

Cytochrome P450 Gene-directed Enzyme Prodrug Therapy (GDEPT) for Cancer

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Abstract: Several commonly used cancer chemotherapeutic prodrugs, including cyclophosphamide and ifosfamide, are metabolized in the liver by a cytochrome P450 (CYP)-catalyzed prodrug activation reaction that is required for therapeutic activity. Preclinical studies have shown that the chemosensitivity of tumors to these prodrugs can be dramatically increased by P450 gene transfer, which confers the capability to activate the prodrug directly within the target tissue. This P450 gene-directed enzyme prodrug therapy (P450 GDEPT) greatly enhances the therapeutic effect of P450-activated anti-cancer prodrugs without increasing host toxicity associated with systemic distribution of active drug metabolites formed by the liver. The efficacy of P450 GDEPT can be enhanced by further increasing the partition ratio for tumor:liver prodrug activation in favor of increased intratumoral metabolism. This can be achieved by co-expression of P450 with the flavoenzyme NADPH-P450 reductase, which increases P450 metabolic activity, by localized prodrug delivery, or by the selective pharmacologic inhibition of liver prodrug activation. P450 GDEPT prodrug substrates are diverse in their structure, mechanism of action, and optimal prodrug-activating P450 gene; they include both established and investigational anticancer prodrugs, as well as bioreductive drugs that can be activated by P450/P450 reductase in a hypoxic tumor environment. Several strategies may be employed to achieve the tumor-selective gene delivery that is required for the success of P450 GDEPT; these include the use of tumor-targeted cellular vectors and tumor-selective oncolytic viruses. Overall, P450-based GDEPT presents several important, practical advantages over other GDEPT strategies that should facilitate the incorporation of P450 GDEPT into existing cancer treatment regimens. A recent report of clinical efficacy in a P450-based phase I/II gene therapy trial for pancreatic cancer patients supports this conclusion.

INTRODUCTION

Chemotherapy using cytotoxic drugs is presently the most commonly used weapon in anticancer warfare. The therapeutic utility of cytotoxic anticancer agents is limited, however, by a moderate therapeutic index associated with nonspecific toxicity toward normal host tissue. Recent advances in molecular medicine and genomic research have provided new opportunities to develop novel, more selective anticancer therapeutics. One such approach involves the application of gene transfer (gene therapy) technologies designed to increase tumor sensitivity and responsiveness to cytotoxic drugs by transfer of a suitable therapeutic gene. Candidate therapeutic genes for cancer treatment include tumor suppressors, cytokines and lymphokines for immunotherapy, and prodrug-activation enzymes. In the latter case, a chemosensitization or "suicide" gene encoding a prodrug-activation enzyme is directly transferred to tumor cells in an effort to sensitize the tumor to drugs that are otherwise non-cytotoxic or less cytotoxic [1,2]. This strategy is referred to as gene-directed enzyme prodrug therapy (GDEPT). Ganciclovir in combination with the herpes

simplex virus thymidine kinase gene (HSV-TK) is a prototypical example of this GDEPT strategy. Cancer cells transduced with *HSV-TK* acquire sensitivity to the prodrug ganciclovir, a clinically proven agent originally designed for treatment of viral infections [3]. Another widely studied example is the bacterial gene cytosine deaminase, which can sensitize tumor cells to the anti-fungal agent 5-fluorocytosine as a result of its transformation to 5-fluorouracil, an established cancer chemotherapeutic drug [4]. Preclinical and clinical gene therapy studies using these prodrug activation genes have yielded some promising findings [1], suggesting that GDEPT will ultimately find clinical applications. However, the successful application of GDEPT is likely to require the development of other, more effective prodrug-enzyme combinations, along with novel strategies to apply them to cancer treatment.

P450-BASED GDEPT USING CYCLOPHOSPHAMIDE AND IFOSFAMIDE

The anticancer prodrug cyclophosphamide (CPA) is commonly used in the treatment of a broad spectrum of human cancers, including breast cancer, endometrial cancer, lung cancer and various leukemias and lymphomas. Ifosfamide (IFA), an isomer of CPA, displays high activity against soft tissue sarcomas, testicular cancer, ovarian and

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breast cancer, among others. Both drugs are alkylating agent prodrugs of the oxazaphosphorine class, and both undergo bioactivation in the liver via a cytochrome P450 (P450 or CYP)-catalyzed 4-hydroxylation reaction (Fig. 1) [5,6]. *In vitro* biochemical studies using purified or cDNA-expressed P450 enzymes have shown that the P450 subfamily 2B enzymes CYP2B1 (in the rat) and CYP2B6 (in humans) are the most active catalysts of CPA 4-hydroxylation. IFA 4-hydroxylation is catalyzed by CYP3A and CYP2B enzymes [7,8]. An alternative P450 reaction, N-dechloroethylation, deactivates the prodrug and generates the neurotoxic by-product chloroacetaldehyde (Fig. 1). Different subsets of liver P450 enzymes catalyze the 4-hydroxylation and the N-dechloroethylation reactions, making it possible to devise pharmacologic strategies to alter the partitioning between these two metabolic pathways [9,10]. The therapeutic metabolites, 4-OH-CPA and 4-OH-IFA, are formed in the liver, are released into the general circulation, and spontaneously decompose, both in circulation and within the target tumor cells, by a β -elimination reaction that yields acrolein and an electrophilic mustard in equimolar amounts. Acrolein can bind covalently to protein and is responsible for some of the bladder toxicity associated with use of these prodrugs, while phosphoramidate mustard (derived from CPA) or isophosphoramidate mustard (from IFA) mediates the DNA cross-linking and cytotoxic effects associated with anti-tumor activity. The systemic distribution of these activated drug metabolites inevitably leads to significant side effects, including cardiotoxicity, renal toxicity, marrow suppression, and neurotoxicity [11,12].

