

Mini-review

Activation of oxazaphosphorines by cytochrome P450: Application to gene-directed enzyme prodrug therapy for cancer

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Abstract

Cancer chemotherapeutic prodrugs, such as the oxazaphosphorines cyclophosphamide and ifosfamide, are metabolized by liver cytochrome P450 enzymes to yield therapeutically active, cytotoxic metabolites. The effective use of these prodrugs is limited by host toxicity associated with the systemic distribution of cytotoxic metabolites formed in the liver. This problem can, in part, be circumvented by implementation of cytochrome P450 gene-directed enzyme prodrug therapy (P450 GDEPT), a prodrug activation strategy for cancer treatment that augments tumor cell exposure to cytotoxic drug metabolites generated locally by a prodrug-activating cytochrome P450 enzyme. P450 GDEPT has been exemplified in preclinical rodent and human tumor models, where chemosensitivity to a P450 prodrug can be greatly increased by introduction of a prodrug-activating P450 gene. Further enhancement of the efficacy of P450-based gene therapy can be achieved: by co-expression of P450 with the flavoenzyme NADPH-P450 reductase, which provides electrons required for P450 metabolic activity; by metronomic (anti-angiogenic) scheduling of the prodrug; by localized delivery of the prodrug to the tumor; and by combination with anti-apoptotic factors, which slow the death of the P450 ‘factory’ cells and thereby enhance the bystander cytotoxic response. P450 GDEPT has several important features that make it a clinically attractive strategy for cancer treatment. These include: the substantial bystander cytotoxicity of P450 prodrugs such as cyclophosphamide and ifosfamide; the ability to use human P450 genes and thereby avoid an immune response to the therapeutic gene; the use of well-established conventional chemotherapeutic prodrugs, as well as bioreductive drugs activated by P450/P450 reductase in a hypoxic tumor environment; and the potential to decrease systemic exposure to active drug metabolites by selective inhibition of hepatic P450 activity. Recent advances in this area of research are reviewed, and two proof-of-concept clinical trials that highlight the utility of this strategy are discussed.

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1. Introduction

Many conventional cancer chemotherapeutic drugs have a low therapeutic index due to a lack of specificity toward tumor tissue, coupled with dose-limiting toxicity towards one or more host tissues. Over the past decade, intensive research efforts have been made to overcome these difficulties by developing novel gene therapeutic approaches using tumor suppressor genes, cytokines and lymphokines for immunotherapy, and genes encoding prodrug-activation enzymes (McCormick, 2001). Introduction of a prodrug-activation gene into tumor cells has proven to be an effective way to sensitize tumors towards cytotoxic anti-cancer prodrugs. This gene-directed enzyme prodrug therapy (GDEPT) makes use of a prodrug-activation enzyme/suicide gene to stimulate in situ bioactivation of prodrugs that are otherwise inactive (Dachs et al., 2005; Aghi et al., 2000; Kirn et al., 2002). Prodrug-activation gene therapy is particularly effective when the activated drug exerts a strong “bystander” cytotoxic effect, whereby the active metabolite is sufficiently lipophilic to diffuse from the site of its formation, i.e., away from the tumor cells that express the prodrug activation gene, and spread throughout the solid tumor mass, thereby amplifying the localized chemotherapeutic response (Denny, 2003). Other gene therapy strategies, such as tumor suppressor gene replacement or oncogene inactivation using antisense methods, are not typically characterized by strong bystander effects, and therefore require gene transfer into a very high fraction of a patient’s tumor cells, which is impossible to achieve using current gene delivery technologies or those likely to be developed over the next few years. Many anti-cancer prodrugs have been introduced with the goal of increasing bioavailability or enhancing local drug delivery (Rooseboom et al., 2004), and some of these agents have been investigated for use in GDEPT treatments for cancer. One important class of anti-cancer prodrugs is activated by cytochrome P450 (CYP) enzymes, which constitute a large family of heme protein monooxygenases and play a key role in hepatic drug metabolism. P450 enzymes are essential for the activation of several established anti-cancer prodrugs, most notably the oxazaphosphorines

cyclophosphamide (CPA) and ifosfamide (IFA) (Sladek, 1988), and are particularly effective when incorporated into a suicide gene therapy for cancer treatment (Wei et al., 1994; Chen and Waxman, 1995a). This article reviews recent studies on the development of a prodrug activation gene therapy based on the use of P450 prodrug-activation enzymes. The reader is also referred to several earlier reviews of work in this field (Waxman et al., 1999; Jounaidi, 2002; Chen and Waxman, 2002; Chiocca and Waxman, 2004).

