2* knock-down leukemia cells and xenografted models.

The whole genome sequencing on paired diagnostic and remission T-ALL samples revealed mutations, associated with a resistance to different therapeutics. In particular, IL7R, JAK1, NRAS, and AKT abnormalities are related to the GC resistance, without affecting the sensitivity to vincristine or L-asparaginase (205). Subsequent functional studies revealed that GC resistance was associated with MEK-ERK and AKT/mTOR axes, and upregulation of the pro-apoptotic MCL1 and BclXL.

RE-PURPOSED DRUGS CAN HELP TO OVERCOME GC- AND CHEMORESISTANCE IN ALL

Metabolic upregulation and apoptosis resistance represent convergent points for various signaling pathways, involved in GC resistance in ALL (chapter 2). Consequently, in this section we will introduce the compounds that target precisely these mechanisms, with the focus on the agents already approved by the Food and Drug Administration (FDA) for treatments of some types of cancer or other diseases that demonstrate promising results in preclinical models of the GC-resistant ALL. These drugs may be divided into two groups: (1) drugs with a novel mechanism of cytotoxicity in ALL, which was not considered at the initial approval; (2) drugs that demonstrate the classical mechanism of cytotoxicity in ALL. The data are summarized in the **Table 2** and drugs effects are shown in the **Figure 2**.

Re-purposed Drugs With a Novel Mechanism in ALL

Antibiotic Tigecycline Can Efficiently Control Infections and Kill Leukemic Cells by Targeting Mitochondria

Tigecycline (TGC) is the first commercially available glycylcicline, belonging to a new class of antibiotics, derived from tetracycline (211). TGC binds the bacterial 30S ribosomal subunit and inhibits the bacterial protein translation. It is extremely effective against a broad spectrum of gram-positive and gram-negative pathogens, including the multidrug-resistant ones. Due to similarities between bacterial and mitochondrial ribosomes, TGC is able to suppress the synthesis of mitochondria-encoded proteins, required for OXPHOS, and is efficient in a suppression of some cancer types (212, 229–234). At the same time, TGC exhibits a low toxicity for healthy tissues (229, 233, 234). In addition to the effect on mitochondrial function, TGC inhibits the Wnt signaling and induces autophagy in cervical and gastric cancers (230, 231). Remarkably, TGC is especially effective against therapy-resistant chronic myeloid leukemia stem cells: it inhibits OXPHOS and proliferation and increases their sensitivity to antileukemic drugs (212). As it was discussed in the previous chapter (see section Metabolic Re-programming and Upregulation of the PI3K/Akt/mTOR Pathway Is Related to GC Resistance in ALL), an increased OXPHOS level is a hallmark of GC resistance in ALL. Thus, a possibility of TGC use in therapeutic protocols against ALL is worth to be explored. Up to now, a single pre-clinical study of TGC cytotoxicity against ALL is reported (213). They demonstrated that TGC inhibited mitochondrial respiration, effectively triggered apoptosis and acted synergistically with standard

TABLE 2 | Candidates for drug repurposing against the GC-resistant ALL.

