

Application of Gene-Directed Enzyme Prodrug Therapy in Cancer Treatment

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ABSTRACT

Gene-directed enzyme prodrug therapy (GDEPT) is an advanced cancer therapy that has potential use against localized and metastasized cancer. This strategy aims to improve the limitations of chemotherapy and existing cancer treatments by specific gene delivery, which allows the conversion of systemically administered non-toxic prodrugs to active chemotherapeutic drugs inside the target tumor cells, thereby resulting in a significant therapeutic index by introducing high concentrations of cytotoxic compounds to the tumor cells while limiting the systemic toxicity. The main attraction of GDEPT is by expanding the toxicity to adjacent non-expressing target cancer cells through local and distal bystander effects, leading to tumor regression. This review focused on the application of the six main GDEPT systems for treating cancer, including herpes simplex virus thymidine kinase (HSV-TK) with ganciclovir (GCV), cytosine deaminase (CD) from bacteria or yeast with 5-fluorocytosine (5-FC), E. coli nitroreductase (NfsB) with 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954), hepatic cytochrome P4150 (CYP450) with cyclophosphamide (CPA), purine nucleoside phosphorylase (PNP) from E. coli with 6-methylpurine deoxyriboside (MEP), and bacterial carboxypeptidase G2 (CPG2) with 4-[(2-chloroethyl)(2-mesloxyethyl)amino] benzoyl-L-glutamic acid (CMDA). In each system, the mechanism of action, clinical trials for the past decades, limitations, and areas that need improvement are discussed.

Keywords

Gene-directed enzyme prodrug therapy (GDEPT), Bystander effect, Thymidine kinase, Cytosine deaminase, Nitroreductase, Cytochrome P450, Purine nucleoside phosphorylase, Carboxypeptidase G2, Ganciclovir, 5-FC, CB1954, Cyclophosphamide, MEP, CMDA.

Introduction

Cancer remains a devastating disease with 18.1 million new cases and 9.6 million deaths being estimated and reported worldwide in 2018. The International Agency for Research on Cancer (IARC) reported the cancers of lung, female breast, and colorectum account for one-third of global cancer incidence and mortality burden. However, lung cancer ranks as the first leading cause of death due to the poor prognosis, followed by colorectal cancer and stomach cancer. Lung cancer followed by prostate cancer and colorectal

cancer are the leading cause of cancer death in men, while breast cancer is the leading cause of cancer death in women, followed by lung cancer and colorectal cancer. A significant proportion of cancer deaths are higher than newly diagnosed cases in developing countries, such as Asia and Africa, due to financial constraints and inadequate health service planning [1].

Cancer is characterized as uncontrolled proliferation of any cell types and organs in the body, which is mostly initiated by an accumulation of genetic mutation in cells over time. Under normal circumstances, cell proliferation is a highly regulated process that occurs for cells to grow and replenish dead or damaged cells due to environmental factors, such as cigarette smoke and ultraviolet light. A normal cell cycle progression is monitored at checkpoints which are responsible for detecting errors in DNA replication or DNA damage caused by exogenous or inherited factors. Activation

of cell cycle checkpoints causes cells to undergo cell cycle arrest, thereby allowing cells to repair and prevent the transmission of potentially oncogenic mutations to daughter cells. Genes associated with cancer development include proto-oncogenes encoded intracellular regulatory proteins as well as growth factors for cell proliferation and differentiation [2], and tumor suppressor genes encoded for proteins that restrict cell division or promote programmed cell death, such as the TP53 gene that encodes for p53 protein [3]. Damage or mutation in both proto-oncogenes and tumor suppressor genes may contribute to tumor development. Hallmarks that promote the development of cancer include uncontrolled cell proliferation, absence of inhibition of the excessive cell growth, avoidance of immune destruction, genome instability, resistance to cell death, tumor-promoting inflammation, replicative immortality, deregulating cellular energetics, activating invasion and metastasis, and new vascularization. These cancer hallmarks are generally promoted by genome instability and tumor-promoting inflammation [4]. Genomic instability provides a rapid accumulation of DNA mutations that promote uncontrolled cell cycle activity and resistance to cell death, thereby generating an abnormal cell mass or tumor. Cancer can be either benign or malignant. A benign tumor remains confined to its original location and does not spread to other body parts. However, a malignant tumor can invade adjacent normal tissue and metastatic spread throughout the body through the circulatory or lymphatic system, resulting in cancer death.

