10

Cytochrome P450-Based Gene Therapies for Cancer

E. Antonio Chiocca and David J. Waxman

1. Introduction: Role of Cytochrome P450s in Anticancer Drug Metabolism and Carcinogen Activation

Cytochrome P450 (CYP) is comprosed of a family of hemeprotein monooxygenases that catalyze reactions as diverse as the biosynthesis of steroid hormones, metabolism of fat-soluble vitamins, oxidation of unsaturated fatty acids, and metabolism of drugs, pollutants, and other xenobiotics (for a review, *see* **ref. *1***). About 55 *CYP* genes, grouped into 17 gene families, are present in the human genome. *CYPs* belonging to gene families 1, 2, and 3 are particularly active in drug and xenobiotic metabolism and are most abundantly expressed in the liver and other tissues that come in contact with foreign chemicals. Large interindividual variations in *CYP* expression and, consequently, CYP-dependent drug metabolism are seen in humans, as a result of both genetic and environmental factors.

In the context of cancer, a large number of investigative studies on *CYPs* have focused on their role as bioactivators of procarcinogens. For instance, CYP1A1 catalyzes the activation of airborne polycyclic aromatic hydrocarbons, such as benzo(*a*)pyrene, in the lung to yield DNA-alkylating carcinogenic metabolites. The finding of functionally important single-nucleotide polymorphisms (SNPs) in *CYP1A1* and in other carcinogen-activating *CYPs* raises the possibility that these SNPs may be linked to interindividual differences in susceptibility to environmental carcinogens in the human population. For example, an A to G polymorphism in exon 7 of *CYP1A1* leads to an Ile to Val substitution that is found more commonly in patients afflicted with lung cancer in a Japanese population ***(2)***.

Cytochrome P450s play an important role in the metabolism of several widely used anticancer drugs (for a review, *see* **ref. *1***). Typically, CYP-catalyzed drug metabolism (including anticancer drug metabolism) leads to drug inactivation. However, in the case of several cancer chemotherapeutic drugs, including cyclophosphamide (CPA) and its isomer ifosfamide (IFA), CYP metabolism converts an inactive prodrug to an

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active chemotherapeutic metabolite ***(3)***. Individual variation in *CYP* expression profiles may thus be associated with individual differences in anticancer drug metabolism and pharmacokinetics and, potentially, individual differences in drug toxicity and clinical response. The potential impact of pharmacogenetics—in particular, allelic variations in *CYP* genes—on the metabolism of anti-cancer drugs such as CPA or IFA is suggested by the finding that two allelic variants of *CYP2C18*, one with a Met(385) and the other with a Thr(385) allele ***(4)***, exhibit up to a sixfold difference in catalytic efficiency in IFA activation ***(5)***. Cancer patients with this SNP who are treated with IFA might exhibit an altered therapeutic index compared to the remainder of the population. Several other anticancer drugs, such as taxol, tamoxifen, and flutamide, are converted into inactive metabolites by *CYPs* ***(6–8)***. Accordingly, SNPs in the *CYPs* that inactivate these drugs may potentially contribute to individual differences in therapeutic indices and anticancer responses encountered in the clinic.

Studies of *CYP*-dependent pharmacogenomics and anticancer pharmacology are thus important to cancer therapeutics and may lead to important advances in our understanding of cancer epidemiology and cancer biology. As will be discussed, the detailed understanding of the role of CYPs in the conversion of anticancer prodrugs into active drug metabolites can be exploited to enhance the responsiveness of tumor cells using a novel gene therapy for cancer treatment. Studies initially reported in 1994 demonstrate that CYPs have the potential to enhance chemotherapeutic responses when incorporated into an anticancer regimen in a gene-based treatment strategy ***(9)***. In this chapter, we review recent studies demonstrating the efficacy of CYP-based cancer gene therapy and discuss how these studies have established a firm preclinical basis for clinical trials that have recently been initiated.

