From bench to bedside for gene-directed enzyme prodrug therapy of cancer

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Gene therapy of cancer offers the possibility of a targeted treatment that destroys tumors and metastases, but not normal tissues. In gene-directed enzyme prodrug therapy (GDEPT), or suicide gene therapy, the gene encoding an enzyme is delivered to tumor cells, followed by administration of a prodrug, which is converted locally to a cytotoxin by the enzyme. The producer cells as well as surrounding bystanders are subsequently killed. Promising results have meant that suicide gene therapy has reached multicenter phase III clinical trials. This review will discuss the development, efficiency, mode of action and pharmacokinetics of seven GDEPT systems *in vitro* and *in vivo*. We will review the latest data of those systems in clinical trials (herpes simplex virus thymidine kinase/gancyclovir, bacterial cytosine deaminase/ 5-fluorocytosine, bacterial nitroreductase/CB1954 and cytochrome P450/cyclophosphamide), as well as the development of more recent and experimental systems which are not yet in clinical trials (P450 reductase/tirapazamine, carboxypeptidase/CMDA, horseradish peroxidase/indole-3-acetic acid or paracetamol and others). *Anti-Cancer Drugs* **16:349–359**

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Introduction

Gene therapy of cancer is now the major use of genetic-based treatments. The July 2004 update of clinical trials showed that cancer trials accounted for two-thirds of all 987 gene therapy trials approved worldwide [1]. The first clinical trial on gene therapy was started in 1989 [2] and the first remarkable cures were reported in 2002 in children with X-linked severe combined immunodeficiency (X-SCID) [3].

Two patients (of five) from the first human gene therapy trial have been followed-up long-term [4]. These adenosine deaminase (ADA)-deficient patients were treated with peripheral blood lymphocytes transduced with ADA-containing retroviral vector. Ten years after the last cell infusion, about 20% of one patient's lymphocytes still expressed the retroviral gene. However, the other patient, who developed an immune response to the gene transfer system, showed no transgene expression, illustrating both the promise and the drawback of gene therapy in humans.

The number of new trials peaked in 1999 with 114 new trials approved, but over the last 5 years the number progressively reduced to 96 in 2000, 80 in 2002 and 74 in 2003. This may reflect a more cautious approach, possibly since the high (public) expectations of this new treatment modality have not (yet) been met and possibly due to two well-publicized negative events. The first death attributable to gene therapy occurred in Pennsylvania in 1999 [5] and two patients from the X-SCID trials developed leukemia in 2003 [6]. These events sent shock waves through the gene therapy community and put several trials on halt. However, severe side-effects are an unfortunate, but not rare, event in a radical new treatment regime, and only underline the need for innovative development of new strategies and further careful preclinical testing.

Gene-directed enzyme prodrug therapy (GDEPT)

The choice of therapeutic genes for gene therapy can be made from several candidates: mutation compensation, genetic immunopotentiation and molecular chemotherapy. In this review, emphasis will be placed on molecular chemotherapy and its recent advances from *in vitro* studies to clinical trials.

In molecular chemotherapy, GDEPT or 'suicide gene therapy', the gene encoding an enzyme is delivered to

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tumor cells, followed by the systemic administration of a prodrug, which is converted locally to a cytotoxin by the enzyme. The enzyme expression can be genetically controlled or its delivery targeted to ensure tumor selectivity. A further advantage of the GDEPT system is the ability to image the correct location and expression of the (harmless) enzyme prior to prodrug administration.

The use of a transgene for enzyme prodrug activation was first described in 1991 [7] and there are currently 74 clinical trials testing GDEPT. Promising results have meant that three thus far have reached phase III multicenter trials [1].

In any gene therapy system, delivery of the gene to all target cells is unlikely. Therefore, it is important that any cytotoxic agents produced by the transfected cell should be able to kill surrounding cells for tumor growth arrest or regression. This effect is termed the bystander effect.

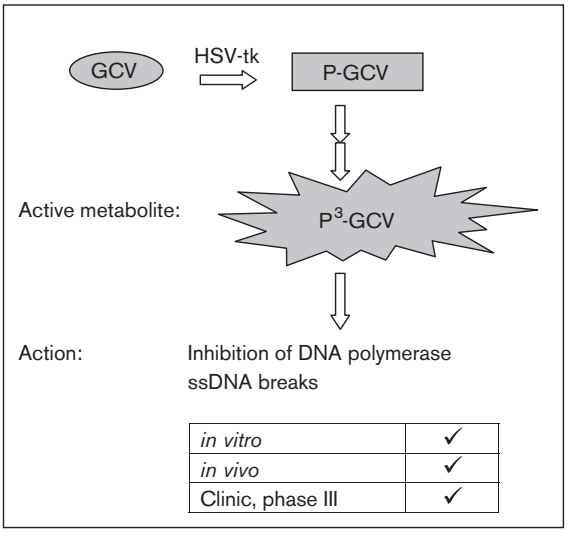
All current GDEPT strategies rely on a bystander element of cytotoxic species transfer, yet there also appears to be an immune response component. Reports agree that in immunocompetent animals, immunity is conferred to gene-modified cells, but not syngeneic cell lines. A significant increase in both $CD8^+$ and $CD4^+$ lymphocytes has been reported in several GDEPT systems. This may be important for metastatic tumors, which were not targeted by the original gene transfer.

