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Enzyme/Prodrug Systems for Cancer Gene Therapy

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Abstract The use of enzyme/prodrug system has gained attention because it could help improve the efficacy and safety of conventional cancer chemotherapies. In this approach, cancer cells are first transfected with a gene that can express an enzyme with ability to convert a nontoxic prodrug into its active cytotoxic form. As a result, the activated prodrug could kill the transfected cancer cells. Despite the significant progress of different suicide gene therapy protocols in preclinical studies and early clinical trials, none has reached the clinic due to several shortcomings. These include slow prodrug-drug conversion rate, low transfection/transduction efficiency of the vectors, and nonspecific toxicity/immunogenicity related to the delivery systems, plasmid DNA, enzymes, and/or prodrugs. This mini-review aims at providing an overview of the most widely used enzyme/prodrug systems with emphasis on reporting the results of the recent preclinical and clinical studies.

Keywords Enzyme prodrug · GDEPT · Suicide gene therapy · Bystander effect · Nitroreductase · Cytosine daminase · Thymidine kinase · Carboxyl esterase · Ganciclovir · Cancer gene therapy · CB1954 · SN-38 · CPT-11

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Introduction

In recent years, cancer treatment methods are becoming more focused on targeted approaches in order to maximize treatment efficacy while reducing side effects. One of these targeted approaches is termed suicide gene therapy, also known as gene-directed enzyme prodrug therapy (GDEPT). The success of suicide gene therapy is dependent on its three major functional components: enzyme, prodrug, and gene delivery system (vector).

The function of the vector is to carry the gene that encodes an enzyme to the target cancer cells for expression. In general, gene delivery systems can be divided into two major groups including viral (e.g., adenovirus, lentivirus, etc.) and nonviral (e.g., synthetic polymers and lipids, bacteria-based and cell-based) (Table 1). Each vector type comes with a series of advantages and disadvantages. Since the focus of this mini-review is not on vectors but on enzyme/prodrug systems, we will not discuss them further here. For more information related to the use of vectors for cancer gene therapy, the readers are referred to excellent recent reviews by Forbes [27], Cattaneo et al. [11], and Mohit and Rafati [63].

The function of the enzyme which is expressed by the transfected cancer cells is to convert the nontoxic/nonfunctional prodrug into its toxic (functional) form resulting in the death of the enzyme-producing cancer cells as well as neighboring cells through a phenomenon known as bystander effect (Fig. 1) [10, 26]. It is worth mentioning that the expression of the enzyme in transfected cells can be regulated by tumor-specific promoters. This regulatory element could enhance the enzyme/prodrug system's safety by restricting the enzyme expression only to the tumor cells. By applying the cancer-specific promoters, the suicide gene could

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**Table 1** Classification of the vectors used in suicide gene therapy of cancer

Vector type	Vector subtype	Advantages	Disadvantages	References
Viral vectors	Retrovirus/lentivirus	Long-term transgene expression Integrates the gene into host genome Low immunogenicity	Safety concerns (insertional mutagenesis)	[7, 21, 115]
	Adenovirus	Effect on dividing and nondividing cells Lower risk of host genome integration	Safety concern (high immunogenicity) Transient transgene expression	[48, 54, 111]
	Adeno-associated virus	Medium to high transgene expression Effect on dividing and nondividing cells No significant immunogenicity	Low DNA loading capacity Safety concerns (possibility of insertional mutagenesis)	[21, 60, 64, 88]
Nonviral vectors	Synthetic polymers and lipids	Ease of preparation Lower cost Lower immunogenicity	Lower transfection efficiency	[18, 43, 106]
	Amino acid-based vectors	Monodisperse and uniform constructs, ability to fine tune structure	Lower transfection efficiency	[8, 69, 108]
	Bacteria-based vectors	Large capacity for suicide enzyme loading Bacterial minicells (BMCs) are noninfectious.	Safety concern (infection by using live bacteria)	[33, 53, 100]
	Cell-based vectors	Tumor tropism Self-isolated cells without the immunogenicity concerns	Low efficiency of tropism High costs Safety concern (unknown fate)	[1, 70, 68]

get selectively expressed in cancer cells, while sparing normal ones. The promoter of human telomerase reverse transcriptase (hTERT) is one of the most widely used promoters in the field and is the only transcriptional control element that has successfully entered into clinical trials [67]. However, the problem associated with the use of hTERT promoter is its low expression activity. In order to enhance promoter's expression activity and also overcome the potential development of resistance by the tumor cells, several groups have tried to create more specific and efficacious ones. Recently, Rad 51, OPN, RAN, BRMS1, and MCM5 promoters were identified through screening of a large panel of normal and cancer cells, and interestingly, some of them showed significantly higher activity than hTERT promoter [14, 15,

