OPINION

Carboxypeptidase G2-based gene-directed enzyme–prodrug therapy: a new weapon in the GDEPT armoury

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Abstract | Gene-directed enzyme–prodrug therapy (GDEPT) aims to improve the therapeutic ratio (benefit versus toxic side-effects) of cancer chemotherapy. A gene encoding a 'suicide' enzyme is introduced into the tumour to convert a subsequently administered non-toxic prodrug into an active drug selectively in the tumour, but not in normal tissues. Significant effects can now be achieved *in vitro* and in targeted experimental models, and GDEPT therapies are entering the clinic. Our group has developed a GDEPT system that uses the bacterial enzyme carboxypeptidase G2 to convert nitrogen mustard prodrugs into potent DNA crosslinking agents, and a clinical trial of this system is pending.

Conventional chemotherapeutic treatments almost invariably produce unwanted, and for the patient often very unpleasant, toxicity owing to affecting characteristic features of cancer cell growth that are also found in some non-neoplastic, self renewing tissues, such as the gut, skin and the bone marrow. The most important toxicity that threatens the survival of the patient is bone marrow suppression, which can cause increased susceptibility to infection and reduced platelet counts, with increased risk of haemorrage[1]. In addition, drugs that target growing cells interfere with gut epithelial renewal and lead to recurrent nausea, vomiting and diarrhoea, and suppress hair follicle renewal leading to alopecia in cancer patients[2]. Combinations of chemotherapeutic drugs are used routinely to treat aggressive tumours and, along with radiation insults to uninvolved tissue, compound the unpleasant side effects experienced by most patients with advanced cancer. Moreover, the use of current chemotherapy may only be a stop-gap, as small populations of primary and metastatic cells that harbour resistance genes may survive and grow into inoperable and chemotherapeutically resistant tumours[3]. Cancer statistics show that the disease is still very prevalent, and although 5-year survival rates have increased across all tumour types[4], overall the annual number of cancer diagnoses has increased[5].

From the point of view of efficiency of treatment, as well as patient welfare, the precise targeting of tumours with cytotoxins is a long-cherished goal. Gene-directed enzyme–prodrug therapy (GDEPT)[6] is a method of treating tumours that should be capable of significantly reducing the undesirable side effects of cancer treatment. This Perspective article will briefly review the goals and mechanisms of GDEPT and will focus on the combination of the bacterial enzyme carboxypeptidase G2 (BOX 1) to convert nitrogen mustard prodrugs, as a means to illustrate how modifications of the vector and suicide enzyme can be used to improve GDEPT.

The principles of GDEPT

GDEPT is a concept aimed at improving the therapeutic ratio (benefit versus toxic side-effects) of cancer chemotherapy. A gene encoding an enzyme that is not naturally expressed in the host (experimental animal or patient) is first introduced into the cells of a tumour by a targeting mechanism ('vector') that leaves the surrounding non-cancerous cells untransformed. The transformed cells of the tumour should then express the enzyme[7,8] (FIG. 1). It is important that the enzyme or a related protein is not normally active in human tissue so that the non-toxic prodrug that is administered to the patient cannot be activated in any tissue other than the tumour tissue. The prodrug should be sufficiently lipophilic to diffuse into tumour cells and should then be cleaved into the cytotoxic drug only by the introduced suicide enzyme. Alternatively, if cleavage of the prodrug takes place extracellularly, the active drug should be capable of diffusing through cell membranes. Moreover, the drug should be able to kill non-dividing as well as dividing cells because it is unlikely that all tumour cells will be in cycle when the drug is activated. It is also unlikely that all the cells within a tumour can be targeted by the vector and thus not all tumour cells will express the suicide enzyme. Therefore, those tumour cells would not be exposed to the active drug. In order to prevent the tumour cells that do not express the enzyme from escaping GDEPT, the active drug has to be designed to diffuse into the intercellular fluid, killing neighbouring cells in the tumour. This action is termed the bystander effect and is a requirement of this type of therapy as it amplifies the effect of the drug.

Vectors

The vector should target tumours by virtue of a characteristic or function either related to their transformed state, such as the expression of specific cell surface proteins or enzymes, or specifically active promoters; or one of the sequelae of tumour formation itself, such as hypoxia. TABLE 1 lists the basic categories of different targeting strategies (for more in depth reviews see REFS 9–11). A wide variety of vectors has been developed, and those in current use include modified microorganisms (such as bacteria or viruses, which we consider here), natural proteins (such as antibodies[8]) or synthetic vectors (such as liposomes[12]).

Box 1 | **Carboxypetidase G2**

Carboxypeptidase G2 (CPG2) from *Pseudomonas* RS-16 is a 42 kDa exopeptidase in the aminoacylase-1/M20 family of enzymes that is not found in mammals. Early nomenclature referred to the *Pseudomonas* enzyme as carboxypeptidase G1 (REF. 101). Like other structurally related peptidases, the active conformation of CPG2 requires homodimerization and the presence of two atoms of zinc per subunit. Each subunit of the active enzyme contains a zinc-binding domain and a dimerization domain, both of which participate in catalysis[102]. The natural substrate of the enzyme is folic acid, but its specificity is broad enough to allow it to hydrolyse glutamate from amidic, urethanic and ureidic bonds[95], which allows it to be used medically as methotrexate rescue treatment[103] and to activate synthetic prodrugs.