Existing cancer treatment strategies remain ineffective and consist of significant limitations, including surgery, radiotherapy, chemotherapy, and hormonal therapy. Cancer surgery is highly dependent on quality pathology technique and imaging technique for accurate diagnosis of early-stage disease and determining the presence of metastatic disease, to provide effective planning of surgical cancer care. Surgery aims to remove solid cancerous tissue, however, it is only limited to localized disease and non-metastatic cancers. Therefore, cancer treatment with surgery alone is only effective for early-stage or localized disease [5]. Moreover, radiotherapy serves as an effective cancer treatment for local and non-metastatic cancers which aims to shrink the tumor cells by exposing them to high energy ionizing radiation. However, increased exposure to high-energy radiation may cause severe adverse effects as well as induced bystander effects by damaging the normal cells adjacent to cancer cells, which are susceptible to produce new DNA damage (chromosomal aberration) and increase the risk for developing new malignancies [6].

Conversely, chemotherapy and hormonal therapies are commonly used to eradicate metastatic cancers [7]. Chemotherapy is performed by delivering chemotherapeutic drugs that non-selectively kill actively dividing cells throughout the body including normal healthy cells, such as bone marrow, hair follicles, and gastrointestinal tract cells. Therefore, it may induce significant negative side effects that may adversely impact patients, such as nausea, vomiting, loss of appetite, hair loss, peripheral neuropathy, fatigue, and anemia. Besides, chemotherapy may disrupt the function of the central nervous system, including cognitive dysfunction, memory loss, reduced attention, anxiety,

and depression [8]. Hormonal therapy is commonly used to treat metastatic or recurrent breast cancer. A significant adverse effect that usually presents with all hormonal therapy is flare reaction. The common manifestation of flare is redness, swelling, nausea, increased thirst, constipation, polyuria, or increased tumor size [8]. Other significant side effects that negatively impact the quality of patient life include psychological changes, decreased lean muscle mass, increased fat mass, increased bone pain, loss of bone mineral density, or hypercalcemia, which may be associated with weight gain and fractures [9].

Gene-directed enzyme prodrug therapy (GDEPT) has emerged as an effective cancer treatment strategy with greater specificity and less toxic treatment options for patients with cancer, which aims to improve the anticancer outcomes and to overcome the limitations of current anticancer modalities. In this review, we summarize the various types of gene-directed enzyme prodrug therapy as well as discuss the application of GDEPT for cancer treatment.

Tumor-specific delivery techniques by activation of prodrugs

Gene-directed enzyme prodrug therapy focused on improving the efficacy of existing cancer treatment as well as reducing the side effects of chemotherapy, in which the enzyme-encoding transgenes are delivered into the targeted tumor cells by a vector, including bacterial, yeast, or viral. Consequently, the selectively administered harmless prodrug can be selectively activated by the expressed enzymes to a cytotoxic agent within the tumor site while minimizing drug exposure to normal tissues [10], resulting in the killing of local cancer cells [11]. The administered prodrug should be a systemically stable substrate for the expressed enzymes, able to be metabolized to effective cytotoxins. Also, the active drug should be highly diffusible and able to spread to adjacent non-transduced cells, preferably able to kill all stages of cancer cells including adjacent tumor cells to achieve the bystander effect [12].

There are three essential components required in GDEPT, including enzyme, prodrug, and vector. There are two categories of enzymes that will be used in GDEPT. The first group includes enzymes that originate in normal human cells, which is less abrupt of triggering immune response but able to observe toxicity in normal non-target cells, such as cytochrome P450 (CYP450). Besides, the second group includes enzymes that originate from bacteria or viruses, which are more likely to induce immune response but less probable to observe toxicity in non-target tissues, such as viral thymidine kinase (TK), cytosine deaminase (CD) originated from bacterial and yeast, purine nucleoside phosphorylase (PNP), and bacterial nitroreductase (NTR).