32]. Another means of improving promoter activity is to artificially design a chimeric promoter. For example, a promoter with a stronger activity can be obtained by constructing the chimeric promoters with the fusion of two promoters [23] or various transcriptional regulatory elements [30, 89].

In the following sections, we provide an overview of the eight most widely investigated enzyme/prodrug systems which are shown to be effective in inducing tumor cell death and inhibiting cancer progression. The purpose of this concise mini-review is to discuss the forte and weaknesses associated with the efficacy and safety of each system. Therefore, the coverage of the literature in this review is not encyclopedic; rather, selected examples have been chosen to highlight certain important points.

Fig. 1 Schematic representation of the mechanism of action in suicide gene therapy. First, cancer cells are transfected with a gene that codes for an enzyme. The enzyme is able to convert the nontoxic prodrug into its cytotoxic form resulting in the death of the transfected cancer cells and the neighboring ones through a phenomenon known as bystander effect
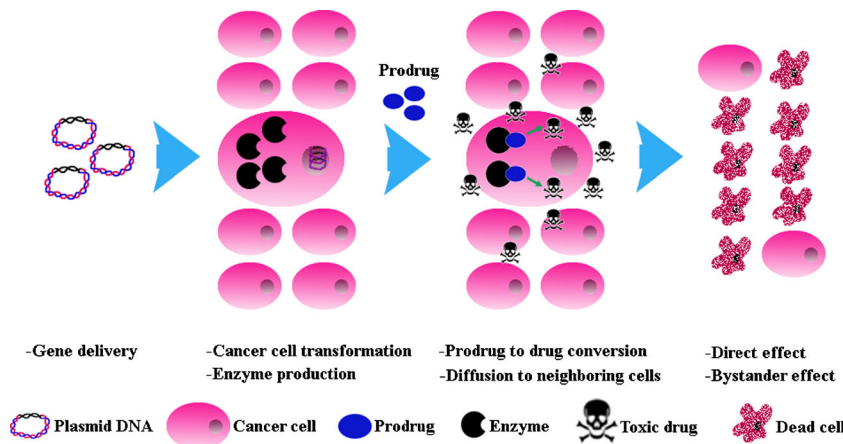
Enzyme/Prodrug Systems

Herpes Simplex Virus Thymidine Kinase (HSV-TK) /Ganciclovir

In this system, gancyclovir (GCV) undergoes a monophosphorylation step by herpes simplex virus thymidine kinase (HSV-TK) enzyme. After the first phosphorylation step, the triphosphate form of the GCV is produced with the help of endogenous kinase (Fig. 2a). In this form, the GCV-triphosphate can cause apoptosis by introducing chain termination and single-strand breaks into DNA [13]. One limitation of using GCV-triphosphate as anticancer agent is that it could only affect the dividing cells [4, 66, 87, 98]. The second drawback of this system is that the GCV-triphosphate transportation to surrounding cells is limited by the presence of gap junctional intercellular communications. Recently, Wu et al. showed that the bystander effect of the GCV-triphosphate can be enhanced by the expression of connexin32 (Cx32), which is responsible for producing gap junctions [112]. Furthermore, HSV-TK has a high affinity toward its natural substrate, thymidine, which leads to the need for using a high dose of GCV in order to win the competition [2]. This higher GCV dose could result in suppression of immune system and damage to bone marrow cells [50]. The enhanced activity of ATP-binding cassette (ABC) transporters in cancer-resistant cells is one of the main reasons of therapy resistance in cancer [55]. Therefore, ABCG2-mediated efflux of GCV in side population cells can limit the efficiency of this enzyme/prodrug system [35].