2. Activation of oxazaphosphorines by P450 enzymes

CPA and IFA are isomeric alkylating agents of the oxazaphosphorine class. Both drugs are widely used in combination chemotherapy to treat a variety of neoplasms. CPA and IFA are therapeutically inactive prodrugs; they both undergo metabolic activation catalyzed by specific liver cytochrome P450 (CYP) enzymes (Clarke and Waxman, 1989), which convert the inactive prodrugs to bioactive, cytotoxic metabolites (Sladek, 1988). Both prodrugs are metabolized by P450 enzymes to yield a 4-hydroxy derivative, which equilibrates with the ring-opened aldophosphamide. This aldehyde, in turn, undergoes chemical decomposition to yield a bifunctional mustard (phosphoramidate or ifosphoramidate mustard) and acrolein. The mustard generates a highly electrophilic aziridinium species that forms DNA cross-links, proposed to be the key cytotoxic lesion induced in tumors treated with oxazaphosphorines (Sladek, 1988; Fleming, 1997). Alternatively, the 4-hydroxy metabolite can be deactivated by aldehyde dehydrogenase enzymes to yield carboxyphosphamide, a therapeutically inactive metabolite (Sladek, 1999). Tumor cell expression of these oxazaphosphorine-inactivating aldehyde dehydrogenase is an important mechanism of resistance to this class of drugs (Bunting and Townsend, 1996a,b).

Studies with isolated liver microsomes and cDNA-expressed P450 enzymes have established that CPA and IFA are activated by multiple, overlapping subsets of liver P450s. Both oxazaphosphorines are metabolized by constitutive P450 enzymes belonging to the CYP2C subfamily, and by drug-inducible enzymes belonging to P450

subfamilies CYP2B and CYP3A. These P450 metabolic patterns are seen in the rat model (Clarke and Waxman, 1989; Weber and Waxman, 1993) and in humans (Chang et al., 1993). CYP2B1 is the most active catalyst of CPA 4-hydroxylation in rat liver, whereas the corresponding human enzyme, CYP2B6, is the most active CPA 4-hydroxylase in human liver. In contrast, human liver IFA 4-hydroxylation is primarily catalyzed by CYP3A enzymes, with minor contributions made by several other CYP enzymes, including CYP2B6 (Roy et al., 1999a). CYP2B6 and CYP3A4 apparently correspond to the high K_m components of CPA and IFA 4-hydroxylation, respectively, seen in human liver microsomes (Chang et al., 1993). Several CYP2C enzymes catalyze these reactions with a lower K_m than the CYP2B and CYP3A enzymes (Chang et al., 1997; Ren et al., 1997) and may contribute to the low K_m oxazaphosphorine 4-hydroxylase activity seen in liver tissue. Certain CYP2B enzymes can also activate CPA and IFA with a low K_m , as was recently shown for the dog liver enzyme CYP2B11 (Chen et al., 2004). Cytochrome P450 catalyzed metabolic pathways for cyclophosphamide and ifosfamide are shown in Fig. 1.

Although CPA and IFA are chemical isomers, each drug exhibits a unique spectrum of anti-tumor activity, host toxicity and drug resistance. CPA is commonly used in the treatment of breast cancer, endometrial cancer, lung cancer and various leukemias and lymphomas, whereas IFA is often prescribed for treatment of soft tissues sarcomas, testicular cancer, ovarian and breast cancer (Sladek, 1988; Peters et al., 1993; Ayash et al., 1992). IFA has greater activity than CPA in several experimental tumor models, produces less myelosuppression, and exhibits little cross-resistance compared to CPA (Goldin, 1982). Some patients treated with IFA develop severe neurotoxicity (Thigpen, 1991; Goren et al., 1986) and urotoxicity (Skinner et al., 1993), which have both been associated with the formation

of chloroacetaldehyde via *N*-dechloroethylation, an alternative P450-catalyzed metabolic pathway (Goren et al., 1986; Norpoth, 1976; Lind et al., 1990) (Fig. 1). In the case of IFA, this alternate pathway deactivates the drug through side chain oxidation yielding the inactive, monofunctional alkylating metabolites 2- and 3-dechloroethyl-IFA, and at the same time produces the neurotoxic byproduct chloroacetaldehyde (Kajiser et al., 1993). This later pathway may consume up to 50% of the therapeutic dose of IFA, whereas in the case of CPA it is generally a minor metabolic pathway, with only ~10% of the prodrug undergoing *N*-dechloroethylation to yield chloroacetaldehyde.

In vitro studies with human liver microsomes demonstrate that CYP3A4 is a major catalyst of CPA and IFA *N*-dechloroethylation (Walker et al., 1994); however, CYP2B6 also mediates a substantial portion of this metabolic pathway, particularly in livers that are high in CYP2B6 content (Roy et al., 1999a; Huang et al., 2000a; Roy et al., 1999b). Cell culture experiments (Bruggemann et al., 1997) and in vivo studies (Borner et al., 2000) suggest that the chloroacetaldehyde released upon *N*-dechloroethylation of IFA may actually contribute to therapeutic activity. The cytotoxicities of 4-OH-IFA and chloroacetaldehyde may be additive, a finding that may help explain the lack of cross-resistance between IFA and CPA and the observed differences in response of some tumors towards these isomeric prodrugs (Brade et al., 1986; Bramwell et al., 1987).