Prodrugs that can be used in GDEPT should be stable under physiological conditions, exhibit low toxicity prior to activation, and highly toxic to cancer cells after activation. Moreover, the activated cytotoxic prodrug should perform a high bystander effect, in order to overcome the low transduction rate of the vectors. Prodrugs can be classified into a direct-linked and self-immolative prodrug. Direct-linked prodrugs can be activated directly to a cytotoxic component in a single reaction, such

as GCV and CB1954 are the prodrugs that target TK and NTR enzymes respectively. However, self-immolative prodrugs can be converted to intermediate components and subsequently activated to cytotoxic components via the fragmentation process, such as doxorubicin prodrug.

Besides, an appropriate vector should have low cytotoxicity, high transfection rate, high tissue specificity, and low cost. The gene delivery system of GDEPT can be categorized into synthetic vector, microorganism-based vector, and cell-based vector. For instance, synthetic vectors include polymeric and lipid-based, whereas cell-based vectors include stem cells or dendritic cells. Viral, bacterial, and yeast are the microorganism-based vectors that can be used in GDEPT. In clinical trials, adenovirus has been reported to be the most common vector used for gene delivery, due to adenovirus can infect both dividing and non-dividing cells, able to induce gene expression into the host genome without integration, and the adenoviral particles can be eliminated by the reticuloendothelial system such as the liver. However, adenovirus can induce a significant immune response against viral capsid proteins, and non-specifically bind to all coxsackievirus and adenovirus receptor (CAR) over-expressing cells [13]. Other preferred vectors for gene delivery are retrovirus and lentivirus, as they can integrate the target genome into the host genome and are less probable to induce an immune response. Furthermore, tumor-specific suicide gene expression can be accomplished and improved by linking a tumor-specific promoter to the transgene in the vector, allowing the suicide gene to be expressed in the target tumor cells to avoid non-specific toxicity. (O) the most common promoters used include human telomerase reverse transcriptase (hTERT) promoter, carcinoembryonic antigen (CEA) promoter, osteocalcin promoter (OC), as well as hypoxia and radiation responsive elements [13].

The main attraction of GDEPT is the local bystander effect, where the activated drug has the ability to kill any neighboring cells that are not expressing foreign enzyme via gap junction, apoptotic vesicles, active transfer, or diffuse to adjacent non-expressing cancer cells [14]. Besides, the release of necrotic material from the dying tumor cells can initiate inflammatory immune responses mediated by T-cells and natural killer cells accompanied by high levels of various cytokines, known as the distant bystander effect [15], which are capable of killing local and distal untransfected cells. (Q) Studies have demonstrated that less than 10% of transduced cells is sufficient to eliminate the whole population of cancerous cells [16].

HSVtk/GCV system

Thymidine kinase gene of the Herpes Simplex virus (HSVtk) in combination with the prodrug Ganciclovir (GCV) was the first potential GDEPT system to be suggested and has been widely investigated [16]. Thymidine kinase from HSV-1 (HSVtk) able to phosphorylate nucleoside analogs including ganciclovir (GCV) or acyclovir (ACV) prodrug to a GCV monophosphate, which normally fails to phosphorylate by both cytosolic and mitochondrial thymidine kinase from human eukaryotic cells [12].

GCV monophosphate can be further phosphorylated by cellular kinase to GCV diphosphate and GCV triphosphate. (S) The active metabolite GCV triphosphate competes with 2'-deoxyguanosine triphosphate (dGTP) to become the substrate for DNA polymerase by incorporating into DNA during the S phase of the cell cycle, resulting in the inhibition of DNA polymerase or DNA synthesis by producing a chain termination, which causes cell death in the dividing cells [12]. Several studies showing that HSVtk/GCV system-mediated cell death can be either by the necrotic mechanism of irreversible cell cycle arrest at the G2-M checkpoint or apoptotic mechanism of S-/G2-phase arrest mediated by the signal pathway of Fas receptor including CD95/APO-1, Fas ligand accompanied with several associated regulators [16]. However, HSVtk system was shown to be no survival benefits over patients, which can be possibly due to poor gene transfer or insufficient dose of ganciclovir tolerated in humans (10 mg/kg/day), whereas approximately 300 mg/kg/day of ganciclovir was used in most preclinical animal experiments [20].

