PRODRUG ACTIVATING SYSTEMS IN SUICIDE GENE THERAPY OF CANCER: A REVIEW

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ABSTRACT
Theoretically, prodrugs are relatively noncytotoxic molecules, capable of being converted to cytotoxic species only at the site of the tumor, affording enhanced antitumor selectivity. One approach aimed at enhancing the selectivity of cancer chemotherapy for solid tumors relies on the application of prodrug-activating systems in suicide gene therapy. Enzyme-activating prodrug therapy, also known as gene-directed enzyme prodrug therapy (GDEPT) or virus-directed enzyme prodrug therapy (VDEPT) is the form of suicide gene therapy approach where the delivered gene encodes an enzyme that activates a prodrug administered systemically causes death of own tumour cells which expresses prodrug converting enzyme. GDEPT couples cancer chemotherapy with gene therapy and offers immense therapeutic potential with more tumor specificity and less systemic toxicity. The enzyme prodrug therapy has been developed for hepatocellular carcinoma, prostate cancer, ovarian, bladder and breast cancer. The peculiarity of this approach for the treatment of cancer is reduction of dose of anti cancer agent required to produce therapeutic benefit because of its bystander effect, this is not seen with any other type of cancer treatment.

Key words: Prodrug-activating systems, suicide gene therapy, cancer, bystander effect.

INTRODUCTION
Cancer is one of major leading cause of death in human as well as animals. There are large number of well-documented and clinically established methods for the treatment of cancer such as chemotherapy, radiotherapy, immunotherapy, gene therapy and surgical correction. Among this cancer chemotherapy is major component of malignant diseases. However, the efficacy of these approaches is often hampered by an insufficient therapeutic index, lack of specificity and the emergence of drug-resistant cell subpopulations. Prodrugs have often been proposed as a solution to the major problem of nonspecific toxicity to normal tissues, such as the bone marrow and lining of the gut,
often associated with conventional cytotoxic chemotherapy. Theoretically, prodrugs are relatively noncytotoxic molecules, capable of being converted to cytotoxic species only at the site of the tumor, affording enhanced antitumor selectivity. One approach aimed at enhancing the selectivity of cancer chemotherapy for solid tumors relies on the application of prodrug-activating systems in suicide gene therapy.

Gene therapies are techniques for modifying the cellular genome for therapeutic benefit. In cancer gene therapy, both malignant and nonmalignant cells may be suitable targets. The possibility of rendering cancer cells more sensitive to chemotherapeutics or toxins by introducing “suicide genes” was suggested in the late 1980s. This approach has two alternatives: toxin gene therapy, in which the genes for toxic products are transfected directly into tumor cells; and enzyme-activating prodrug therapy, in which the transgenes encode enzymes that activate specific prodrugs to create toxic products. The latter approach, known as gene-directed enzyme prodrug therapy (GDEPT) [1,2] or virus-directed enzyme prodrug therapy (VDEPT) [3]. Gene-directed enzyme prodrug therapy (GDEPT) is a suicide gene therapy approach where the delivered gene encodes an enzyme that activates a prodrug administered systemically. The prodrug is non-toxic as such but produces an active metabolite due to action of the expressed enzyme.

Components of Gene-directed enzyme prodrug therapy (GDEPT)

Gene-directed enzyme prodrug therapy (GDEPT) is comprised of three components;
1. The prodrugs to be activated
2. The enzymes used for activation and
3. The delivery systems for corresponding gene

THE PRODRUGS TO BE ACTIVATED

GDEPT or VDEPT are two-step treatments for solid tumors. In the first step, the gene for a foreign enzyme is delivered and targeted in a variety of ways to the tumor where it is to be expressed. In the second step, a prodrug is administered that is selectively activated to the drug by the foreign enzyme expressed in the tumor. Ideally, the gene for the enzyme should be expressed exclusively in the tumor cells and should reach a concentration sufficient to activate the prodrug for clinical benefit. The catalytic activity of the expressed protein must be adequate to activate the prodrug under
physiological conditions. Because expression of the foreign enzymes will not occur in all cells of a targeted tumor in vivo, a bystander effect (BE) is required, whereby the prodrug is cleaved to an active drug that kills not only the tumor cells in which it is formed, but also neighboring tumor cells that do not express the foreign enzyme[^4].