2. Clinical Uses of CPA

Cyclophosphamide is an oxazaphosphorine alkylating agent prodrug used in two distinct clinical settings (*see* **Fig. 1**). CPA is a widely used anticancer agent, and it is a pleiotropic immunosuppressive drug that can rapidly decrease lymphocyte counts, affecting B-cell maturation and antibody production and decreasing macrophage/monocyte, natural killer (NK) cells, and cytokine generation ***(10,12)***. CPA can affect multiple arms of the immune system, but it is more effective in its inhibition of the innate responses as well as of the adaptive humoral responses. Accordingly, CPA is effective in treating autoimmune disorders and as a countermeasure against organ rejection in transplant recipients.

As an anticancer drug, CPA and its chemical isomer IFA are typically used in combination with other chemotherapeutic agents. CPA is a cell-cycle-phase-nonspecific anticancer agent ***(10)***. It is an important component of chemotherapeutic regimens currently employed in the treatment of breast, lung, and ovarian cancers, sarcomas, and a variety of childhood cancers ***(10)***. CPA is also employed in the treatment of other cancers, including certain brain tumors. CPA diffuses rapidly across cell membranes, including in the liver, where it is subject to CYP-catalyzed activation to yield the polar metabolite 4-hydroxy-CPA (*see* **Fig. 2** and **Subheading 3.**) ***(13)***. Like many anticancer drugs, CPA has a relatively narrow therapeutic index, and careful dosing and schedules must be

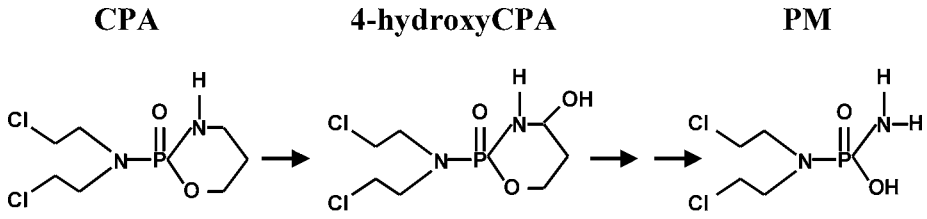

Fig. 1. A summary of the pathway of CPA activation. CPA is activated by members of the cytochrome P450 family into 4-hydroxycyclophosphamide, which spontaneously decomposes into the active metabolite, phosphoramide mustard.

employed to minimize harmful side effects. Chemically activated forms of CPA have been synthesized (e.g., 4-hydroperoxy-CPA and mafosfamide), but these are of limited clinical utility because of dose-limiting toxicities ***(14,15)***.

3. Activation of CPA and IFA by CYP2B Enzymes

Cyclophosphamide is inactive until it is converted into its active metabolites in a reaction mediated by a CYP-catalyzed hydroxylation reaction at C-4 (*see* **Fig. 1**). The resulting metabolite, 4-hydroxy-CPA (4-OH-CPA), equilibrates with the ring-opened aldophosphamide, which spontaneously decomposes in a β-elimination reaction to yield equimolar amounts of acrolein and phosphoramide mustard. The latter metabolite is the therapeutically active anticancer agent responsible for DNA alkylation and tumor cell toxicity, whereas the former contributes to some of the host toxicities of CPA, including hemorrhagic cystitis and other side effects ***(10)***. CPA metabolism primarily occurs in the liver and is catalyzed by CYP enzymes. The finding that the rate of hepatic CPA activation can be induced by the liver CYP inducer phenobarbital provided the first clue that this metabolism was catalyzed by a phenobarbital-inducible liver CYP enzyme. In studies of CPA 4-hydroxylation using liver microsomes isolated from rats treated with phenobarbital, reconstituted enzyme systems, enzyme CYP2B1 was shown to be the most active of 11 rat CYPs examined (2B1, 2B2, 2C11, 2A2, 2C6, 1A1, 2C12, 2A1, 2C13, 3A1, 1A2) ***(11)***. Activation of CPA was established using a variety of enzymatic assays, including measuring the binding to bovine serum albumin (BSA) of [^{14}C]acrolein, formed from [^{14}C]CPA, and the alkylation of calf thymus DNA by [^{3}H]phosphoramide mustard derived from [*chloroethyl*-^{3}H]CPA. CYP2B1 activation of CPA (turnover rate of approx 26 nmol metabolite/min/nmol P-450) proceeded at a ≥ 10-fold higher rate than that catalyzed by the other CYPs.