In the past decades, several attempts have been made in order to improve the efficiency of TK/GCV system. In one approach, modifications of enzyme active sites have resulted in generation of mutated forms of the enzyme, such as mutant 30 (L159I/I160L/F161A/A168Y/L169F), SR39 (L159I/I160F/F161L/A168F/L169M) [5, 50], A168H, and a mutated form of A168H (TK007) [78]. All these mutants have shown enhanced enzyme affinity toward GCV in comparison to wild type. In a different approach, an MDR-1-targeted small hairpin RNA (shRNA) was used to inhibit the ABC transporters which led to the accumulation and increase in drugs effect [73]. Other groups have also examined the use of other drugs such as acyclovir and valacyclovir instead of GCV and have observed more efficacy [93, 113].

TK/GCV system has also been used in combination with other therapeutic modalities in order to enhance its anticancer efficacy. For example, TK/valacyclovir in combination with surgery and accelerated radiation showed an increase in patient survival rates in malignant glioma patients [16]. This approach has also shown to not only increase the efficacy of the treatment but also decrease the toxicity in pancreatic adenocarcinoma [4]. Currently, there are a couple of clinical trials

in progress for patients with high-risk acute leukemia (NCT00914628) and in patients with recurrent prostate cancer (NCT01913106) (www.clinicaltrials.gov).

Cytosine Deaminase (CD)/5-Fluorocytosine

In this system, 5-fluorocytosine (5-FC) that is a nontoxic prodrug is converted to its active form, 5-fluorouracil (5-FU), by CD activity (Fig. 2b) [37]. Although 5-FU has been used for cancer chemotherapy, at its therapeutic dose, it causes significant side effects such as mucositis, myelosuppression, dermatitis, and cardiac toxicity. Due to these side effects, its application as prodrug in the form of 5-FC and in combination with cytodeaminase enzyme has gained momentum in the past decades. Two forms of cytosine deaminase, bacteria (bCD) and yeast (yCD), have been investigated in the context of cancer therapy. After activation by CD, 5-FU can be further changed into potent pyrimidine antimetabolites by other cellular enzymes. 5-FU can inhibit thymidylate synthase which consequently leads to the cell cycle arrest and apoptosis. Since 5-FU traverse into the neighboring cells independent of gap junctions and even can pass the blood-brain barrier by diffusion, it induces a significant bystander effect [25, 45]. Because of its unique properties, CD/5-FC system has been used for the treatment of different types of cancer, such as colon carcinoma, glioma, and pancreatic cancer [24, 41, 42]. Having been combined with radiotherapy, CD/5-FC has shown quite a promising result due to the radio-sensitizing effect of 5-FU on the treated cells [65]. In comparison to HSV-TK/GCV, CD/5-FC has shown better results in renal and colorectal carcinoma probably due to its more potent bystander effect [92, 99].

Although use of CD/5-FC system has several strengths as mentioned above, there are some drawbacks that yet need to be addressed. The first drawback is due to the presence of normal flora in the gut which is able to convert 5-FC to 5-FU resulting in side effects. Several groups have worked to create bCD mutants in order to improve its kinetic showing promising results [24, 40, 41]. Yeast cytosine deaminase is another form of the enzyme which provides a few advantages over bCD including high affinity and better prodrug conversion kinetics. In addition, yCD can be fused with E. coli uracil phosphoribosyl transferase (UPRT) which is absent in mammalian cells. UPRT can directly convert 5-FU to 5-FdUMP [107] resulting in significant improvement of activity and enhanced cancer cell killing efficiency in prostate, ovarian, colon, and breast cancers [62, 81]. Some studies have also shown that yCD:UPRT/5-FC system may not get affected by acquired 5-FU resistance and could potentially kill both proliferating and nonproliferating cancer cells [52, 81].