Because tumor cells do not express high levels of CPA- or IFA-activating P450 enzymes, it was hypothesized that the transfer to a tumor cell of a gene encoding a CPA- or IFA-activating P450 enzyme 1) would result in a direct, intratumoral activation of the prodrug and 2) could lead to more efficient tumor cell killing without a major increase in systemic toxicity. Initial investigations confirmed both

hypotheses, and demonstrated that cultured tumor cells, as well as solid tumors grown in animal models, can be rendered highly sensitive to CPA or IFA cytotoxicity by CYP2B1 gene transfer [13-15]. The CYP2B1-dependent chemosensitization effect seen in these experiments could be blocked by the CYP2B1 enzyme inhibitor metyrapone [13,15], supporting the conclusion that the prodrug-activating P450 *per se* is responsible for the tumor cell's increased chemosensitivity.

In one study, a significant enhancement of CPA anti-tumor activity was demonstrated by using CYP2B1-encoding retroviral producer cells for the treatment of experimental brain tumors in athymic mice [14]. Intrathecal administration of CPA prevented the development of meningeal neoplasia and also led to partial regression of the parenchymal tumor mass in mice receiving CYP2B1-producing retrovirus, but not in mice receiving control retrovirus. The striking therapeutic effect of intra-cranial P450 expression observed in this study indicates that this strategy may be useful for circumventing the inhibition of tumor access to liver P450-activated CPA imposed by the blood-brain barrier. Brain tumors may thus be an ideal target for P450-based gene therapy.

Other experiments showed, however, that the P450 GDEPT concept can also be applied to the treatment of solid gliosarcomas grown subcutaneously in rats, and to the treatment of human breast tumors grown as xenografts in nude mice [13,15,16]. The substantial chemosensitization of these systemic solid tumors was somewhat unexpected because the tumors are already exposed to high levels of circulating 4-OH-CPA formed in the liver by endogenous P450 enzymes, even in the absence of P450 gene transfer. The observed increase in chemosensitivity therefore indicates a significant proximity effect. Thus, the *effective* intratumoral concentration of alkylating metabolites *in vivo* is substantially higher in P450-expressing tumor cells than

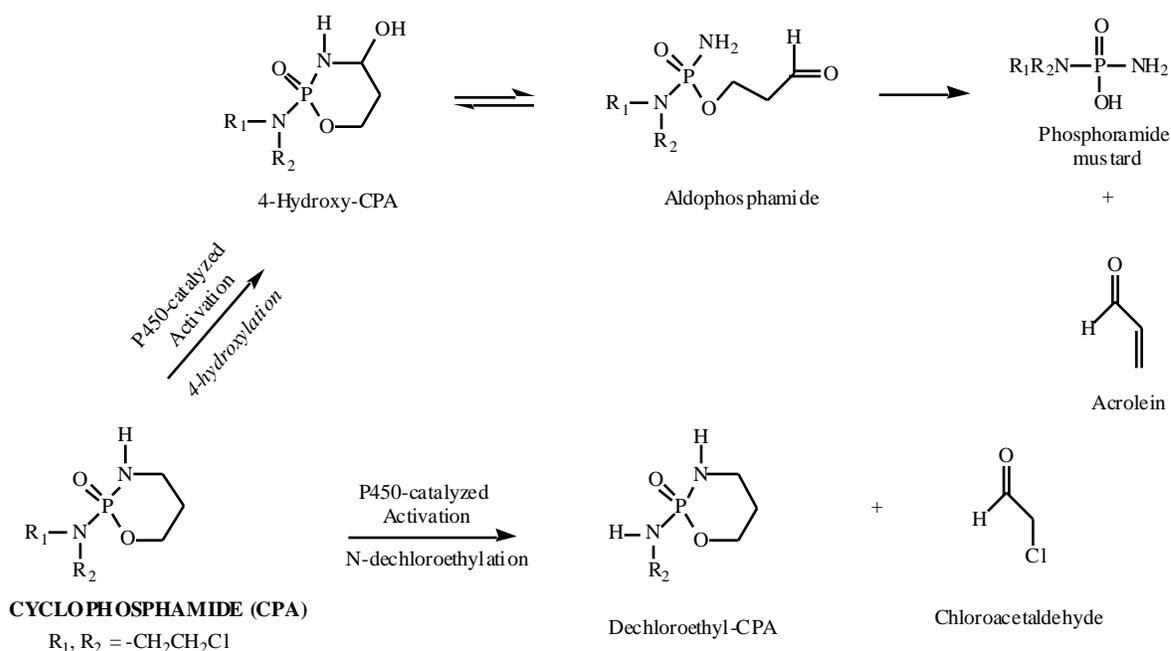


Fig. (1). P450-catalyzed activation of CPA and IFA via 4-hydroxylation.

in P450-deficient tumor cells, despite the much higher inherent P450 metabolic capacity of the liver compared to the P450-transduced tumors. Possible explanations for this striking proximity effect of the P450-activated prodrug include the following: 1) When formed in the liver, the primary metabolite 4-OH-CPA (which exists in equilibrium with its ring opened form, aldophosphamide, and as a plasma protein-stabilized sulfhydro adduct [5]) may have limited access to the tumor vasculature, or perhaps a much lower degree of tumor cell membrane permeability than is generally assumed. 2) Substantial decomposition of the primary metabolite ($t_{1/2} = 3-5$ min in rat and human plasma [17]) may occur before CPA activated in the liver reaches the tumor mass and then diffuses into individual tumor cells. 3) Acrolein, a byproduct of CPA activation, may synergize with and potentiate the cytotoxic effects of phosphoramidate mustard when the primary 4-hydroxy metabolite decomposes directly within the tumor cell.