Compound and original mechanism	Original indications	ALL Model	Mechanism/ Effects in pre-clinical experiments with ALL	Considerations	References Clinical trials identification number*
2.A. Repurposed drugs with novel mechanisms described in ALL					
Cannabidiol The mechanism is uncertain or there are multiple mechanisms. Different receptors/ targets were proposed as candidates: cannabinoid receptors CB1/CB2; orphan receptors GPR55, serotonin 5-HT_{1A} receptors, μ– and σ– opioid receptors, some ion channels.	FDA approved for treatments of Lennox-Gastaut and Dravet epileptic syndromes: Epidiolex® (oral solution); Sativex® (spray, equal amount of CBD and THC) Arvisol® (oral tablets with pure CBD)	GC-resistant and GC-sensitive continuous T-and B-ALL cell lines	*The novel mechanism*: targets the VDAC channel in the outer mitochondrial membrane, promotes the formation of mPTP and disturbs calcium homeostasis. *Effects*: enhances autophagy (at low concentrations) and the MPT-related necrosis (at high concentrations), decreases migration.	Routes of administration, vehicle, concentrations, and synergism with other drugs should be considered	(206, 207) *Clinical trials.* As a single agent: NCT02255292 (solid tumors); in a combination with surgery/ radiation: NCT04428203 (prostate cancer); in a combination with chemo- and radiotherapy: NCT03246113, NCT03529448 (glioma); NCT03607643 (gastrointestinal malignancies, glioblastoma multiforme and multiple myeloma).
Ivermectin Milbemycin Moxidectin	FDA approved (Stromectol®) for the treatment of intestinal parasites	B and T lymphoblasts from relapsed patients, cocultured with stromal cells and xenografts	*The novel mechanism*: promotes the intracellular chloride increase and mitochondrial permeabilization *Effects*: induction of the intrinsic apoptosis	Synergism with BH3 mimetics and GCs	(208)
Mebendazole Benzimidazole anthelminticagent; binds to the β-tubulin and inhibits cell proliferation.	FDA approved (Vermox™) for the treatment of gastrointestinal worm infections	GC-resistant and GC-sensitive T cell lines.	*The novel mechanism:* promote Notch1 and Hes1 suppression. *Effects:* enhances the GR autoregulation.	Relatively safe even at high doses, effective in nM concentrations, can be administrated by via oral	(73, 209) *Clinical trials* NCT03925662 (colon cancer); NCT02644291 (brain tumors); NCT03628079, (gastric cancer); NCT01729260, NCT01837862 (glioma); NCT02201381 (different cancers)
Niclosamide	FDA approved (Niclocide ®) for the treatment of tapeworm infections	GC-resistant and GC-sensitive T cell lines (CCRF-CEM, CEM/ADR5000).	*The novel mechanism*: binds to the glutathione synthetase and limits the NFAT expression. *Effects:* ROS accumulation, decreases the proliferation, interleukin production.	Effective doses are achievable and safe	(210) *Clinical trials* NCT03123978, NCT02807805, NCT02532114 (prostatic cancer); NCT02687009, (colon cancer); NCT02519582 (cancer colorectal); NCT04296851 (adenomous polyposis)
Tamoxifen A non-steroid competitive antagonist of nuclear estrogen receptors.	FDA approved (Nolvadex®) for the treatment of metastatic ER-positive breast cancer	GC-resistant cell line (Jurkat)	*The novel mechanism*: binding to the GPER and "off-target" effects *Effects*: causes the autophagy and reverses the sensitivity to GC (non-toxic concentrations); decreases proliferation, causes cell death (at high concentrations)	Effective doses, the protocol for application in pediatric patients	(166) *Clinical trials* NCT00108069 (glioma); NCT00256230, NCT00492505 (melanoma); NCT00710970; NCT02197897 (bladder cancer).

(Continued)

TABLE 2 | Continued

Compound and original mechanism	Original indications	ALL Model	Mechanism/ Effects in pre-clinical experiments with ALL	Considerations	References Clinical trials identification number*
Tigecycline The glycylcycline, a broad spectrum tetracycline antibiotic derivative; binds to the bacterial/organellar ribosome and suppresses the protein synthesis	FDA approved (Tygacil®) for the treatment of complicated skin and skin structure infections, complicated intra-abdominal infections and the community-acquired bacterial pneumonia in adults.	GC-sensitive and GC-resistant T-ALL cell lines Primary samples derived from ALL patients. Xenograft mouse models	*The novel mechanism*: inhibits mitochondrial respiration, causing the energy crisis, oxidative stress, and apoptotic cell death. A synergistic effect with doxorubicine and vincristine	Effective doses	(211–213) *Clinical trials* NCT01332786 (R/R AML)
2.B. Candidate drugs suggested to be extended for therapeutic application in ALL					
Azacitidine **5-Azacytidine** An hypomethylating agent	FDA approved (Vidaza®) for treatment of the myelodysplastic syndrome. *The mechanism*: a hypomethylating agent.	The ALL-7R cell line: GC-resistant, GR-positive	*The mechanism*: a conventional hypomethylating agent. *Effects*: decreases the DNA methylation in the BIM region, increases BIM expression, and reverts the GC resistance.	A toxicity due to an unspecific action and activation of multiple genes; caspase 1 activation, and GR cleavage	(48, 214–216) *Clinical trials* NCT02828358; NCT01861002 (relapsed/ refractory ALL)
BEZ235 **NVP-BEZ235** **Dactolisib;** A dual inhibitor of the class I PI3K and mTOR kinases by capturing their ATP-binding sites.	Phase I Study in adult R/R ALL patients. Phase Ib study in patients with advanced renal cell carcinoma: an early termination due to the toxicity and a lack of clinical efficacy.	GC-resistant and GC-sensitive continuous T- and B-ALL cell lines. Primary T- and B-ALL cells co-cultured with hBM HS5 stromal cells. Systemic *in vivo* models of T-ALL (including a patient-derived xenograft).	*The mechanism*: a conventional dual inhibition of class I PI3K and mTOR kinases by capturing their ATP-binding sites. *Effects*: enhances the dexamethasone-induced apoptosis in ALL cells (preferentially T-ALL); down regulates Mcl-1 and increases BIM expression; enhances DEX efficiency in T-ALL xenograft models (the tumor load and burden decreases, the EFS increases).	Toxicity	(190, 217–224) *Clinical trials* Next clinical trials were closed due drug toxicity: NCT01453595, NCT01658436, NCT01717898. No one clinical trial registered at the Clinical.trials.gov page provided results about drug safety.
Venetoclax (ABT-199/GDC-0199) A specific Bcl-2 protein suppressor	FDA approved (Venclexta®) for adult chronic lymphocytic leukemia and small lymphocytic leukemia	GC-resistant and GC-sensitive continuous T- ALL cell lines Primary R/R and ETP ALL	*The mechanism*: a conventional, specific Bcl-2 protein suppressor. *Effects*: the mitochondria-dependent apoptosis	Effectiveness in ALL with the upregulation of multiple pro-survival Bcl-2 proteins; safety	(225–228) *Clinical trials* in R/R ALL: NCT03181126, NCT03808610, NCT03504644, NCT03576547, NCT03319901