While past clinical trials with CD/5-FC system alone have reported limited success [44], there are several new clinical trials in progress which could produce interesting outcomes. For instance, in one trial, genetically modified neural stem
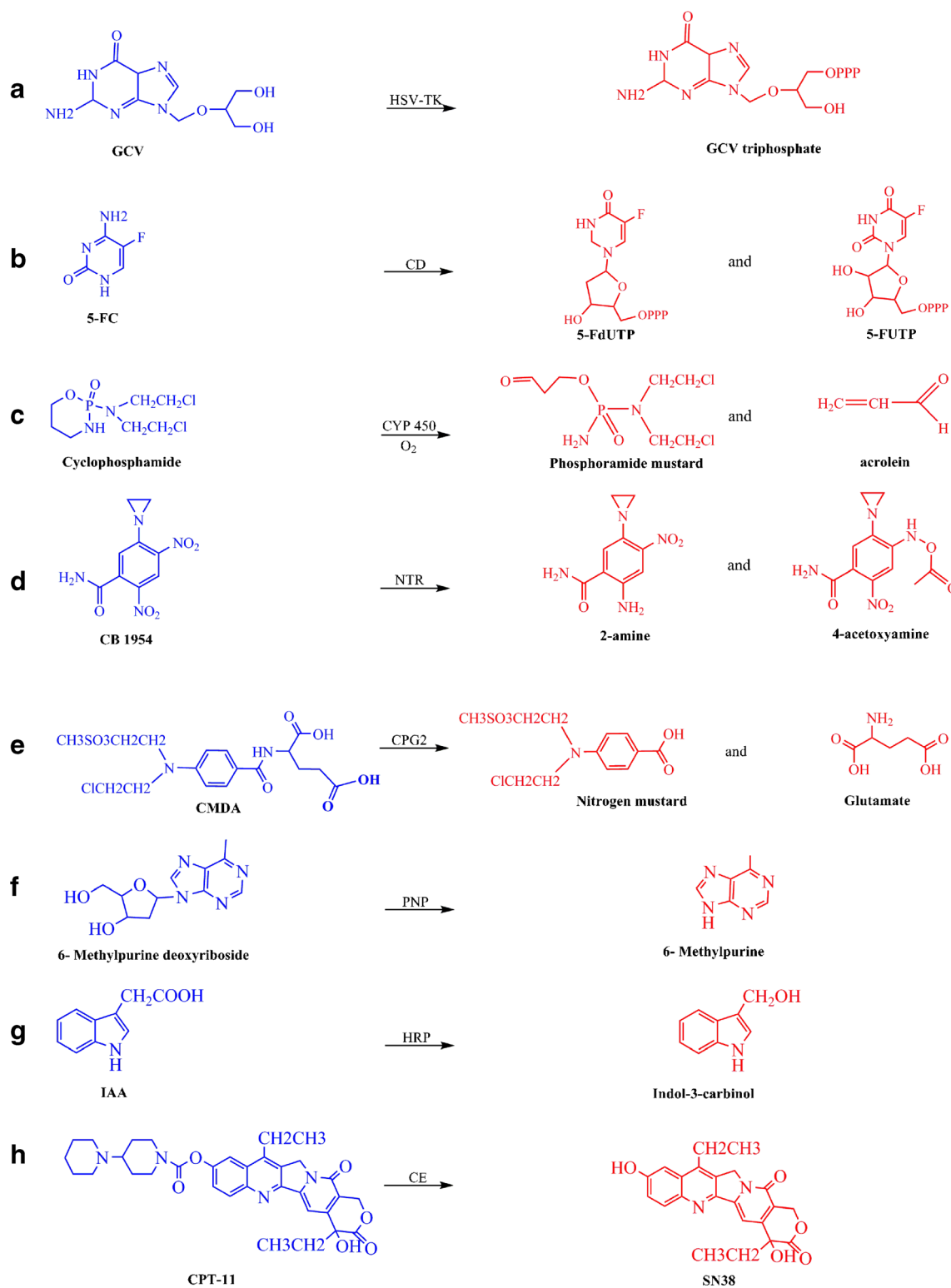
Fig. 2 Chemical structures of the inactive prodrugs and their cytotoxic forms after activation by the corresponding enzyme

cells expressing bCD are used in combination with orally administered 5-FC for the treatment of recurrent gliomas (NCT01172964). In another phase I–II study, an investigational drug APS001F is currently being examined in combination with a recombinant bacterium (*Bifidobacterium longum*) which can produce CD (NCT01562626). Finally, there is a phase I study in progress for the treatment of grade III/IV gliomas by using retroviral replicating vector (Toca

511) in combination with 5-FC (NCT01470794). The results of these ongoing trials are not reported but can be followed at www.clinicaltrials.gov.