An interesting method for increasing the bystander effect is the use of the herpes simplex virus (HSV-1) structural protein VP22 [8]. The VP22 protein is unusual in that cells infected with HSV show localization of the VP22 protein in a diffuse pattern in the cytoplasm, yet VP22 is able to spread to surrounding cells (up to 200 per infected cell *in vitro*), where it is taken up and transported to the nucleus. Fusion proteins retain this transport property and have been used for GDEPT [9].

HSV thymidine kinase/gancyclovir (HSV-tk/GCV)

The HSV-tk/GCV combination represents the clinical standard in GDEPT. GCV and related compounds are widely used for the treatment of HSV infections, as they are poor substrates for the human monophosphatase kinase and hence give few side-effects. GCV is a derivative of acyclovir with the addition of a methoxy group at the 3′ carbon acyclic side-chain which gives increased activity, especially against HSV. Unfortunately, this addition also increases hematopoietic toxicities. After phosphorylation by HSV-tk, GCV undergoes a series of intracellular reactions resulting in the formation of a triphosphate (Fig. 1; see [10,11] for recent reviews). This competes with deoxyguanosine triphosphate in DNA elongation during cell division, resulting in inhibition of DNA polymerase and single-strand breaks. This combination of enzyme and prodrug therefore has some specificity for rapidly dividing tumor cells invading normal quiescent tissue. However, as it is S phase specific, the cells must be actively dividing at the time of drug administration, which is a potential drawback in tumors containing regions of non-proliferating hypoxic cells.

Fig. 1



Activation of GCV by HSV-tk. GCV is phosphorylated by HSV-tk to its monophosphate (P-GCV), which is subsequently converted to di- and triphosphates (P^3-GCV) by cellular kinases. The triphosphates are incorporated into DNA during cell division, causing single-strand DNA (ssDNA) breaks and inhibiting DNA polymerase. The HSV-tk/GCV combination has reached multicenter, phase III clinical trials in glioblastoma patients.

In vitro studies showed that stable expression of HSV-tk could increase cell sensitivity to GCV by up to 2000-fold [12], although results vary according to the assay and cell type used. The HSV-tk/GCV combination relies on gap junctions for its bystander effect, as the highly charged triphosphate is lipid insoluble and therefore incapable of diffusing from one cell to another. The reliance on gap junctional communication for the bystander effect is a drawback, since expression of connexins is often decreased in neoplastic tissues and can also be decreased by hypoxia [13].

The doses of GCV used in animal studies vary from 25 mg/kg twice daily (b.i.d.) to 100 mg/kg b.i.d. [14] and 150 mg/kg/day [15] with varying toxicities. The *in vivo* effect of HSV-tk/GCV has been demonstrated in many tumor models either using stably transfected cell lines or following viral gene delivery.

Table 1 Pharmacokinetic parameters for oral GCV

F (%)	t_{max} (h)	$t_{1/2}$ (h)	Cl (l/h/kg)	V_{ss} (l/kg)	f_e (%)	CSF (%)
3–6	~1	2–3	12–18	0.5–1	91	24–70

F, bioavailability; t_{max}, time to maximum concentration; CL, total body clearance; V_{ss}, volume of distribution in the steady state; f_e, urinary recovery of the parent drug; CSF, cerebrospinal fluid concentration as a percentage of that in plasma [16].

The pharmacokinetic profile of GCV in humans is represented by an open two-compartment model (Table 1 [16]). The majority of the drug is excreted unchanged in the urine within 12 h of administration. Oral bioavailability is very low, resulting in the need for i.v. administration in patients with viral infections. Central exposure to the drug varies with cerebrospinal fluid (CSF) levels 24–70% of plasma concentrations [17].

The HSV-tk/GCV combination is currently the most widely tested GDEPT system in clinical trials by far: 82 out of 95 trials recorded up to July 2004 [1]. It is the only GDEPT approach to have reached phase III clinical testing. Most HSV-tk trials were being conducted on brain tumors, with leukemias/lymphomas and prostate being the next most frequently targeted tumor types. The phase I HSV-tk clinical trials have demonstrated safety in a range of tumors with up to 10^{13} virus particles delivered, including malignant gliomas [18–20], metastatic colorectal carcinomas in the liver [21], melanoma [22], prostate [23] and ovarian cancer [24,25].

Clinical effects are still modest. In addition to poor transfection efficiencies, the slower growth of human tumors compared to xenografts used in animal models and also the limited dose of GCV tolerated due to bone marrow toxicity may have adversely affected treatment outcome. Nevertheless, several trials have shown that injection of virus-producing cells directly into glioblastoma tumors followed by GCV treatment showed partial responses and increases in survival time, although not in all patients [26,27]. This response was thought to be mainly due to the bystander effect, as retroviral transduction efficiencies were very low. Similarly, HSV-tk delivery by adenovirus, but not by retroviral packaging cells, improved the mean survival times of patients with recurrent glioblastoma tumors [28]. A phase I/II study in prostate cancer patients with local recurrence after radiotherapy showed significant biological responses, according to prostate-specific antigen levels, not only after one, but also after a repeat cycle of adenoviral treatment [23].