Apoptotic Cell Death Mechanism

Cell cycle analysis of MCF-7 human breast carcinoma cells expressing CYP2B1 revealed that CPA treatment leads to a delayed S-phase progression and G₂-M arrest [15]. Thus, the DNA damage induced by CPA activated intracellularly allows tumor cells to exit from G₀-G₁ into S-phase, but prevents the cells from traversing from G₂-M into G₀-G₁. This process ultimately leads to tumor cell death by a mechanism that has recently been characterized as apoptosis, and is mediated by the mitochondrial, caspase 9-dependent cell death pathway [18]. The cytotoxicity of P450-activated CPA and IFA is therefore subject to modulation by the level of expression of apoptotic and anti-apoptotic factors, such as Bcl-2, which is likely to be an important determinant of tumor responsiveness to conventional therapy using CPA and IFA, and also to P450 GDEPT using these drugs. Investigation of the utility of Bcl-2-targeting strategies (e.g., Bcl-2 anti-sense [19]) in combination with P450-based GDEPT may thus be warranted.

Bystander Effect

As with other prodrug-activation gene therapies, an important feature of P450-based GDEPT is the bystander cytotoxic effect, which amplifies the cytotoxic action of the drug to include tumor cells that are in the vicinity of tumor cells transduced with the therapeutic P450 gene. This amplification is essential to the success of any GDEPT strategy, as it eliminates the need (unachievable using current gene delivery technologies) to transduce 100% of the target tumor cell population with the therapeutic gene. Unlike several other GDEPT systems (e.g., HSV-TK/ganciclovir), tumor cells that express CYP2B1 are able to sensitize adjacent tumor cells to CPA toxicity by a mechanism that does not require direct contact between the P450-expressing 'donor' cell and the bystander/recipient tumor cell [13]. This bystander effect is mediated by soluble cytotoxic metabolites released by P450-expressing cells, e.g., 4-OH-CPA, which readily diffuses across cell membranes. Phosphoramidate mustard, the ultimate cytotoxic

metabolite derived from CPA, is not membrane-permeable, and is thus not likely to contribute to the lethal effect of CPA on neighboring, P450-deficient tumor cells. Other possible bystander killing mechanisms, including direct cell-cell contact leading to the transfer of apoptotic signals from dying P450 tumor cells, could also play a role. Of note, the apoptotic cell death mechanism that is induced by CPA in P450-expressing tumor cells is a relatively slow process, requiring 2-3 days to be fully manifest [18]. This provides for the possibility of prolonged prodrug activation, and hence a greater bystander effect, when compared to prodrugs that induce a more rapid cytotoxic response.

STRATEGIES TO ENHANCE P450 GDEPT: INCREASING TUMOR: LIVER PRODRUG PARTITION RATIO

P450 prodrugs are actively metabolized in the liver, where cytochrome P450 enzymes are the most highly expressed. It should therefore be possible to increase the efficacy of P450 GDEPT by altering the partitioning of prodrug substrate between the liver and tumor to favor increased tumor cell-catalyzed prodrug metabolism. In principle, this goal may be achieved 1) by further increasing tumor cell P450 activity and/or 2) by selectively decreasing the fraction of prodrug that is activated in the liver, i.e., without attenuating intratumoral prodrug activation and tumor cell killing. The latter step should lead to an increase in GDEPT activity by increasing the extent to which prodrug is available for activation within the tumor. At the same time, it may help minimize systemic drug toxicity associated with the formation of cytotoxic drug metabolites in the liver. Several approaches to these goals have been investigated and are described below.

1) Enhancing Tumor P450 Activity by P450 Reductase Gene Transfer

Liver microsomal cytochrome P450 activities require two enzyme components, the heme-containing cytochrome P450 and the flavoprotein NADPH-cytochrome P450 reductase (P450R). Both proteins are embedded in the phospholipid bilayer of the endoplasmic reticulum. P450R, an FAD- and FMN-containing flavoenzyme, encoded by a single gene, catalyzes the transfer of electrons required for all microsomal P450-dependent reactions, including prodrug activation. A total of two electrons from NADPH are transferred, first to the FAD of P450R and then to the FMN cofactor, before being transferred one at a time to the P450 hemeprotein [20]. Cytochrome P450, in turn, utilizes these reducing equivalents for various monooxygenase reactions, including the hydroxylation reactions associated with the activation of CPA and IFA.

Initial studies using P450-based GDEPT were based on the premise that P450R gene transfer is unnecessary, since P450R is expressed in essentially all mammalian cell types, including a broad range of human tumor cells [21]. Experiments designed to test this supposition revealed, however, that overexpression of P450R leads to a substantial augmentation of P450-mediated CPA chemosensitivity,

both *in vitro* and *in vivo* [22]. In 9L gliosarcoma cells transduced with *CYP2B1* or with the human gene *CYP2B6* [16], P450 metabolic activity and the associated cytotoxicity of CPA and IFA were increased in rank order to the increase in P450R expression [23]. Thus, despite the ubiquitous basal expression of P450R in tumor cells, the discovery that P450R gene transfer substantially augments P450-dependent prodrug activation and cytotoxicity provides a simple approach to enhance P450-based GDEPT. Quantitation of the degree of enhancement using a colony formation-based tumor excision assay indicates up to a 10-fold increase in CPA-induced tumor cell kill *in vivo* by co-expression of P450R [22].

2) Localized Prodrug Delivery

A second approach to augment intratumoral prodrug activation at the expense of liver P450 activation involves the localized delivery of prodrug using controlled release polymers that are implanted intratumorally. In a recent mouse study using the replicating P450 herpes virus rRp450 to deliver *CYP2B1* (see below) [24], implantation of a CPA-loaded polymer within a solid subcutaneous tumor resulted in a 250-fold increase in intratumoral 4-OH-CPA levels when compared to intraperitoneal CPA administration. This demonstration of the utility of a prodrug slow release polymer is an important finding and should be explored further.