Drug metabolism in the liver leads to the systemic formation and distribution of high levels of alkylating metabolites in patients treated with CPA and IFA. This may lead to serious side effects, including cardiotoxicity, bone marrow suppression, nephrotoxicity and neurotoxicity. Another limitation of oxazaphosphorine therapy is the inherent inability of phosphoramidate mustard, the key therapeutically active cytotoxic metabolite, to cross cell membranes. Given the short intrinsic half-life of 4-

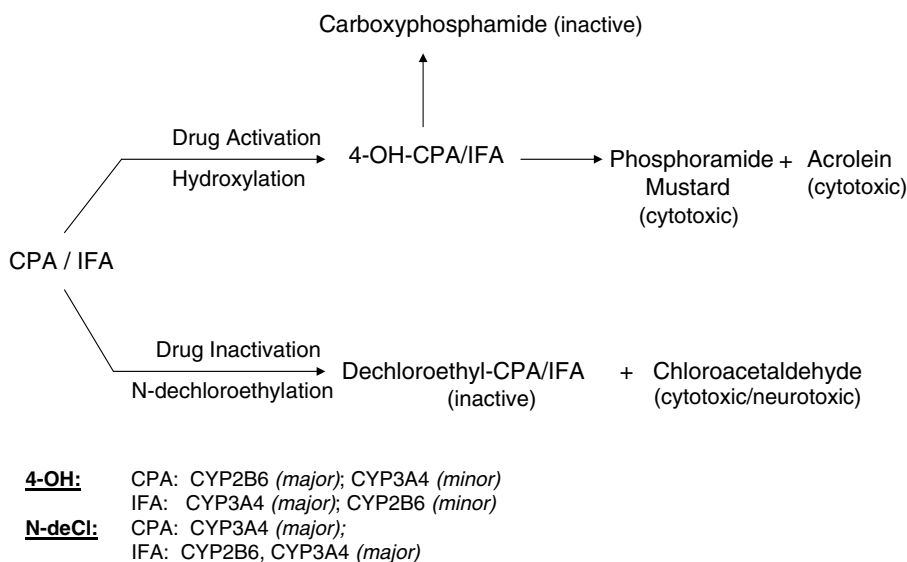


Fig. 1. P450 catalyzed metabolic pathways of CPA and IFA.

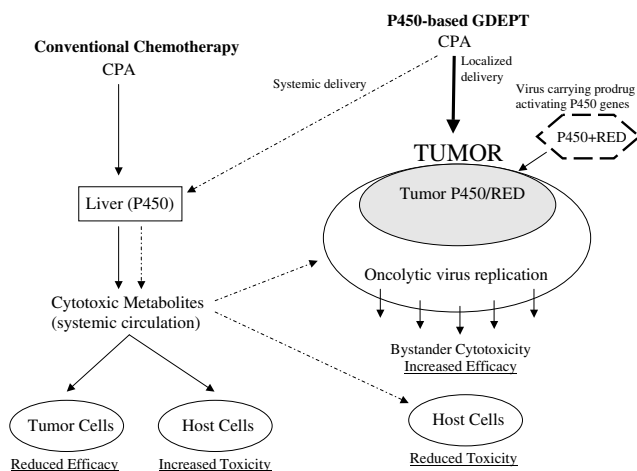


Fig. 2. Proposed therapeutic benefit from P450-based GDEPT compared to conventional chemotherapy. RED, P450 reductase.

hydroxy-cyclophosphamide in plasma ($t_{1/2} = 5.2$ min) (Hong et al., 1991), a major fraction of the 4-hydroxy metabolite formed in the liver is likely to decompose in the bloodstream to phosphoramidate mustard, which does not enter tumor cells efficiently. P450 GDEPT aims to overcome these limitations by targeting prodrug-activating P450 genes to tumor cells, with the goal of inducing localized, intratumoral prodrug activation (Fig. 2).

3. Tumor cell death and the bystander effect

In practice, only a small fraction of the target tumor cell population is transduced in a typical *in vivo* gene transfer study. A strong bystander cytotoxic effect is therefore required if meaningful tumor regression and durable clinical responses are to be achieved. In the case of GDEPT using the herpes simplex virus thymidine kinase (HSV-TK) gene and the prodrug ganciclovir, the active drug metabolite is a phosphorylated nucleoside analog, which does not readily cross cell membranes. Consequently, the bystander effect is rather limited, and is dependent on direct, gap junction-mediated contact between the HSV-TK-expressing donor cell and the bystander (recipient) tumor cell (Nicholas et al., 2003). By contrast, in the case of P450 GDEPT using CPA and IFA, there is a strong bystander effect mediated by cell permeable, cytotoxic 4-hydroxy metabolites generated by the P450-expressing tumor cells (Chen and Waxman, 1995a). This enables the active metabolites of CPA and IFA to diffuse within a tumor cell mass and to alkylate both dividing and quiescent cancer cells.