**ClinicalTrials.gov, https://clinicaltrials.gov.*
EFS, the event free survival.
ER, estrogen receptors.
ETP ALL, ALL of early T cell precursors.
GPER, G protein coupled estrogen receptors.
MPT, the mitochondrial permeability transition.
R/R ALL, Relapsed/ Refractory ALL.
R/R AML, Relapsed/ Refractory acute myeloid leukemia.
VDAC, the voltage-dependent anion channel.

chemotherapeutic drugs vincristine and doxorubicin in multiple GC sensitive and GC resistant ALL cell lines. TGC is also efficient against both newly diagnosed and treatment-refractory clinical samples. Importantly, TGC causes less cytotoxicity in normal hematopoietic cells from leukemia patients. Considering the TGC effectiveness against life-threatening bacterial and fungi infection as well as its good tolerance in ALL patients, including children (235, 236), one may presume that TGC may have a dual function in antileukemic protocols, by targeting heterogeneous populations of leukemic cells, perhaps even primitive leukemia-initiating ones and, at the same time, controlling bacterial and fungal infections.

A Multi-Target Drug Tamoxifen May Be Effective Against the GC Resistant ALL

TAM is widely recognized as the gold standard in treatments of the ER positive breast cancer over half a century. However, antiproliferative and cytotoxic effects of TAM against tumor cells of different histogenesis, which do not express classical ERs, including brain and pancreatic cancers, pediatric rhabdoid tumors, melanoma, uterine carcinoma, and T-ALL were reported. Successful *in vitro* experiments and clinical trials represent a solid fundament to reveal the underlying mechanisms and search for new TAM prescriptions as an anticancer drug. TAM easily permeates biological membranes and multiple "non-classical" intracellular TAM targets were reported. TAM suppresses protein kinase C and PI3K/Akt/mTOR pathways and causes a direct suppression of multidrug resistance proteins. In mitochondria, TAM affects membrane fluidity and interacts with pore proteins of the inner membrane, electron transport chain proteins and proteins of the Bcl-2 family. As a result, cell metabolism and proliferation are decreased, and apoptosis is triggered on. TAM also targets lysosomes: it increases the permeability of the lysosomal membrane, causing a release of cathepsine D and activation of autophagy [reviewed in (237)].

Several studies reported a cytotoxic effect of TAM in non-breast cancers, such as melanoma, bladder, and lung ones [reviewed in (238)]. Importantly, TAM shows a synergistic effect with chemotherapeutic drugs, acting via different mechanisms. In particular, TAM enhances the anticancer effect of protein phosphatase 2 inhibitors in pancreatic cancer cell lines through the inhibition of the protein kinase C (239). TAM also enhances the therapeutic effect of a nucleoside analog gemcitabine in the cholangiocarcinoma (240). In the metastatic malignant melanoma, treatment with TAM in a combination with an alkylating agent dacarbazine is more successful than with dacarbazine alone (241). In rhabdoid tumor cells, pan-inhibitor of cyclin-dependent kinases flavopiridol inhibits tumor growth more efficiently, when it is combined with TAM (242).

As it was discussed previously, a GC resistant phenotype in ALL possesses efficient mechanisms for a rapid adaptation to glycolysis inhibition, caused by GCs, by a switch to mitochondrial OXPHOS, with an up-regulation of both glycolysis and mitochondrial metabolism (see section Metabolic Re-programming and Upregulation of the PI3K/Akt/mTOR Pathway Is Related to GC Resistance in ALL). Additionally, autophagy is involved in this switch, but an excessive autophagy observed in a GC-sensitive phenotype is related to a subsequent cell death (see section Effects of GCs on Sensitive Lymphocytes). Thus, TAM, which targets mitochondria and lysosomes and efficiently provokes autophagy, may represent a favorable candidate for ALL treatments.