The patient trials thus far were performed using strong viral promoters to direct tk expression. Targeted gene expression, using osteocalcin promoter-driven HSV-tk expression, which should restrict expression to prostate cells, was analyzed in 11 men with recurrent and metastatic hormone-refractory prostate cancer [29]. Although no significant tumor responses were reported, apoptosis and mediation to fibrosis was observed, and some patients described a resolution of pain.

However, a phase III, multicenter, randomized, controlled trial on HSV-tk/GCV in glioblastoma multiforme consisting of 248 patients with previously untreated tumors was disappointing [30]. Comparison of standard therapy (surgical resection and radiotherapy) and standard therapy with adjuvant gene therapy showed no significant differences. Possible reasons were quoted to be the poor rate of gene delivery and the use of non-migratory fibroblasts as retroviral delivery vehicles.

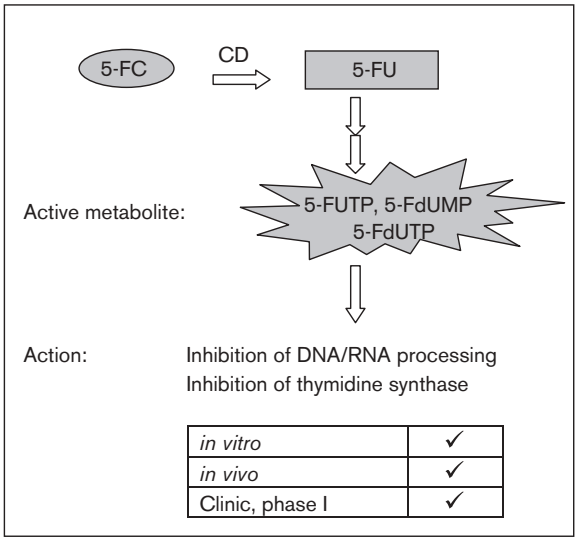
Several studies have started to analyze possible mechanism of action in patients. Positron emission tomography (PET) with a fluorinated HSV-tk substrate has been successfully used to track gene expression non-invasively [31]. The extent of gene expression determined by PET seemed to predict therapeutic outcome in recurrent glioblastomas. Interesting data from another trial on malignant glioma showed hemorrhagic necrosis due to endothelial cell transfection and subsequent vessel necrosis [18]. This effect was accompanied by local inflammation. A trial in prostate cancer reported systemic T cell response after gene therapy, suggesting the potential for activation of cell-mediated immune response [32]. A study performed in recurrent ovarian cancer indicated that the gene therapy treatment eliminated differentiated cells first and induced fibrosis [33]. Findings from these mechanistic studies together with better vector designs will, no doubt, improve patient outcome.

Cytosine deaminase/5-fluorocytosine (CD/5-FC)

5-Fluorouracil (5-FU) is a widely used anticancer agent, especially active against colon cancer, but its side-effects and high dose levels required for response limit its use. The CD enzyme of some bacterial and fungal cells is capable of converting the far less toxic 5-FC to 5-FU [10,11] (Fig. 2). 5-FU undergoes further enzymatic conversion to 5-FUTP, which is incorporated into DNA and prevents nuclear processing of ribosomal and mRNA, and to 5-fluorouridine-5′-monophosphate, which irreversibly inhibits thymidylate synthase. The toxicity of 5-FU is not cell cycle specific, but showed decreased *in vitro* cytotoxicity under hypoxic conditions (own observations).

The bystander effect of CD/5-FC is not dependent on gap junctions, as 5-FU is capable of non-facilitated diffusion into, and out of, cells. *In vitro* studies show that the presence of the CD gene can increase sensitivity to 5-FC significantly, although to what degree is highly

Fig. 2



Activation of 5-FC by prokaryotic CD to form 5-FU. 5-FU is converted by cellular enzymatic steps to 5-fluorouridine-5-triphosphate (5-FUTP), 5-fluorodeoxyuridine-5-monophosphate (5-FdUMP) and 5-fluorodeoxyuridine-5-triphosphate (5-FdUTP), which are involved in induced cytotoxicity.

dependent on the cell type used, varying from 0.2 to 600 μM to reduce viability by 50% (see reviews in [10,11]).

Dosing schedules for 5-FC in animals are more consistent than those for GCV, with 500 mg/kg b.i.d. being the predominant dose chosen. *In vivo* effects of this system appear to be slightly contradictory and some studies found that expression of the CD enzyme alone was sufficient to cause an immune response capable of causing tumor regression [34]. Tumors grown from stable hepatocellular carcinoma transfectants showed 75% eradication by 5-FC, when 20% of the inoculated cells contained the CD gene, similar to results obtained with HSV-tk/GCV [12]. On the other hand, a study demonstrated that 4% CD expressing cells was sufficient to give a 60% cure rate, while for the same level of regression to be achieved with the HSV-tk/GCV system, 50% of cells needed to be HSV-tk$^+$ in a colorectal carcinoma model [35]. The antitumor activity of the CD/5-FC system has since been demonstrated in a range of tumor models.

As is the case with HSV-tk/GCV, there is a level of immunity against tumor rechallenge in animals treated with CD/5-FC. In addition, rats bearing experimental liver metastases subsequently vaccinated with CD$^+$ expressing cells s.c. or beneath the liver capsule followed by 5-FC treatment showed a 70% reduction in volume of the original liver tumor [36].

