Minireview

Strategies for Enzyme/Prodrug Cancer Therapy

Guang Xu and Howard L. McLeod

Washington University School of Medicine, Departments of Medicine, Molecular Biology & Pharmacology, and Genetics and the Siteman Cancer Center, St. Louis, Missouri 63110

Abstract

The selective activation of prodrug(s) in tumor tissues by exogenous enzyme(s) for cancer therapy can be accomplished by several ways, including gene-directed enzyme prodrug therapy (GDEPT), virus-directed enzyme prodrug therapy (VDEPT), and antibody-directed enzyme prodrug therapy (ADEPT). The central part of enzyme/prodrug cancer therapy is to deliver drug-activating enzyme gene or functional protein to tumor tissues, followed by systemic administration of a prodrug. Although each approach (GDEPT, VDEPT, and ADEPT) has been tested in clinical trials, there are some potential problems using the current delivery systems. In this article, disadvantages and advantages associated with each approach (GDEPT, VDEPT, and ADEPT) and future perspective for improving current systems are discussed.

Introduction

Chemotherapy is an important treatment for cancer patients. However, its success is limited by several drawbacks, including insufficient drug concentrations in tumors, systemic toxicity, lack of selectivity for tumor cells over normal cells, and the appearance of drug-resistant tumor cells (1–7). A number of strategies have been used to overcome these problems, including alternative formulations (e.g., liposomes; Ref. 8), resistance modulation (e.g., PSC833; Ref. 9), antidotes/toxicity modifiers (e.g., ICRI-187; Ref. 10), and gene therapy. One promising area for improving tumor selectivity is enzyme prodrug therapy.

Enzyme-activating prodrug therapy is a two-step approach. In the first step, a drug-activating enzyme is targeted and expressed in tumors. In the second step, a nontoxic prodrug, a substrate for the expressed enzyme in tumors but not be activated by endogenous enzyme in nontumor tissues, must be long enough to induce a bystander effect but short enough to avoid the drug leaking out into the systemic circulation (13).

Currently, delivery methods for an enzyme/prodrug strategy can be divided into two major classes: (a) delivery of genes that encode prodrug-activating enzymes into tumor tissues (GDEPT, VDEPT, etc.); and (b) delivery of active enzymes onto tumor tissues (ADEPT). An overview of these strategies is shown in Fig. 1. The aim of this review is to summarize some of the areas of recent progress in enzyme-activating prodrug therapy and discuss areas of future development.

Approaches to Deliver Genes Encoding Prodrug-activating Enzymes into Tumor Cells

GDEPT. GDEPT, also known as suicide gene therapy, is a technique that involves physical delivery of a gene for a foreign enzyme to tumor cells where a systemically administered nontoxic prodrug can be activated after expression of the enzyme (5, 13–16). Many GDEPT studies have used liposomal gene delivery, but the challenge of vector delivery is common for all areas of gene therapy and has been exhaustively reviewed elsewhere (3, 11, 13). An early example of GDEPT is the combination of HSV-TK and GCV. GCV is an antiviral drug, which is phosphorylated by HSV-TK and then by cellular kinases to produce GCV triphosphate, which disrupts DNA synthesis during S phase, leading to cell death (1, 4, 16–20). A second early example is the combination of the bacterial CD and the antifungal drug 5-FC, which was effective to kill tumor cells expressed only at low concentrations in normal tissues (11, 12). The protein must achieve sufficient expression in the tumors and have high catalytic activity (13). The prodrug should be a good substrate for the expressed enzyme in tumors but not be activated by endogenous enzyme in nontumor tissues. It must be able to cross the tumor cell membrane for intracellular activation, and the cytotoxicity differential between the prodrug and its corresponding active drug should be as high as possible. It is preferred that the activated drug be highly diffusible or be actively taken up by adjacent nonexpressing cancer cells for a “bystander” killing effect, the ability to kill any neighboring nonexpressing cells (13). In addition, the half-life of active drug should be long enough to induce a bystander effect but short enough to avoid the drug leaking out into the systemic circulation (13).

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3 The abbreviations used are: GDEPT, gene-directed enzyme prodrug therapy; ADEPT, antibody-directed enzyme prodrug therapy; CB1954, 5-(aziridin-1-yl)-2,4-dinitrobenzamide; CD, cytosine deaminase; CEA, carcinoembryonic antigen; CES, carboxylesterase; CMDA, 4-(2-chloroethoxyethyl)4-(2-mesyloxyethyl) aminobenzyl-1-glutamic acid; CPA, carboxypeptidase A; CPG2, carboxypeptidase G2; CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] benzoxyloxy-camptothecin (irinotecan); CYP, cytochrome P450 isozyme; 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; GCV, ganciclovir; GPAT, genetic prodrug activation therapy; HSV, herpes simplex virus; IFA, ifosfamide; MTX, methotrexate; NTR, nitroreductase; ODC, ornithine decarboxylase; RED, flavoenzyme NADPH-p450 reductase; TK, thymidine kinase; TRE, tumor-specific transcriptional regulatory elements; VDEPT, virus-directed enzyme prodrug therapy.
after the conversion by CD to active 5-FU (20, 21). In addition to the above classical examples, many other enzyme/prodrug combinations have been used for GDEPT (Table 1; Refs. 13 and 22).