In our hands, TAM causes mitochondrial dysfunction and autophagy, induces cell cycle arrest and reduces cell viability in GC-resistant Jurkat cells. Autophagy is triggered through the novel membrane G protein-coupled estrogen receptor, GPER. Remarkably, being added in sub-toxic concentrations, TAM partially reverses GC resistance. Healthy lymphocytes are less sensitive to TAM treatment (166).

Although TAM treatment may cause a rapid decrease of the BM cellularity, it shows only a minor effect on a steady state hematopoiesis (243). As TAM has a long history in its clinical use and now proved to exert the antileukemic activity, it may be considered as an appropriate repurposed drug for ALL treatments. But, the application of TAM to pediatric patients requires a more careful consideration.

Several clinical trials, which evaluate the safety and efficacy of TAM for different tumors were successfully undertaken or are in course (**Table 2**).

Cannabidiol Targets Mitochondria

A non-intoxicating cannabinoid cannabidiol (CBD) has a long-term safety and treatment efficacy in pediatric and adult patients with treatment-resistant epilepsies (206). Accordingly, it has been recently approved by FDA for treatments of Lennox-Gastaut and Dravet syndromes [(244); **Table 2**]. For a long time, CBD was considered as a palliative agent, to improve negative effects of the anticancer therapy, such as pain, nausea, and appetite loss (245–247). At the same time, antineoplastic properties of cannabinoids have been also reported in numerous experimental cancer models (248, 249). In contrast to tetrahydrocannabiol, CBD shows a low affinity for classical cannabinoid receptors CB1 and CB2 and has no undesirable effects on CNS (250). Consequently, its use in anticancer protocols is widely discussed (248–251). On the other hand, the mechanism of CBD cytotoxicity is uncertain. Due to its high lipophilicity, CBD can readily permeate biologic membranes and therefore targets both surface and intracellular structures. Among putative CBD molecular targets some members of the TRP channels family, the orphan cannabinoid receptor GPR55 and mitochondrial VDAC channel have been suggested (207, 248, 251).

Importantly, VDAC acts as a main gatekeeper in the outer mitochondrial membrane that mediates exchange of principal metabolites and ions between mitochondria and cytosol [(143), discussed in Metabolic Re-programming and Upregulation of the PI3K/Akt/mTOR Pathway Is Related to GC Resistance in ALL]. It may adopt different substates, e.g., the completely open one, favoring the transport of metabolites, or the "closed state," facilitating the mitochondrial Ca^{2+} uptake and preventing the ATP export. A moderate increase of intramitochondrial Ca^{2+} is optimal for the TCA enzymes. Therefore, VDAC exerts the coordination between the aerobic glycolysis in cytosol and OXPHOS in mitochondria, ensuring the metabolic plasticity of a cancer cell. Additionally, VDAC interacts with Bcl-2 family

proteins, being involved also in the maintenance of the apoptosis-resistant status. As it was mentioned previously, an upregulation of the aerobic glycolysis and OXPHOS as well as an unpaired apoptosis are classical features of the GC-resistant phenotype in ALL (57, 143).

In our recent study we have tested the CBD efficiency against ALL (207). We have demonstrated that CBD suppressed the viability and impaired the migration of leukemic cells, wherein the T-ALL cell lines were significantly more sensitive than the B-ALL ones. In case of the T-ALL cell line Jurkat mitochondria are proved to be a direct CBD target. CBD seems to directly interact with VDAC channel in the outer mitochondrial membrane, favoring its Ca^{2+}-permeable configuration. The resulting Ca^{2+} overload promotes the formation of the mitochondrial transition pore (MTP), membrane potential collapse, and cell death via the MTP-driven necrosis. In our experiments, CBD demonstrates a similar efficiency in both GC-sensitive and GC-resistant cell lineages.

Remarkably, cannabinoids (and CBD in particular) were shown to decrease the P-gp expression and to reverse the MDR activity in ALL cell lines (252). They also inhibit the multidrug transporter ABCG2 (253).

Obviously, the vehicles and routes of the CBD administration, which are necessary to reach the effective concentration in a chemotherapeutic protocol, will differ from those used for the epilepsy treatment. In general, cannabinoids possess a low solubility in aqueous solutions and are relatively unstable (sensitive to oxidation, light and temperature) that should be taken into a consideration during the development of formulations for chemotherapeutic protocols. The effectiveness of CBD, encapsulated in polymeric microparticles, was demonstrated recently in the experimental model of breast cancer [(254), and references therein].