Table 2 Pharmacokinetic parameters of 5-FC in healthy subjects

Dose	K_a (h^{-1})	K_{el} (h^{-1})	AUC (mg/l·h)	$t_{1/2}$ (h)	Cl_p (ml/min)
500 mg p.o.	5.08	0.226	54.3	3.1	
500 mg i.v.		54		3.8	154

K_a, absorption constant; K_{el}, elimination constant; AUC, area under the curve; Cl_p plasma clearance (after [37]).

Kinetics of 5-FC in humans show that it is greater than 80% bio-available in patients with normal renal function [37]. A single 500 mg oral dose of 5-FC has the pharmacokinetic properties described in Table 2. Treatment of fungal infections usually relies on 4 g/day, which results in peak serum levels of 88–94 μg/ml [38]. Unlike GCV, 5-FC will accumulate after multiple dosing. A 2 g oral dose administered every 6 h will give a steady-state plasma concentration of 60–80 μg/ml [39].

It is unlikely that gene therapy would ultimately be used as a single treatment modality in patients. Combination with radiotherapy is advantageous especially for 5-FC, which can act as a radiosensitizer. *In vivo* results have shown both a significant bystander effect [40] and effects at clinically relevant dose regimes of 2 or 5 Gy per fraction over 1 week [41].

There are currently 10 clinical trials being conducted using CD, four of which as fusions with HSV-tk [1]. Two phase I clinical trials have reported the safety of the CD/5-FC combination, one using adenoviral delivery to target colon carcinoma metastatic to the liver [42], and one using a combination of a lytic adenovirus, HSV-tk and CD genes in prostate [43]. Both trials showed signs of biological activity and the prostate trial had two of 16 patients clear of carcinoma at 1-year follow-up. A trial has also been conducted using *Salmonella* for cellular delivery of CD rather than attempting *in vivo* modification of the tumor cells [44,45]. Data showed evidence of bacterial colonization and increased levels of 5-FU without significant adverse events.

Nitroreductase (NTR)/CB1954

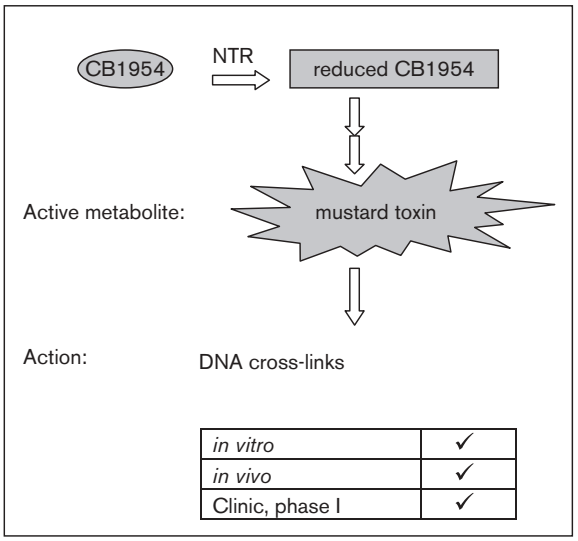
The compound 5-aziridinyl-2,4-dinitrobenzamide (CB1954) was the first single-agent cure in preclinical studies on the Walker rat carcinoma. On this basis a phase I clinical trial was carried out at the Royal Marsden Hospital involving 30 patients in the 1970s [46]. Unfortunately, there was no effect in patients. The Walker carcinoma is a poor preclinical model because it is immunogenic and further investigation of this sensitive rat tumor line showed that it expressed high levels of rat NAD(P)H dehydrogenase (DT-diaphorase) able to convert CB1954 to 5-aziridynyl-4-hydroxylamino-2-nitrobenzamide [47]. On the other hand, CB1954 is a poor substrate for human DT-diaphorase. The mustard metabolite was shown to be a potent DNA cross-linking agent,

causing cell death [47]. Interestingly, a latent nitroreductase, DT-diaphorase 2 (DTd2), which requires the non-biogenic compound dihydronicotinamide riboside as a co-substrate, was recently discovered in some human tumors [48]. Upon activation DTd2 was 3000 times more effective than human DT-diaphorase in the reduction of CB1954.

A study assessed the ability of other reductases to metabolize CB1954 and found that the two-electron nitroreductase from *Escherichia coli* (NTR) was catalytically superior to the rat DT-diaphorase [49] and all further work has utilized the bacterial enzyme (Fig. 3).

The NTR/CB1954 combination is effective under hypoxia and anoxia, and against many cell lines *in vitro*, and IC_{50} values ranged from 1 to 20 μM depending on the cell line used (see reviews in [10,11]). A sensitization of 500-fold was achieved in ovarian carcinoma cells expressing the NTR enzyme [50]. The bystander effect has been shown to be due to a freely diffusible metabolite [51] and cell killing to be cell cycle independent [46], both characteristics important for future clinical application.

Fig. 3



Activation of CB1954 by *E. coli* NTR. Secondary activation occurs via non-enzymatic acetylation to yield 5-(aziridin-1-yl)-4-acetoxyamino-2-nitrobenzamide, the mustard toxin, which is a potent DNA cross-linking agent.