Mitochondria play an essential role in the regulation of apoptosis by initiating the mitochondrial damage pathway and the accumulation of p53 protein. As a consequence, the up-regulation of CD95 mediated by p53 causes the formation of the CD95 death-inducing signaling complex as well as the caspase cascade activation, which in turn triggers the mitochondrial perturbations. For instance, loss of the mitochondrial Trans membrane potential and the release of apoptogenic factors, such as cytochrome c from mitochondria into the cytosol, initiating the activation of apoptosis effector system [21]. Besides, the adjacent HSVtk non-expressing cells can be phagocytosed by the toxic metabolites from apoptotic vesicles generated in the HSVtk expressing cells. (P) Cytochrome c may induce the activation of procaspase-9 and the cleavage of caspase-activated DNase (CAD), DNA fragmentation factor, and downstream caspases including caspase-3, resulting in nuclear fragmentation [21]. Moreover, HSVtk/GCV system can induce a distant bystander effect by triggering the immune system mediated by T-cells and natural killer cells accompanied by high levels of cytokines through the recognition of tumor antigen or the presence of dying cells of the treated tumor. Thus, local treatment with the HSVtk/GCV system can prevent the possibility of metastasis. It was also demonstrated that treatment with GCV may achieve 100% of cell death when only 10% of HSVtk expressing cells in the culture and complete tumor regression when 10-50% of HSVtk expressing cells in an in vivo model [16].

In recent studies, HSV-tk system has been used as a reporter gene for clinical non-invasive imaging, as HSV-tk can phosphorylate radiolabelled nucleoside analogs, thereby allowing to detect of the location of active HSV-tk in patient's body using nuclear imaging technologies, such as positron emission tomography (PET) [21]. However, a major drawback of HSVtk/GCV system is that the GCV prodrug can passively diffuse into target cells, but the cytotoxic GCV triphosphate is highly charged and insoluble in lipid membranes. Hence, the bystander effect is limited to the diffusion of GCV triphosphate into adjacent untransfected tumor cells [19]. Attempts have been made to overcome the limited

bystander effect of HSVtk/GCV system, which include the use of co-expression of connexins Cx32 and Cx26 with HSVtk to increase HSVtk/GCV mediated bystander effect as well as the construction of fusion proteins consisting of HSVtk and 8-11 amino acids from the human HIV-1 TAT protein, to induce intercellular export or import, independent of gap junctions. Additionally, several drugs that may positively regulate the formation of gap junctions can be utilized to improve the bystander effect, including retinoic acid, lovastatin, and apigenin [16].

Cytosine deaminase/5-fluorocytosine prodrug system

Cytosine deaminase (CD) can catalyze the deamination of cytosine to uracil, which can be found in many bacteria and fungi but not in mammalian cells. Low toxic pyrimidine prodrug, 5-fluorocytosine (5-FC), can be catalyzed to a highly toxic 5-fluorouracil (5-FU) by cytosine deaminase (CD) with the production of toxic metabolites including 5-fluoro-2'-deoxyuridine 5'-monophosphate (5-FdUMP) and 5-fluorouridine 5'-triphosphate (5-FUTP). Normal mammalian cells are not able to convert 5-FC prodrug to 5-FU and 5-FU to 5-FdUMP due to the lack of CD and uracil phosphoribosyltransferase (UPRT) respectively. The cytotoxic 5-FU may induce growth inhibition as well as apoptotic cell death of solid tumors including gastric, hepatocellular cancer, [22] breast, head and neck, glioma, colorectal, (N), and prostate cancer [23]. Moreover, 5-FC can diffuse through the blood-brain barrier; hence the CD/5-FC strategy has an advantage for treating brain tumors, such as glioblastoma [14].

Likewise, 5-FUTP can be converted to 5-fluorodeoxyuridine diphosphate (5-FdUDP) by ribonucleotide reductase, which can be further dephosphorylated to 5-FdUMP [24]. 5-FdUMP can irreversibly inhibit thymidylate synthase and thus interferes with DNA synthesis by forming a ternary covalent complex that prevents the formation of thymidylate, which is a precursor of thymidine-5'-triphosphate (dTTP), leading to the inhibition of DNA replication [25]. Furthermore, 5-FUTP has the ability to incorporate into the uridine 5'-triphosphate (UTP) of RNA, which inhibits the nuclear processing of mRNA and rRNAs, resulting in the inhibition of protein synthesis [22]. Moreover, 5-FdUMP can be converted to 5-FdUTP, which may lead to DNA damage by incorporating into DNA [26]. Studies have shown that high mRNA inhibition can lead to protein starvation in non-dividing tumor cells at a high concentration of 5-FU [14].