Many of the enzyme/prodrug systems applied in GDEPT, including HSV-TK/GCV, Escherichia coli CD/5-FC, and E. coli NTR/CB1954, act intracellularly by converting prodrugs into active drugs within cells (2). This mode of action requires cell-cell contact to mediate maximal killing. Recently, an extracellular cytotoxic effector system was reported in which a secreted form of lysosomal human β/H9252-glucuronidase, which converts an inactive glucuronidated derivative of doxorubicin (HMR 1826) to the cytotoxic doxorubicin, was introduced into tumor cells (2). There was evidence that a bystander effect resulted in a significant tumor cell killing both in vitro and in a human xenograft model in nude mice (2). This mode of action requires cell-cell contact to mediate maximal killing. Recently, an extracellular cytotoxic effector system was reported in which a secreted form of lysosomal human β/H9252-glucuronidase, which converts an inactive glucuronidated derivative of doxorubicin (HMR 1826) to the cytotoxic doxorubicin, was introduced into tumor cells (2). There was evidence that a bystander effect resulted in a significant tumor cell killing both in vitro and in a human xenograft model in nude mice (2). The advantage of this extracellular system is that a hydrophilic prodrug was converted into a lipophilic, cell-permeable cytotoxic drug outside cells so that both transduced and nontransduced cells were targeted. As cell-cell contact is not required for a bystander effect, the killing ability of this system is enhanced. To explore the mechanism of a bystander effect of the NTR/CB1954 system, hydroxylamine metabolites of CB1954 were found to be able to cross cell membranes freely and are responsible for a bystander effect, independent of gap junctions (23). Although the cell-permeable active metabolites of prodrugs for GDEPT have advantages in certain situations, such as tumors with few gap junctions, the downside of this mode is the cytotoxicity to adjacent normal tissues and the risk of systemic diffusion (23).

GDEPT can also be used to improve the selectivity of currently used agents. CYP-based prodrug activation systems is one example that shows promise for clinical use (27). Members of the CYP enzyme superfamily convert the chemotherapeutic agents cyclophosphamide and IFA to active alkylating agents that cause cell death (27). The expression of CYP is generally high in liver but lower in tumor cells (27), providing a potential mechanism for intrinsic drug resistance. Tumor cells are highly sensitive to cyclophosphamide or IFA after the delivery of the CYP2B1 gene into tumor cells both in vitro and in vivo (28). To additionally improve and increase the efficiency of this system, a two enzymes/one prodrug system was developed. In this study, CYP2B1 was coexpressed with the RED gene and used to

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**Table 1** Selected examples of GDEPT

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Prodrugs</th>
<th>Model systems</th>
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<td>Human β-glucuronidase</td>
<td>HMR 1826</td>
<td>Tumor cells and xenograft model in nude mice</td>
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<td>Bacterial nitroreductase</td>
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<td>Carboxypeptidase</td>
<td>MTX-α-peptide</td>
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<td>CYP2B1 and p450 reductase</td>
<td>Cyclophosphamide</td>
<td>Rat 9L gliosarcoma cells</td>
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<tr>
<td>Rabbit CYP4B1</td>
<td>2-AA or 4-IM&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Human, rat glioma cells and in nude mice tumor model</td>
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<tr>
<td>Thymidine phosphorylase</td>
<td>5-FU or 5'-DFUR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>LS 174T human colon carcinoma cells</td>
<td>1</td>
</tr>
<tr>
<td>Rabbit and human carboxylesterase</td>
<td>Frinotecan</td>
<td>Glioblastoma and rhabdomyosarcoma cells and preclinical mouse xenograft model</td>
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<tr>
<td>E. coli β-galactosidase</td>
<td>Anthracycline</td>
<td>Human melanoma cells</td>
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<tr>
<td>Cytosine deaminase</td>
<td>5-FC</td>
<td>Murine fibroblast cells</td>
<td>21</td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>GCV</td>
<td>Cisplatin-resistant human ovarian carcinoma cells</td>
<td>26</td>
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</table>

<sup>a</sup> 2-AA, aminosanthracene; 4-IM, 4-ipomennol; D'FUR, 5'-deoxy-5-fluorouridine.
activate prodrug cyclophosphamide in tumor cells (18). The rationale is that CYP-catalyzed prodrug activation is dependent on electron reduction from the flavoenzyme RED, an enzyme widely expressed in many cell types, including tumor cells. Despite the significant level of endogenous basal expression, the authors found that RED gene transfer greatly enhanced the cyclophosphamide sensitivity of tumor cells transfected with CYP2B1. Thus, endogenous RED levels in tumor tissues may be an important determinant for the sensitivity of tumor cells to CYP2B1. Consequently, endogenous RED levels in tumor tissues may be an important determinant for the sensitivity of tumor cells to CYP2B1. The result also suggested that CYP-based cancer gene therapy might be particularly effective for tumors that express high endogenous levels of RED.