Initial *in vivo* studies varied from those of other strategies in that transgenic mice were utilized. T cell localization of the NTR enzyme was achieved in mice through the use of the CD2 locus promotor [52]. When CB1954 was administered there was a decrease in the cell number of the spleen and thymus to 14–16% of controls. When NTR was expressed in the luminal cells of mice mammary glands, these cells were selectively destroyed by CB1954 administration [53].

Both human and laboratory animal kinetics have been performed with CB1954 [54] and the results are summarized in Table 3. A dose-escalation study was performed concomitantly with the human pharmacokinetic phase I study [54]. This showed that dose-limiting toxicities were seen with a dose of 37.5 mg/m^2 when administered i.v., presenting as diarrhea and elevated levels of transaminase. Below this dose, patients tolerated doses every 3 weeks for a maximum of six cycles.

There is currently considerable interest in the design of new and more potent prodrugs for NTR [55]. Based on the analysis of CB1954 metabolites and their diffusion properties through multicellular layers, a new dinitrobenzamide nitrogen mustard, SN 23862, was designed with superior cytotoxic potency and diffusion properties [56].

The NTR/CB1954 combination is currently being tested in four clinical trials in the UK [1]. Initial data has been published on 18 patients with resectable liver cancer showing minimal side-effects following intratumoral injection of up to 5×10^{11} virus particles [57]. Dose-related increases in tumor NTR expression levels were detected by immunohistochemistry. Although the enzyme levels recorded were capable of activating the prodrug, tumor effects were not yet seen since CB1954 was not co-administered in this study. Future clinical data will show whether this enzyme/prodrug combination lives up to its promise.

Table 3 Pharmacokinetic parameters for CB1954

Species	Dose	Route of administration	C_{pmax}	$t_{1/2}$ (h)	Cl_p (ml/min)	*F* (%)
Mouse	50 mg/kg	i.v.	100 μg/ml	1.4–2		
Mouse	50 mg/kg	i.p.	3- to 5-fold lower than i.v.	1.4–2		85
Dog	25 mg/kg	i.v.	27 μg/ml	2.5–4		
Dog	25 mg/kg	p.o.	3- to 5-fold lower than i.v.	2.5–4		40
Human	24 mg/m^2	i.v.	6.3 μM	α 9 min; β 110 min	376	

The two half-lives shown for humans are due to the bi-exponential delay, thought to be due to hepatic clearance of the drug [54]. C_{pmax}, maximum plasma concentration; Cl_p, plasma clearance; *F*, bioavailability.

Cytochrome P450/cyclophosphamide (CYP/CPA)

The oxazophorines CPA and iphosphamide (IPA) are cancer chemotherapeutic prodrugs, which need to be activated by liver CYP enzymes. In human liver the CYP2B6 and CYP3A4 forms are catalytically active for both CPA and IPA [58]. The rat CYP2B1 is the most catalytically efficient isoform [59] identified to date.

Metabolism of oxazophorines gives rise to a 4-hydroxy compound which is in equilibrium with its open-ring aldo-tautomer (Fig. 4). This breaks down to a phosphoramide mustard and acrolein in equimolar amounts. The mustard is an alkylating agent able to form DNA cross-links in a cell cycle-independent manner.

Expression of CYP enzymes is able to sensitize cells to both CPA and IPA in a range of cell lines *in vitro* (see reviews in [10,11]). The bystander effect is mediated through a soluble compound, likely to be 4-hydroxy-CPA. It has been proposed that acrolein sensitizes cells to the mustard [60].

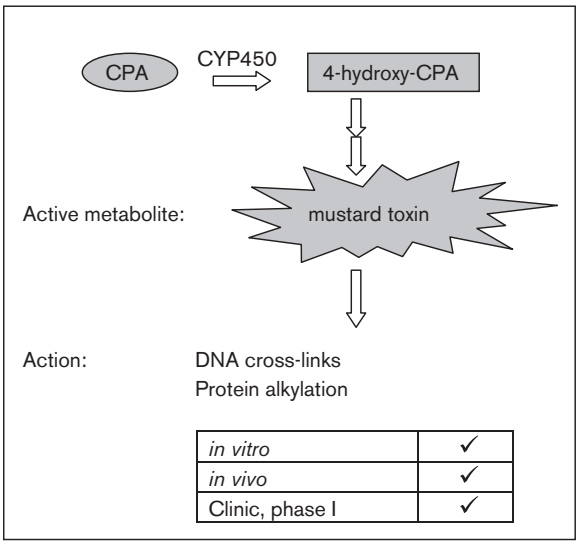
The use of a multicellular spheroid model has demonstrated the ability of macrophages to deliver the CYP2B6 gene controlled by a hypoxia-regulated element (HRE) [61]. Both a reduction in clonogenic survival and spheroid size were seen after CPA treatment.

In animal models, s.c. tumors grown from stable transfectants demonstrated prolonged growth delay after a single 150 mg/kg dose of CPA [62]. This was accompanied by some toxicity, seen as a decrease in body weight. Later studies showed that lower multiple dosing may be more effective and this was demonstrated in a rat gliosarcoma model where tumors regressed [62].