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**Figure 1**

Showing mechanism of prodrug-activating systems in suicide gene therapy
Design of prodrugs for GDEPT

The design of prodrugs for GDEPT should address several issues related to clinical situations. These include the following:

- Penetration of prodrugs into cancer cells for intracellular activation.
- Effective activation of prodrug by the expressed enzyme with favorable activation kinetics.
- High cytotoxicity of the active drug.
- Ideally, the released drugs should be effective in both cycling and noncycling cells.
- The released drugs should induce a bystander effect.
- Lipophilic prodrugs are required to penetrate across cell membranes, or prodrugs could be synthesized that are taken up by active transport.
• Low cytotoxicity for the prodrugs is essential since they will also be taken up by normal cells.
• It is an advantage if the prodrugs are activated to drugs with high cytotoxicity and optimum half-lives to maximize the benefits for GDEPT. The half-lives should be short enough not to allow the drug to leak out from the tumor but long enough to induce a bystander effect.

Table 1: Depicts the prodrugs and enzymes used in GDEPT

<table>
<thead>
<tr>
<th>No.</th>
<th>Prodrug</th>
<th>Enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6-methoxypurine arabinonucleoside</td>
<td>Thymidine kinase</td>
<td>[5]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Varicella zoster virus)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Ganciclovir, Acyclovir, 1-(2'-deoxy-2'-fluoro-β-D- arabinofuranosyl)-5-iodouracyl</td>
<td>Thymidine kinase</td>
<td>[6,7,8,9]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Herpes simplex virus)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5-fluorocytosine</td>
<td>Cytosine deaminase</td>
<td>[10,11,12]</td>
</tr>
<tr>
<td>4</td>
<td>Cyclophosphamide, ifosfamide</td>
<td>Cytochrome P450</td>
<td>[13,14]</td>
</tr>
<tr>
<td>5</td>
<td>9-(β-D-2'-deoxy-erythro-pentafuranosyl)-6-methylpurine</td>
<td>Purine nucleoside phosphorylase</td>
<td>[15]</td>
</tr>
<tr>
<td>6</td>
<td>5'-deoxy-5-fluorouridine</td>
<td>Thymidine phosphorylase</td>
<td>[16]</td>
</tr>
<tr>
<td>7</td>
<td>Arabinosyl cytosine, 2-chloro-2'-deoxyadenine, 2-fluoro-9-((β-D- arabinofuranosyl)cytosine, 2',2'-difluorodeoxycytidine</td>
<td>Deoxycytidine kinase</td>
<td>[17]</td>
</tr>
<tr>
<td>8</td>
<td>(2-chloroethyl)(2-mesyloxyethyl)aminobenzoyl-L-glutamic acid</td>
<td>Carboxypeptidase G2</td>
<td>[1]</td>
</tr>
<tr>
<td>9</td>
<td>5-aziridinyl-2,4-dinitrobenzamide</td>
<td>Nitroreductase</td>
<td>[2]</td>
</tr>
<tr>
<td>10</td>
<td>6-thioxanthine, 6-thioguanine</td>
<td>Xanthine-guanine phospho-ribosyltransferase</td>
<td>[18]</td>
</tr>
</tbody>
</table>

Activation of the prodrugs is one of the critical steps in GDEPT. It is an advantage if prodrug activation proceeds directly by the expressed enzyme without the requirement for further catalysis by endogenous enzymes. The involvement of host enzymes in prodrug activation may lead to resistance of tumor cells if the endogenous enzymes are deficient or defective. Furthermore, cells may differ in the levels of the
endogenous enzymes they express and thus individual tumors may differ in their sensitivity to prodrugs, even if they express equivalent amounts of exogenous protein. With ara-M, GCV (Ganciclovir), and ACV (Acyclovir) the toxic metabolites are generated by mammalian adenosine monophosphate deaminase, AMP- and/or GMP-kinases following initial activation by TK (Thymidine Kinase). With 5-FC (Fluorocytosine), which is converted to 5-FU (fluorouracil) by CD (Cytosine Deaminase), the active metabolite is 5-FdUMP (fluorodeoxyuridine-5'-monophosphate) or 5-FUTP (fluorouridine-5'-triphosphate), which results from 5-FU conversion by a number of mammalian enzymes involving a complicated activation pattern. The complex activation pathway of these antimetabolites is partially responsible for their propensity to induce resistance [19].

The prodrugs CP (cyclophosphamide) and IF (ifosfamide), are dependent on fewer endogenous enzymes for activation than those cited above. CP, after the primary activation by CYP (Cytochrome P450), requires a second step by 3',5'-exonuclease to generate the active metabolite. Good kinetic parameters (higher k_{cat}) seem favorable. This effect was noticed when the activity of CP (higher k_{cat}, lower IC_{50}) was compared with that of IF (lower k_{cat}, higher IC_{50}) [20]. Improving the kinetic parameters of TK (Thymidine kinase) by mutagenesis has resulted in enzymes that make cells more sensitive to prodrugs than wild-type enzymes [21].