3) Improved P450 Catalysts of Prodrug Activation

P450 GDEPT activity may be increased by the identification and selection of prodrug-activating P450s whose enzyme kinetic properties toward the prodrug substrate are improved (i.e., increased V_{max}/K_m ratio) compared to *CYP2B1* and *CYP2B6*. In the case of CPA and IFA, *CYP2B1* and *CYP2B6* both exhibit maximal turnover numbers $\leq 100 \text{ min}^{-1} \text{ P450}^{-1}$ and K_m values of $\sim 1\text{-}2 \text{ mM}$ [25], leaving much room for improvement. Increased P450 catalytic efficiency might be achieved by selection of a more active, naturally-occurring prodrug-activation P450 (c.f., lower K_m values exhibited by some *CYP2C* enzymes with CPA or IFA; [26]), or by site-directed mutagenesis based on the available mammalian *CYP2C* X-ray structure [27] and insights provided from extensive earlier active-site P450 mutagenesis studies [28]. Studies of HSV-TK have shown that a substantial improvement in prodrug activity can be achieved using suitably selected mutant enzymes [29].

4) Selective Inhibition of Liver P450-Catalyzed Prodrug Activation

In one study [30], the anti-thyroid drug methimazole was used to selectively decrease hepatic expression of P450R, whose expression in liver [31], but not 9L gliosarcoma, is thyroid hormone-dependent. Pharmacokinetic studies demonstrated that this decrease in liver P450R leads to a corresponding decrease in the rate of liver P450/P450R-dependent CPA activation *in vivo*. Moreover, anti-thyroid treatment was accompanied by a corresponding decrease in

the host toxicity of CPA (body weight loss and hematuria) in the context of P450 GDEPT without loss of the intratumoral P450-dependent anti-tumor effect [30]. Further studies are needed to ascertain whether anti-thyroid drugs alter human liver P450R expression and CPA pharmacokinetics in cancer patients in a similar manner. Independent of their potential utility for enhancing P450 GDEPT, several beneficial effects of anti-thyroid drug-induced hypothyroidism have been reported in both preclinical and clinical cancer studies [32-34].

A second study was designed to identify liver P450-specific chemical inhibitors that might be used to selectively block liver prodrug activation but not tumor cell P450-catalyzed prodrug activation. This strategy is based on the premise that the biochemical properties, including chemical inhibitor sensitivities, of the subset of P450 enzymes that activate CPA in the liver are distinguishable from those of *CYP2B1* transduced into tumor cells. Direct inhibition of the liver P450 catalysts of CPA activation using P450 form-specific enzyme inhibitors could be very useful in improving CPA's therapeutic index. However, the P450 inhibitors examined did not display sufficient P450 form-selectivity to accomplish the desired degree of liver P450 inhibition when tested *in vivo* in a rat model [35]. A more systematic examination of P450 form-selective inhibitors [36] should be undertaken to identify liver-specific P450 inhibitors that 1) do not interfere with prodrug activation in the tumor, 2) are non-toxic and 3) do not adversely affect the metabolism of other concurrently administered drugs. With the advent of antisense technologies, a possible alternative approach is to employ antisense oligonucleotides or ribozymes to specifically block expression of the relevant P450 enzymes in hepatic tissue.

P450-BASED GDEPT HAS SEVERAL UNIQUE ADVANTAGES

P450-based GDEPT presents several unique advantages for clinical applications in cancer treatment when compared to other prodrug-activation systems (Table 1). These include:

1) Implementation Using Human P450 Genes

P450-based GDEPT can be carried out using human or other mammalian P450 genes, i.e., rat *CYP2B1* for studies using rat tumors in rodent models, and human *CYP2B6* for treatment of human cancer patients. The demonstrated feasibility of using human P450 genes for GDEPT [16] is a significant advance, insofar as it insures that complications associated with adverse host immune responses toward a prodrug-activation enzyme of foreign origin can be avoided. Other widely studied GDEPT enzymes are of microbial origin, and include HSV-TK (viral origin), cytosine deaminase (bacteria or fungi), carboxypeptidase G2 and nitoreductase (bacteria). Consequently, human patients treated with gene therapy vectors bearing these foreign genes may develop immune responses, including antibodies and cytotoxic T lymphocytes, which could interfere with delivery and/or expression of the prodrug-activation gene, in

Table 1. Comparison of P450-Based GDEPT with Two other Commonly Used GDEPT Systems

	P450/Cyclophosphamide	HSV-TK/Ganciclovir	Cytosine deaminase/5-fluorocytosine
Origin of gene	Human (<i>CYP2B6</i>), Rat (<i>CYP2B1</i>)	Herpes simple virus	Bacteria or fungi
Host immune reaction	Unlikely	Likely	Likely
Repeated application	No immune interference	Possibly complicated by immune interference	Possibly complicated by immune interference
Primary clinical use of the prodrug	Cancer	Viral infection	Bacterial or fungal infection
Other prodrug substrates	Structurally and mechanistically diverse (see Table 2)	Limited to analogs	Limited to analogs
Experience with prodrug use in cancer patients	Extensive	Little to none	Little to none
Tumor cell killing by activated drug	Wide spectrum; kills tumor cells in cell cycle-independent manner	Kills rapidly dividing tumor cells	Kills rapid dividing tumor cells
Bystander killing effect	Independent of cell-cell contact	Dependent on cell-cell contact	Independent of cell-cell contact
Integration with existing anticancer regimen in clinic	Feasible at present	Further testing required	Further testing required

particular if repeated gene delivery is required for effective clinical treatment, as seems likely.

2) Compatibility with a Wide Range of Established and Investigational Anticancer Prodrugs

P450-based GDEPT can be applied to a broad class of widely used, clinically established anticancer P450 prodrugs [37,38], of which CPA (CYTOXAN) and IFA (IFEX) are the best-studied examples. Both of these drugs have been used in the cancer clinic for many years and their pharmacokinetics and other properties, including absorption, distribution, metabolism, elimination and toxicity (ADMET) in cancer patients are well understood [6,39]. Other established anticancer prodrugs whose activity requires P450 metabolism (Table 2) include procarbazine, dacarbazine [37,40] and tegafur (activated to 5-fluorouracil by several P450s) [41]. Investigational prodrugs, including MMDX, a methoxymorpholinyl derivative of doxorubicin (activated by CYP3A enzymes) [42] and the furane mold toxin 4-ipomeanol (activated by rabbit CYP4B1 but not by the orthologous human CYP4B1) [43] are also subject to activation by liver P450 metabolism, and are candidate agents for further development of P450-based GDEPT.