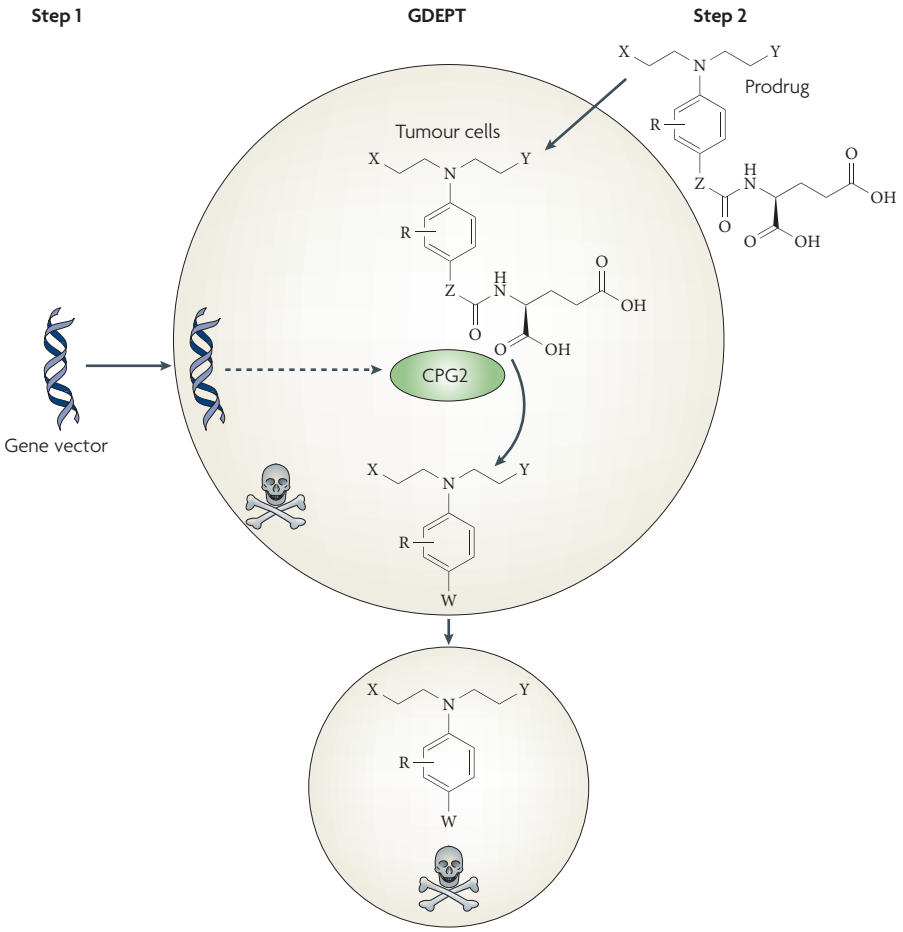


Figure 1 | **Principle of gene-directed enzyme–prodrug therapy (GDEPT).** A gene encoding an enzyme that is not naturally expressed in the host is introduced into the cells of a tumour by a vector-targeting mechanism that leaves the surrounding non-cancerous cells untransformed (step 1). The tumour cells that have been transduced with the vector should then express the enzyme that is not normally expressed in mammalian cells (carboxypeptidase G2 (CPG2) in the figure). This enzyme then cleaves a specific prodrug (step 2; in this case a nitrogen mustard produg), such that the drug only becomes active in cells that express the enzyme. The active drug kills these cells and other cells in the vicinity.

Bacteria

The most prevalent organisms used in this category are species of *Salmonella* and *Clostridium*, but others, such as *Bifidobacterium* and *Escherichia coli* have also been used.

Salmonella. A well studied *Salmonella typhimurium* clone is VNP20009 (REF. 13) (Vion Pharmaceuticals Inc., New Haven, Connecticut, USA). This bacterium is attenuated in its pathogenicity by deletion of the *msbB* gene, which is essential for the terminal myristoylation of lipid A in the external lipopolysaccharide. Myristoyl lipid A induces the production of tumour necrosis factor α in the host, leading to toxic shock syndrome. Inhibiting this modification therefore reduces the toxicity of the bacterium. VNP20009 is also auxotrophic for purines by deletion of the *purI* gene. As solid tumours are partially necrotic, intracellular contents accumulate in the interstitial spaces, providing a source of purines for bacterial growth that is not present in normal tissues. These mutations have produced a bacterium that is much safer to use than the parental strain, and one that grows preferentially within solid tumours. In mouse models, VNP20009 accumulates in various human solid tumour xenografts to concentrations that are many times higher than those found in the liver, the second-most-infected organ. Systemically administered VNP20009 has been seen to accumulate in xenograft tumours in mice using non-invasive imaging with green fluorescent protein[14] and by positron emission tomography (PET) scanning[15]. *Salmonella typhimurium* has been engineered to express suicide enzymes, such as cytosine deaminase (CD)[16] from *E. coli* or *Saccharomyces cereviciae* and *Herpes simplex* thymidine kinase (HSV-TK)[13]. Administration of the relevant prodrugs (5-fluorocytosine (5-FC) or ganciclovir, respectively) leads to specific localization of the cognate drugs and tumour growth suppression or regression greater than that seen with the vector alone[16]. This effect has been called tumour amplified protein expression and targeting (TAPET).

One caveat to the use of VNP20009 as a vector is that it can still manifest systemic toxicity in animals, although only when present at markedly higher bacterial numbers than are required for the wild-type bacterium.

Clostridium. Species of *Clostridium* have been known to proliferate preferentially in tumours since the 1950s. The initial, but lethal, experiments used *C. tetani*[17], but later studies using non-pathogenic *C. butyricum*[18] also showed tumour localization. Clostridia are obligate anaerobes that survive in aerobic conditions by developing a spore that remains quiescent until conditions allow it to germinate. When spores are injected into animals, they may be present in aerobic tissues without apparent effects but will germinate and produce replicating bacteria in the hypoxic regions that develop within even small tumours. Therefore, using spores rather than replicating bacteria provides a targeting mechanism. This effect has been successfully used to deliver therapeutic genes in GDEPT systems.

C. beijerinckii has also been used to deliver *E. coli* nitroreductase[19] and CD[20] to mouse xenograft tumours, but unfortunately the associated prodrugs (CB1954 and 5-FC respectively) did not become activated in the infected xenografts. Liu *et al.*[21] suggested that the lack of activity could be due to the low levels of viable bacteria in tumours because of the strain used, and went on to show that systemically administrated spores of *C. sporogenes,* transformed with *S. cerevisiae* CD, led to the production of the enzyme exclusively within an abdominal wall squamous cell carcinoma of mice (SCCVII). Injection of 5-FC produced a tumour growth delay for about 7 days in the animals treated with spores compared with untreated controls or mice treated with 5-FC or spores alone. However, tumour growth recommenced after this period at the same rate as that in the controls, and no further response to the prodrug was seen. This suggests that the bacteria had a limited lifespan within the tumours and that multiple administration of spores might be necessary to maintain the anti-tumorigenic effect, as observed recently by Theys *et al*[22].