Ideally the released drug should be effective against both cycling and noncycling cells. Unfortunately, the majority of prodrugs used in GDEPT release active drugs that are antimetabolites, which require cycling cells (S phase) for cytotoxicity. It has been speculated that the resistance to GCV in GDEPT is not an acquired one but resulted from tumor cells being in G0 during the time of GCV administration [22]. This argument is supported by the fact that the tumors which grew out remained sensitive to GCV. The use of prodrugs that are activated to alkylating agents should have an advantage over purine nucleosides or 5-FC, since they are cytotoxic to both cycling and noncycling cells and this has been shown to be true for the alkylating agent CB 1954 [2]. Alkylating agents also have the advantage that they are less prone than other anticancer compounds to induce resistance [23, 24].
The rationale for their use and the results obtained with each of them are discussed below.

**6-Methoxypurine Arabinonucleoside (Ara-M) / Varicella Zoster Virus Thymidine Kinase (VZV-TK):**

The rationale for this prodrug choice was that ara-M is a good substrate for VZV-TK but a poor substrate for the major mammalian nucleotide kinase \[^{5, 25}\]. The prodrug is activated by the viral enzyme to ara-M-monophosphate (ara-MMP), which is then further converted by the mammalian AMP-deaminase to inosine arabinonucleoside monophosphate (ara-IMP). This metabolite is further catalyzed by mammalian adenylsuccinate synthetase lyase into adenine arabinonucleoside monophosphate (ara-AMP), which is then phosphorylated by the AMP-kinase and subsequently by the cellular nucleoside diphosphate kinases to the anabolite adenine arabinonucleoside triphosphate (ara-ATP). An alternate possible pathway is through hypoxanthine arabinonucleoside (ara-H), which involves the adenosine deaminase-catalyzed demethoxylation of ara-M. Ara-ATP exerts its cytotoxic effects on dividing cells, by inhibiting DNA synthesis.

**Purine Nucleosides: ACV (Acyclovir), GCV (Ganciclovir), and 1-(2'-Deoxy-2'-fluoro-D-arabinofuranosyl)-5-iodouracyl (FIAU) / Herpes Simplex Virus TK (HSV-TK):**

This system is based on the metabolic activation by the HSV-TK of certain purine nucleosides which have previously demonstrated clinical efficacy for the treatment of herpes in humans. HSV-TK catalyzes the phosphorylation of the purine nucleoside analogues ACV, GCV, and FIAU, which are poor substrates for VZV-TK \[^{25}\]. The conversion is to the corresponding nucleoside monophosphates, which are catalyzed to nucleoside triphosphates by mammalian nucleoside monophosphate kinases. These latter nucleotides are able to inhibit DNA synthesis and thus kill dividing cells \[^{26}\]. Evidence has been obtained for the inhibition of DNA polymerase by ACV-triphosphate, resulting in inhibition of the elongation of newly synthesized DNA. Incorporation of ACV-triphosphate into DNA is also a chain-terminating event, and GCV-triphosphate also acts as a chain terminator \[^{26, 27}\]. Cells transfected with the HSV-TK gene and treated with GCV have been shown to die by apoptosis. This process is probably mediated by
induction of DNA damage and occurs via a pathway that is independent of p53. The GCV/HSV-TK system is the most intensively studied.

5-Fluorocytosine (5-FC) / E. coli Cytosine Deaminase (CD):

CD is an E. coli enzyme, which has homology to human cytidine deaminase but different substrate specificity. CD converts the nontoxic 5-FC to 5-fluorouracil (5-FU), which is then transformed by cellular enzymes to a potent pyrimidine antimetabolite. Its cytotoxicity depends upon cellular anabolism to 5-fluorodeoxyuridine-5'-monophosphate (5-FdUMP) and then to 5-fluorouridine-5'-monophosphate (5-FUMP) by endogenous enzymes. 5-FdUMP is a powerful inhibitor of thymidylate synthase (TS) through the formation of the covalently linked ternary enzyme-inhibitor-cofactor complex, resulting in an irreversible inhibition of TS, a key enzyme for DNA biosynthesis and leading to cell death. 5-FUMP is phosphorylated to 5-fluorouridine-5'-triphosphate (5-FUTP), resulting in the formation of "fraudulent" (5-FU) RNA. This affects RNA maturation and processing and may sometimes be an essential event for cytotoxicity. 5-FUMP is also converted into the corresponding triphosphate (5-FdUTP), by diphosphate kinase, which is incorporated into DNA, interfering with its biosynthesis.