Recently, an approach to improve the CPA/MTX-α-peptide system was described (4). CPA is a zymogen that becomes catalytically active after its propeptide is removed by trypsin (4). Activated CPA converts MTX-α-peptide prodrug into active MTX that inhibits dihydrofolate reductase and causes cell death. Because trypsin is localized in the small intestine but is absent in tumors, the prodrug activation by CPA is limited to the intestine, causing local toxicity and low drug concentration in tumors. To activate the prodrug intratumorally in a trypsin-independent manner, a battery of CPA mutants were created in which the trypsin cleavage sites were mutated at the recognition sites for mammalian propeptidases. These constructs were cotransfected into COS-1 cells with propeptidases, endogenous cellular enzymes present in tumor cells. They found that one mutant, CPA95, had a high level of endogenously activated enzyme in cotransfected cells, and it was able to secrete and sensitize tumor cells to MTX-α-peptide in a manner similar to that of trypsin-activated wild-type CPA (4). This mutant CPA/MTX prodrug system might represent a novel approach in activating prodrug intratumorally attributable to bypassing an activation step. An additional advantage of this system is the potential use for other antifolates, such as the α-peptide conjugates of thymidylate synthase inhibitors.

**VDEPT.** VDEPT is a pharmacologically oriented gene therapy strategy that uses viral vectors to deliver a gene that encodes an enzyme that is capable of converting a systemically administrated nontoxic prodrug into a cytotoxic agent within tumor cells (20, 29). The NTR/CPA95 combination was an initial example of VDEPT in which colorectal and pancreatic cancer cells were found to be sensitized to CB1954 after retroviral transduction and expression of the *E. coli* NTR gene (20, 30). Currently, several viruses have been used for VDEPT, including retroviruses, adenoviruses, HSV (31), adenov-associated virus (32–34), lentivirus, and EBV (35). Over the years, many drug-activating enzyme/gene/prodrug combinations have been delivered into tumors in vitro or in vivo by VDEPT, the majority using CD/5-FC or HSV-TK/GCV with the involvement of retroviral and adenoviral vectors. These examples were reviewed elsewhere (29, 36). Several recent illustrations of VDEPT are described below and are also listed in Table 2 to highlight novel therapeutic strategies.

Recently, recombinant retroviruses were used to individually deliver six different cyclophosphamide- or IFA-metabolizing human CYP genes to 9L gliosarcoma cells (43). It was found that CYP2B6 and CYP2C18 transfection yielded pronounced cytotoxicity after cyclophosphamide treatment, with more efficient prodrug activation and cytotoxicity observed after cotransfection with RED. Antitumor activity was also seen after VDEPT in immunodeficient mice bearing tumor xenografts. Taken together, the results indicated that either CYP2B6 or CYP2C18 plus RED may be an excellent combination for use with prodrug cyclophosphamide in CYP-based cancer gene therapy. A retroviral gene transfer approach has also been used for NTR/CPA95 enzyme/prodrug therapy by two different groups (20, 30). In female BALB/c nude mice bearing s.c. NTR-expressing human pancreatic carcinoma cells, administration of CB1954 resulted in tumor regression, growth delay, and significantly increased median survival (20). In another study, the *E. coli* NTR gene was introduced into colorectal and pancreatic cancer cell lines by retroviral delivery. It was shown that NTR-expressing clones of both cell lines were more sensitive to cytotoxic effects mediated by the prodrug CB1954 (30), suggesting that NTR and CB1954 may be an attractive combination for VDEPT.

NTR has also been delivered into tumor cells by a replication-defective adenovirus vector containing an NTR expression cassette (40). In this study, ovarian carcinoma cells were grown at low-serum condition, mimicking the low rate of cell proliferation in human tumors. It was shown that NTR-expressing cells were sensitive to CB1954. Additionally, cisplatin-resistant cells that expressed NTR were also susceptible to CB1954, suggesting this system might be useful for patients with cisplatin-resistant tumors. An adenoviral vector was used

**Table 2** Selected examples of VDEPT

<table>
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<tr>
<th>Viral vectors</th>
<th>Enzymes delivered</th>
<th>Prodrugs</th>
<th>Model system</th>
<th>References</th>
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<tr>
<td>Adenovirus</td>
<td><em>Herpes simplex virus thymidine kinase</em> Human carboxylesterase</td>
<td>GCV Irinotecan</td>
<td>Mouse prostate cancer cell line and clinical trials Human lung adenocarcinoma cell lines and nude mice tumor model</td>
<td>19, 37, 38 39</td>
</tr>
<tr>
<td><em>E. coli</em> nitroreductase</td>
<td>CB1954</td>
<td>Ovarian tumor cells and animal model of disseminated intraperitoneal carcinoma</td>
<td>40, 41</td>
<td></td>
</tr>
<tr>
<td>Retrovirus</td>
<td><em>E. coli</em> nitroreductase</td>
<td>CB1954</td>
<td>Colorectal, pancreatic, ovarian cancer cells and xenografts of human ovarian &amp; pancreatic cancer</td>
<td>20, 30, 41</td>
</tr>
<tr>
<td>Yeast cytosine deaminase</td>
<td>5-FC</td>
<td>SCC VII murine squamous carcinoma cells and YCD-expressing tumors</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Human CYP and p450 reductase</td>
<td>Cyclophosphamide and IFA</td>
<td>Gliosarcoma cells and <em>in vivo</em> tumor model</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>EBV Nitroreductase</td>
<td>CB1954</td>
<td>EBV-positive B-cell lines</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>
to carry the human CES gene, driven by the cytomegalovirus promoter, to infect human lung adenocarcinoma cells in vitro and in vivo (39). It was shown that CES was efficiently expressed in these cells. In the presence of CPT-11, the growth of adenovirus-infected cells was greatly inhibited, and additional cell mixing studies indicated the presence of a bystander effect in this system. Results from in vitro studies using nude mice bearing s.c. A549 tumors and receiving CPT-11 at three sites within the tumor indicated that there was a significant reduction in tumor size.