Table 1 | **Vector targeting strategies**

Strategy	Target tumour characteristics	Type of vector	Refs
Transductional targeting*			
Vector affinity for a tumour cell surface antigen	FGFR	AdV5 adenoviral vector with bifunctional conjugate retargeting fibre knob domain	104
	ERBB2	AdV5 adenoviral vector with genetically modified fibre protein	105
	ERBB2	AdV adenoviral vector with bispecific adaptor protein	106
Inherent tropism enhanced by genetic modification of the vector or by artificial selection	Actively dividing cells	Replication competent retrovirus locally infused	107
	Active DNA synthesis by proliferating cell nuclear antigen	Herpes simplex virus with virulence factor deletion	69
	Anaerobic conditions	*Clostridia* bacterial spores	22
	Leaky vasculature, as in tumour, may be necessary to allow large virions to escape vessels	Vaccinia	74,108
	Availability of nutrients and environmental conditions within the tumour	*Salmonella*	13
Transcriptional targeting‡			
Promoter driving vector replication	hTERT expression	Adenovirus	96,97
	Human melanoma inhibitory activity (hMIA) expression	AAV	51
Promoter driving suicide-gene transcription	Secretory leukoprotease inhibitor (SLPI) expression	Adenovirus	105
	Carcinoembryonic antigen	Adenovirus	112
	Human hexokinase II (hHK2) expression	Lentivirus	113
Translational targeting§			
	eIF4E overexpression	Adenovirus	114
	eIF4E overexpression	Lentivirus	115

Transductional and transcriptional strategies are not mutually exclusive; ideally their effects compound to refine selectivity[105]. In some cases targeting has been shown with a reporter protein standing in for a therapeutic gene product such as a GDEPT enzyme suicide gene. *In transductional targeting, the vector selectively infects and delivers a suicide gene to tumour cells rather than normal cells. ‡In transcriptional targeting, the suicide gene may be delivered to any cell but promoters selectively switch it on in tumour cells. For general reviews see REFS 109–111. §In translational targeting, suicide gene mRNA is translated to protein in tumour cells where initiation factor is present corresponding to an upstream sequence. There are many methods for targeting adenoviruses for GDEPT and gene therapy in general, which are covered in depth in REFS 108,116. AdV, adenovirus; AAV, adeno-associated virus; eIF4E, eukaryotic translational initiation factor 4E; FGFR, fibroblast growth factor receptor.

Bifidobacterium. This genus of gram-positive anaerobes is commonly added to yogurt cultures and is regarded as beneficial to the digestive system.

Bifidobacterium longum was found by Yazawa *et al.*[23,24] to accumulate in B16-F10 murine melanoma xenografts, such that after 96–168 hours following intravenous administration, viable bacilli could be detected only in tumour tissue. Nakamura *et al.*[25] cloned *E. coli* CD into *B. longum* and showed that the transformed bacterium expressed the enzyme, suggesting that this organism could be used as a gene vector for solid hypoxic tumours. Sasaki *et al.* also used this system and were able to demonstrate tumour-site-specific prodrug activation and efficacy against autochthonous mammary tumours in rats[26]. The minimal pathogenicity of *Bifidobacterium* compared with *Salmonella* and *Clostridium* has been cited as advantageous to its possible use in suicide gene therapy. However, there is a report in the literature of a patient in an acupuncture clinic suffering from *B. longum* sepsis following treatment for a herniated vertebral disk[27]. The conclusion from this is that although an organism can be apparently non-pathogenic in some situations and routes of administration, there is no guarantee that it will be safe to use without attention being given to possible complications from its use.

E. coli. A strain of *E. coli* that is auxotrophic for the cell wall component diaminopimelic acid, making it non-replicating, has been engineered with the *inv* gene from *Yersinia pseudotuberculosis* and the *hly* gene from *Listeria monocytogenes*[28]. The *inv* gene product, invasin, increases selective uptake of *E. coli* into cells that express β1-integrin. After internalization the *hly* gene product binds to and perforates the phagosomal membrane, releasing the bacterium into the interior of the cell. In C57Bl/6J mice, this engineered strain of *E. coli* expressing purine nucleoside phosphorylase (PNP) was delivered to Panc-2 allograft tumours by intratumoral injection, followed by intraperitoneal injection of the prodrug, 6-methyl-purine-2′-deoxyriboside (6-MPDR). The tumours were reduced to approximately half the volume of those in control animals treated with bacteria and PBS, or PBS and prodrug, or PBS alone. However, this vector is limited to tumour masses that include β1-integrin-expressing cells, such as macrophages and dendritic cells, and must be injected intratumorally as it has no systemic targeting mechanism and would otherwise bind to all tissues that express β1-integrin.

Viruses

The use of viruses as gene vectors is very popular at present, as many papers and reviews testify. See, for example REFS 29,30. Opinions differ as to the type and specificity of virus that best fulfils the requirements of a suitable vector. Viral vectors can be non-replicating, such as those that, for safety reasons, only deliver a suicide gene, or replicating, such as those that are oncolytic in addition to delivering a gene, the peak of the latter state being a conditionally replicating vector that targets tumours precisely.

Adenovirus. Adenovirus (AdV) is a non-enveloped, double-stranded DNA virus that exists in episomal form in infected cells, reducing the risk of insertional mutagenesis, where genetic material becomes inserted at random in the host's genome, a known tumorigenic event[16]. Moreover, the genome of AdV is well characterized and can be readily manipulated to express targeting factors[31]. The capsid (the protein coat of the virus that contains its genetic material) is large, so sizeable amounts of foreign DNA can be introduced. Adenoviruses have been used extensively for GDEPT, and it is not possible to review all of this work in this Perspective. A brief summary is provided below, but

Table 2 | **GDEPT in clinical trials**

Enzyme	Prodrug	Tumour	Phase	Refs
Adenovirus				
HSV-TK	Ganciclovir	Hepatocellular carcinoma	I	117
		Glioma	I	118
		Ovarian	I	119
		Prostate	I	120–124
		Colorectal	I	125
CD with HSV-TK	Ganciclovir and 5-FC	Prostate	I	126–128
Nitroreductase	CB1954	Primary and metastatic hepatocellular carcinoma	I	129
Retrovirus				
CYP2B6	Cyclophosphamide	Breast, melanoma	I	130
		Thyroid	I	131
HSV-TK	Ganciclovir	Melanoma, breast, non-small-cell lung and osteogenic sarcoma	I	132
		Glioma	I/II; III	61,62, 133

CD, cytosine deaminase; CYP2B6, a member of the cytochrome P450 group of enzymes; 5-FC, 5-fluorocytosine; GDEPT, gene-directed enzyme–prodrug therapy; HSV-TK, Herpes simplex virus-thymidine kinase.

more indepth information on these vectors and on their preclinical and clinical use can be found in REFS 32,33.