IFA and CPA exhibit unique cytotoxic activities. 4-OH-IFA displays several-fold lower intrinsic cytotoxicity than 4-OH-CPA in many tumor cell lines (Jounaidi et al., 1998; Jounaidi and Waxman, 2004). This may be related to the distinct nature of the DNA cross-links induced by each drug: seven atom cross-links are formed by IFA vs. five atom cross-links by CPA. Another difference may

relate to the mechanism of cell death triggered by each drug. 4-OH-CPA induces apoptosis (Schwartz and Waxman, 2001), whereas 4-OH-IFA is reported to induce necrosis (Karle et al., 2001) or apoptosis (Schwartz and Waxman, 2001). These responses are likely to affect not only the inherent cytotoxicity of each drug, but also the bystander effect and anti-tumor immune responses. Tumor cell death via necrosis may trigger local inflammation and a rapid immune response, whereas apoptosis may induce the formation of membrane-enclosed apoptotic bodies that are phagocytosed by macrophages or bystander cells and are non-immunogenic. Apoptotic tumor cell death mediated by P450-activated CPA is modulated by the balance between the expression of apoptotic and anti-apoptotic factors, such as Bcl-2, which may be an important determinant of tumor responsiveness both in conventional therapy and in P450-mediated GDEPT using CPA (Schwartz and Waxman, 2001).

As noted above, the bystander effect of P450-activated CPA and IFA does not involve the mustard metabolites, which do not readily diffuse across cell membranes, but is mediated by other metabolites, such as 4-OH-CPA and 4-OH-IFA, aldophosphamide, and perhaps the cytotoxic byproducts chloroacetaldehyde and acrolein. Diffusion of these reactive metabolites from their site of formation within tumor cells may, however, be seriously limited by the expression of enzymes and other factors that inactivate these metabolites and confer drug resistance, including certain aldehyde dehydrogenases (Sladek, 1999), glutathione S-transferases and glutathione (Chen and Waxman, 1995b), which may metabolize and thereby inactivate these cytotoxic metabolites. Oxazaphosphorine resistance may be minimized by treatment with small molecule inhibitors of aldehyde dehydrogenases (Sladek, 1999; Quash et al., 2002) or by targeting aldehyde dehydrogenase using antisense sequences (Moreb et al., 2000). If this inhibition can be achieved in a tumor cell-selective manner *in vivo*, the effective exposure of tumor cells to cytotoxic metabolites would be increased and a stronger bystander effect would be obtained. Tumor cell death induced by P450-activated CPA is a relatively slow process, one that requires 2–3 days to fully manifest (Schwartz and Waxman, 2001). During this time the production of bystander cytotoxic metabolites continues, albeit at a reduced rate as the cells enter apoptosis (Schwartz et al., 2003).

4. Strategies to improve P450 GDEPT

Several strategies have been investigated to improve the effectiveness of P450 GDEPT. In one approach, NADPH-P450 reductase is co-expressed with the P450 gene to augment P450 metabolic activity. In a second approach, the balance of prodrug activation is shifted from the liver to the tumor, in an effort to increase *in situ* prodrug activation and thereby increase tumor cell killing, while at the same time decreasing hepatic production and systemic release of host cell toxic metabolites. Finally, in a third approach,

an anti-apoptotic factor that delays, but does not block tumor cell apoptosis can be introduced together with the therapeutic P450 gene in order to increase the production of bystander cytotoxic metabolites.