Tumor response may also be modified by co-expression with CYP reductase (P450R) and administration of bioreductive agents, such as tirapazamine [62]. It was shown that despite a requirement of the CYP enzymatic function for oxygen, oxygen concentrations of 1% were sufficient to result in the formation of the active compound. Administration of inhibitors of liver CYP isoforms may be beneficial in targeting prodrug activation to the tumor and reducing global toxicity, especially as CPA is capable of auto-induction of the CYP enzymes. The CYP2B1 gene has been inserted into a replication competent HSV-1 vector which led to additive effects in a model of liver metastasis [63].

CPA administration for cancer therapy in humans is usually given in combination, e.g. in breast cancer the two main regimes involving CPA are 'AC' and 'CMF'. In AC, adriamycin (also referred to as doxorubicin) (60 mg/m^2) and CPA (600 mg/m^2) are administered i.v. every 3 months. With CMF, CPA is administered orally at 100 mg/m^2 on the first 14 days of every month in combination with the antimetabolite methotrexate and 5-FU [64].

Fig. 4



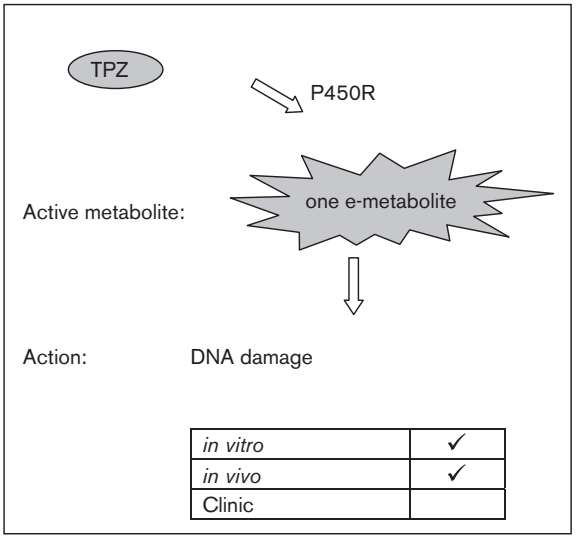
Activation of CPA by liver CYP450 to 4-hydroxy-CPA. This intermediate metabolite undergoes spontaneous conversion resulting in the phosphoramide mustard (mustard toxin) and acrolein, which cause DNA cross-links and protein alkylation.

Three clinical trials are currently open in Germany using CYP2B1 showing remarkable early success. The studies use microencapsulated allogeneic cells transfected with rat CYP2B1 to activate IPA. In a trial of 14 patients with inoperable pancreatic cancer, median survival was doubled in the treatment group compared to historical controls and 1-year survival improved 3-fold [65,66]. Another seven trials are being conducted worldwide analyzing transfer of P450 genes for cancer vaccination purposes [1].

P450R/bioreductives

Inadequate oxygenation in solid tumors is a poor prognostic indicator. Hypoxic cytotoxins, or bioreductives, are prodrugs activated within the reducing environment of the oxygen-deprived cells and can hence exploit this tumor characteristic. The redox-sensitive flavoprotein NADPH P450R is an important activator of many bioreductives. It was shown that the toxicity of tirapazamine (TPZ), a benzotriazene-di-*N*-oxide, under hypoxia, was strongly correlated with P450R activity in a panel of breast cancer cell lines [67]. In addition, sensitivity to TPZ could be restored in a resistant cell line (A549c50) by transfection with P450R [68] (Fig. 5).

Fig. 5



Activation of TPZ by P450R to the toxic one-electron reduced metabolite.

Co-transfection of CYP/P450R in gliosarcoma cells was shown to lead to increased toxicity and tumor growth delay following treatment with CPA and TPZ compared with either compound alone [62]. A bystander effect of the P450R/TPZ combination was apparent.

Other bioreductives, including E09 [69] and RSU1069 [70], show increased activity in cell lines overexpressing P450R. Placement of the P450R enzyme under the control of hypoxia response elements resulted in hypoxia-dependent expression leading to increased sensitivity to RSU1069 in a human fibrosarcoma model [71]. When tumor-bearing animals were treated with RSU1069 and 10 Gy radiation, 50% tumor-free survival was reported, compared with 100% mortality in empty vector controls. Similarly, when hypoxia-targeted P450R was delivered by adenoviral vector followed by TPZ and radiation, complete tumor regression was reported [72]. These results show that the use of hypoxia-targeted gene expression combined with bioreductive drugs can provide tumor-specific targeting of prodrug activation.

The diffusion of TPZ through a multilayered cell model has been used to estimate the penetration of the drug through cells in a tumor cord [73]. It was estimated that levels in cells distant from blood vessels would be approximately 10% of the blood concentration and that cell kill would be limited to the first 75 μm of tissue surrounding a blood vessel, due to metabolism of TPZ by cells closer to the vessel at intermediate oxygen tensions. This has implications for targeting chronically hypoxic regions which may lie at a greater distance from the vessel than this estimate.

Pharmacokinetics of TPZ have been determined in mouse and man. In mice there is a steep dose–lethality relationship, with only 9 mg/m^2 separating the LD_{10} (dose lethal to 10% of animals) and LD_{50} [74]. An increase in dose leads to a disproportionately high increase in AUC in both mice and men [74], although it is more exaggerated in mice. In a phase I clinical trial the MTD was found to be 390 mg/m^2, with dose-limiting ototoxicity being the primary symptom, and muscle cramp and nausea also reported [74].