The most commonly used adenoviruses are those in serogroups 2 and 5, and the furthest advanced in the clinic is the restricted replication adenovirus Onyx 015, a chimaera of the two serogroups (Onyx Pharmaceuticals, Inc., Richmond, California, USA). Onyx 015 has had its early-expressed gene *E1B* deleted as its method of tumour targeting. When expressed in the host cell, E1B sequesters p53, allowing viral replication to commence. In the absence of E1B, viral replication should only take place in cells where p53 is non functional, a condition frequently found in tumours, although the efficiency of this targeting has been questioned[34,35]. AdV infection of cells requires expression by the host cell of the cell surface coxsackie-adenovirus receptor (CAR) and αv integrins, which interact with protein fibres protruding from the capsid, although the relative importance of these receptors is also uncertain[36]. A disadvantage of the use of AdV is the fact that the virus induces a potent immune response[37] such that there is concern that it can become ineffective as a vector after the initial or second injection. Thus the effects of systemic AdV GDEPT in wild-type animals and in humans could be less successful than in athymic or severe combined immune deficient (SCID) mice, but circulating anti-AdV antibodies could be beneficial if the virus is injected intratumorally, preventing collateral toxicity[38].

Several methods have been used to aid viral targeting, as shown in TABLE 1. Replacing the E1 promoter with the human telomerase reverse transcriptase (hTERT) promoter restricts viral replication to cells that express telomerase. This enzyme is often re-expressed in tumour cells, where it replaces the ends of chromosomes that are normally gradually lost during cell division and allows the cells to replicate indefinitely[39–41]. Modification of the structure of the capsid fibres to target receptors other than CAR broadens the range of cell types that AdV can infect[42,43]. Cationic liposome conjugation partially protects adenovirus from anti-AdV antibodies, possibly allowing multiple administration[44]. Engineering for overexpression of adenovirus death protein, ADP, a viral nuclear membrane glycoprotein needed in late-stage infection for efficient cell lysis, appears to increase the effect of this vector in GDEPT[45,46]. Freytag *et al.*[47] have recently reported that an adenoviral vector carrying a *CD/mutTK*$_{SR39}$*rep–adp* fusion suicide gene construct, when injected intrapancreatically in dogs, could be visualized within the target organ using a positron-emitting substrate of HSV1 TK and positron emission tomography. The signal remained exclusively in the pancreas, highlighting the safety of the vector. Another study in the same system suggests that GDEPT might augment the effectiveness of radiotherapy for pancreatic cancer without excessive toxicity[47].

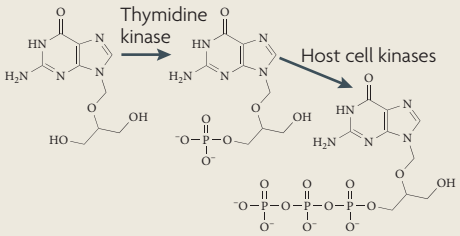
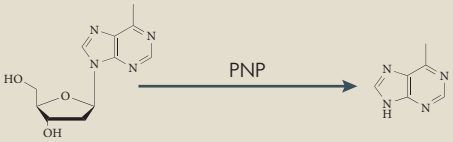
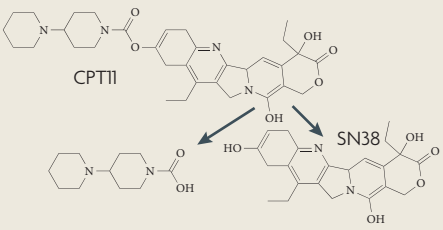
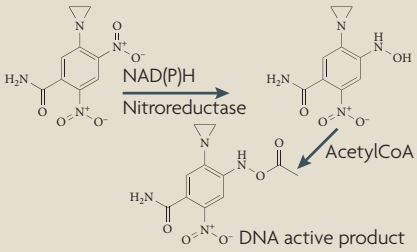
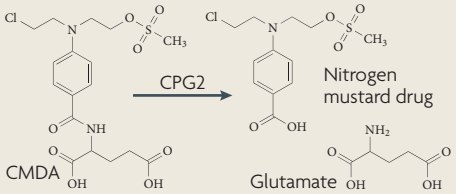
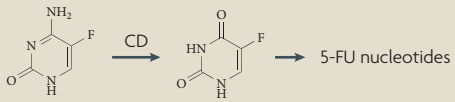
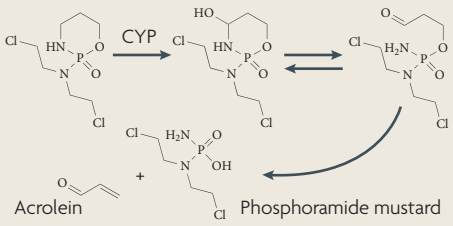
Adenoviral vectors have been used in GDEPT clinical trials, as shown in TABLE 2, despite worries about their safety following the death, from 'a systemic inflammatory response', of a patient being treated for an inherited enzyme deficiency in 1999 (REF. 48).

These trials were in general well tolerated, with no treatment-related deaths reported, although the clinical benefits have so far been modest.