4.1. NADPH-P450 reductase as an adjuvant for P450 gene therapy

All microsomal P450 monooxygenase reactions require two distinct enzyme components, the flavoprotein NADPH-cytochrome P450 reductase (P450 reductase) and the heme-containing cytochrome P450. P450 reductase, a FAD- and FMN-containing flavoenzyme, catalyzes the transfer of two electrons from NADPH to the P450 hemoprotein, and is a rate-limiting component of the overall monooxygenase reaction. Initial studies using P450-based GDEPT were based on the premise that P450 reductase gene transfer is unnecessary, since P450 reductase is ubiquitous in nearly all mammalian cell types including a broad range of human tumor cells (Yu et al., 2001). Subsequent studies revealed, however, that the overexpression of P450 reductase leads to a substantial increase in P450-dependent chemosensitivity to CPA, both in vitro and in vivo (Chen et al., 1997). In rat 9L gliosarcoma cells transduced with cDNA encoding rat CYP2B1 (Chen et al., 1997) or human CYP2B6 (Jounaidi et al., 1998), P450 metabolic activity and the associated cytotoxicity are substantially increased with P450 reductase co-expression. P450 reductase increases the production and release of activated CPA metabolites, leading to a stronger bystander effect. This was exemplified in a tumor excision study utilizing 9L gliosarcoma cells, where a 50–100-fold overall increase in tumor cell kill was observed by co-expression of P450 with P450 reductase (Chen et al., 1997). The effectiveness of P450 reductase co-expression appears to be strongest when the cDNA for P450 reductase is expressed together with the P450 cDNA as a bicistronic mRNA linked by an IRES (internal ribosome entry signal) sequence (Jounaidi and Waxman, 2004). Thus, despite the ubiquitous expression of P450 reductase in tumor cells, P450-dependent prodrug activation and cytotoxicity can be increased by supplementation of P450 reductase, providing a simple way to increase the efficacy of P450 GDEPT. The P450 and P450 reductase DNA sequences may also be expressed as a single coding sequence that generates a P450–P450 reductase fusion protein (Tychopoulos et al., 2005).

4.2. Selective inhibition of liver P450-catalyzed prodrug activation

Efforts to suppress liver P450 metabolism in a selective manner by using anti-thyroid drugs, such as propylthiouracil (Ross et al., 1995) and methimazole (Waxman et al., 1989), have been described. These drugs inhibit hepatic P450 reductase transcription and thereby decrease hepatic P450 reductase protein and activity, which in turn sup-

presses overall P450 metabolic activity. In studies conducted in the rat model, methimazole treatment decreased the expression of hepatic P450 reductase by about 75% without affecting the level of P450 reductase expressed in 9L gliosarcomas. This led to an increase in CPA-induced tumor growth delay in 9L tumor xenografts expressing CYP2B1 and P450 reductase, consistent with there being an increase in the partitioning of CPA metabolism from the liver to the tumor (Huang et al., 2000b). An additional benefit of this approach is that the anti-thyroid drug methimazole moderates several of the systemic toxicities that accompany CPA treatment. Furthermore, methimazole exhibits some intrinsic anti-cancer activity, independent of CPA.

In an alternative approach to suppress hepatic but not tumor cell-associated P450 activity, 1-amino benzotriazole (ABT), a suicide substrate for multiple hepatic P450 enzymes, was examined for its ability to selectively suppress liver CPA 4-hydroxylation (Huang and Waxman, 2001). In rats given ABT in vivo, followed by CPA treatment, ABT induced a 4-fold decrease in C_{max} and a 7-fold increase in the apparent half-life of 4-hydroxy-CPA, consistent with there being substantial inhibition of hepatic CPA metabolism. However, the area-under-the-curve (AUC) for 4-OH-CPA was only reduced by ~50%, indicating that a substantial fraction of CPA is still metabolized by the 4-hydroxylation pathway in the ABT-treated animals, albeit at a slower rate than in the absence of ABT. Moreover, no increase in CPA- and P450-dependent tumor growth delay was observed in a 9L/P450 2B1 gliosarcoma xenograft model, suggesting that ABT treatment in vivo did not increase the tumor/liver partition ratio for CPA metabolism (Huang and Waxman, 2001). Most likely, ABT does not have sufficient P450 form-selectivity to achieve the desired specificity of liver P450 inhibition in vivo. A more systematic investigation is needed to identify inhibitors that are truly selective and can be used to inhibit prodrug activation in the liver without inhibiting prodrug activation catalyzed by a tumor cell-expressed P450 transgene.

4.3. Use of anti-apoptotic factors to delay tumor cell death

4-Hydroxy-CPA induces tumor cell death by a mechanism that involves mitochondrial transition stimulated by drug-induced DNA damage followed by activation of the caspase 9-dependent apoptotic pathway (Schwartz and Waxman, 2001). This cell death pathway is blocked in tumor cells that over-express the mitochondrial anti-apoptotic factor Bcl-2, which inhibits the release of cytochrome C from mitochondria and thereby confers drug resistance to CPA-treated tumor cells. By contrast, when the caspase 9 pathway is blocked in P450-expressing tumor cells at a step that is down-stream of the critical step of mitochondrial transition/cytochrome C release, CPA-induced cell death is delayed but, ultimately, is not blocked (Schwartz et al., 2002). Caspase activation is, therefore, not required for CPA to induce tumor cell death. Although the use of pro-apoptotic factors, such as Bax, p53, and various casp-