Another important issue to be considered should be the combined effect of CBD with the conventional anti-cancer therapy. The effectiveness of the CBD-loaded microparticles as a potent formulation to improve the doxorubicin- and paclitaxel-based chemotherapy was recently reported (254). Similarly, CBD acts synergistically with the TNF-related apoptosis-inducing ligand (TRAIL) and enhanced the effectiveness of the photodynamic therapy against the colorectal cancer in preclinical models (255, 256). The synergism of CBD with temozolomide and radiotherapy was reported against the glioblastoma (257–259). The effect of CBD in a combination with the conventional therapy was also studied in preclinical models of hematological neoplasms. A synergistic effect of CBD with ibrutinib was demonstrated in cell lines of the diffuse large B-cell lymphoma and mantle cell lymphoma (260). Similarly, a synergism with vincristine and vinblastine was reported in studies with T-ALL and myeloid leukemia- derived cell lines (261). Notably, CBD decreases the cardiocytotoxicity of doxorubicin, which is also used in anti-ALL chemotherapy (262, 263). Thus, the inclusion of CBD in existing anti-leukemic protocols may improve the outcome. However, low CBD concentrations stimulate the T-ALL cells proliferation (207). Thus, the issues of tissue distribution, specific targeting, and safety should be also considered. Additionally, the CBD use in infants and pediatric patients needs to be evaluated. There are several clinical trials in course, which evaluate the safety and efficacy of CBD as a single agent and in a combination with chemo- and radiotherapy against different tumors (**Table 2**).

Anthelmintic Compounds Show Antileukemic Activity

Anthelmintics possess a disruptive activity over the parasite's microtubules, altering the parasite vital functions. Several anthelmintics such as flubendazole, albendazole, and niclosamide demonstrate antitumor properties in several cancer types, including resistant leukemias (73, 208–210, 264–266), albeit the affinity of anthelmintics to the mammalian tubulin appears to be weaker than to the helminthic one (265). The anticancer potential of anthelmintics is also evidenced by several clinical trials, studying safety and efficacy of mebendazole and niclosamide against colorectal, gastric, hepatic, and brain tumors (**Table 2**). The antileukemic activity of anthelmintics seems to rely on diverse mechanisms. Albendazole alters the MAPK signaling, promotes the mitochondrial dysfunction, such as $\Delta\Psi m$ loss, ROS production, cytochrome c (Cyt-c) release, and causes the intrinsic apoptosis (266). Niclosamide limits the antioxidant system and promotes ROS production by glutathione synthetase inhibition and reduces the NFAT signaling, a vital pathway for leukemic progression (210). Mebendazole was found to inhibit T-ALL by decreasing Notch 1 signaling (reviewed in section Notch Activation) and limiting the *NR3C1* repressor HES1 (73). Mebendazole in both GC resistant and sensitive leukemias represses c-Myc, a key regulator of glucose transporters and cell metabolism. Several groups independently reported that mebendazole targets glucose uptake, reduces cell metabolism, and promotes apoptosis (209). Recently, Mezzatesta and colleagues, using B and T cells from relapsed leukemic patients and patient-derived xenografts for *ex-vivo* experiments, screened 2487 FDA-approved compounds (208). Of the tested anthelmintics, three (ivermectin, moxidectin and milbemycin) display a high cytotoxic effect against leukemic blasts with IC50 values in a low micromolar range, independently on the ALL phenotype. Moxidectin exhibits synergistic effects with DEX and ABT-263 (208).

It should be noted that cancer patients, receiving chemotherapy, show an increased vulnerability to parasite infections [reviewed in (267)] so that the usage of anthelmintics may be justified also by this fact.

Drugs With a Conventional Mechanism in ALL

Hypomethylating Agents May Restore the Expression of the Pro-poptotic BIM Protein

Hypo- and hypermethylation can act as a promoter or a repressor of expression of certain genes, which favor the oncogenic phenotype of a certain cancer, e.g., an overexpression of anti-apoptotic genes, conferring the resistance to cell death induced by chemotherapy, or the inactivation of tumor suppressor genes. Indeed, the methylation profile can be helpful for the diagnosis and prognosis of the patient outcome (268). An aberrant DNA hypermethylation, associated with drug resistance and early

relapse, was described in hematologic disorders, in particular, in the myelodysplastic syndrome (214).

Hypomethylating agents (HMA) were proposed, therefore, to be included into chemotherapy protocols. The cytotoxic drug azacitidine (5-Azacytidine, 5-AZA) was shown to act at lower concentrations as a DNA methyltransferase inhibitor, which induces a global DNA hypomethylation (215). 5-AZA (Vidaza^{TM}) was approved by FDA for treatments of the myelodysplastic syndrome, where it prolongs the time to the leukemia transformation (216). GC resistance in some cases of ALL is determined by the hypermethylation in lymphocyte-specific open regions of DNA, resulting in a decreased accessibility and a prevention of the correct docking of GC-GR complexes with target genes as the pro-apoptotic BIM [(48), discussed in Effects of GCs on Sensitive Lymphocytes)]. In this study, a gradual decrease of the DNA methylation in the *BCL2L11* region was observed in the GC-resistant ALL-7R cell line during 6 days of the exposure to 5-AZA. Importantly, the combined (5-AZA + DEX) treatment significantly increases BIM expression already at 48 h, causes a synergistic cytotoxicity *in vitro*, decreases the bone marrow infiltration and increases the survival in ALL-7R engrafted mice. However, it should be noted, that the demethylating effect of the HMA is unspecific and can lead to the activation of undesirable genes. For example, the administration of HMA can increase the expression of caspase 1, capable to cleave the GR [(116), discussed in GR May Be Cleaved by Upregulated Caspase 1]. Several clinical trials with a participation of patients with relapsed/ refractory ALL are going on, or are concluded, but their results are still awaiting the FDA approval (**Table 2**).