Subsequent studies of human CYP enzymes demonstrated that CYP2B6, together with several CYP2C enzymes, plays a key role in the activation of CPA in human liver tissue ***(16,17)***. By contrast, IFA activation in human liver was found to be primarily carried out by CYP3A4 ***(16)***. Therefore, species-specific differences in the metabolism of these prodrugs are readily apparent and need to be taken into account in considering CYPs for therapeutic exploitation.

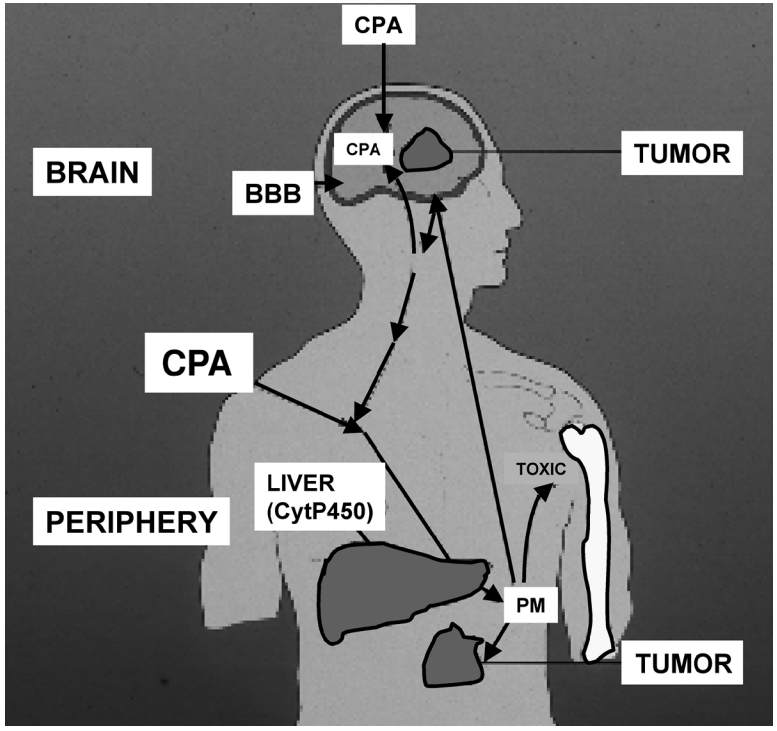

Fig. 2. CPA is activated by hepatic cytochrome P450 enzymes into 4-hydroxycyclophosphamide. The latter gives rise to phosphoramide mustard, which acts as an anticancer agent. However, transport of this metabolite across the blood brain barrier is not very efficient. In this case, the gene therapy paradigm includes: (1) endogenous tumor cells in the brain that are transduced to express one of the cytochrome P450 enzymes, responsible for CPA bioconversion (such as CYP2B1), and (2) local CPA administration. This may provide a means for more effective chemotherapy of the brain tumor.