P450 GDEPT may also be applicable to those established anticancer agents, which while not absolutely requiring P450 metabolism for activity, are nevertheless transformed to a more active derivative when metabolized by P450. Examples of this class of drugs include the following prodrug-P450 gene combinations: thio-TEPA, which is activated by a CYP2B-catalyzed oxidative desulfuration reaction [44], and perhaps also by metabolism leading to release of reactive aziridine moieties [45]; etoposide (VP-16), which is converted to a reactive catechol by a P450-catalyzed

O-demethylation reaction [46,47]; and tamoxifen, which is converted by P450 2D6 to 4-hydroxy-tamoxifen [48], a metabolite that is a 100-fold more potent anti-estrogen than tamoxifen itself [49] (Table 2).

P450 prodrugs are thus diverse in terms of their structure and their mechanisms of action, suggesting that combinations of P450 prodrugs, as well as the combination of a P450 prodrug with a non-P450 prodrug [50,51] may exhibit enhanced GDEPT anti-cancer activity. It should be recognized, however, that P450 enzymes exhibit broad intrinsic substrate specificities, and consequently, intratumoral expression of a prodrug-activating P450, such as CYP2B6, could lead to an undesirable localized increase in the P450-dependent inactivation of other drugs administered to the cancer patient [37,38], potentially comprising their therapeutic effect. Appropriate tailoring of the choice of drug combinations given to P450 GDEPT patients should help avoid this type of drug interaction. Moreover, this issue may not present a major challenge in the case of GDEPT using CYP2B6, because CYP2B6 does not appear to play a major role in human drug metabolism, with the exception of CPA and a limited number of other drugs [52].

In contrast to the clinically established anticancer prodrugs that can be used in P450 GDEPT discussed above, prodrugs employed in several other GDEPT systems have either been used primarily (or exclusively) for non-cancer applications or have not yet been approved for human use. Ganciclovir (activated by HSV-TK) is currently labeled for treatment of viral infections, while 5-fluorocytosine (activated by cytosine deaminase) is labeled as an anti-fungal agent. Other GDEPT prodrugs, such as CMDA (2-chloroethyl, 2-mesyloxyethylaminobenzoyl-L-glutamic acid, activated by carboxypeptidase G2) and CB1854 (activated by

Table 2. Anti-Cancer P450 Prodrugs. See Text for Details and References

P450 Prodrug	Activated Metabolite(s)	Drug/Chemical Class	Cellular Target/ Mechanism of Action	Prodrug-activating P450 enzyme(s)
CPA	4-OH-CPA	Oxazaphosphorine	DNA crosslinking	CYP2B, CYP2C
IFA	4-OH-IFA	Oxazaphosphorine	DNA crosslinking	CYP3A, CYP2B1
Procarbazine	Azoxy metabolites, Methyldiazonium ion	Hydrazine	Alkylating agent	Unknown
Dacarbazine	Methyldiazonium ion	Triazenyylimidazole	Alkylating agent	CYP1A2
Tegafur	5-Fluorouracil	Nucleoside antimetabolite	Thymidylate synthetase inhibitor	Multiple CYPs
MMDX	Unknown	Anthracycline	Unknown	CYP3A
4-Ipomeanol	Furan ring epoxide	Furylpentanone	Alkylating agent	Rabbit CYP4B1
thio-TEPA	TEPA, aziridine	Thiophosphoramidate	Polyfunctional Alkylator	CYP2B1
Etoposide (VP-16)	Catechol, ortho-Quinone	Epipodophyllotoxin	Topoisomerase II Inhibition; O ₂ free radicals	CYP2B1
Tamoxifen	4-OH-tamoxifen	Triphenylethylene anti- estrogen	Estrogen receptor antagonist	CYP2D6
Tirapazamine	Nitroxide radical	Bioreductive benzotriazine	DNA strand scission	CYP2B/P450R
AQ4N	4e ⁻ reduced AQ4N	Bioreductive anthraquinone	Topoisomerase II inhibitor; Radiation enhancer	CYP3A/P450R

nitroreductase) have not been approved for human use. The ultimate clinical effectiveness of these agents in treating human cancers thus remains to be established. The ability to proceed directly to clinical trials of P450-based GDEPT using proven and tested anticancer prodrugs such as CPA and IFA should be viewed as a significant advantage.

3) Extension to Include Bioreductive Drugs

P450-based GDEPT can be extended to include bioreductive drugs [53], several of which have been shown to undergo P450/P450R-dependent activation. Two examples are tirapazamine, an aminobenzotriazine-di-N-oxide [54] and AQ4N, an alkylaminoanthraquinone-di-N-oxide radiation enhancer [55]. Implementation of P450/P450R-based GDEPT using bioreductive drugs such as these, which are preferentially activated under the hypoxic conditions that characterize many solid tumors [56], is supported by three important observations [54]: 1) P450 GDEPT using CPA retains full activity in a cell culture model under hypoxic conditions, the intrinsic P450 requirement for O₂ notwithstanding; 2) When activated by P450/P450R under hypoxic conditions, tirapazamine exhibits bystander cytotoxicity; and 3) a significant increase in anti-tumor activity can be achieved by combining CPA with tirapazamine in the context of P450 GDEPT. This latter effect indicates that the potential competition between CPA and tirapazamine for metabolism within a tumor cell by the

same P450/P450R enzyme couple either does not occur, or is outweighed by the intrinsic enhanced activity of this drug combination. The strong activity of this drug combination may reflect the fact that CPA and tirapazamine kill tumor cells by two distinct mechanisms: DNA cross-linking in the case of CPA, and DNA strand scission induced by a one electron reduced nitroxide radical metabolite under hypoxic conditions in the case of tirapazamine [57]. Studies of AQ4N suggest that this bioreductive drug may be even more efficacious than tirapazamine when combined with CPA [58], a supposition that needs to be tested more directly in a P450 GDEPT model.