BH3 Mimetics Inhibits the Anti-apoptotic Members of the Bcl-2 Family

The failure of apoptosis is a hallmark of many types of tumors, including ALL. Proteins of the Bcl-2 family represent a complex network in the apoptosis regulation. The apoptosis execution is ensured by the oligomerization of BAK and BAX proteins in the outer mitochondrial membrane, which mediates its permeabilization and a liberation of Cyt-c and other pro-apoptotic factors into the cytosol. In a pro-survival mode, anti-apoptotic members of the Bcl-2 family proteins (Bcl-2, Bcl-XL, Mcl-1, BFL-1/A1, or Bcl-A1) sequester the BAK and BAX, preventing their oligomerization and apoptosis. Apoptotic and stress stimuli differentially activate other Bcl-2 family members, namely, small proteins, possessing only the BH3 domain ("BH3 only" proteins), such as BIM, Bid, Noxa, and Puma, among others. A balance and interactions between pro-survival and pro-apoptotic proteins determines the threshold for the apoptotic response. Based on this idea, synthetic small molecules that structurally mimic "BH3 only" proteins ("BH3 mimetics") were developed. BH3 mimetics are capable to bind to and inhibit anti-apoptotic proteins and, accordingly, lower the threshold for apoptosis in cancer cells. Multiple BH3 mimetics with a different specificity were developed. For example, venetoclax (ABT-199/GDC-0199) possesses a high selectivity for the Bcl-2 protein, navitoclax (ABT-263) is dual inhibitor of Bcl-2 and Bcl-XL, whereas a broad spectrum obatoclax (GX15-070) and sabutoclax (B1-97C1) efficiently bind to Bcl-2, Bcl-XL, Mcl-1, and A1 with submicromolar IC50 values. At present, only venetoclax is approved by the FDA (Venclexta®) for treatmentd of adult patients with chronic lymphocytic leukemia (CLL) and small lymphocytic leukemia [(269), and references therein].

Serious alterations in the Bcl-2 proteins profile were found in the GC resistant ALL (discussed in Bcl-2 Superfamily). Bcl-2 is upregulated in the highly aggressive early T precursor (ETP) leukemia, which underlies its sensitivity to venetoclax (225). A mature GC resistant phenotype is characterized by the overexpression of different pro-survival members, including Bcl-2, Bcl-XL, Mcl-1 and, in some highly malignant cases, A1 (57, 139, 162, 194). At the same time, the pro-apoptotic "BH-3 only" BIM protein is downregulated due to the hypermethylation of corresponding gene *BCL2L11* [(48), discussed in Bcl-2 Superfamily]. BIM possesses a high binding affinity to and can efficiently antagonize all members of the anti-apoptotic Bcl-2 proteins family. Its down-regulation results in enhanced levels of all of them. Thus, the pharmacologic strategy to restore the apoptosis triggering in a GC resistant phenotype would consist in (a) application of HMA to restore the BIM expression (see section Hypomethylating Agents May Restore the Expression of the Pro-apoptotic BIM Protein); (b) antagonization of the pro-survival Bcl-2 proteins, using the synthetic BH3 mimetics (269), and (c) a combination of both. However, the narrow anti-Bcl-2 spectrum of venetoclax might reduce its efficiency in malignant cells, which express other anti-apoptotic Bcl-2 family proteins. Therefore, the use of broad spectrum BH3 mimetics looks more promising. Unfortunately, broad BH3 mimetics may cause severe collateral effects. The Bcl-XL targeting causes the thrombocytopenia. In turn, Mcl-1 plays an important physiologic role in hepatic and cardiac tissues, neurons, and pluripotent stem cells. Thus, for effective BH3 mimetics use in ALL treatment one needs to verify first the therapeutic window and their safe tolerability profile (270).