Cytochrome P450 (CYP450)/Oxazaphosphorine

Cytochrome P450 (CYP) enzymes are important factors in xenobiotics metabolism as they are responsible for deactivation of the toxic drugs in the liver. It has also been shown that these enzymes play an important role as an activating factor for oxazaphosphorine agents [72]. Oxazaphosphorine is a class of anticancer drugs that includes cyclophosphamide (CPA) and ifosfamide (IFA). Both of these two akylating agents are the substrates of P450 enzymes which metabolizes these compounds to yield a 4-hydroxy derivative. Then, the resulting metabolites continue their chemical reaction to produce phosphoramide or ifosphoramide mustard and acrolein (Fig. 2c). Ultimately, they form DNA cross-links and kill the cells. The resulted active reagents can be transported to the surrounding cells, independent of gap junctions, which gives them potent bystander effect [85]. Another advantage of this system in comparison to the other enzyme/prodrug systems is its less immunogenicity due to the presence of P450 enzymes in human body [70].

One major drawback of this system is the natural metabolism of these prodrugs by the liver P450 enzymes that generates active toxic metabolites in the whole body and eventually causes various side effects, including cardiotoxicity, bone marrow suppression, neurotoxicity, and nephrotoxicity. Some of these toxicities can be associated with the chloroacetaldehyde formation from CPA and IFA via N-dechloroethylation [12]. Therefore, the big challenge in this system is the inhibition of P450 enzyme activity in the liver so that the prodrug gets activated only in the tumor sites [29].

A few methods have been used to improve the cancer treatment efficacy of P450 GDEPT and to reduce its side effects. In one approach, combined or in parallel with CYP450/oxazaphosphorine treatment, anti-thyroid drugs, such as propylthiouracil and methimazole were used to inhibit hepatic P450 reductase activity and improve the prodrug activation in tumor cells [84]. In another approach, a CYP2B6/RED fusion protein was designed so that it can transfer electrons from NADPH to the heme moiety of the protein. This modification increases the efficiency of CYP2B6 substrate metabolism into active drugs [102].

In a phase I/II clinical trial, a recombinant retroviral vector that contains human CYP450 type 2B6 genes (CYP2B6), neomycin resistance marker gene and *E. coli lacZ* has been tested. In this study, gene-based delivery of CYP2B6 to the tumor site led to prodrug activation locally and produced higher concentrations of the active drug at the target site. The promising results of this study inspired the use of this approach in other cancer treatment studies [7]. However,

regardless of the exciting results, there are still serious concerns about using this system for cancer therapy because of normal activity of CYP450 in human cells and difficulty in the delivery of the GDEPT elements to the targeted cancer cells without causing side effects on normal healthy cells [51, 82].

Nitroreductase/CB1954

This enzyme/prodrug system contains group of nitroreductase (NTR) enzymes that can activate prodrugs, such as CB1954 (5-(aziridin-1-yl)-2,4-dinitro-benzamide) (Fig. 2d). Two classes of enzymes and four types of prodrugs have been studied in this system. The two classes of enzymes are categorized based on their sensitivity to oxygen. Type I NTR can produce nitroso, hydroxylamine, and/or amine terminated products in the presence of molecular oxygen, while type II, on the other hand, can only produce these products when oxygen is absent [110]. The four classes of prodrugs include dinitroaziridinyl benzamides, dinitrobenzamide mustards, 4-nitrobenzyl carbamates, and nitroindolines. CB1954, which is the most widely used prodrug, is from dinitrobenzamide group [91]. The metabolites of this prodrug can freely diffuse to the surrounding cells which induce a strong bystander effect. They can damage DNA by their chelating property leading to p53 and cell cycle-independent apoptosis in both dividing and nondividing cells. This is an important advantage of the system over the ones that can only target the dividing cells [105]. While several studies have reported promising results, the low activation rate of CB1954 is one major limitation associated with this enzyme/prodrug system. At least two general approaches have been tested to improve the efficacy of this system. One is the engineering and application of alternative CB1954-activating nitroreductases, such as AzoR, NFsA, NemA, and flavin reductase I (FRase I) [79, 97]. The other approach is the synthesis of other nitroreductase prodrugs such as nitro-CBI-DEI and PR-104A [31, 80]. Both approaches have shown promising results in vitro and in vivo. Effective use of this enzyme/prodrug system has been demonstrated in a few clinical trials for the treatment of prostate and liver cancers [76]. However, the CB1954 low conversion rates and dose-dependent hepatotoxicity remained as major challenges for clinical application of this GDEPT method [46, 61]. For additional and more in depth information related to the nitroreductase/CB1954 system, readers are referred to an excellent review by Williams et al. [110].