Oxazaphosphorines | Cyclophosphamide (CP), Ifosfamide (IF) / Rat Liver Cytochrome P450 Isoenzyme (CYP2B1) or Human Isoenzyme (CYP2B6):

CP and IF are good candidates for GDEPT, since they are prodrugs and their biochemical and pharmacological properties are known. CP is activated in rat liver by microsomal CYP to 4-hydroxy-CP, which exists in equilibrium with its ring-opened tautomer aldophosphamide. There is evidence that the significant circulating and pharmacologically important metabolite is 4-hydroxy-CP/aldo-CP. The cytotoxic metabolite is the corresponding phosphoramidate mustard, which is generated together with acrolein from the decomposition of aldo-CP. The spontaneous β-elimination of acrolein from aldo-CP is a base-catalyzed process (involving hydroxide ions, carbonate anion, and organic or inorganic phosphate). However, data suggesting that 3', 5'-exonucleases (and especially those related to DNA polymerase) catalyze the release of the phosphoramidate mustard, and this could be the basis of its relative specificity to the cancer cells. The
activation of IF occurs in a similar way\textsuperscript{[31]}. These bifunctional alkylating agents kill both cycling and noncycling cells by cross-linking DNA.

6-Methylpurine-2'-deoxyribonucleoside (6-MePdR) / \textit{E. coli} Purine Nucleoside Phosphorylase (PNP):

PNP (or purine nucleoside orthophosphate transferase) catalyzes the reversible phosphorolysis of purine ribonucleotides\textsuperscript{[32]}. These enzymes are found in bacteria and in eukaryotes, but there are important differences in substrate specificity between the bacterial and the human enzymes. For instance, adenine is a good substrate for bacterial PNP but not for the human enzyme. Purine arabinosides have at least 10-fold greater activity with \textit{E. coli} PNP compared to the human enzyme. On the other hand, mammalian PNP is more efficient in the synthesis of nucleosides from 2-oxo-6-substituted purines and their analogues than is the bacterial PNP.

5'-Deoxy-5-fluorouridine (5'-DFUR) / Thymidine Phosphorylase (TP):

Human TP has been proposed for GDEPT in connection with the activation of 5'-DFUR\textsuperscript{[16]}. TP catalyzes the reversible phosphorolytic cleavage of deoxythymidine, deoxyuridine, and some of their analogues to the corresponding bases and deoxyribose-1-phosphate. Although TP is implicated in the activation of 5'-DFUR, there has been no clear demonstration that TP of human origin can cleave the glycosidic bond of 5'-DFUR to yield 5-FU.

\textbf{Arabinosylcytosine} [1-(\beta -D-Arabinofuranosyl)cytosine (Ara-C), 2-Chloro-2'-deoxyadenosine (CdA), 2-Fluoro-9-(\beta -D-arabinofuranosyl) cytosine (Fara-C), 2-Fluoro-9-(\beta-D-arabinofuranosyl) adenine (Fara-A), and 2', 2'-Difluorodeoxycytidine (dFdC) / Deoxycytidine Kinase (dCK)]:

Human dCK is an enzyme involved in the salvage pathway of deoxyribonucleotides biosynthesis. The phosphorylation reaction is the limiting step in this pathway\textsuperscript{[33]}. The prodrugs ara-C, CdA, Fara-C, Fara-A, and dFdC can all be activated by a phosphorylation reaction to the corresponding deoxynucleotide monophosphates which, after further phosphorylation to the corresponding triphosphates, are biosynthetically incorporated into RNA and DNA, leading to DNA fragmentation and consequent cell death.
2-Chloroethyl-2-mesyloxyethyl aminobenzoyl-L-glutamic Acid (CMDA) /

_Pseudomonas Sp. Carboxypeptidase G2 (CPG2):_

CPG2 is a bacterial enzyme from a *Pseudomonas* species \[^{[17]}\] for which there are no known human homologues, and it is therefore a good candidate for GDEPT. CPG2 catalyzes the scission of amidic, urethanic, and ureidic bonds between an aromatic nucleus and L-glutamic acid \[^{[34]}\]. It can therefore be used to activate prodrugs such as CMDA to aromatic bifunctional alkylating agents.

CMDA, benzoylglutamate mustard, is cleaved by CPG2 to benzoyl mustard which can cross-link DNA. Since no endogenous enzymes are required for this activation, the chance of developing resistance is greatly reduced. The released drug is also active against cycling and noncycling cells and therefore has an advantage over antimitabolite toxins (e.g., GCV or 5-FC). CPG2 has been expressed stably in a variety of human tumor cell lines (breast, ovarian, and colon carcinomas) which rendered the cells sensitive to the prodrug CMDA. The cell lines expressing CPG2 were found to be 10-100 times more sensitive to CMDA than appropriate controls _in vitro_. Total cell kill could be achieved when 4-12% of the cells expressed CPG2, depending on the cell line tested \[^{[1]}\].