Adeno-associated virus. Adeno-associated virus (AAV) is a small virus that is not known to cause disease in humans and thus could be seen as a possible suicide gene vector. As the name suggests, this virus is often found in cells where adenovirus is also present. Unlike adenovirus, AAV becomes inserted in the human genome but at a specific site on chromosome 19, designated AAVS1, reducing the risk of insertional mutagenesis[49]. It has been found to target ovarian tumour cells in culture, delivering the prodrug-activating oxidoreductase DT-diaphorase[50], and melanoma tumours in mice, expressing HSV-TK using the tissue-specific melanocyte inhibitory activity promoter to target the virus[51]. Vermeij *et al.*[52] found AAV on its own to have very poor rates of transfection in ovarian cancer cells. Using an AAV engineered to express green fluorescent protein (GFP), so that the presence of virus in cells can be visualized, the virus alone transfected <1% of cells in culture, and the presence of an AdV only increased this to a meagre 15%. By contrast, AdV–GFP transfection in the same cell type resulted in the infection of about 50% of the cells. Transgene expression appears to depend on the promoter driving it, as Veldwijk *et al.*[53] observed consistently better GFP fluorescence in a range of cell lines with an *EF1α* promoter-driven expression vector compared with one using the CMV promoter. Kanazawa *et al.*[54] found that AAV transgene expression in head and neck cancer xenografts in nude mice was increased by γ-irradiation. The size of AAV might be a disadvantage in its use, as it only has the capacity for about 4.7 kb of DNA, limiting the size of therapeutic genes that it can deliver.

Retroviruses. Retroviruses are enveloped, single-stranded RNA viruses that rely on the activity of reverse transcriptase to produce double stranded DNA that then becomes lysogenic, replicating with the host[55]. For example, Vesicular stomatitis virus is a retrovirus that is less susceptible to the mutating effects of active 5-FU than DNA

Table 3 | **Mechanism of prodrug activation**

Enzyme	Prodrug	Drug	Structures	Action	Refs
HSV-TK	Ganciclovir	Ganciclovir monophosphate		Intracellular metabolism to triphosphate nucleotide which competes with dGTP for DNA polymerases	134
Purine nucleoside phospho-rylase (PNP)	6-methylpurine deoxyriboside	6-methylpurine		Inhibition of RNA, protein and DNA synthesis	135
Carboxy-esterases	Irinotecan (CPT 11)	SN38 (Camptothecin)		Binding to nuclear enzyme topoisomerase I-DNA adducts leads to single strand breaks	136
Nitrore-ductase	CB1954	5-(azirdin 1-yl) 4-hydroxyl-amino 2-nitro benzamide		DNA interstrand crosslinking	22,81
Carboxy-peptidase G2 (CPG2)	Nitrogen mustard L glutamates, such as CMDA (4 [(2-chloroethyl)(2-mes yloxyethyl)amino] benzoyl L-glutamic acid)	Nitrogen mustards 4 [(2-chloroethyl)(2-mesyloxyethyl)amino] benzoic acid		DNA interstrand crosslinking	137
Cytosine-deaminase (CD)	5-fluorocytosine	5-fluorouracil (5-FU)		Intracellular metabolism to 5-fluorodeoxy-uridylate, which inhibits thymidylate synthetase and thereby DNA synthesis; other metabolites inhibit DNA and RNA synthesis	138
Cytochrome P450 (CYP450)	Cyclophosphamide	4-hydroxycyclo-phosphamide		Spontaneously disintegrates to acrolein (potential cytotoxin) and a bifunctional alkylating oxazaphosphorine mustard drug (DNA crosslinker)	80

HSV-TK, Herpes simplex virus thymidine kinase.

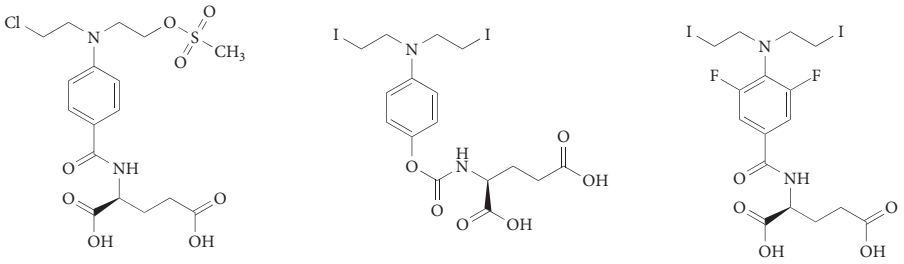


Figure 2 | **Structure of nitrogen mustard prodrugs.** The structures of three common nitrogen mustard produgs used in gene-directed enzyme–prodrug therapy (GDEPT) are shown[93].

viruses, and so might more effectively be used with a CD and 5-FC combination[56,57]. Haemagglutinating virus of Japan (HVJ, or Sendai virus) is a retrovirus that is non-pathogenic to humans, primarily infecting rodents[58]. HVJ fuses with the cell membrane rather than undergoing endocytosis, minimizing cell damage. Lentiviruses such as HIV-1 fall within the retrovirus group, and although there are safety concerns regarding their use, they have several advantages. In particular, they do not elicit a host immune response, and target both dividing and quiescent cells. The use of these viruses as suicide gene vectors also has the obvious advantage of potentially long-lived expression of a prodrug-activating enzyme[59] owing to retroviral insertion into the host DNA. However, there are risks. As the incorporation of the viral genome into that of the host occurs at random, retroviral infection could lead to insertional mutagenesis. Using generator cells that express viral proteins from constructs that are not encapsulated in the viral progeny allows viruses to infect a single cell and deliver the suicide gene[60]. This reduces the possibility of generating a replicative viral clone by recombination but does not eliminate the risk entirely. Despite these risks, retrovirally-mediated HSV-TK–ganciclovir GDEPT has entered clinical trials for malignant glioma, using vectors derived from the Moloney murine leukaemia virus[61,62].

Herpes simplex virus. Herpes simplex virus (HSV) is an enveloped, double-stranded DNA virus known to infect tumours and tumour cells *in vitro*. HSV, and replication-competent vectors derived from it, have been investigated as vectors for the treatment of glioma[63,64] and colon cancer, including liver metastases[65,66].