As compared to the HSVtk/GCV system, the CD/5-FC system appears to have a stronger bystander effect, due to the 5-FU molecules are small in size and neutrally charged which can freely diffuse through cellular membranes, while GCV mono, di-, and triphosphate transport through cells via gap junctions. CD/5-FC system appears to be more effective as CD-mediated deamination does not require energy sources or any cellular cofactors and CD reaction can take place in the extracellular space. Conversely, TK reaction must take place inside the cell and it requires a phosphate donor to activate GCV prodrug. In the CD/5-FC system, the mechanism of the bystander effect is the uptake of 5-FU that effluxes from the transfected cells by neighboring non-transfected

cells in the interstitial space. In contrast, the bystander effect of non-transfected cells in HSVtk/GCV systems is encountered via the phagocytic uptake of apoptotic bodies from the dying transfected cells [24]. Several studies have shown that a significant increase in both CD4+ and CD8+ lymphocytes can be identified in both the HSVtk/GCV and CD/5-FC systems [11].

Several drawbacks of the CD/5-FC system include side effects, the inefficiency of cytosine deaminase-catalyzed conversion of 5-FC into 5-FU, as well as the use of adenovirus or tumor-tropic cells including mesenchymal stem cells (MSC) or neural stem cells as the carriers of CD/5-FC to the targeted tumor locations [23]. In several studies, the use of cytosine deaminase from yeast *Saccharomyces cerevisiae* (yCD) demonstrated to have a 15-fold higher amount of 5-FU produced as compared with bacterial cytosine deaminase (bCD), due to yCD has a significantly higher affinity for 5-FC. (AA) Recently, Toca 511 is a non-lytic retroviral vector that has been introduced for the heat stabilized yCD suicide gene therapy [14]. Numerous attempts were made to improve the prodrug conversion efficiency by constructing adenovirus containing CD and uracil phosphoribosyltransferase (UPRT) gene with 5-FC administration [28]. The bacterial UPRT can directly convert 5-FU to 5-UMP and subsequently to 5-FUDP, which can be further metabolized to 5-FUTP and 5-FdUMP. Due to the lack of UPRT in mammalian cells, 5-FU is converted to 5-FUMP through a two-step pathway which is only activated at a high concentration of intracellular 5-FU [29]. Research demonstrated that the simultaneous expression of CD and UPRT genes by adenovirus can generate a synergic effect which dramatically enhanced the ability of glioma cells to metabolize 5-FU to 5-FUMP in the first step of 5-FU activating pathway compared with the transduction of the CD gene alone as well as increased the antitumor effect of CD/5-FC gene therapy [29]. Additionally, the 5-FU/UPRT system promotes a strong bystander effect due to the passage of 5-FU through the gap junction between neighboring cells.

Side effects are the major problem that persists with the CD/5-FC system, as 5-FC may produce minor side effects including nausea, diarrhea, and vomiting as well as adverse effects such as bone marrow depression and hepatotoxicity [23]. In recent studies, a new approach ICD/5-FIC system is established to have better potential than the CD/5-FC system, as novel isocytosine deaminase, which is termed as Vcz, may specifically convert 5-fluoroisocytosine (5-FIC) into 5-fluorouracil (5-FU). Moreover, mesenchymal stem cells (MSC) have proven to be a successful carrier of the Vcz/5-FIC system in reducing tumor aggressiveness. Research reported that the Vcz/5-FIC system is 10% to 20% more efficient than 5-FU and may alleviate the toxic side effects that normally persist in the CD/5-FC system [23].