5-Aziridinyl-2,4-dinitrobenzamide (CB 1954)/ _E. coli_ Nitroreductase (NR) :

The prodrug CB 1954, a monofunctional alkylating agent, was found to be extremely potent against the Walker carcinosarcoma in rats but inactive against a panel of tumors used in screening anticancer agents \[^{[35]}\]. It has subsequently been shown that the sensitivity of the Walker cell line was due to DT-diaphorase present in these cells, which activates CB1954 into the bifunctional alkylator 5-aziridinyl-4-(hydroxylamino)-2-nitrobenzamide \[^{[36]}\]. This is probably converted by acetyl-CoA or other endogenous enzymes to a more potent electrophile, for example, the corresponding acetate, which then reacts with DNA. Since the active drug is an alkylating agent, it is able to kill both cycling and noncycling cells, an advantage compared to antimitabolite toxins. Human DT-diaphorase is much less able to activate CB 1954 than the rat enzyme.

**THE ENZYME USED FOR ACTIVATION**

The enzymes proposed for GDEPT can be characterized into two classes:
(a) One class consists of foreign enzymes is of nonhuman / animal origin, e.g., viral TK (Thymidine kinase), *Escherichia coli* CD (cytosine deaminase), *Pseudomonas sp.* carboxypeptidase, *E. coli* purine nucleotide phosphorylase, *E. coli* xanthine-guanine phosphoribosyltransferase, and *E. coli* nitroreductase. They may elicit cell-mediated immune responses in humans / animals, which may be an advantage, producing more extensive bystander cytotoxicity.

(b) The other class of enzymes is of human / animal origin and is not present or is expressed only at low concentrations in tumor cells, e.g., deoxycytidine kinase, thymidine phosphorylase, and cytochrome P450. Their main advantage resides in the reduction of the potential for inducing an immune response. However, their presence in normal tissues is likely to preclude specific activation of the prodrugs only in tumors.

The genes can be engineered to express their products either intracellularly or extracellularly in the recipient cells [37]. There are potential advantages to each approach. When the enzyme is intracellularly expressed, the prodrug must enter the cells for activation and subsequently the active drug must diffuse through the interstitium across the cell membrane to elicit a bystander effect (BE). Cells in which the enzyme is expressed (tethered to the outer surface) are able to activate the prodrug extracellularly. A more substantial BE could therefore be generated with extracellular gene product expression, but spread of the active drug into the general circulation is a possible disadvantage [1, 37].

<table>
<thead>
<tr>
<th>No.</th>
<th>Enzyme</th>
<th>Source</th>
<th>Reaction catalyzed by the enzyme</th>
<th>Origin</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thymidine kinase</td>
<td>Herpes simplex virus</td>
<td>Phosphorylation of GCV, ACV, and FIAU</td>
<td>exogenous, viral origin</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Varicella zoster virus</td>
<td>Phosphorylation of ara-M (6-methoxypurine); poor activity against GCV, ACV, and FIAU</td>
<td>exogenous, viral origin</td>
<td>[38, 39]</td>
</tr>
<tr>
<td>2</td>
<td>Cytosine deaminase</td>
<td><em>E. coli</em></td>
<td>Conversion of cytosine to uracil (or 5FC to 5FU)</td>
<td>exogenous, bacterial origin</td>
<td>[28, 40]</td>
</tr>
<tr>
<td>3</td>
<td>Cytochrome P450</td>
<td>Rat</td>
<td>Activation of oxazaphosphorines (CP, IF, etc.) by hydroxylation of the oxazaphosphorine cycle</td>
<td>exogenous, CYP2B1, mammalian origin; its human counterpart, CYP2B6 is expressed in liver but not in tumor cells</td>
<td>[11]</td>
</tr>
</tbody>
</table>

Table 2: Shows the enzymes used for activation in GDEPT
4  Purine nucleoside phosphorylase  |  *E. coli*  |  Reversible phosphorylation of purine nucleosides  |  exogenous; human counterpart expressed in erythrocytes but presents important differences in specificity  |  [32]  

5  Thymidine phosphorylase  |  Human  |  Reversible phosphorolytic cleavage of thymidine, deoxyuridine, and their analogues  |  endogenous, low level in tumour cells  |  [16]  

6  Deoxycytidine kinase  |  Human  |  Phosphorylation of deoxycytidine (but as well some purine deoxynucleosides)  |  endogenous, low level in tumour cells  |  [33]  