Besides the above in vitro viral delivery systems, either retroviral or adenoviral vectors have been used to deliver HSV-TK into human tumors in Phase I/II clinical trials (44–48). The targeted human tumors included recurrent glioblastoma multiforme (44, 46), adenocarcinoma of the prostate (45), and malignant mesothelioma in the pleural cavity were selected as a model for treatment of a localized malignancy (37). Adenoviral vector-mediated intrapleural HSV-TK injection (1 × 10^8 – 1 × 10^12 plaque-forming units), followed by systemic administration of GCV for 2 weeks, was given to these patients. HSV-TK gene transfer was observed in 11 of 20 patients in a dose-related manner, with minimal side effects, suggesting treatment of localized malignancy using this vector is feasible. No evidence of clinical activity was noted in this study (37). The main clinical challenge appears to be overcoming the failure to achieve cDNA expression in a sufficient number of human tumor cells.

Despite extensive use of retroviral and adenoviral vectors to deliver prodrg-activating enzyme genes, both vectors have some disadvantages, which limit the use of VDEPT (42). The major disadvantage associated with a retroviral vector is that recombinant retroviruses only target dividing cells, whereas most human tumor cells are slowly dividing, yielding a low transduction rate (2–10% Ref. 42). When this strategy uses HSV-TK/GCV or CD/5-FC to generate antimitables, which also require cell division for activity, it is not surprising that the results are less than dramatic. However, this drawback could be beneficial in some case. Brain tumors, where only tumor cells are proliferating, allow for a high tumor:normal transfection differential for retroviral delivery (48). The low retrovirus titer, leading to decreased infection efficiency, is another drawback. Some researchers have been trying to increase retrovirus titer for VDEPT. It was found that prolonged low speed centrifugation during viral preparation was a simple way to concentrate recombinant retrovirus ~100-fold (20). Another disadvantage is that retroviruses produced from murine or dog cells are all sensitive to human serum when applied in human subjects, whereas viral particles generated from human cells are more resistant (13). To solve this problem, efforts have been made to develop a variety of packaging cell lines that produce high-titer recombinant retroviruses resistant to human serum. In addition, a soluble protein called GaLa1–3Gal was found to protect retroviruses from human serum when coadministered with retroviruses (13). The other disadvantages associated with retroviral vectors include immunogenicity, risk of insertional mutagenesis, risk of reversal to wild-type virus, envelope-induced complement-mediated inactivation, difficulties in producing high-titer viruses, and only targeting dividing cells (11, 13). Compared with retroviruses, adenoviruses have some advantages, including higher titers capable of generating infections in both dividing and nondividing cells (13). The disadvantages of adenoviral vectors include immunogenenicity, reversal to wild type, and short periods of gene expression in dividing cells (11). In addition to adenoviral and retroviral vectors, an EBV-based viral vector has been used to deliver exogenous enzyme-encoding CD or NTR into EBV-positive B-cell lines to activate 5-FC or CB1954, respectively (35). Both enzyme systems were effective to kill tumor cells in vitro in a prodrug-dependent manner.

Both the adenoviral and retroviral vectors mentioned above are replication incompetent, which limit their ability to infect additional cells subsequent to the initial infection event (49). Although the bystander effect increases the degree of tumor cell killing, additional strategies are needed to enhance the therapeutic efficacy of VDEPT. Viral prodrg-activating enzyme constructs have been engineered to retain viral oncolytic potential and replication capability. The most well-studied example is Onyx-015, an E1B-55D-deleted adenovirus that selectively replicates and lyses p53-deficient cancer cells, while sparing cells with functional p53 (50–53). Over 10 clinical trials (Phase I–III) in >200 patients with recurrent squamous cell carcinoma of the head and neck indicated that this single viral agent alone has antitumor activity (50, 54, 55). There is a suggestion that this activity is additionally enhanced when Onyx-015 is combined with conventional cytotoxic chemotherapy (51). Onyx-015 has been used to express HSV-TK for the treatment of human colon cancer xenografts in nude mice (49). E1B-55D-deleted adenovirus carrying HSV-TK alone was as effective as a standard replication-deficient adenoviral vector expressing HSV-TK in combination with GCV. The addition of GCV enhanced the antitumor effect of E1B-55D-deleted adenovirus carrying HSV-TK by 5-fold (49). Recently, combined oncolysis and prodrug bioactivation was studied using a HSV-1 mutant in which the viral ribonucleotide reductase gene was inactivated by insertion of transgene sequences encoding yeast CD (31). In cultured human colon carcinoma cells, the virus effectively destroyed cells by oncolysis and simultaneously induced conversion of the prodrug 5-FU to 5-FU by yeast CD, resulting in enhanced cytotoxicity when compared with either approach alone (31). The similar results were also observed in mice bearing colon cancer xenografts. The combination of direct viral oncolysis and suicide gene system appears to offer an advantage over either approach alone and may represent an important future direction for the integration of VDEPT into clinical practice.