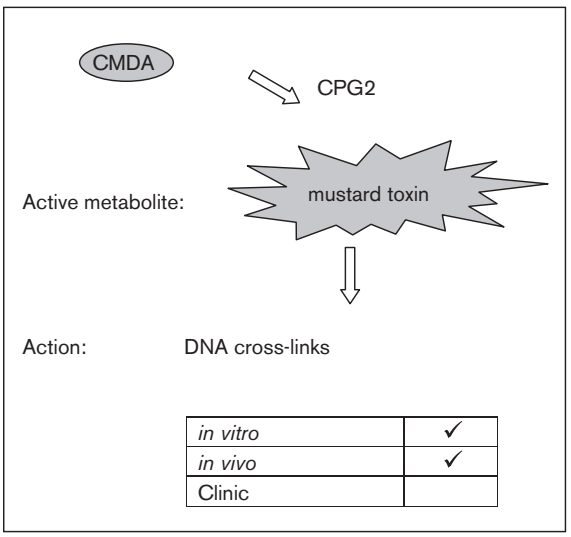
TPZ is in clinical trials as a bioreductive agent both in combination with chemotherapy and radiotherapy. The P450R/TPZ combination has not as yet entered clinical trials for gene therapy.

Carboxypeptidase G2 (CPG2)/CMDA

CPG2 is a bacterial enzyme with no human analog, able to catalyze the conversion of 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoyl-L-glutamic acid (CMDA) to the DNA cross-linking mustard, 4-[(2-chloroethyl)(2-mesyloxyethyl)amino]benzoic acid [75] (Fig. 6).

Results from *in vitro* studies showed significant variation in sensitivity to CMDA and was shown to be due to limited CMDA entry into cells. This could be overcome by genetically tethering the CPG2 enzyme to the cell surface [76]. This also resulted in a significant bystander

Fig. 6



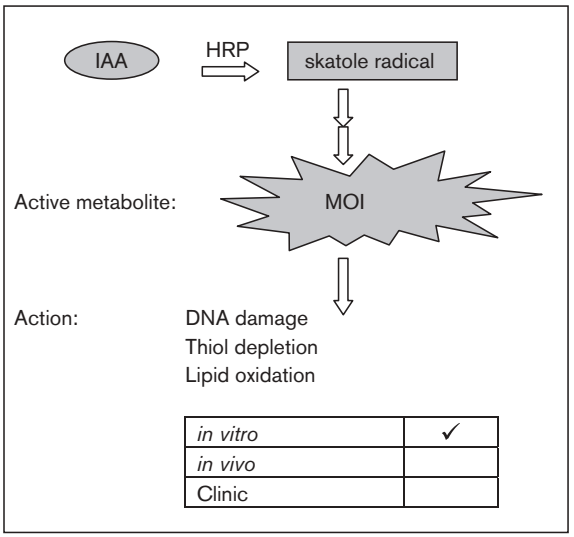
Activation of CMDA by CPG2 to 4-[(2-chloroethyl)(2-mesyloxyethyl) amino]benzoic acid (mustard toxin).

effect with only 12% of WiDr monolayers required to express CPG2 to obtain 100% cell kill.

In vivo experiments have utilized surface tethered CPG2 to show tumor regression and cures in five of six mice when all cells expressed the CPG2 enzyme [77]. The dose regime for this study was a single i.p. administration of 500 mg/kg once per week for 3 weeks. Some non-specific toxicity was associated with CMDA, as demonstrated by a significant decrease in body weight, although this was reversed upon cessation of treatment.

This enzyme/prodrug combination is now almost exclusively used for antibody-directed enzyme prodrug therapy (ADEPT), in particular with anti-CEA antibodies. Using an ADEPT strategy against colon carcinoma models, tumor response could be greatly enhanced (16-fold) by addition of the vascular targeting agent DMXAA [78]. A phase I clinical trial using this combination for ADEPT [79] showed promising results, with one patient showing a partial response and six with stable disease for 4 months. Hematological disorders were seen, but were easily managed. Clinical trials of the GDEPT combination have not yet been reported.

Fig. 7



Activation of IAA by HRP. IAA is oxidized by HRP to a radical cation which rapidly fragments to form the skatole radical, which probably forms adducts under hypoxia. In air, this radical is converted by further steps to 3-methylene-2-oxindole (MOI) to cause cellular damage.

Horseradish peroxidase/indole-3-acetic acid (HRP/IAA)

HRP is a heme enzyme isolated from the roots of the horseradish plant. The active site of the HRP molecule resembles that of hemoglobin and myoglobin. IAA is a plant auxin involved in the regulation of plant cellular growth, division and differentiation. It is also a natural metabolite in mammals of the amino acid tryptophan by monoamine oxidase. Its excretion via urine is increased in upper gastrointestinal tract cancers and its use as a marker of disease progression in gastric cancer has been proposed [80].

The reaction of HRP with IAA is characterized by the formation of a radical cation, which undergoes scission of the carbon–carbon bond to give a carbon-centered skatolyl radical and after subsequent oxic steps forms the toxin 3-methylene-2-oxindole (MOI) [81] (Fig. 7; reviewed by [82]).