HSV has been shown to deliver the gene for *Saccharomyces cerevisiae* CD to HT29 human colon cells *in vitro* and MC26 mouse colon carcinoma allografts, producing significant cell kill, tumour regression and an increase in survival time following 5-FC treatment[67]. Recently, a conditionally replicating HSV mutant has been shown to produce similar results with *E. coli* CD in murine Neuro-2a neuroblastoma allografts[68]. The vector HSV1716, which selectively replicates in dividing cells, has been tested safely in phase I trials in patients with glioma and metastatic melanoma[69].

Other potential viral vectors. Other vectors that have been tested for the delivery of suicide genes *in vitro* and *in vivo* include Baculovirus and Vaccinia. Baculovirus has evolved to suit insect hosts, and its own promoters do not function in mammalian cells; thus it can express engineered constructs but not replicate. An advantage is that mammals are unlikely to have prior exposure and immunity to an insect infection[70,71]. Vaccinia has inherent tumour specificity[72] and a track record of safe use for smallpox vaccination[73,74]. Although the mechanism of this natural tropism is unidentified, Vaccinia tumour selectivity can be increased by deletion of the viral thymidine kinase and other genes[75,76], making viral replication more dependent on the nucleotide-rich environment in dividing cells. Vaccinia also seems to require leaky vasculature to access target cells, which is more common in tumours than normal tissues.

The vectors described above all have their advocates, but none is without some disadvantage in either its safety, targeting or efficacy. Approaches that combine different treatment modalities, such radiotherapy, with vector-delivered suicide gene systems might, in some cases, improve preclinical and perhaps clinical outcomes. Work is currently in progress in the development of safe, targeted vectors for GDEPT and gene therapy in general (TABLE 1).

Carboxypeptidase G2-based GDEPT

Many suicide gene therapy systems have been investigated, and those in current use are shown in TABLE 3. See Portsmouth *et al.*[77] for a recent review. We have considerable experience in working with nitroreductase and CB1954 (REFS 78,79), but our group is currently working with *Pseudomonas* carboxypeptidase G2 (CPG2; expressed in various vectors) and nitrogen mustard prodrugs, and the rest of this Perspective documents the progress made in this area.

CPG2 catalyses the hydrolysis of nitrogen mustard prodrugs, releasing glutamic acid and the cognate drug. The system is illustrated in FIG. 1. The CPG2–nitrogen mustard prodrug system has several advantages over other GDEPT systems: CPG2 has no mammalian equivalent, unlike carboxylesterase, purine nucleoside phosphorylase or CYP450; no additional activating steps are required, unlike the CD–5FC combination[80], where the 5FU produced must be metabolized to its cognate nucleotides, or nitroreductase–CB1954, where the 4-hydroxylamine derivative of the drug requires acetylation by reaction with acetylCoA to become the active DNA crosslinking[81] species; and both dividing and quiescent cells are killed by the drug. Moreover, gap junctions are not required for a bystander effect, unlike HSV-TK–ganciclovir, where the activated drug (ganciclovir triphosphate) is charged and therefore cannot pass through cell membranes. CPG2-activated nitrogen mustard drugs are relatively lipophilic and can pass directly through cell membranes.

Our experience with antibody-directed enzyme-prodrug therapy (ADEPT) using the combination of a human anti-carcinoembryonic antigen (CEA) antibody conjugated with CPG2 and nitrogen mustard prodrugs[8,82–84] has established the suitability of this enzyme–prodrug combination, such that it has been taken forward into GDEPT studies. The first report of CPG2-based GDEPT was that of Marais *et al.*[85], who tested the system *in vitro* using a range of human cancer cell types that had been transformed with the bacterial gene encoding CPG2. The enzyme produced was found to have a K_m for methotrexate (the chemotherapeutic drug that is used as a model substrate to assay CPG2 activity) that was almost identical to that found in bacterial cultures. It was not secreted but remained in the cytoplasm of the cells and was designated CPG2*. CPG2*-expressing cells were found to have between 11-fold and 95-fold greater sensitivity to the nitrogen mustard prodrug 4-[(Mesyloxyethyl)(2-chloroethyl)amino] benzoyl-L-glutamic acid (CMDA) than control cells expressing bacterial β-galactosidase, an enzyme irrelevant to the therapy. Sensitivity was dependent on cell type, with A2780 ovarian adenocarcinoma

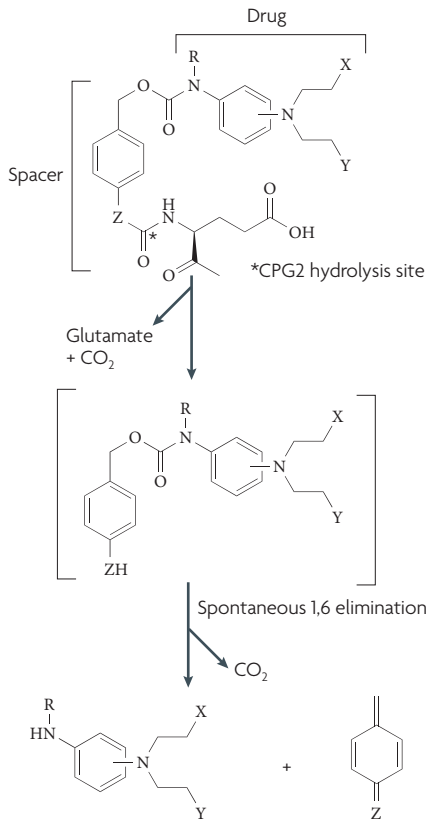


Figure 3 | **Activation of self-immolative prodrugs.** Separating the carboxypeptidase G2 (CPG2) hydrolysis site from the 'effector' end of a nitrogen mustard produg by a spacer that spontaneously undergoes 1,6-elimination to release the active drug has enabled an increase in the number and range of active drug structures that can be formulated as CPG2-activated prodrugs. Depending on the structure of the linking group, the lipophilicity and hence the bioavailability of the prodrugs can be changed without affecting the activation kinetics[90,92,95].

and LS174T colon carcinoma being very much more sensitive than SK-OV-3 ovarian adenocarcinoma and WiDr colon carcinoma. Moreover, by treating cell cultures containing various proportions of normal and transfected cells, GDEPT showed a large bystander effect — when ~2% of the cells expressed CPG2* 90% of them were killed.