**GPAT.** GPAT is a variation of GDEPT, which uses known transcriptional differences between normal and tumor cells to drive the selective expression of a drug-metabolizing enzyme to convert a nontoxic prodrug into a toxic moiety. TREs are placed upstream of the enzyme gene, driving selective expression (11, 13, 56). A number of tumor-specific TREs have been used, including genes that are amplified in tumor cells compared with normal cells or genes that express tumor-associated antigens, such as CEA for colorectal cancer or N-myc for neuroblastoma. Alternatively, TREs of tissue-specific genes can also be used for GPAT (Refs. 11 and 56–58; Table 3).

The goal of GPAT is tumor-selective therapy, and this
strategy has been used for breast and pancreatic cancer therapy, using TREs of the MUC1 and erbB2 genes. The level of MUC1 gene product, the polymorphic epithelial mucin, is increased in most carcinomas, and the level of the erbB2 product is overexpressed in breast and pancreatic cancers (57). The TRE of the MUC1 or erbB2 gene was placed upstream of the HSV-TK gene, and this expression cassette was placed in a retroviral vector (57). Transduction with retroviruses containing only the MUC1 promoter and HSV-TK gene increased GCV sensitivity in MUC1-positive cells. The cytotoxic effect was additionally enhanced by transduction with retroviruses containing chimeric MUC1/erbB2 promoters and HSV-TK gene, suggesting a useful role of multiple TREs to drive expression of the suicide gene. In a recent study, the ODC promoter was used to up-regulate expression of the rabbit CES, a drug-activating enzyme for the prodrug CPT-11, in N-myc overexpressing neuroblastoma cells (Ref. 59; Fig. 2). This strategy was based on the fact that MYC family proteins (c-, N-, and L-) are activators of the ODC promoter by dimerizing with the transcription factor MAX (59). Introducing proteins (c-, N-, and L-) are activators of the ODC promoter by dimerizing with the transcription factor MAX (59), suggesting a useful role of multiple TREs to drive expression of the suicide gene. In a recent study, the ODC promoter was used to up-regulate expression of the rabbit CES, a drug-activating enzyme for the prodrug CPT-11, in N-myc overexpressing neuroblastoma cells (Ref. 59; Fig. 2). This strategy was based on the fact that MYC family proteins (c-, N-, and L-) are activators of the ODC promoter by dimerizing with the transcription factor MAX (59). Introducing proteins (c-, N-, and L-) are activators of the ODC promoter by dimerizing with the transcription factor MAX.

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### Table 3 Selected examples of GPATs

<table>
<thead>
<tr>
<th>Promoters</th>
<th>Enzyme genes</th>
<th>Model system</th>
<th>References</th>
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<tbody>
<tr>
<td>Ornithine decarboxylase</td>
<td>Rabbit carboxylesterase</td>
<td>Neuroblastoma cells overexpressing N-myc</td>
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<tr>
<td>Regulatory elements of MUC1 and erbB2</td>
<td>Herpes simplex virus thymidine kinase</td>
<td>MUC1-positive cells</td>
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</tr>
<tr>
<td>CEA tumor antigen</td>
<td>Herpes simplex virus thymidine kinase</td>
<td>CEA-producing gastric cancer cell lines</td>
<td>11</td>
</tr>
<tr>
<td>α-fetoprotein enhancer</td>
<td>Herpes simplex virus thymidine kinase</td>
<td>Hepatocellular carcinoma cell lines</td>
<td>11</td>
</tr>
<tr>
<td>erbB2</td>
<td>Cytosine deaminase</td>
<td>Breast and pancreatic tumor cells</td>
<td>11</td>
</tr>
<tr>
<td>Human tyrosine promoter</td>
<td>Purine nucleoside phosphorylase</td>
<td>Melanoma cell lines</td>
<td>11</td>
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**Other Genetic Approaches.** In addition to the above approaches, genetically modified cells have been used to express drug-activating enzyme genes in tumors. In this approach, drug-activating enzyme genes are stably encapsulated by cellulose sulfate. The engineered cells are then introduced into tumors by injection in an immunoprotected environment to produce enzymes in tumors (7). This method was developed as a novel approach that combines gene/cell therapy with chemotherapy (7). It was also considered as a safe and easy application for clinical use, because delivery of suicide gene-transfected/encapsulated cells is a feasible clinical approach without involving direct gene therapeutic interference in patients.

Using this approach, CYP2B1 was delivered into mice for tumor therapy (7, 60). In these studies, encapsulated human embryonal kidney epithelial 293 cells expressing CYP2B1, under the control of the cytomegalovirus immediate early promoter, were administered into mice by two routes: (a) to deliver the capsules directly into the tumors in nude mice (7); and (b) to implant capsules adjacent to pre-established pancreatic tumors in nude mice (60). Low doses of the prodrug IFA were administered to tumor-bearing mice every 3rd day for 2 weeks in both studies. Tumor regression was achieved after 3 weeks, with no tissue reaction or pancreatitis observed 7 days after injection (7, 60). A similar result was observed when Feline kidney cells...
were used for CYP2B1 expression (7). Human breast cancer cells (MDA-MB-361) have also been used to express enzymatically active surface-tethered bacterial CPG2(Q)-3 (61). In this study, engineered breast cancer cells were mixed with nonexpressing cells, and the resultant mixtures were injected into nude mice that had a breast carcinoma xenograft. After 4 days, the prodrug CMDA was administrated into those mice. Expression of the drug-activating enzyme was able to convert the prodrug into the cytotoxic moiety in vivo, resulting in either cures or tumor regression in all surface-tethered CPG2(Q)-3-expressing groups (61). Furthermore, CPG2 activity was not detected in blood samples, indicating there was no significant shedding of the enzyme into the blood circulation, and high level of selectivity for the surface-tethered approach was achieved.