Carboxypeptidase G2/Nitrogen Mustard

The enzyme carboxypeptidase G2 (CPG2) is derived from the *Pseudomonas* strain RS-16 and has no human analog. This enzyme cleaves glutamic acid from nitrogen mustard-based drugs, such as 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA)

and bis-iodo phenol mustard (ZD2767P), into DNA alkylating agents, without any further modification (Fig. 2e). The resulted active drug can cause inter- and intra-strand DNA cross-linking, leading to apoptosis in both dividing and nondividing cells. It can also freely diffuse through cell membranes independent of gap junctions which offer potent bystander effect. Being independent from host enzymes for prodrug activation gives this system an outstanding benefit of a higher prodrug conversion rate, in comparison to the other enzyme/prodrug systems [22]. While different nitrogen mustard reagents have been developed and tested in the past two decades, 4-[(2-chloroethyl)(2-mesyloxyethyl)amino] benzoyl-l-glutamicacid (CMDA) is among the first successful ones. Several attempts have been made to increase the potency of CMDA. For example, ZD2767P is 300 times more potent than CMDA [28].

To increase the enzyme efficiency, a mutated form of the CPG2 gene was fused to vascular endothelial growth factor (VEGF). This modification resulted in binding of secreted CPG2 to the VEGF receptor, which kept CMDA around tumor mass and significantly reduced systemic side effects [96]. This enzyme prodrug system was used in attachment with tumor-specific antibodies in different clinical studies. However, some issues, like immunogenicity against mouse antibodies and CPG2 and also insufficient localization of enzyme\antibody conjugate, limited its success in cancer therapy [28, 56, 58].

Purine Nucleoside Phosphorylase/6-Methylpurine Deoxyriboside

In this system, prodrugs such as 6-methylpurine 2-deoxyriboside and fludarabine can be converted to 2-deoxyribose-1-phosphate by the *E. coli* purine nucleoside phosphorylase (PNP) (Fig. 2f). The advantage of using PNP/MEP is its high bystander activity based on gap junction-free transport of activated drugs, its effect on both proliferating and nonproliferating cells, and its unique mechanism of action that is independent of DNA synthesis. PNP catalyzes the cleavage in glycoside bond of (deoxy) adenosine-based substrates that produces either (MeP) or 2-fluoroadenine (F-Ade). Then, these substrates are converted to their triphosphate forms by cellular monophosphate and diphosphate kinases, which can inhibit both RNA and protein syntheses [74, 94]. Three studied prodrugs of this system are 6-methylpurine-2′-deoxyriboside (MeP-dR), 2-F-2′-deoxyadenosine (F-dAdo), and arabinofuranosyl-2-F-adenine monophosphate (F-araAMP). Among them, F-araAMP is clinically approved for chronic lymphocytic leukemia treatment [95]. These (deoxy) adenosine-based analogs are only the substrates of *E. coli* PNP and cannot be recognized by humane PNP. Thus, the prodrugs cannot be converted into toxic metabolites in normal tissues and consequently limits the off-target toxicities. However, the application of bacterial PNP can induce host immune response. A few groups have worked on the development of human PNP that can consume the (deoxy) adenosine-based prodrugs as its substrate [3, 75]. Among the recorded clinical trials, there is only one completed phase I clinical trial using this system. In this trial, the safety of *E. coli* PNP/fludarabine phosphate in patients with head and neck cancers has been studied. It was shown that localized production of fluoroadenine within tumor tissues, using fludarabine and *E. coli* PNP system, is successful in eradicating tumors without causing any significant toxicity [83].