A potential problem with the expression of CPG2* is the release of the enzyme from lysed cells and into the general circulation, possibly producing generalized toxicity. To address this, we have expressed CPG2 in a cell-surface-tethered form by creating a fusion protein with the transmembrane region of the epidermal growth factor receptor family member ERBB2. This initially led to surface expression but reduced activity, resulting from N-glycosylation that occurs in mammalian membrane targeting. By point-directed mutation of three asparagine residues to glutamines (producing stCPG2(Q)3), glycosylation was circumvented and the kinetics of the enzyme resembled those of CPG2*[86].

This improves the efficacy of the system *in vitro*, as illustrated by a 10-fold reduction in the IC_{50} of CMDA in MDA MB 361 human mammary tumour cells. A good bystander effect was seen with the surface-tethered CPG2 both *in vitro*[86] and *in vivo*[87]. The efficiency of a surface-tethered CPG2 GDEPT system has been borne out by work by others, such as Cowen *et al.*[88], who used a glycosylphosphatidylinositol (GPI)-anchored enzyme delivered by an adenovirus vector. In general terms, it is possible that in some GDEPT systems where the prodrug is too hydrophilic to cross the cell membrane the enzyme and substrate will not meet if the enzyme is only within the cytoplasm, so the technology of surface-tethering has wide applicability.

Nitrogen mustard prodrugs. The most widely used prodrugs in this class of compounds are CMDA and 4-[bis(2-iodoethyl)amino]phenoxycarbonyl-L-glutamic acid (ZD2767P, previously known as CJS149) (FIG. 2). The active drugs are potent alkylating agents that form inter-and intra-strand linkages in DNA. ZD2767P emerged as the best candidate compound from structure–activity relationship studies[82], and the active drug produced from it (ZD2767D) was found to be at least 300-times more potent than the benzoic acid mustard drugs used in the first ADEPT studies[89]. Using transfected cells has allowed us to validate the CPG2 GDEPT system and to test new nitrogen mustard prodrugs both in optimizing their kinetic parameters for the enzyme and in their efficacy in ablating cells *in vitro* and xenograft tumours[90–94].

Varying the types and number of groups around the heterocyclic ring affects the potency of the compound as a prodrug in killing human MDA MB 361 tumour cells *in vitro* and as xenografts in nude mice[93]. An analogue of ZD2767P with an amide linkage to the glutamate and para-difluoro groups on the ring was over 227 times more effective at killing surface-tethered CPG2 transfected cells than cells expressing β-galactosidase. By comparison, CMDA only exhibited a 19-fold difference in toxicity between the two cell types.

We have also developed self-immolative compounds that can widen the range of active drug structures that can be formulated as CPG2-activated prodrugs without introducing unfavourable steric or electronic effects. This was achieved by separating the CPG2 hydrolysis site from the 'effector' end of the molecule by a spacer that spontaneously undergoes 1,6-elimination to release the active drug. Depending on the structure of the linking group, the lipophilicity and hence the bioavailability of the prodrugs can be changed without affecting the activation kinetics[90,92,95]. The self-immolative mechanism is illustrated in FIG. 3.

Targeting tumours in model systems. For GDEPT to be used as a clinical therapy, it must be shown to be effective *in vivo*, with the suicide gene delivered to and expressed within the target tumour. To this end, we have carried out systemic GDEPT in athymic mice bearing human hepatocytic carcinoma tumours (Hep3B and HepG2)[96]. The gene for CPG2* was delivered by intravenous injection of a replication-competent adenovirus in which the essential early-expressed gene *E1* was under the control of the *TERT* promoter (AdV.hTERT–CPG2*). This resulted in the AdV infection and expression of CPG2* in vastly greater titre in tumours than in the liver, the next most infected tissue. Although this virus is oncolytic in its own right, addition of a once-weekly administration of ZD2767P for 6 weeks led to a significant decrease in tumour growth rate and prolonged survival of the animals (FIG. 4).

We have also recently tested a similar therapy using SW620 human colorectal adenocarcinoma cells[97]. *In vitro*, comet assays for DNA crosslinking showed that AdV.hTERT–CPG2 with ZD2767P was as effective as cisplatin and free ZD2767D nitrogen mustard, whereas the other control groups were not significantly different from untreated cells. There was a 160-fold increase in sensitivity to ZD2767P in cells infected with the virus compared with uninfected cells. A good bystander effect was seen in mixed cultures of SW620 cells, which are infected with AdV, and WM266.4 cells, which are not: approximately 80% of the cells were killed in co-cultures with 25% SW620 cells.

In xenografts we observed good tumour regression and increased survival in the GDEPT group of animals compared with the control, virus- and prodrug-alone groups. We sampled a wide range of tissues (tumour, liver, lungs, kidney, spleen, gut, ovaries, femoral muscle, brain and bone marrow) to determine CAR and CPG2 expression. Although the tumour expressed less CAR than lung, gut, liver or ovaries, the level of CPG2 in the tumour was 20-fold greater than in the liver, with little or no expression in any other sampled tissue. Interestingly, in

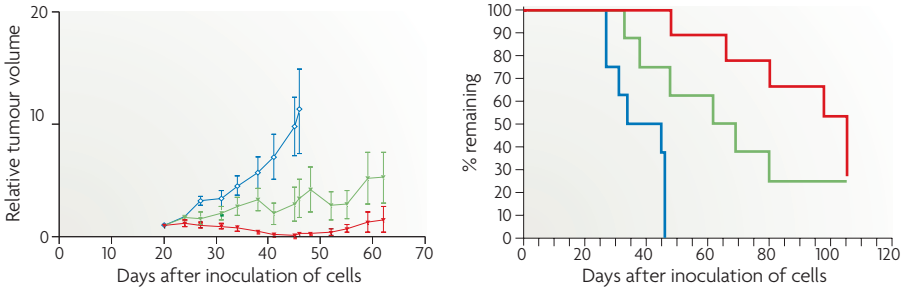


Figure 4 | **Efficacy of carboxypeptidase G2 gene-directed enzyme–prodrug therapy (GDEPT) in hepatocellular xenografts. a** | Hep3B human hepatocellular carcinoma xenografts in athymic mice. Animals received subcutaneous injections of human tumour cells that were allowed to grow before receiving intravenous virus. A week later prodrug treatment commenced and continued for 6 doses at weekly intervals. Tumours were measured twice a week using manual calipers. These growth curves clearly show that treatment of mice with both vector and drug (AdV.hTERT–CPG2* ZD2767P; red line) decreases growth of the xenograft compared with vector alone (green line) and control (blue line). **b** | Kaplan–Meier plot for Hep3B human hepatocellular carcinoma xenografts in athymic mice. Treatments are as described above. Mice treated with the full GDEPT protocol (red line) have improved survival compared with vector alone (green line) and control (blue line) mice.