### Approaches to Deliver Prodrug-activating Enzymes into Tumor Cells or Tissues

ADEPT is a strategy in which a tumor-associated monoclonal antibody is linked to a drug-activating enzyme to create a systemically administered conjugate that only targets tumor tissues. Nontoxic prodrug is then administrated systemically and is converted by the pretargeted enzyme localized on the tumor surface into a toxic drug, resulting in cytotoxic effects in tumor cells (6, 62–67). The ideal drugs for ADEPT are small molecules that can diffuse within the tumor tissues, including both antigen-positive and antigen-negative tumor cells, and cause a bystander effect (62–64). When ADEPT is applied clinically, the interval between enzyme and prodrug administrations should be optimized so that the conjugate is only accumulated in tumors rather than in blood and normal tissues, to avoid systemic toxicity. ADEPT has been used to deliver many drug-activating enzyme genes to tumors in vitro and in vivo, and recent examples are described below and are also listed in Table 4.

<table>
<thead>
<tr>
<th>Therapy route</th>
<th>Enzymes</th>
<th>Antibodies</th>
<th>Prodrugs</th>
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<tr>
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<td>Human β-glucuronidase</td>
<td>Humanized CEA-specific binding region</td>
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<tr>
<td>Human β-glucuronidase</td>
<td>Single-chain anti-CD20 antibody</td>
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<td>Fused protein</td>
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<tr>
<td>Human β-glucuronidase</td>
<td>Humanised Fab fragments of the anti-CEA Mab</td>
<td>Doxorubicin</td>
<td>Fused protein</td>
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<tr>
<td><strong>In vivo</strong></td>
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</tbody>
</table>

Because the interval between enzyme and prodrug administrations is important for ADEPT, some studies were performed to explore the optimal interval in animals. Linking the enzyme CPG2 to the anti-CEA antibody A5B7, a rapid clearance of conjugate from the circulation was demonstrated, allowing the prodrug CMDA to be safely given 48 h or 72 h after antibody-enzyme administration (63). In human subjects, 7 days were needed for adequate clearance of antibody-enzyme conjugate from the plasma before the prodrug may be administrated safely, to avoid activation of prodrug in plasma and subsequent systemic toxicity (62). Recently, this CMDA/CPG2 prodrug/enzyme system has been used in a Phase I clinical trial of 10 patients with colorectal carcinoma (70). The bacterial enzyme CPG2 was conjugated to the F(ab’)$_2$ fragment of murine A5B7 monoclonal Ab, and a galactosylated second clearing Ab against CPG2 was also used to lower levels of conjugate in the circulation and other nontumor tissues. The aim of the trial was to measure plasma levels of the prodrug CMDA and active drug CJS11, a bifunctional alkylating agent, released from prodrug by the action of CPG2 localized in tumors. CPG2 activity was found in metastatic tumor biopsies, but not found in normal tissues, after applying the clearing agent. The rapid appearance of the active drug with half-life of 36 ± 14 min in plasma was observed in this system (70). This initial example provides promise for the use of ADEPT to improve the selectivity of current therapy for solid tumors.

Like GDEPT and VDEPT, there are many clinical limitations associated with ADEPT. In poorly vascularized tumors, delivery of the large conjugate is restricted, and it is not possible to deliver antibody/enzyme conjugate to all of the tumor cells (5, 70). Because the enzyme level is low, it is very difficult to generate adequate quantities of active drug to reach the lethal concentration. Furthermore, the binding of the conjugate to the cell surface is limited by antigen heterogeneity. Other drawbacks of ADEPT include cost and difficulties with development and purification of antibodies, immunogenicity of antibodies, accessibility of tumor to the enzyme/antibody conjugate, and the conversion of prodrugs in nontumor tissues (69). The main problem with ADEPT is the immunogenicity of the antibody-enzyme conjugate, which limits multiple cycles of its application (5, 62). To solve this problem, several solutions have been...
tried, including the use of humanized proteins and concomitant administration of immunosuppression (62).