Venetoclax has shown an activity against primary ETP samples (225). Despite its narrow specificity to the Bcl-2 protein, venetoclax is also effective against T-ALL cell lines (226). Moreover, it demonstrates very promising results in a combination with classical chemotherapy in clinical trials with the refractory/relapsed T-ALL and ETP patients (227, 228). It turns out that venetoclax therapy is safe, with no clinically significant tumor lysis syndrome and no early patients' death. However, a moderate myelosuppression was reported. There are several ongoing clinical trials, evaluating the Bcl-2 inhibition as a therapeutic strategy for relapsed or refractory ALL (**Table 2**). The combination of HMA with venetoclax was suggested as a safe and most promising strategy in the AML therapy (271). Thus, it may be considered also for ALL treatments.

The pan-active inhibitor obatoclax sensitizes the GC-resistant ALL cell lines to DEX and causes apoptosis, autophagy, and autophagy-dependent necroptosis (163, 164). Similarly, obatoclax efficiently kills leukemic cells, derived from infants, diagnosed with ALL in *in vitro* assays. It promotes multiple death scenarios, including apoptosis, necroptosis, and autophagy. Importantly, obatoclax acts synergistically with conventional drugs, including DEX (272). Several clinical trials, evaluating the

obatoclax safety and effectiveness in hematologic malignances, are in course.

PI3K/AKT/mTOR Pathway Inhibitors Are Effective Against ALL

The blockade of the PI3K/AKT/mTOR signaling pathway, which is upregulated in different types of tumors, including the GC-resistant ALL (see section Metabolic Re-programming and Upregulation of the PI3K/Akt/mTOR Pathway Is Related to GC Resistance in ALL), is proposed as a rational therapeutic approach (153, 273). Allosteric mTOR1 inhibitors (rapamycin and its analogs, rapalogs) display promising effects in preclinical models of T-ALL (274, 275) and in a combination with GC synergistically decrease the ALL cells viability (276). Such effects were attributed to the capacity of mTOR to regulate the balance between pro- and anti-apoptotic proteins (188). However, mTOR encompasses two distinct complexes, mTORC1 and mTORC2, which differ in their structure, substrate specificity, and function (277, 278). While mTORC1 induces cell growth by affecting the translational regulators S6K1 and 4E-BP1, mTORC2 mediates cell proliferation and survival via the Akt phosphorylation (279, 280). Therefore, rapalogs could hyperactivate the Akt due to feedback loops between mTORC1, PI3K, and Akt (155). Accordingly, the imidazoquinoline derivative NVP-BEZ235, which inhibits class I PI3K as well as mTORC1/mTORC2 kinases by capturing their ATP-binding sites, may be preferable for treatments (281).

In a panel of T-ALL cell lines and patient-derived T lymphoblasts, NVP-BEZ235 causes cell cycle arrest and apoptosis, and, importantly, also synergizes with the first-line chemotherapeutic agents such as cyclophosphamide, cytarabine, and DEX (282). Activation of the PI3K/Akt/mTOR pathway leads to autophagy (283). It is not surprising that NVP-BEZ235 causes the autophagy activation in T-ALL, and, importantly, the NVP-BEZ235-induced autophagy is not protective against apoptosis (282). However, considering that autophagy may play both pro- and anti-tumor functions, this phenomenon should be studied in more detail.

Using B-ALL patient-derived long-term cultures, the effectiveness of dual inhibitors NVP-BEZ235 (dactolisib) and NVP-BGT226 was tested and compared with those of the pan-PI3K inhibitor NVP-BKM120, combined mTORC1/mTORC2 inhibitors Torin1, PP242, KU-0063794, and the allosteric mTORC1 inhibitor RAD001. Dual PI3K/mTOR inhibitors exerted pronounced antiproliferative and pro-apoptotic effects on ALL cells of different genetic subtypes (284). Yet, a rather variable response of different human B-ALL xenografts was observed in the alternative study, where some xenografts responded better to the single mTOR inhibition (285).

A synergistic antileukemic effect of DEX and NVP-BEZ235 was observed in T-ALL, including *in vitro* (continuous cell lines and primary T-ALL) and systemic *in vivo* models (patient-derived xenograft), but not in B-ALL (190, 286). In T-ALL, NVP-BEZ235 and DEX, added simultaneously, are able to increase BIM and decrease Mcl-1 expression (190). However, rapamycin strongly blocks the GR phosphorylation at Ser211, which is required for its translocation to the nucleus (287).

Up to date, many research groups continue to test dual inhibitors NVP-BEZ235 and NVP-BGT226 in experimental cancer models. Phase I clinical trials were undertaken in patients with different cancers (217–223). Beneficial effects were observed in a small group of relapsed ALL patients (223). However, the unacceptable toxicity of the drug was reported by various researchers (272–277, **Table 2**). Taking into account that the PI3K/AKT/mTOR pathway is a central regulator of so many metabolic functions in healthy cells and tissues, the clinical perspective for its inhibitors is highly questionable (224).