4) Mechanism-Based Wide-Spectrum Tumor Killing

P450-activated CPA and IFA kill tumor cells in a cell cycle-independent manner. Phosphoramidate mustard derived from 4-OH-CPA induces DNA cross-linking, leading to activation of a caspase 9-dependent apoptotic pathway [18], with tumor cell killing manifest at whichever point in time the tumor cells begin to replicate. Indeed, cell cycle profiling of CYP2B1-transduced MCF-7 human breast cancer cells demonstrated that the activation of CPA prevents cells traversing to mitosis after DNA replication [15]. In contrast, the prodrugs ganciclovir and 5-fluorocytosine need to be incorporated into the tumor cell's chromosomal DNA; these GDEPT prodrugs thus require that the cells be in the DNA

synthesis (S phase) of the cell cycle to exert their anti-tumor effect.

5) Cell Contact-Independent Bystander Killing

Studies on the bystander cytotoxicity of HSV-TK/ganciclovir indicate that the killing of non-HSV-TK-transduced cells is mediated by gap junctions and thus requires direct cell-cell contact [59]. By contrast, as noted above, the bystander killing of non-transduced tumor cells by P450/CPA occurs even in the absence of direct cell-cell contact. This mode of bystander killing is of therapeutic significance, because it suggests that eradication of the tumor, in principle, can be achieved even if only a small fraction of tumor mass expresses the prodrug activation P450 enzyme.

6) Efficacy without an Increase in Host Toxicity

Despite the striking enhancement of CPA's anticancer effect by P450 GDEPT, *in vivo* studies using rodents have shown that no additional host toxicity is associated with the expression by a tumor of a prodrug-activating P450 enzyme. This is most evident in a preclinical study that implemented a novel, anti-angiogenic schedule of CPA administration [60], one that involves the repeated injection of CPA every 6 days [61]. Full tumor regression leading to the eradication of 6 of 8 CYP2B6/P450R-transduced 9L gliosarcomas grown in *scid* mice was reported, with little or no overt CPA toxicity detected. Indeed, major tumor regression (>95%) could safely be achieved using the CPA/6 day schedule in mice bearing large, late-stage P450/P450R-expressing tumors (up to 4-5 gr in size at the time of initial CPA treatment) [60]. The striking therapeutic effect of GDEPT-directed CPA activation seen in these studies reflects the rapid onset of tumor cell cytotoxicity *in vivo* (within 4-7 days), which contrasts to the much slower onset of tumor regression that occurs in the absence of P450 expression (11-18 days after initial drug treatment) [60]. The added cytotoxic effects of P450 GDEPT are localized rather than systemic, in large part because hepatic P450 activation continues to dominate the overall systemic profile of prodrug activation. As a result, P450-based GDEPT under conditions that are sufficient to confer a therapeutically significant increase in localized anti-tumor activity is unlikely to increase substantially host toxicity associated with the systemic circulation of activated drug metabolites.

EXPERIMENTAL APPROACHES TO IMPLEMENTATION OF P450 GDEPT: APPLICATION OF TUMOR-SELECTIVE GENE THERAPY VECTORS

Several approaches can be undertaken to translate P450-based anticancer GDEPT research from the laboratory into the cancer clinic, where the goal is to generate a clinically meaningful increase in the level of activated metabolites in the tumor, and thus an increase in tumor cell killing, without significantly increasing the patients' risk. In this regard, the most important issue to address is how to achieve the selective and efficient delivery and expression of

P450 (and P450R) in the target tumor tissue (Fig. 2). A wide variety of gene therapy vectors, including viral and non-viral vectors, has been investigated or can be explored toward this goal.

1) Replication-Defective Retrovirus

Recombinant retroviruses expressing *CYP2B1* or *CYP2B6* have been tested as gene therapy vectors in rodent tumor models [14,62,63]. Retroviruses exert a certain degree of intrinsic tumor targeting because they selectively transduce dividing cells and thus can be used to deliver a therapeutic P450 gene to a rapidly growing tumor cell population. However, several problems may limit their use in the clinic for human GDEPT applications, including difficulties associated with the manufacture of a large quantity of high titer retroviral vector, the inability of retroviruses to infect dormant tumor cells, and the risk of retroviral integration into host chromosomes, in particular, the risk of integration into germline cells.

2) Replication-Defective Adenovirus

Adenovirus infects both dividing and non-dividing cells with high efficiency, including a wide variety of cancer cells. The viral DNA does not integrate into host chromosomes. Deletion of the adenoviral E1A and E1B genes results in a replication-defective adenoviral vector, also referred to as a first generation adenoviral vector. Replication-defective adenovirus can be used to deliver P450 genes and thereby sensitize human cancer cells of breast, prostate, ovarian, and brain origin to CPA [15]. In contrast to retrovirus, adenovirus can readily be produced in large quantity and at high titer ($\geq 10^{12}$ pfu/ml). A first-generation adenovirus expressing HSV-TK is now in early phase clinical trial for cancer and was reported to be free of severe adverse effects in patients [64]. First generation adenoviral vectors are also being tested in clinical trials for the treatment of genetic diseases and for vaccine applications. One problem encountered with these vectors is that they are strongly immunogenic, and this may prevent repeated virus administration. However, progress in vector formulation, including PEGylation to mask the virus' immunogenic epitopes [65], the ability to employ other adenovirus serotypes, and the engineering of helper-dependent adenoviral vectors ("gutless" adenoviral vectors) by deletion of additional portions of the viral genome may help solve this problem.