Because of the problems mentioned above, many ways have been tried to improve ADEPT. The first way to improve ADEPT is to use a three-phase system to speed up the removal of enzymes from the circulation without affecting the enzyme activity in tumor tissues (62, 66). In this approach, a galactosylated anticongjugate antibody was applied after the administration of conjugate and prodrug as a clearing agent that reacted with the conjugate in the plasma, thus decreasing its blood levels, but retaining enzymatic activity in tumors (62). A second way to improve ADEPT is to use a conjugate containing an enzyme and a partial fragment of antibody, which would be cleared more rapidly from the circulation, with the prodrug given earlier, whereas the enzyme level within the tumor is at the peak concentrations (67). The third way to improve ADEPT is to combine ADEPT with an antivascular agent, a drug that selectively inhibits tumor blood flow and causes extensive necrosis. In a study of nude mice bearing a colorectal tumor xenograft, a conjugate containing the bacterial CPG2 and the F(ab’)_2 fragment of anti-CEA antibody to activate the prodrug CB1954 was combined with the antivascular agent 5, 6-dimethylxanthenone-4-acetic acid at 20 h postconjugate injection, resulting in killing a larger part of tumor, doubling the concentration of antibody-enzyme conjugate retained in tumor, and significantly prolonging the tumor growth inhibition caused by ADEPT alone (66). Furthermore, 5, 6-dimethylxanthenone-4-acetic acid also increased prodrug retention within the tumor by 16-fold. The fourth way to improve ADEPT is to use mutant form(s) of human enzymes to avoid systemic toxicity caused by the use of wild-type human enzyme(s) and decrease immune responses caused by the use of nonhuman enzyme(s) (6). A mutant form of human CPA conjugated to a tumor-associated antibody was effective to activate several prodrugs, including thymidylate synthase inhibitors GW 1031 and GW 1843 and the dihydrofolate reductase inhibitor MTX, whereas all these prodrugs were not efficient substrates for endogenous CPA. The use of mutant human enzymes may provide less immunogenicity than nonendogenous enzymes and less systemic toxicity than endogenous enzymes (6). The final way to improve ADEPT is to use recombinant DNA technology to produce a fusion protein with defined characteristics and to avoid additional antibody purification steps, which may cause reduced enzymatic activity or decreased antibody binding of the conjugate (67). An antibody-human β-glucuronidase fusion protein was studied in mice bearing a human tumor xenograft and found to be rapidly cleared from the blood, with a tumor: blood ratio > 100:1 at 7 days after injection. The combination of this fusion protein with a doxorubicin prodrug resulted in superior growth inhibition when compared with prodrug alone (68). Recently, an expression plasmid for the production of a fusion protein containing the single-chain Fv anti-CD20 mouse monoclonal antibody and human lysosomal enzyme β-glucuronidase was found to bind CD20-expressing lymphoma cells in a specific manner and was able to activate the prodrug N-[4-daunorubicin-N-carbonyl (poxymethyl)phenyl][O-b-glucuronyl carbamate at a rate similar to that of purified human β-glucuronidase (67).

Which Approach Is Better: GDEPT, VDEPT, or ADEPT?

All three enzymatic-prodrug strategies have practical advantages for optimizing the treatment of human cancer. GDEPT and VDEPT have an advantage over ADEPT in that most enzymes need cofactor(s) that is present only inside the cells. Therefore, enzymes delivered by ADEPT may need to gain access inside the cells before they can optimally activate prodrugs. This requirement is limited by the poor penetration of large-sized antibody-enzyme conjugates. In GDEPT, gene-encoding enzymes can be specifically delivered to target tissues by the use of tissue-specific elements, to drive the expression of the enzyme within the target cells (60). Despite this idea, some theoretical risks for GDEPT, including insertional mutagenesis, anti-DNA antibody formation, local infection, and tumor nodule ulceration, restrict its use (56). The other limiting factors include immunogenicity in ADEPT and difficulties with the selective delivery and expression of genes in GDEPT (5). Regarding VDEPT, most viral vectors are engineered to be replication deficient. However, there is a slight risk of reversion to wild-type virus. Furthermore, retrovirus vectors are inserted into the host-cell DNA, which may cause mutagenesis of the host’s genome (11). Another drawback associated with retroviral vectors is that they only target dividing cells (40, 41). Even in a rapidly growing tumor nodule, only 6–20% of cells are in a proliferating state and in S phase (71). Thus, the majority of the tumor would not be sensitive to killing mediated by retroviral VDEPT. On the basis of these variables, the choice for GDEPT, VDEPT, or ADEPT should depend on the clinical scenario and is determined by how developers view the risks associated with each approach. Recent clinical trials of enzyme/prodrug therapy are summarized in Table 5.

Future Perspective

There are three major aspects that need to be improved for enzyme/prodrug combination therapy in the future.

Improved Prodrugs. The design, synthesis, and clinical trials of prodrugs were recently reviewed elsewhere (73). One limitation for the prodrug/enzyme approach is that only a small part of the tumor cells become activation competent with current strategies. To overcome this problem, the design of appropriate prodrugs that can diffuse efficiently and can kill activation-incompetent cells via a bystander effect is necessary (5). Because hypoxia and lower pH are a common environmental feature in solid tumors, there is a need to design prodrugs, which can be activated under these conditions (5). Currently available systems, including HSV-TK/GCV and CD/5-FC, are dependent on ongoing DNA replication in proliferating cells (20). Because the majority of tumor cells are in a nonproliferating state, these two commonly used systems are not very effective in killing tumor cells. The ideal active drugs should be effective against both dividing and nondividing cells. Unfortunately, most of the prodrugs used now are antimetabolites and target only dividing cells for cytotoxicity (13, 20). Alkylation agents derived from the prodrugs CB1954 or IFA are not cell phase specific (74) and cannot be activated under these conditions (5). Currently available systems, including HSV-TK/GCV and CD/5-FC, are dependent on ongoing DNA replication in proliferating cells (20). Because the majority of tumor cells are in a nonproliferating state, these two commonly used systems are not very effective in killing tumor cells. The ideal active drugs should be effective against both dividing and nondividing cells. Unfortunately, most of the prodrugs used now are antimetabolites and target only dividing cells for cytotoxicity (13, 20). Alkylation agents derived from the prodrugs CB1954 or IFA are not cell phase specific (74) and may represent a prototype for the development of other novel prodrugs in this class. In a series of CB1954 derivatives, evaluated in a Chinese hamster cell line transfected with the E. coli NTR, 4 of 20 analogues were more potent cytotoxic agents than the parent compound (15). Another novel prodrug amino-seco-
immunogenicity of nonhuman proteins. However, this does not avoid the concern of carcinogenicity of human squamous carcinoma cells and in a mice model of squamous cell cancer of the head and neck (42). However, this does not avoid the concern of carcinogenicity of nonhuman proteins.