Nitroreductase/CB1954 system

Nitroreductase (NTR) is a flavoenzyme bounded with a flavin mononucleotide (FMN) cofactor, promoting the reduction of nitro residues on aromatic rings by either NADH or NADPH. Nitroreductase (NTR) from *Escherichia. Coli* (NfsB) is an enzyme that has been studied extensively on nitroreductase-based

GDEPT. It can efficiently reduce the non-toxic DNA alkylating prodrug, 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) to a potent cytotoxic agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, by using NADH or NADPH as an electron donor via ping-pong mechanism [30]. In the first step, NADH or NADPH reacts with nitroreductase and transfer two electrons to the FMN cofactor, which leads to the formation of NAD⁺ or NADP⁺. (N) Next, the nitroaromatic substrate including CB1954 or quinones binds to nitroreductase and subsequently activates by the reduction of 4-nitro group, resulting in the generation of cytotoxic 2-hydroxylamine and 4-hydroxylamine metabolites [31]. Consequently, the cytotoxic 4-hydroxylamine is further metabolized via cellular acetylation pathway in cells [32], causing extensive DNA damage and apoptotic cell death by forming interstrand crosslinks in DNA in both dividing and non-dividing cells. (N) Although both hydroxylamine components are generated at the same rate with equal proportions, the 4-hydroxylamine component is more cytotoxic compared to the 2-hydroxylamine component [31]. In the current clinical trials, PR-104 is a hypoxia-activated prodrug that can be metabolized to cytotoxic PR-104A by nitroreductase, which is further metabolized to hydroxylamine PR-104H and amine PR-104M by reducing the nitro group in hypoxic cells, causing DNA interstrand cross-linking and resulting in cytotoxicity [33].

Previous studies determined several distinct advantages of the NTR/CB1954 system over the HSVtk/GCV and CD/5-FC systems. For instance, NTR/CB1954 system can target both quiescent and actively dividing cancer cells, due to NTR requiring NADH or NADPH for 4-nitro group reduction. However, the active metabolites of HSVtk/GCV and CD/5-FC systems are only effective at eliminating actively dividing cells; due to GCV and 5-FC are nucleotide analogs [20]. Both the activated 2-hydroxylamine and 4-hydroxylamine metabolites by nitroreductase can freely diffuse across the cell membrane, while GCV triphosphate is not cell-permeable, whereby the bystander effect of GCV system is primarily dependent on cell-cell contacts, such as gap junction. Hence, the NTR/CB1954 system has the ability to induce a potent bystander effect on the cell cycle, which is primarily independent of cell-cell contacts [30]. In the present study, increased cell apoptosis and reduced level of surviving cells can be observed in the cell group that received both the synergistic effect of the NTR/CB1954 treatment and gamma-ray radiation. [30]. A new modality has been developed by generating a combined expression of *E. coli* nitro reductase and heat shock protein 70, which promotes a strong immune response, cell death and efficiently inhibit the tumor outgrowth [34].

Hepatic cytochrome P450-2B1

Oxazaphosphorines are the prodrugs that have been extensively studied in P450-based GDEPT, such as cyclophosphamide (CPA) and ifosfamide (IFO). Cyclophosphamide (CPA) is an anticancer prodrug that is commonly used in the treatment of breast cancer, ovarian cancer, endometrial cancer, lung cancer, leukemia, multiple myeloma, retinoblastoma, neuroblastoma, and lymphoma. However, Ifosfamide (IFO) is an isomer of CPA; it performs high

activity against soft tissue sarcomas, ovarian cancer, testicular cancer, and breast cancer [35]. Both alkylating agent prodrugs are activated to 4-hydroxy-cyclophosphamide (4-OH-CPA) or 4-hydroxy-ifosfamide (4-OH-IFA) via 4-hydroxylation reaction in the liver that catalyzed by various hepatic cytochromes P450 (CYP450) enzymes, such as CYP2B6 and CYP3A4 respectively [36]. Studies revealed that the CYP2B6 in humans and CYP2B1 in rats are the most active catalysts of the 4-hydroxylation reaction [35]. The activated metabolite, 4-OH-CPA or 4-OH-IFA, is released into the circulation and distributed throughout the body. However, both the unstable 4-OH-CPA and 4-OH-IFA undergo spontaneous β -elimination reactions to yield cytotoxic phosphoramidate mustard and acrolein metabolites [37]. Phosphoramidate mustard derived from CPA or isophosphoramidate mustard derived from IFO is a strong cytotoxic DNA alkylating agent that diffuses into cancer cells and causes cell death [24], while acrolein can bind covalently to alkylate proteins and cause urotoxicity. Besides, both CPA and IFO can be catalyzed by CYP3A4 to generate another cytotoxic component such as chloroacetaldehyde via N-dechloroethylation reaction, which induces anticancer activity, severe neurotoxicity, and urotoxicity [38].