3) Conditionally-Replicating Oncolytic Viruses

These viruses are designed to replicate with much higher efficiency in cancer cells than non-cancer cells and to ultimately effect tumor cell lysis; they are thus referred to as oncolytic viruses [66]. Oncolytic viral vectors can be prepared in several ways. One approach involves the deletion of viral genes required for viral replication in normal cells but not tumor cells. One example of this is ONYX-015, an adenovirus with the viral E1B-55kDa gene deleted. As a result of this deletion, ONYX-015 can only replicate in tumor cells that lack p53 function [67]. At least 50% of

Strategies for Achieving Tumor-Selective Killing

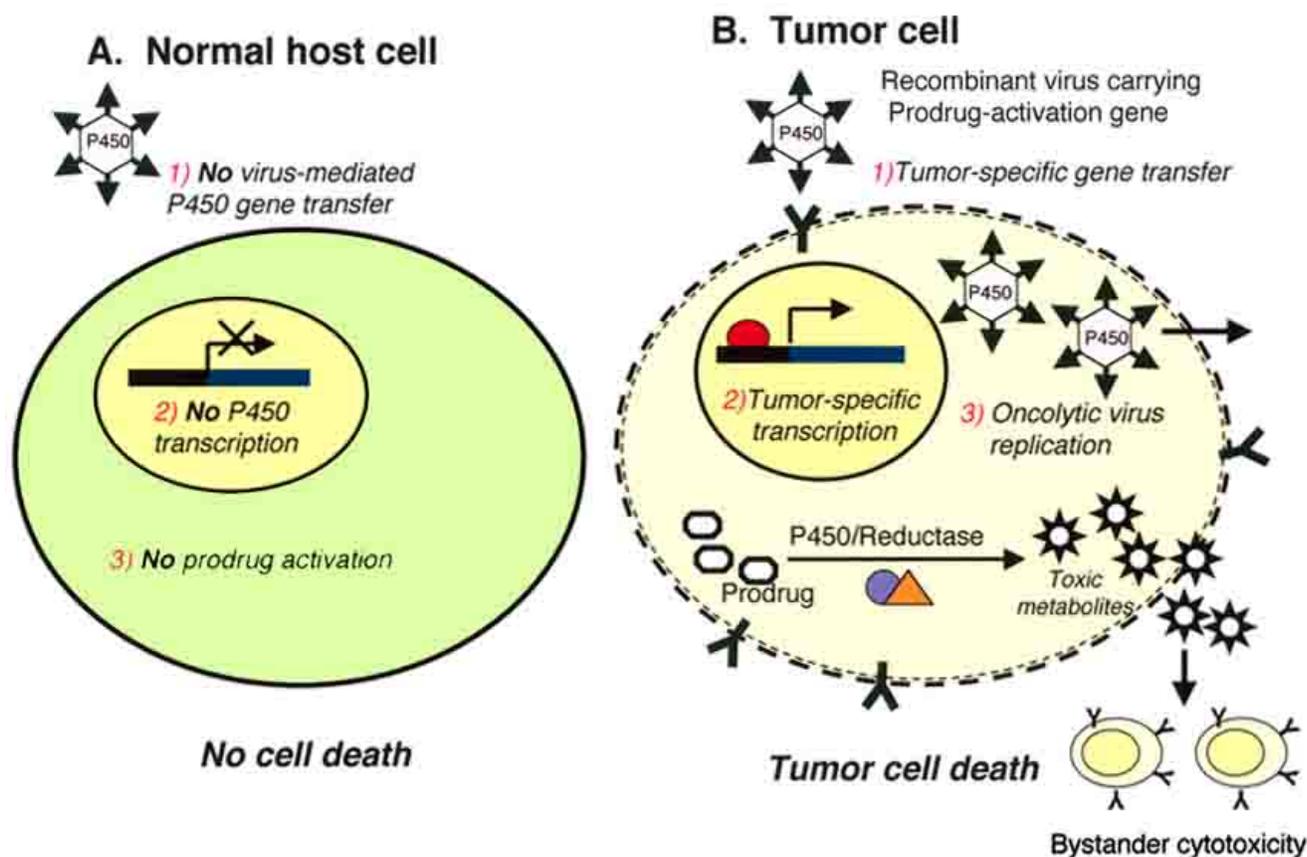


Fig. (2). Multiple strategies for achieving selective tumor killing. 1) Use of the vector's natural tropism or an engineered tropism to enable targeted P450 gene transfer to tumor cells. 2) Use of *cis*-acting promoter/enhancer elements to direct transcription of a therapeutic P450 gene in a tumor cell-selective manner. 3) Viral vectors may replicate conditionally in tumor cells as the result of a loss of function (e.g., p53) or a gain of function (e.g., tumor-specific expression of essential viral proteins). See text for further details.

human tumors are deficient in p53 function, suggesting that ONYX-015 may be useful for delivery of P450 genes to a broad range of human tumors. Initial clinical trials show this virus to be safe, with no dose-limiting toxicities observed [68]. Combination of ONYX-015 with cisplatin-based chemotherapy in human tumor xenografts results in superior anticancer activity [69], suggesting the potential utility of this virus as a clinically effective vector for P450 gene delivery.

A second strategy uses tumor cell-specific DNA regulatory elements (promoters and enhancers) to regulate the expression of essential viral genes, resulting in tumor cell-specific virus replication. For example, the androgen-regulated, prostate-specific PSA promoter can be used to regulate the expression of adenoviral E1 genes in a manner that limits viral replication to the prostate [70], while a similar strategy using the α -fetoprotein promoter can be used to generate a hepatocellular carcinoma-specific oncolytic adenovirus [71]. The potential utility of these two types of replicating viral vectors for P450 gene delivery has not been explored.

A third example of a conditionally replicating oncolytic virus is herpes simplex virus with deletion of the viral ribonucleotide reductase gene, which is essential for viral replication in quiescent cells. Insertion of the P450 gene *CYP2B1* in place of the viral ribonucleotide reductase results in a replicating oncolytic herpes virus vector, designated rRp450 [72]. This virus has demonstrated preclinical P450 gene therapy activity when used in combination with CPA to treat gliomas and hepatocellular carcinoma [50,72,73]. A high degree of tumor selectivity can be achieved with this virus (3-4 log tumor specificity in the case of hepatocellular carcinoma). Importantly, virus replication in tumor cells is not substantially impaired by the P450 prodrug CPA [73]. This may reflect the comparatively small size of the viral genome.