the animals treated with the full GDEPT, we found that the presence of prodrug led to a significantly increased viral copy and enzyme activity (around 20 and 3-fold respectively) in the tumours compared with those treated with virus alone. This suggests that our GDEPT system, while inducing cytotoxicity in the target tumour, presumably by nitrogen mustard-induced DNA crosslinking, does not affect the viral genome, but directly or indirectly increases adenovirus replication. This finding corroborates that of Bernt *et al.* [98], who observed similar effects of a β-glucuronide prodrug, of 9-aminocamptothecin, a topoisomerase I inhibitor, and 5-FC on adenoviral replication *in vivo* and a range of cytostatic agents *in vitro*. However, some agents (5-fluorouracil (5-FU) and hydroxyurea) seemed to have a biphasic effect on viral reproduction, being stimulatory at low doses and inhibitory at high doses. Conversely, Schaak *et al.*[99] found that inhibition of topoisomerase I with camptothecin stopped adenoviral DNA replication in cells *in vitro* immediately. McCart *et al.*[100] found that mice with subcutaneous MC38 tumours (murine colon adenocarcinoma) were protected from Vaccinia virus toxicity in the presence of a CD–5-FC GDEPT system, suggesting that in this case, the generated 5-FU inhibited viral replication as well as tumour growth. By contrast, Nakamura *et al.*[67] found that the replication of a HSV-1 mutant, carrying yeast CD in addition to its native TK, was significantly more inhibited by ganciclovir than by 5-FC. In our system the prodrug appears to exert a positive cooperative effect on the vector, which could further increase the bystander effect. The increased survival of mice with virus alone compared with the controls seen in our data suggests that either the virus has a relatively low toxicity to non-tumour cells or that the hTERT targeting mechanism restricts its distribution largely to the tumour. In the light of these reports of apparent counter-productivity of combining GDEPT systems with oncolytic viral vectors, our paradigm seems, perhaps somewhat fortuitously, to exhibit synergistic behaviour. The reason for this differential action on cellular and viral DNA is unclear and we have not investigated it, but it underscores the potential of CPG2-based GDEPT as a cancer therapy.

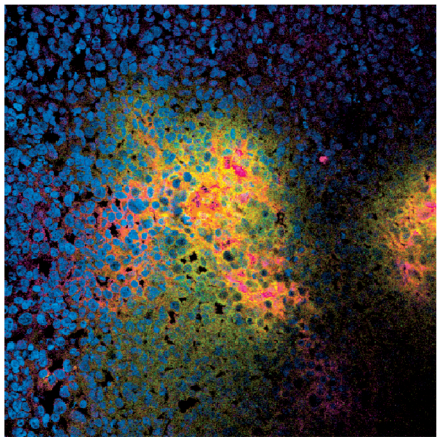


Figure 5 | **Gene-directed enzyme–prodrug therapy (GDEPT) in action.** Immunohistochemical staining of sections of SW620 human colorectal xenograft tumours following GDEPT treatment. Nuclei were stained with TO-PRO3. Adenovirus was probed with a rabbit anti-AdV5 hexon polyclonal antibody and stained with a goat anti-rabbit polyclonal antibody conjugated to Alexa 568 (red). Carboxypeptidase G2 (CPG2) was probed with a rat anti-CPG2 monoclonal antibody and stained with a chicken anti-rat polyclonal antibody conjugated to Alexa 488 (green). Yellow areas show co-localization of virus and enzyme. There are areas of CPG2 staining (green) that extend further than the areas of viral staining (red), indicating an alternative bystander effect mechanism.

Immunohistochemical staining of tumour slices revealed foci of virus surrounded by a halo of CPG2, giving an *ex vivo* correlate for the *in vivo* observations (FIG. 5). We are presently exploring the efficacy of this CPG2 GDEPT system on other model cell types, such as human head-and-neck cancer cells as a prelude for clinical studies.

Clinical submission. We are currently preparing for a Phase 1 clinical trial of the adenovirus-mediated CPG2-based GDEPT system in head-and-neck cancer patients at the Royal Marsden National Health Service Trust in Surrey, UK. This tumour type was chosen so that intratumoral injection of AdV.hTERT–CPG2, rather than systemic administration, can be performed. This will ensure tumour targeting and should reduce the risk of the release of a genetically modified organism into the environment. Tumour biopsies from treated patients will be analysed for enzyme activity to give an indication of the optimum timing for prodrug administration and their blood will be tested for anti-AdV and anti-CPG2 antibodies. Patients will be dosed with ZD2767P to assess the pharmacokinetics and toxic dose limit of the prodrug, and tumour growth will be measured for any GDEPT response.

Conclusion

GDEPT has the promise of delivering target-specific cancer therapy with reduced systemic toxicity and thus better prognoses for patients. A wide range of gene vectors are currently being assessed, some of which are already in clinical trials. The CPG2-based system that we have developed has shown efficient cell kill *in vitro*, potent bystander effect and effective targeting in xenograft tumours in experimental animals, leading to decreased tumour growth rates and prolonged survival. The preclinical data have allowed us to prepare this therapy for the clinic with the promise of a finely tuned treatment. The development of new vectors and prodrugs should allow GDEPT to become another 'magic bullet' for the twenty-first century.

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doi:10.1038/nrc2247
Published online 18 October 2007

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Acknowledgements

Thank you to Frank Friedlos for the immunofluorescence experiments. This work is funded by Cancer Research UK (grant numbers C309/A2187 and C309/A8274).

Competing interests statement

The authors declare competing financial interests: see web version for details.

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