ases, to stimulate tumor cells to undergo apoptosis is presently being explored by many laboratories in gene therapies designed to enhance tumor cell death, this strategy is not suitable for GDEPT (Waxman and Schwartz, 2003). In the case of prodrug-activation gene therapy, such as P450 GDEPT, any acceleration of the apoptotic death of a tumor cell transduced with a prodrug-activating enzyme will decrease the net formation of active drug metabolites. This, in turn, will compromise the bystander effect that is critical to the success of the overall GDEPT strategy. Recent studies demonstrate, however, that anti-apoptotic factors can be combined with P450 GDEPT to prolong the longevity of the prodrug-activating tumor cells. In particular, retroviral expression of the pan-caspase inhibitor p35 was shown to substantially delay the death of P450-expressing tumor cells treated with CPA, thereby increasing bystander killing by the P450-activated prodrug. Moreover, the introduction of p35 delayed, but did not completely block, the ultimate death of the tumor cells (Schwartz et al., 2002). The inhibition of caspase-dependent apoptosis in tumor cells expressing p35 may trigger the tumor cells to undergo necrotic cell death in response to CPA treatment, instead of apoptotic death in response to CPA treatment, and this may potentially enhance systemic (immune system-based) bystander activity (Waxman and Schwartz, 2003).

5. Vectors for delivery of prodrug activating P450 genes

The vectors used for gene delivery are critical to the success of any gene therapy strategy. In the case of P450 and other prodrug activation genes, gene expression in the tumor must be high enough to generate an intratumoral level of activated metabolites that is sufficient to induce tumor cell death and efficient bystander tumor cell killing. Both viral and non-viral vectors have been investigated and have shown varying degrees of success in P450 GDEPT applications.

5.1. Replication-defective retroviruses

Replication-defective retroviruses are attractive for cancer gene therapy because of their intrinsic selectivity for dividing cell populations. CYP2B1 and CYP2B6 have been used in ecotropic retroviral vectors to transduce several rodent tumor cell lines (Wei et al., 1994; Jounaidi et al., 1998). Retroviral vectors carrying a P450 cDNA can be directly injected into solid tumor masses, leading to expression of CYP2B6 and a CPA-induced anti-tumor response (Kan et al., 2001). Retroviruses present several challenges that may limit their ultimate utility in P450 GDEPT applications in the clinic. These include: (1) the difficulty of obtaining high viral titers, which may translate into a low transduction efficiency and necessitate repeated treatments with retrovirus *in vivo* to achieve a sufficient level of transgene expression and efficacy; (2) the risk of retroviral integration into germ line cells, which may lead to oncogenesis

(Yi et al., 2005); and (3) the restriction of viral infection to replicating tumor cells. This latter problem may be addressed using lentiviral vectors for delivery of genes to both replicating and non-replicating tumor cells.

5.2. Replication-defective adenoviruses

The adenoviral E1A and E1B genes are required for viral replication, and deletion of these genes generates a replication-defective adenoviral vector. Distinct advantages of replication-defective adenoviruses for gene therapy include: (1) their ability to infect both rapidly dividing and quiescent tumor cells; (2) the viral DNA does not integrate into host chromosomal DNA, eliminating the risk of insertional mutagenesis associated with retroviruses; and (3) adenoviruses can be easily produced in large quantities and at high titers. A P450 transgene may be incorporated into the adenoviral genome under the control of a strong viral promoter, e.g., the cytomegalovirus promoter, to ensure strong expression in a wide range of tumor cells. Replication-defective adenovirus has been used to deliver P450 genes into a variety of human cell lines (Chen et al., 1996). A serious drawback, however, is that adenoviral particles are rapidly cleared from circulation and elicit a strong innate immunogenic response, which limits the opportunity for repeated virus administration (Muruve, 2004). Several strategies are being developed to overcome this difficulty, including advances in vector formulation (e.g., PEGylation to mask immunogenic viral epitopes), the use of alternate adenoviral serotypes, and the engineering of helper-dependent adenoviral vectors with deletions of highly immunogenic portions of the viral genome. Adenoviral vectors with genetically modified capsid proteins have also been developed to circumvent the requirement for the coxsackie-adenovirus receptor for viral entry, in view of the low levels of this receptor protein found on many tumor cells (Kanerva and Hemminki, 2004).

5.3. Conditionally replicating oncolytic viruses

Tumor cell-replicating viruses have been designed to facilitate selective gene delivery to malignant cells *in vivo*. These viruses typically have the ability to lyse the cells in which they replicate, providing an added therapeutic advantage, hence their designation as oncolytic viruses (Everts and van der Poel, 2005; Lin and Nemunaitis, 2004). Oncolytic viruses can be engineered in several ways. One approach involves the deletion of viral genes required for replication in normal cells but not tumor cells. A well-studied example is the replicating adenovirus designated Onyx-015, which has a deletion of the adenoviral E1B-55kDa gene (Cohen and Rudin, 2001). Normally, the E1B-55kDa protein inactivates the tumor suppressor protein p53, which prevents adenovirus-infected cells from activating the p53-dependent apoptotic response that serves to limit viral replication. Consequently, when