The bystander killing effect of this system is independent of the cell to cell contact, as it is induced by the release of cytotoxic metabolites from P450-expressing cells, such as 4-OH-CPA, and the transfer of apoptotic signals from the dying P450-expressing tumor cells. Furthermore, 4-OH-CPA can trigger apoptotic cell death, whereas 4-OH-IFO triggers necrotic or apoptotic cell death [38]. Research showed a delayed S-phase progression and G2-M phase arrest in CYP2B1-expressing breast carcinoma cells, which leads to apoptotic cell death mediated by the mitochondrial caspase 9-dependent cell death pathway. However, the systemic distribution of phosphoramidate mustard and acrolein may cause side effects, including renal toxicity, cardiotoxicity, neurotoxicity, and bone marrow suppression [35]. Conversely, there are numerous advantages of this system, including the ability to bypass any immune response against the transgene as well as selectively inhibiting the hepatic CYP activity to potentially reduce the systemic exposure to activated drug metabolites [26]. Attempts have been established to improve the prodrug activation in cancer cells, which include the use of anti-thyroid drugs such as propylthiouracil and methimazole, allowing the inhibition of hepatic P450 reductase activity. Also, anti-apoptotic factors can be used to delay tumor cell death and to prolong and enhance the bystander effect.

Purine nucleoside phosphorylase/6-methylpurine deoxyriboside 740 (PNP/MEP) system

Purine nucleoside phosphorylase (PNP) from *E. coli* is a hexameric enzyme that is responsible to convert the purine ribonucleoside prodrug, including 6-methylpurine-2-deoxyriboside, to 2-deoxyribose-1-phosphate and 6-methylpurine (6MP) [14]. Other trials have used fludarabine phosphate which is an antileukemic agent as a prodrug. Fludarabine is converted to 2-fluoroadenine (2FA) which inhibits the ATP-involving reactions, resulting in killing dividing and non-dividing cells by inhibiting protein and

RNA synthesis. Studies revealed that 2FA is a relatively 100-fold more potent cell growth inhibitor than 6MP [26]. The cytotoxic compounds, 6-methylpurine (6MP) or 2-fluoroadenine (2FA), can freely diffuse across cell membranes from PNP-expressing tumor cells to non-expressing tumor cells. The bystander effect of the PNP system is facilitated by the nucleotide and nucleobase transporters across cell membranes, which are independent of gap junction or cell-cell contact [14]. Moreover, this system can induce apoptotic cell death by the PNP-fludarabine system, which is independent of the Fas/FasL pathway and p53 status. According to *In vitro* studies, 1% of PNP-expressing tumor cells is efficient for occurring total cell death [26]. Hence, PNP-based systems have been reported to have higher efficiency for the treatment of local and metastatic prostate cancer as compared to HSVtk/GCV systems.

Carboxypeptidase G2/4-[(2-chloroethyl) (2-mesyloxyethyl) amino] benzoyl-L-glutamic (CPG2/CMDA) system

Bacterial enzyme Carboxypeptidase G2 (CPG2) derived from *Pseudomonas* RS-16 can cleave glutamic acid from nitrogen mustard-based prodrug to release its cytotoxic drug form. For instance, the first developed nitrogen mustard-based (NM) prodrug is 4-[(2-chloroethyl) (2-mesyloxyethyl) amino] benzoyl-L-glutamic acid (CMDA). CPG2 catalyzes the hydrolysis of CMDA, which allows the release of glutamic acid and a toxic DNA alkylating agent, 4-[(2-chloroethyl) (2-mesyloxyethyl) amino] benzoic acid drug [14], resulting in DNA inter- and intra-strand crosslinking and subsequently cause cell death in both dividing and quiescent cells. There are several advantages of the CPG2/CMDA system over other GDEPT systems. For instance, no additional activating procedures are required due to CPG2 has a mammalian equivalent. Moreover, CPG2-activated alkylating metabolite is lipophilic which can directly diffuse through cell membranes, resulting in the formation of intra- and inter-strand DNA crosslinking. Studies revealed that the CPG2/CMDA system can induce a significant bystander effect, as 2% of CPG2-expressing tumor cells are efficient for occurring 90% of total cell death [39].