7  Carboxypeptidase G2  |  *Pseudomonas sp.*  |  Cleavage of the amidic bond between an aromatic nucleus and glutamic acid  |  exogenous, bacterial origin  |  [17]  

8  Xanthine-guanine phosphoribosyl-transferase  |  *E. coli*  |  Catalyzes the conversion of hypoxanthine, xanthine, and guanine to IMP, XMP, and GMP  |  bacterial origin with human counterpart  |  [41]  

9  Nitroreductase  |  *E. coli*  |  Reduction of aromatic nitro groups  |  exogenous, bacterial origin having a human counterpart with different substrate specificity  |  [42]  

THE DELIVERY SYSTEMS FOR CORRESPONDING GENE

Gene delivery vehicles, also known as vectors, are required for successful deployment of prodrug-activating systems in suicide gene therapy, and their performance sets the boundaries for what can be attempted in prodrug-activating systems in suicide gene therapy. The two broad categories of gene delivery vehicle are nonviral and viral.

**Nonviral Vectors**

Nonviral vectors are based on plasmid DNA that is grown in bacterial hosts such as *Escherichia coli*. Plasmids are circular DNA molecules that carry an antibiotic resistance marker gene and a bacterial origin of replication to facilitate their amplification in *E. coli*. A mammalian expression cassette comprising a therapeutic gene with its associated regulatory elements can be inserted into the plasmid. An alternative approach to achieving in vivogene delivery to liver or muscle by using naked plasmid DNA is the so-called hydrodynamic approach, in which the DNA is injected into the circulation in a
large volume of fluid [43]. Applying an electric current to the target site (electroporation) can further enhance the efficacy of gene transfer using naked plasmid DNA. However, for more efficient gene delivery to human / animal tissue, plasmid DNA must be incorporated into a fully synthetic gene therapy vector, eg, using microprojectiles or cationic lipid-protein formations [44]. With the gene gun approach, DNA is coated onto microscopic gold or tungsten particles (microprojectiles) that are accelerated toward mammalian cells or tissues using a device known as a gene gun. The microprojectiles penetrate the cytoplasmic and nuclear membranes of the target cells and deliver their plasmid DNA cargo to the cell nucleus with reasonable efficiency. This approach may be useful for gene transfer to explanted tumor cells or to easily accessible tissues such as skin where the target site is relatively well circumscribed. But compared to viral vectors nonviral gene transfer efficiencies remain low.

**Clostridia-directed enzyme prodrug-therapy (CDEPT)**

This is based on the principle of exploiting tumour hypoxia where anaerobic bacteria like Clostridia sp. will proliferate selectively in tumour because of its hypoxia state. In the eighties, advances in Clostridia genetics made gene manipulations of Clostridia available and created the novel concept of CDEPT [45]. At first, the saccharolytic strain Clostridium acetobutylicum and later the proteolytic strain Clostridium sporogenes were used to produce an enzyme which can metabolize a systemically applied non-toxic product into a cytotoxic metabolite at the tumour site, to a large extent sparing healthy tissues and organs. The Clostridia were transformed with a recombinant plasmid carrying the Escherichia coli gene for encoding the enzyme.

**Construction of a BDEPT vector**

A gene encoding a prodrug converting enzyme is used to arm a bacterial gene therapy vector that has the ability to localize to tumour sites. This gene expresses the therapeutic enzyme inside the bacterial cell. The expression of the enzyme carboxypeptidase G2 is shown as an example below.
Figure 3

Showing bacterial-directed enzyme prodrug-therapy (BDEPT)

In a first successful experiment, a clone of *Clostridium sporogenes* which is closely related to *Clostridium oncolyticum* and carrying a recombinant plasmid with the *Escherichia* colgene encoding the bacterial enzyme cytosine deaminase (CD) could be tested with tumour-bearing mice. This enzyme can convert the systemically applied non-
toxic prodrug 5-fluorocytosine (5-FC) to the anti-cancer drug 5-fluorouracil (5-FU) after intravenously injected recombinant *clostridial* spores had yielded high efficiency of tumour colonization which remained undiminished 14 days after a single injection of spores. A significant growth delay of the tumours was achieved by 5 times a week systemic injections of 5-FC. The tumour extract from recombinant spore-treated mice increased the *in vitro* cytotoxicity of 5-FC for tumour cell cultures by the factor of $10^3$. This anti-tumour effect was greater than with 5-FU alone \[46\]. The efficient turnover of 5-FC carried out by the *Escherichia coli* CD could be seen as an essential step toward the tumour targeting strategy with *Clostridial* recombinant spores as selective delivery vectors.