normal host cells are infected with the Onyx-015 virus, the p53-dependent apoptotic response is activated and the host cell is killed prior to viral replication. As a result of this response, host tissues cannot support a sustained infection by Onyx-015. By contrast, tumor cells deficient in a functional p53 pathway do not undergo p53-dependent apoptosis and readily support Onyx-015 replication and viral spread (McCormick, 2003; Bischoff et al., 1996). Almost 90% of human tumors are deficient in p53, or in p53 function. As a result, Onyx-015, or the related Onyx-017 (E3-wild-type and E1B-55kDa-deleted) (Jounaidi and Waxman, 2004) can be used to deliver P450 genes to a broad range of human tumor cells. The P450 gene can be incorporated directly into the genome of the replicating adenovirus, or alternatively, it may be delivered via a second, replication-defective adenovirus that is injected together with the replicating virus. In this latter strategy, the replicating virus serves as a helper virus that co-amplifies and facilitates tumor cell spread of the replication-defective P450 virus (Jounaidi and Waxman, 2004). Clinical trials using the oncolytic adenovirus Onyx-015 have demonstrated safety with no apparent dose-limiting toxicity. Superior anti-cancer activity is seen when this virus is combined with cisplatin-based chemotherapy in human tumor xenografts (Nemunaitis et al., 2001).

A second example of a conditionally replicating oncolytic virus is herpes simplex virus with a deletion of the viral ribonucleotide reductase gene. This latter gene is essential for viral replication in non-dividing cells. Insertion of the CYP2B1 cDNA in place of the ribonucleotide reductase gene generates a replicating oncolytic viral vector, designated rRp450 (Chase et al., 1998). This virus shows good preclinical P450 GDEPT activity when used in combination with CPA to treat gliomas, hepatocellular carcinomas and liver metastases (Aghi et al., 1999; Pawlik et al., 2000; Pawlik et al., 2002). Importantly, CPA treatment has minimal effect on the replication of this virus. This virus displays a high degree of tumor selectivity and enhanced therapeutic effectiveness (Pawlik et al., 2000).

Replication of a lytic virus in host tissues is likely to induce serious host toxicity and may potentially be lethal to the patient. Accordingly, it is essential to engineer appropriate safeguards into any replicating viral vector to prevent systemic infection. A good example is provided by the replicating herpes virus rRp450, which retains the endogenous herpes virus thymidine kinase gene, and consequently, replication of this virus can be strongly inhibited by the prodrug ganciclovir. Any host cell viral replication can therefore be blocked by treatment with ganciclovir once P450-dependent prodrug therapy is complete. Gene therapy using this virus can thus be carried out using a combination of two prodrugs, CPA and ganciclovir.

Another strategy that may be exploited for P450 GDEPT is to utilize tumor cell-specific DNA regulatory elements, such as promoter and enhancer sequences, to drive the expression of viral genes required for replication resulting in tumor-specific virus replication (Saukkonen

and Hemminki, 2004). This strategy has been implemented using the prostrate-specific antigen promoter to regulate the expression of adenoviral E1 genes in a manner that limits viral replication to the prostrate (Chen et al., 2001). Others have used alpha-fetoprotein gene regulatory sequences to achieve selective replication in hepatocellular carcinoma cells (Hallenbeck et al., 1999). These strategies do impart substantial tumor specificity but may not eliminate basal viral replication in non-target tissues.

6. Unique advantages of P450-based GDEPT

Discussed below are several advantages that P450 GDEPT has over other prodrug-activating systems.

6.1. Use of human P450 genes

P450-based GDEPT using the rat CYP2B1 gene has been shown to enhance the selectivity and the cytotoxicity of oxazaphosphorine prodrugs in a variety of rodent and human tumor models (Wei et al., 1994; Chen and Waxman, 1995a; Chen et al., 1997, 1996). Jounaidi et al. subsequently established the feasibility of using human P450 genes for GDEPT, including CYP2B6 and CYP3A4 (Jounaidi et al., 1998). The use of human P450 genes may limit the potential for host immune responses against a prodrug activating enzyme of foreign origin. Many other widely used GDEPT enzymes are of non-mammalian origin, including the viral HSV-TK system, cytosine deaminase from bacteria and fungi, and the bacterial enzymes carboxypeptidase G2 and nitroreductase (Kirn et al., 2002).