To improve the bystander effect, the CPG2 enzyme was tethered to the mammalian cell surface and expressed as stCPG2(Q)3 by creating a fusion protein with the trans membrane region of the ERBB2 to restrict the enzyme localization to the cytosol and reduce the enzyme activity while maintaining the surface expression, thereby leading to extracellular prodrug activation [39]. As a result, a greater bystander effect can be observed in cells expressing CPG2 on their surface against non-expressing cells [29]. Besides, self-immolative prodrugs have been developed without inducing unfavorable steric or electronic effects. The self-immolative mechanism includes the separation of the CPG2 hydrolysis site from the 'effector' end of a nitrogen mustard-based prodrug by a spacer that spontaneously triggers 1,6-elimination, leading to the release of active drug. The prodrug bioavailability can be changed based on the structure of the linking groups, without affecting the activation kinetics [39].

The potential problem for the CPG2-based system is that the release of the activated drug, 4-[(2-chloroethyl) (2-mesyloxyethyl) amino]

benzoic acid has a long half-life, causing myelosuppression due to the reverse diffusion of the activated drug into the circulation which can potentially induce systemic toxicity [40]. However, the CPG2/NM system has been extensively used in Antibody-Directed Enzyme Prodrug Therapy (ADEPT) by conjugating MFCEP1, an antibody-enzyme recombinant fusion protein of CPG2, and a bis-iodo phenol mustard prodrug (ZD2767P). The activated drug produced from ZD2767P was revealed to have a shorter half-life with fast clearance and reduced system toxicity [41]. Additionally, the activated cytotoxic drug produced from ZD2767P was reported to be approximately 300 times more potent than the benzoic acid mustard drug used in the initial ADEPT studies [39]. Unfortunately, there have been no clinical trials utilizing this system in the past decade.

Future perspectives & Conclusions

Gene-directed enzyme prodrug therapy (GDEPT) represents the new promising approach that has the potential to improve both localized and metastasized cancer treatment outcomes in patients, in which the cytotoxic chemotherapeutics agents can be delivered directly to the target cancerous cells with reduced systemic toxicity. However, numerous issues are limiting the progress of GDEPT, including the limited drug delivery, limited prodrug uptake, poor conversion rate of prodrugs, immunogenicity problems and difficulties in selective targeting, low transgene expression, and the bystander effect. Thus, the overall progress of the GDEPT clinical trials appears to be slow, mainly due to ineffective vectors in delivering therapeutics transgenes to tumor cells, especially in solid tumors where the inner tumor cells are not easily accessible. Therefore, a combination of conventional and newly emerged approaches is recommended to effectively eliminate cancerous cells. In recent studies, cancer nanotechnology in combination with anti-cancer prodrugs has been developed to facilitate the efficiency of drug delivery as well as prodrug localization at target sites or tissues. In recent studies, nanotechnology-based strategies have been reported to have efficient drug delivery, which may improve vector delivery. Several drug delivery strategies have been developed but yet to be fully explored and understood for the future development of new prodrug-based nanotechnology, including active targeting functionalization, the combination of drug therapy, and stimuli-sensitive drug release. In recent years, new advances in aptamer conjugation to Nano carriers have emerged for cancer treatment with lower toxicity and better efficacy, as it is low cost, small size that enables solid tumor penetration, higher affinity to bind with a wide range of targets, high selectivity, low toxicity, and immunogenicity. Hence, aptamers can be used as guiding molecules for delivering various drugs, macromolecules, or Nano carriers to target tumor cells. Currently, aptamer-functionalized nanoparticles have effectively been achieved in guiding various types of drugs into target tumor cells. However, there are several efforts needed to be improved, including the investment in aptamers, the improvement of chemical modification, and the ability to design prodrugs, to aid in better aptamer molecules as well as the successes of the GDEPT approach in the clinical and diagnostic application [42].

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