One potential concern is the safety of conditionally replicating viruses when used in cancer patients, which are generally immune suppressed. The replication of viruses in vital host tissues is highly undesirable and could be devastating to these patients. The efficacy of oncolytic viruses may, however, be limited by their own inability to

penetrate and spread in a solid tumor mass and by the effects of host antiviral defenses. One potential solution to the problem of host cell viral replication would be to incorporate a prodrug activation gene such as P450 into the virus. Not only would the P450 gene enhance the degree of tumor cell killing when combined with prodrug treatment; its presence could potentially provide a safeguard mechanism to prevent virus replication from getting out of control. It will be important to achieve a balance between the therapeutically beneficial replication of virus within the tumor target and the need to eventually shut off any systemic virus replication in the patient. Further studies will be needed to determine the utility of P450 prodrugs, or perhaps other prodrugs, in blocking replication of different oncolytic viruses. The finding that ganciclovir, but not CPA, significantly blocks replication of the P450-expressing oncolytic herpes virus rRp450 (which retains its natural HSV-TK gene) [73] indicates that in that case ganciclovir may be used to shut off viral replication once P450-dependent prodrug therapy is complete. Retention of the HSV-TK gene in the vector rRp450 not only provides the opportunity for combination prodrug therapy (CPA + ganciclovir), it can serve as an important safety feature as well.

4) Non-Viral Vectors

Non-viral gene delivery, e.g., using cationic liposomes, polymers, particle bombardment, and electroporation, have been explored to deliver plasmid DNA into solid tumors. However, the efficiency of non-viral mediated gene transfer *in vivo* remains far below the level that can be achieved using viral vectors. It will be several years before these non-viral approaches become practical for human use.

5) Cell Encapsulation

One novel approach to P450-based gene therapy involves the microencapsulation of mammalian tissue culture cells that have been engineered to express P450, followed by implantation of the capsules directly within the tumor [74]. This approach allows the use of allogeneic cells, which when encapsulated in cellulose sulphate, survive the host's immune response. These microencapsulated cells can serve as small "drug factories" which activate the prodrug locally when implanted within a tumor. Delivery of the capsules can be achieved by direct injection into the tumor mass, or angiographically, by delivery of the capsules via the tumor vasculature. A recent phase I/phase II clinical trial implementing this cellular P450 vector reported remarkable efficacy when used in advanced pancreatic cancer patients treated with the P450 prodrug IFA [75].

6) Transcriptional Targeting and Systemic P450 Gene Delivery

Most GDEPT procedures used in preclinical studies and in current clinical trials, including P450 GDEPT clinical trials (see below), have been based on direct injection of the gene therapy vector into a solid tumor mass. A more ideal approach, however, would be to employ systemic P450 vector delivery, with the goal of achieving targeted

expression of the prodrug activation genes in tumor cells, including metastatic sites, to the exclusion of normal host cells and tissues. Several approaches are therefore under development to provide multiple layers of selectivity in gene therapy (Fig. 2) [76]. One approach exploits natural viral tropisms or uses engineered vectors with tumor cell surface-selective ligands or antibodies to facilitate tumor cell binding and thereby enable gene transfer to tumor cells in a selective manner. A second approach uses tumor-specific or tissue-selective promoters to direct gene expression in a tumor-specific manner. Examples of this latter approach include the regulation of prodrug activation gene expression by transcription regulatory sequences derived from genes whose expression is tumor-specific, such as DF3/MUC1 (breast and ovarian cancer), ERBB2 (breast cancer), PSA (prostate cancer), α -fetoprotein (liver cancer), and tyrosinase (melanoma). Both approaches should be directly applicable to P450 GDEPT. Strategies for regulating P450 gene expression can also be envisaged using either telomerase promoter sequences [77] or hypoxia response elements [78], both of which are selectively activated in tumor cells. Hypoxia-regulated adenoviral vectors have been used to transduce human macrophages with *CYP2B6*, which in a cell culture spheroid model leads to infiltration of hypoxic tumor cells, P450 gene induction and enhanced CPA cytotoxicity [79]. Tumoricidal macrophages may therefore find use as effective cellular vectors for regulated P450 delivery. It is likely that the combination of vector targeting with transcriptional regulation will confer the highest degree of tumor selectivity (Fig. 2).

P450-BASED GDEPT CLINICAL TRIALS

Several clinical trials applying the principles of P450-based GDEPT have recently been initiated. In the UK, an improved retroviral vector expressing *CYP2B6* ('MetXia') is presently being tested in patients with advanced breast cancer and ovarian cancer in studies sponsored by Oxford BioMedica, PLC. Initial results reported by the sponsor indicate that the vector was safe and well tolerated, and resulted in gene transfer to the tumors. In the USA, studies designed to evaluate the feasibility of incorporating P450-based GDEPT into replicating oncolytic herpes viruses (rRp450, see above) are underway, and are presently at the preclinical stage. In Germany, microencapsulated mammalian cells expressing *CYP2B1* have been tested as a vehicle for P450 delivery in 14 patients with inoperable pancreatic carcinoma undergoing IFA treatment [75]. The treatment protocol was well tolerated and showed encouraging signs of efficacy. Tumor regression was observed in 4 patients, while stable tumors were reported for 10 other individuals. One-year survival rates were increased 3-fold compared to historic controls. These striking findings are most encouraging. Multi-center trials with a much larger pancreatic patient population will be needed to rigorously test the merits of this therapeutic approach to P450 GDEPT.

FUTURE PERSPECTIVE

P450-based GDEPT has emerged as a practical prodrug-activation cancer gene therapy with many unique advantages

that can readily be incorporated into current cancer treatment regimens. Although P450-based GDEPT is at present being explored in the clinic using retrovirus and microencapsulated cells, future research should be directed to the evaluation of other gene transfer vectors with improved safety and greater gene transfer efficiency for more practical clinical applications, including systemic administration of tumor-targeted oncolytic viruses. The combination of P450-based GDEPT with other therapeutic modalities, such as radiation therapy, antibody therapy, and other chemotherapies are important areas for future research.

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