![Clostridium-directed enzyme prodrug therapy (CDEPT)](image)

**Figure 4(a)**

Showing *Clostridium*-directed enzyme prodrug therapy (CDEPT)
Advantages of nonviral vectors include the high genome capacity of 30 to 40 kb and their lack of immunogenicity [it is difficult to induce an immune response against plasmid (DNA)]. An additional advantage relative to viral vectors is the perception of a lower risk of harmful adverse effects. Important disadvantages of nonviral vectors include their relatively low transduction efficiencies and their transient expression profile, which typically peaks within 48 hours but is thereafter rapidly extinguished by 7 days. However, in some situations this may be an advantage, and it may be possible to prolong the expression profile by using plasmid DNA replicons incorporating mammalian origins of replication, eg, from the Epstein-Barr virus 32.

**Viral Vectors**

Many viruses efficiently deliver their nucleic acid genomes to mammalian cells as the initial critical step in their life cycle. Therefore, they have been perfected throughout millions of years of evolution for the task of gene delivery. The key to exploiting viruses as gene delivery vehicles is to introduce therapeutic genes into their...
genomes while concurrently removing the native viral genes that code for harmful viral proteins by recombinant means. The recombinant virus then functions purely as a vector that delivers the therapeutic gene to the nucleus of the target cell without causing cellular damage or subsequent virus propagation.

Virtually any virus can be exploited as a gene delivery vehicle. The majority of the proposals have been for viral vectors that have been modified to be deficient in replication functions, although the use of replication-competent viruses has also been considered \[47\]. For applications such as \textit{ex vivo} infections, direct administration of vectors to target tissues \textit{in vivo} or for locoregional delivery, the ability to target malignant cells specifically may not be necessary. However, if systemic delivery is used, targeting to malignant cells is an absolute necessity. Some viruses have restricted tropisms that may be of value. For example, the minute virus of mice is oncotropic and can deliver DNA to transformed cells; similarly, the human parvovirus B19 targets primary hematopoietic progenitor cells. The B19 tropism can be transferred to adeno-associated virus by recombinant means. The two groups of viruses that have been most extensively studied, retroviruses and adenoviruses have much less restricted tropisms, and efficient targeting in the clinic. However, at present, the most widely used viral vectors are derived from the following viruses: retrovirus (and lentivirus), adenovirus, adeno-associated virus (AAV), and herpes simplex virus. Each viral vector has distinct characteristics that may make it more or less suitable for a particular gene therapy application.

\textbf{Transductional targeting}

There are 3 broad strategies whereby vectors can be targeted to accumulate at predetermined sites or selectively transduce a particular target cell population. In the first approach, the target cells are isolated and transduced in the tissue culture dish. In the second approach, regional delivery is used to ensure accumulation of vector at a particular site in the body e.g., aerosol delivery to airways, a stereotactically guided injection into the brain or painting vector onto vascular structures during surgical exposure. The third approach is to modify the vector (intrinsic targeting) such that it recognizes and transduces the target cells with high specificity but is incapable of transducing non-targeted cells.
Bystander Effect and Acquired Immunity

Since expression of the foreign enzymes will not occur in all cells of a targeted tumor in vivo, a bystander cytotoxic effect is required whereby the prodrug is cleaved to an active drug that kills not only tumor cells but also neighboring nonexpressing tumor cells [48]. This means that expression in <100% of tumor cells can still give total tumor cell kill. It was demonstrated, in vivo, that when as few as 2% of the tumor cells expressed the foreign enzyme, subsequent treatment with suitable prodrug gave long-term animal survivors [48]. Therefore, an expression efficiency of 10-20% should be enough to achieve 100% cell kill in tumors, and efficiencies of 1-5% are considered sufficient for a therapeutic benefit [48]. Even with lower transfer efficiencies, beneficial results were obtained if the transduced cell population was allowed to expand before prodrug administration.

The mechanisms responsible for the bystander effects are not completely understood. Several explanations are plausible and there is the possibility that more than one mechanism is involved. The toxic metabolites are formed during prodrug activation. These are released by efflux from dead and dying genetically modified cells. This assumption is supported by the fact that no cell to cell contact is required to obtain a bystander effect in these systems. This is postulated for 5-FU formed from 5-FC, for the metabolites of CP or IF, aldophosphamide, phosphoramidic mustards or acrolein [20] and for 6-MeP, formed from the corresponding deoxynucleoside.
For purine or pyrimidine nucleosides, where the toxic metabolites, being phosphorylated, are not diffusible across cell membranes, direct cell to cell contact is required to achieve a bystander effect. Since purine and pyrimidine nucleotides formed during activation cannot cross cell membranes, it was assumed that gap junctions are involved in the transfer of toxic metabolites from cell to cell. Another suggestion is that the TK-enzyme itself is transported by apoptotic vesicles or through gap junctions.