6.2. Compatibility with established and investigational anti-cancer prodrugs

P450-based GDEPT can be applied to a wide range of anti-cancer P450 prodrugs. P450 GDEPT was originally developed for use with the oxazaphosphorine prodrugs CPA and IFA, which are widely used in the clinic and have well established pharmacokinetic and pharmacodynamic properties (Fleming, 1997; Moore, 1991). Investigational P450 prodrugs, such as MMDX, a methoxymorpholinyl derivative of doxorubicin (Quintieri et al., 2000; Lu and Waxman, 2005) and the bioreductive prodrugs AQ4N (McErlane et al., 2005; McCarthy et al., 2003) and tirapazamine (Jounaidi and Waxman, 2000) are candidates for further development of P450-based GDEPT. Several other common anti-cancer drugs that are biotransformed by P450 enzymes to more active metabolites are also potential candidates for P450-based GDEPT (Chen and Waxman, 2002). These include thio-TEPA, which is activated by CYP2B-mediated oxidative desulfuration (Ng and Waxman, 1990); etoposide (VP-16), which is metabolized to a reactive catechol by P450-mediated O-demethylation reaction (van Maanen et al., 1987; Relling et al., 1992) and tamoxifen, which is converted by P4502D6 to 4-hydroxy-

tamoxifen (Dehal and Kupfer, 1997), a far more potent anti-estrogen than the parent drug (Borgna and Rochefort, 1981).

7. Prodrug delivery

Localized prodrug delivery may potentially be used to increase the tumor specificity of P450 GDEPT while minimizing host toxicity. In one study, localized prodrug delivery was achieved using a CPA-loaded controlled release polymer implanted in a solid subcutaneous tumor mass. A 250-fold increase in the intratumoral level of 4-hydroxy-CPA was achieved compared to intraperitoneal CPA administration (Ichikawa et al., 2001), with a corresponding increase in anti-tumor effect. In another study, the schedule of CPA administration was shown to be a critical determinant of the therapeutic effectiveness of P450 GDEPT. Administration of CPA on a 6-day repeating 'metronomic' schedule has been shown to induce a strong anti-angiogenic response (Browder et al., 2000). Application of this CPA treatment schedule to tumors expressing CYP2B6 and P450 reductase induced extensive regression leading to eradication of both small and large tumors with minimal host toxicity (Jounaidi and Waxman, 2001). Tumor regression, but not tumor eradication, was seen when the same CPA treatment schedule was applied to tumors deficient in P450.

8. Clinical trials of P450-based GDEPT

Several initial clinical trials utilizing the P450-based GDEPT strategy have been reported. In a phase I/II clinical trial conducted in Germany, 14 patients with inoperable pancreatic carcinoma were administered cellulose sulfate capsules containing cells that were engineered to express CYP2B1, followed by IFA treatment. The polymer encapsulated cells were introduced directly into the vasculature leading to the tumor, in an effort to achieve a high localized concentration of the IFA-activating P450 enzyme (Lohr et al., 2001, 2003). The treatment protocol was well tolerated and good indications of efficacy were obtained: of the 14 patients undergoing treatment, 2 showed partial remission and 11 had stable disease, i.e., no further tumor growth. Moreover, a three-fold increase in one year survival compared to historic controls was reported. The data obtained in this small phase I/II clinical trial are promising and indicate that further clinical trials involving larger number of patients are warranted.

A separate clinical trial, sponsored by Oxford BioMedica in the UK, used an engineered retroviral gene delivery vector encoding CYP2B6, designated MetXia (Kan et al., 2001). Results from an initial Phase I/II trial of MetXia in patients with advanced breast cancer or melanoma indicated that the product was safe and gene delivery was readily detected in the treated tumors (Hunt, 2001). In addition, a clinical benefit associated with an apparent anti-tumor immune response was seen in a subset of the treated patients

(Kan et al., 2002; Braybrooke et al., 2005). If the systemic anti-tumor immune responses seen in these studies are confirmed, vectors such as MetXia may potentially be useful for the treatment of a wide range of solid tumors, potentially including disseminated metastatic disease. A follow-up Phase I/II trial in patients with breast cancer, melanoma and urethral cell carcinoma has been carried out, with the results confirming the safety and anti-tumor immune activity seen in the initial trial, thus establishing proof-of-concept for this approach.

9. Future work

P450-based GDEPT has emerged as a promising gene-based prodrug activation therapy for cancer treatment. This therapy can readily be tested in the clinic in combination with established chemotherapeutic prodrugs and may lead to improved therapeutic responses. Preclinical studies, both in vitro and in vivo, have demonstrated the potential of this strategy, and recent clinical trials strongly support its clinical utility as an adjuvant for conventional chemotherapy. Moving forward, the effectiveness of P450 GDEPT may be further increased using improved vectors for P450 gene delivery and disease-targeted promoters for focused gene expression at the target site. In addition, there is the potential to engineer site-specifically modified P450 enzymes showing improved enzyme kinetics with prodrug substrates (Chen et al., 2004). Novel P450 prodrug substrates may also be developed. Combination treatments based on a P450-based therapy together with radiation, anti-angiogenic therapies, cytokine therapy or other GDEPT strategies may also be envisioned and hold promise for future research and development.

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