In vitro studies have demonstrated that mammalian cells transiently transfected with the HRP cDNA and then exposed to IAA show reduced colony-forming ability compared to controls [83]. A strong bystander effect was induced in air and hypoxia, requiring only 5% of transfected cells to kill 70% of cells, probably due to the production of a freely diffusible cytotoxin [84]. Hypoxic sensitization was observed with the 5-bromoindole-3-acetic acid (5Br-IAA) analog producing over 2 logs of cell kill under anoxia after only 2 h incubation with 1 mM 5Br-IAA [84]. Subsequently, in a three-dimensional spheroid model, 5Br-IAA displayed selectivity for spheroids with a large hypoxic fraction following short exposure times [85].

It is unlikely that non-specific activation of IAA would take place in mammalian tissues, since IAA is a poor substrate for mammalian peroxidases, including myeloperoxidase [86,87].

The combination of HRP/IAA and radiation has been studied, because the HRP/IAA system is capable of reducing glutathione levels [88] and inducing DNA strand breaks [89]. Both IAA and the 1-methylindole-3-acetic acid (1Me-IAA) analog were able to selectively sensitize HRP-modified human bladder carcinoma cells to ionizing radiation in air and under anoxia, where radical fixation is less likely to occur [90]. HRP expression has been placed under the control of a variety of hypoxia-regulated and radiation-inducible elements. After the appropriate stimuli, hypoxia and/or 0–7 Gy, a significant decrease in cell survival was reported after only 0.5 mM IAA [90].

IAA has been given to patients and volunteers in the 1950s, with no major toxicities reported after administration of 3–10 g [91,92]. Unfortunately, no pharmacokinetic data is available from those early trials. However, in mice, 300 mg/kg of IAA led to side-effects, including myotonia and hypothermia.

High-performance liquid chromatography analysis of a single dose of 250 mg/kg in mice IAA showed that the

Table 4 Pharmacokinetic parameters of indoles following a single i.p. administration of IAA at 250 mg/kg or 5Br-IAA 150 mg/kg

Drug	Plasma $t_{1/2}$ (min)	Plasma K_{el} (min^{-1})	Plasma $AUC_{0-240min}$ (mmol/l·min)	Tumor $AUC_{0-240min}$ (mmol/l·min)	Liver $AUC_{0-240min}$ (mmol/l·min)	Muscle $AUC_{0-240min}$ (mmol/l·min)
IAA/10% EtOH	23	0.03	257	117	149	57
IAA/5% DMSO	69	0.01	403	373	336	117
5Br-IAA/10% EtOH	87	0.008	367	179	212	70

Data are from three or four mice per time point. K_{el}, elimination constant; AUC, area under the curve; DMSO, dimethylsulfoxide; EtOH, ethanol.

prodrug is able to quickly distribute between most tissues (Table 4, own unpublished data). The pharmacokinetic profile of IAA differed according to the solvent used. The plasma half-life was 3 times longer when DMSO was used in place of ethanol and all tissues received a greater exposure to the prodrug. 5Br-IAA was slowly eliminated from the blood, with a half-life 4 times that of IAA in ethanol. Early *in vivo* data has shown a modest growth delay in tumors stably expressing HRP following IAA treatment (own unpublished data).

HRP can oxidize a range of other substrates and recent work showed that incubation of human cells expressing HRP with paracetamol significantly reduced clonogenic survival in air and anoxia, but had little effect on control cells [93].

New enzyme/prodrug combinations

A number of novel enzyme/prodrug combinations have been designed for their use in cancer gene therapy. Both prokaryotic and mammalian genes are being investigated. The *E. coli* purine nucleoside phosphorylase-encoding gene has been mutated to increase substrate specificity and has been shown to efficiently cleave the novel prodrug, 9-(6-deoxy-α-L-talofuranosyl)-6-methylpurine, to a toxic agent, causing xenograft regression *in vivo* [94]. The methionine-αγ-lyase gene from *Pseudomonas putida* was used to convert the physiologic compound selenomethionine into the highly toxic methylselenol and, in combination with doxorubicin, showed a significant increase in tumor doubling times *in vivo* [95].

The CYP isozyme CYP4B1 was utilized to convert the inert prodrug 4-ipomeanol into a toxic alkylating agent and when placed under radiation-inducible gene control could potentiate radiation-induced cell kill *in vitro* [96]. Another CYP isozyme, CYP3A4, has been used to direct activation of the bioreductive drug AQ4N to the tumor [97]. When combined with radiation, local tumor control was achieved in one-third of tumor-bearing animals.

Concluding remarks

In conclusion, even though gene therapy has had a difficult half-decade, the opportunities offered by this (still) novel treatment regime have not diminished. Of the array of candidate genes evaluated for gene therapy of cancer, those encoding prodrug-activating enzymes are especially appealing as they complement ongoing clinical chemotherapy and radiation schedules. Even though the two-component GDEPT system may appear cumbersome, it requires only a fraction of the target cells to be genetically modified for tumor destruction, due to the transfer of cytotoxicity to neighboring cancer cells. Several of the newer enzyme prodrug combinations in clinical trials have shown real promise which supports the further development of this treatment regime.

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