Phagocytosis of the active drug or the activating enzyme from the dying cells (e.g., hydrolases or other lytic enzymes) by the non-enzyme-expressing cells has been suggested as a mediator of the bystander effect. More recent findings suggest that the bystander effect in vivo can be mediated through the release of cytokines [49].

**Clinical evidences**

Paclitaxel (TaxolR; TAX) is currently one of the most important anti-cancer drugs in chemotherapy of ovarian cancer. One of TAX prodrugs, 2'-ethylcarbonate-linked paclitaxel (TAX-2'-Et) was generated and examined regarding its pharmacological aspects. The prodrug of TAX-2'-Et converts into active form TAX by carboxylesterase (CES) suggesting that this combination therapy is a potential GDEPT strategy for ovarian cancer in the future [50].

A targeted systemic therapy for hepatocellular carcinoma for GDEPT was developed [51]. Adenoviruses had been used to target cancer cells because it have an intrinsic tropism for liver, and are efficient gene vectors. Oncolytic adenoviruses produced clinical benefits, particularly in combination with conventional anticancer agents and were well tolerated. The oncolytic adenovirus was used to deliver the prodrug-activating enzyme carboxypeptidase G2 (CPG2) to tumors in a single systemic administration. GDEPT enhanced the adenovirus-alone therapy to elicit tumor regressions in the hepatocellular carcinoma models, showing that the combined approach holds enormous potential as a tumor-selective therapy for the systemic treatment of hepatocellular carcinoma.

Gene-directed enzyme prodrug therapy (GDEPT) based on the ovine atadenovirus which expresses *E. coli* enzyme purine nucleoside phosphorylase (PNP) represents a new approach for treating slow growing tumours like prostate cancer (PCa). Expressed enzyme converts a systemically administered prodrug, fludarabine phosphate, to a toxic
metabolite, 2-fluoroadenine. Infected and neighbouring cells are killed by a bystander effect that results from the inhibition of DNA and RNA synthesis\textsuperscript{[52]}.

Replication-competent retroviral (RCR) vectors are a potentially efficient gene delivery method and that the RCR vector mediated PNP gene transfer and fludarabine phosphate treatment might be a novel and potentially therapeutic modality for bladder cancer\textsuperscript{[53]}.

Genetic prodrug activation therapy has been developed for breast cancer. Breast cancer patients received the transcriptionally targeted CD gene using direct, intratumoural injection of plasmid DNA, combined with systemic administration of the prodrug. The approach was shown to be safe and resulted in targeted expression of the CD gene in 90% of cases. Significant levels of expression of the suicide gene were detected and this expression was restricted to erbB-2-positive tumour cells\textsuperscript{[54]}.

**Advantages of enzyme prodrug therapy**

- As an anticancer strategy, GDEPT couples chemotherapy with gene therapy and offers immense therapeutic potential with more tumor specificity and less systemic toxicity.
- Another additional benefit of such an approach is the bystander effect by which the active cytotoxic metabolite diffuses into neighboring, non-transfected tumor cells and kills / radiosensitizes them thereby the dose of drug can be reduced.
- Gene-directed enzyme prodrug therapy (GDEPT) involves the treatment concept of having maximal efficacy and minimal adverse effects.

**Limitation of prodrug-activating systems in suicide gene therapy of cancer**

As described above there are a large number of prodrug / enzyme combinations that have shown efficiency in vitro and in vivo. There remain some hurdles to overcome before GDEPT will become a clinically efficient treatment of solid tumors. These include the development of better and more efficient delivery systems for the exogenous genes together with restricted expression in the tumour alone; the synthesis of prodrugs that are better substrates for the transfected genes, which are not substrates for endogenous enzymes and that exhibit good bystander effects; and the use of mutated genes that express enzymes with higher specificity for their substrates.
CONCLUSION

The clinical evidence showed that gene-directed enzyme prodrug therapy of cancer causes reduced non-specific toxicity to normal cells compared to conventional chemotherapy. The peculiarity of this approach for the treatment of cancer is reduction of dose of anti-cancer agent required to produce therapeutic benefit because of its bystander effect, this is not seen with any other type of treatment. If we able to develop a 100 percent tumor specific prodrug activating enzyme delivery system, then it is very clear that prodrug therapy of cancer will provide highly enhanced cytotoxicity to tumor cells than normal cells. Based on all this evidence we can state that prodrug-activating systems in suicide gene therapy of cancer will replace conventional chemotherapy in future days.

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