Horseradish Peroxidase/Indole-3-Acetic Acid

This system consists of the horseradish peroxidase (HRP) enzyme and indole-3-acetic acid (IAA) as the prodrug. HRP enzyme oxidizes IAA at neutral pH to its cationic form that produces the carbon-centered skatolyl radical. This radical can be changed to peroxyl radical in the presence of oxygen which would turn into by-products, such as indole-3-carbinol, oxindole-3-carbinol, and 3-methylene-2-oxindole (Fig. 2g). These reactive by-products can induce DNA strand breaks, leading to apoptosis and cell death. This system also has a potent bystander effect because of the transportation of the activated drug via diffusion to the untransfected neighboring cells [30]. HRP/IAA system has been tested in several in vitro *and* in vivo models, including bladder, melanoma, and pancreatic cancer [36, 39, 47]. However, the major concern of using HRP/IAA system is the low expression level of the enzyme in transfected cells [19, 101]. The second drawback of this system is the origin of the enzyme which comes from plants, because it results in undefined mixture of isoenzyme with different glycosylation patterns which can cause side effect in body. To avoid this problem, different recombinant forms of HRP isoenzymes are produced, which have shown significant effects on breast and bladder carcinoma [6]. Although some promising results have been obtained from HRP/IAA system, there has been no clinical trial with this system by the time of writing this manuscript (www.clinicaltrials.gov).

Carboxylesterase (CE)/Irinotecan

Carboxylesterases are active endogenous enzymes in human body that play roles in hydrolysis of different ester and amide-containing chemicals [34]. Because of this property, they can convert irinotecan (CPT-11), a less cytotoxic agent, into its highly toxic form, SN-38 (Fig. 2h). SN-38 inhibits topoisomerase I and thus alters the DNA relaxation machinery [34, 77]. This enzyme/prodrug system has been used for the treatment of colorectal cancer [104], glioma [17], and various other tumor

models [49, 57, 114]. While this enzyme has different isoforms, carboxylesterase-1 (CE1), which is mostly localized in the liver, is the most widely used one. Unfortunately, CE1 suffers from the low catalytic efficiency that limits the conversion rate of CPT-11 to SN-38. To overcome this deficiency, another isoform of this enzyme also known as CE2 has been studied. CE2 isoform is commonly expressed in intestine. CE2 has a higher catalytic efficiency than CE1 [38, 86] and has been used for the treatment of pancreatic and colon cancer [9, 71]. The rabbit analog of CE1 (rCE1) has also been engineered and used for conversion of CPT-11 to SN-38. While this isoform has shown a higher catalytic efficiency in comparison to human CE1 [20], it can cause immune response in human host. To avoid this problem, mutant CE1 (hCE1m6) was engineered using homology alignment of human and rabbit version of CE1 followed by replacing eight amino acids in human CE1. This change in sequence resulted in higher conversion efficiency [109].

Given that SN-38 has poor water solubility, its bystander activity may get reduced [103]. In an attempt to improve the bystander effect and/or overall anticancer efficacy, secretory form of CE2 has been genetically engineered [71]. This approach has shown promising success in several preclinical models [59, 90]. Currently, there are a few ongoing clinical trials using this enzyme/prodrug system. For instance, there is a phase I clinical trial in progress in which the patients with rrecurrent high-grade gliomas are undergoing treatment. Here, genetically modified neural stem cells which can express carboxylesterase are used in combination with irinotecan hydrochloride (NCT02192359).

Conclusion

Significant amount of preclinical work have been performed in the past two decades on gene-directed enzyme prodrug therapy of cancer. Based on the preclinical data, numerous clinical trials have been performed. While substantial progress has been made, no cancer suicide gene therapy protocol has been approved for clinical use. Important issues that have yet to be addressed include development of effective vectors for efficient and targeted gene transfer to the tumor cells, design of prodrugs that can be efficiently converted into the active drugs with potent bystander effect, and engineering enzymes with low immunogenicity and high affinity toward prodrugs. Overcoming these barriers may open new doors into effective eradication of many of yet incurable and stubborn cancers through GDEPT.

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