High-Throughput Drug Screening Reveals GC Sensitizers Against ALL

The high-throughput screening (HTS) is an efficient strategy for drug discovery. Novel class of drugs with a thioimidazoline moiety, capable to sensitize ALL to GC, was revealed recently by this method (78, 288, 289). In particular, the compound J9 in low nontoxic concentrations is able to increase the GR expression (290). Accordingly, the gene expression pattern in GC-resistant cells co-treated with J9 and DEX is similar to that caused by GCs in sensitive cells. In another study, compound GCS-3 significantly increases the BIM enhancer binding to GRs, resulting in upregulation of BIM and downregulation of C-Myc expression (288, 289). Importantly, GCS-3 is effective against GC-resistant and GC-sensitive xenografts of B-ALL, T-ALL (including ETP), and Philadelphia chromosome positive ALL (288, 289). The knowledge of the action mechanism of effective drugs on their molecular targets, approximation of their interaction mechanisms, and consequent HTS of FDA-approved drugs may reveal new repurposed drugs candidates for ALL treatments.

CONCLUSIONS AND FUTURE PERSPECTIVES

In general, mechanisms responsible for GC resistance can be divided into the two large groups: those associated with a reduced expression of functional GRs and those that are not. Among the latter, attention should be paid to: (a) general metabolic up-regulation, including glycolysis and OXPHOS, and mechanisms of a flexible switch between them; (b) resistance to apoptosis due to a specific pattern of the Bcl-2 family proteins, including upregulation of different pro-survival members and downregulation of pro-apoptotic proteins (mainly of BIM); (c) upregulation of MDR transporters. A decreased level of the GR expression determines GC resistance by itself, but not necessarily the unresponsiveness to other anticancer compounds. These are abnormalities unrelated to changes in the GR expression that link the GC resistance to the resistance to other drugs and an overall poor prognosis. Then the strategy to improve the outcome for the patients with the GC-resistant ALL is to invoke alternative mechanisms, including the use of some repurposed drugs.

One of the strategies already used in the therapy against the AML is a simultaneous application of BH3 mimetics to inhibit the pro-survival Bcl-2 members and HMA to enhance the BIM expression (see sections Hypomethylating Agents May Restore

the Expression of the Pro-apoptotic BIM Protein and BH3 Mimetics Inhibits the Anti-Apoptotic Members of the Bcl-2 Family). As a result, the pro-apoptotic balance among Bcl-2 members will be reached, to restore the apoptosis development in a response to chemotherapy. Another strategy may be metabolic inhibition, to lower a threshold for the regulated cell death, different from apoptosis, such as the autophagy-related cell death, necroptosis, and MTP-related necrosis. In this regard, TAM, a traditional drug, used as an ER antagonist for the chemotherapy of breast cancer, may be an option, due to its numerous "off target" anticancer effects. TAM is proposed as an adjuvant in the therapy in different types of cancers and demonstrates promising antileukemic effects in the preclinical model of T-ALL (discussed in A Multi-Target Drug Tamoxifen May Be Effective Against the GC Resistant ALL). CBD is another highly attractive candidate (discussed in Cannabidiol Targets Mitochondria). CBD, which targets mitochondria in ALL, triggers different antileukemic processes, such as inhibition of glycolysis and OXPHOS, mitochondrial damage, and induction of the MTP-related necrosis. In addition, CBD and TAM inhibit MDR proteins and demonstrate a cardioprotective effect. In case of CBD, its reduced bioavailability maybe the problem. However, a synergistic effect, observed with different chemotherapeutic drugs, allows a significant lowering of the CBD effective concentration. Providing, CBD is integrated in conventional chemotherapeutic protocols, it would also improve a general status and life quality of patients, due to its palliative and cardioprotective effects. Yet additional experiments are required to determine the CBD formulation, administration routes, and dosage. The antibiotic TGC also targets mitochondria and causes cytotoxicity in preclinical ALL models. In addition, it demonstrates an extraordinary effectiveness against drug-resistant infections and good tolerance in ALL patients (see section Antibiotic Tigecycline Can Efficiently Control Infections and Kill Leukemic Cells by Targeting Mitochondria). In a conclusion, the use of the BH3 mimetics and HMA agents as well as repositioning of TGC, TAM, CBD and some anthelminthics (see section Anthelmintic Compounds Show Antileukemic Activity) may substantially improve chemotherapeutic protocols for treatment of the GC-resistant ALL in future. It is expected that the list of FDA-approved compounds for anti-ALL treatments will be extended and new repurposed drugs candidates will be revealed by means of the HTS technology.

AUTHOR CONTRIBUTIONS

MO-A and OD contributed to the concept and design of the review. MO-A, LT-L, IP and OD wrote the first draft. MO-A and OD composed the tables. MO-A designed the figures. All authors contributed to the manuscript revision, have read and approved the submitted version.

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