CBI-TMI, a potent minor groove alkylating agent, was synthesized and tested in human ovarian carcinoma cells, where this novel drug gave a 10–21-fold increase in cytotoxicity in the presence of E. coli B NTR (74). However, all of these new prodrugs have the additional hurdle of FDA approval for use in humans, as well as evaluation in combination with enzyme system.

In addition to alkylating agents, other classes of prodrugs have been developed as well. Some effort was made to improve the water solubility, stability in blood, and susceptibility to enzymatic cleavage for camptothecin (1), an antitumor alkaloid which acts by inhibiting the activity of topoisomerase I (75). The other efforts were aimed at the synthesis of a series of new prodrugs of daunorubicin and doxorubicin to find a better substrate for enzyme β-glucuronidase (65, 76).

**Improved Enzymes.** The techniques used to improve enzymes to activate prodrugs are reviewed elsewhere (77). Use of substrates for human enzymes may allow prodrug activation in nontumor tissues. One solution to this problem is to develop a mutant form of human enzymes by site-directed mutagenesis to avoid immune response against nonhuman protein and improve the kinetics of the enzymes for the prodrugs (77, 78) or make the prodrug a highly specific substrate for the enzyme (3, 13). In addition, because certain prodrugs may be activated by a cascade of several enzymes, the cotransfection of genes for each member of the pathway is an alternative to increase the yield of active drugs (3). Finally, use of enzymes from different species may provide another way to improve enzymatic activity (13), e.g., yeast CD is more efficient at converting 5-FC to 5-FU than bacterial CD after retroviral infection in murine squamous carcinoma cells and in a mice model of squamous cell cancer of the head and neck (42). However, this does not avoid the concern over immunogenicity of nonhuman proteins.

**Improved Methods to Deliver Prodrug-activating Enzymes into Tumor Tissues.** Using current delivery systems, only 10–55% of cells can be targeted, depending on the tumor and the delivery route (61). The physical/chemical delivery methods, such as electroporation, direct intracellular injection, and calcium phosphate coprecipitation have been successful in vitro, but clinically, they are only suitable for transfection of tissues that can be removed from the body and then easily returned (11). Therefore, the improvement in design of delivery vectors of therapeutic genes into tumor cells and development of nonviral vectors are expected (43, 79–81). Because of the risks of VDEPT and multiple steps involved to generate functional enzymes in GDEPT, developing nonviral vectors that are able to deliver active enzymes rather than genes into tumors are beneficial for clinical application. Because antibody-enzyme conjugates are large molecules that are difficult to penetrate into tumors using ADEPT, developing a novel approach that is quick, efficient, and involves a small molecule as a targeting agent is needed.

### Table 5 Examples of recent clinical trials of enzyme/prodrug therapy

<table>
<thead>
<tr>
<th>Approach</th>
<th>Enzyme</th>
<th>Prodrug</th>
<th>Percentage of CJS11 in CBI-TMI</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>GPAT</td>
<td>GPAT CD cDNA directed by erbB-2 promoter</td>
<td>5-FC</td>
<td>Plasmid construct was intratumorally injected in 10–20 breast cancer patients. Expression was targeted to erbB2-positive cells and occurred in 90% of cells.</td>
<td>56</td>
</tr>
<tr>
<td>ADEPT</td>
<td>CPG2 linked to F(ab′)2 fragment of murine ASB7 monoclonal antibody</td>
<td>CMDA</td>
<td>The concentration of active drug (CJS11) in plasma of 10 patients with colorectal carcinoma was evaluated. On biopsies, CPG2 activity was only localized in metastatic tumor.</td>
<td>70, 72</td>
</tr>
<tr>
<td>VDEPT</td>
<td>Adenoviral transduction of HSV-TK</td>
<td>GCV</td>
<td>Recombinant adenovirus containing HSV-TK was injected into the pleural cavity of 21 patients with mesothelioma. HSV-TK was detected in tumors of 11 patients.</td>
<td>37, 38</td>
</tr>
<tr>
<td>Retroviral transduction of HSV-TK</td>
<td>GCV</td>
<td>Gene therapy in combination with surgery was applied to 48 patients with GBM. No significant side effects were observed, and the 12-month survival rate was 27%.</td>
<td>44, 46–48</td>
<td></td>
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* PSA, prostate-specific antigen; GBM, glioblastoma multiforme.

### References