4. *CYP* Gene Transfer Confers on Tumor Cells a Novel Chemosensitivity to CPA

In the late 1980s, a novel prodrug activation gene therapy strategy for cancer treatment was introduced ***(18–20)***. The thymidine kinase enzyme encoded by herpes simplex virus type 1, HSV-TK, phosphorylates and thereby activates nucleoside analogs such as acyclovir and ganciclovir (GCV). This phosphorylation enables the nucleoside to ultimately be metabolized to a triphosphate, which can be incorporated into replicating viral DNA, thus terminating the growing DNA chain, leading to an effective antiviral therapy. This same strategy can be applied to kill human tumor cells: In tumor cells that are stably transfected with *HSV-TK* and then treated with a nucleoside analog, phosphorylated acyclovir (or phosphorylated GCV) becomes incorporated into the replicating tumor cell's DNA, leading to tumor cell death. The emerging field of cancer gene therapy became enamored with this strategy: Viral and nonviral vectors

could be genetically engineered to deliver the *HSV-TK* gene into tumors, thereby rendering these neoplasms chemosensitive to GCV. The preclinical and clinical application of this treatment strategy to a variety of cancers and proliferative disorders has now been tested ***(21,22)***. Although the basic concept has been verified and validated, the poor delivery of *HSV-TK* into tumors has proven to be a major limitation and has led to the failure of such trials ***(23)***.

Investigation of other possible prodrug-activation gene therapy strategies for treatment of brain tumors led to the consideration of the CYP-activated prodrug CPA. CPA can be used to treat brain tumors, but its use is hampered by the requirement that CPA be activated in the liver, followed by transport to the tumor of the activated metabolite 4-OH-CPA and/or its ring-opened derivative aldophosphamide. Furthermore, to be effective, 4-OH-CPA needs to diffuse across cell membranes and then decompose intracellularly to yield phosphoramide mustard, a hydrophilic compound with poor capacity for diffusion across lipid membranes. In particular, the blood–brain barrier (BBB) can effectively hamper CPA's anticancer effects in treating brain tumors. In fact, the level of phosphoramide mustard assayed in brain tissue when CPA is activated in the liver is quite low (*see* **Fig. 2**) ***(13)***. One solution to this problem would be to provide for delivery and activation of CPA within the brain, directly within the tumor itself.

The finding that CYP2B1 is an active catalyst of CPA activation ***(11)*** provided the opportunity to evaluate whether the use of CYP2B1 to activate CPA within tumor cells might serve as an effective prodrug-activation gene therapy strategy. To test this proposal, rat *CYP2B1* cDNA was transfected into rat glioma cells, which thereby acquired chemosensitivity to CPA. CYP2B1 was then cloned into a retroviral vector and retroviral producer cells were generated. Treatment of subcutaneous and brain tumors by vector producer cell injection resulted in an enhanced therapeutic effect compared to control treatment ***(3)***. These experiments demonstrated that a strategy coupling *CYP2B1* gene transfer into tumors with CPA treatment had the potential for exploitation as an alternative to *HSV-TK*/GCV.

Further exploitation of this strategy against rat 9L gliosarcoma and human MCF7 breast carcinoma cells, stably transfected with *CYP2B1* and grown in vivo as subcutaneous tumors, was also demonstrated ***(24,25)***. Thus, *CYP*-based gene therapy not only has the potential for applications in the treatment of brain tumors but could also be used to enhance the chemosensitivity of systemic tumors. The large extent of chemosensitization of the systemic tumors was somewhat unexpected, given that these tumors are already exposed to high levels of circulating 4-OH-CPA generated by hepatic CYP enzymes. In other studies, an adenoviral vector expressing *CYP2B1* was developed and shown to sensitize a variety of rodent and human tumor cells to both CPA and IFA ***(25)***. Intratumoral CYP-dependent CPA activation led to an initial accumulation of tumor cells in the S-phase of the cell cycle, followed by an even more substantial accretion of cells at the G2-M-phase at later time-points. This finding is consistent with CPA-metabolite-induced damage to cellular DNA, which is manifest as G2-M cell cycle arrest. It is not known if this arrest is mediated by p21 and/or by proteins involved in the repair of DNA damage. However, recent studies have established that CYP-activated CPA induces a caspase 9-dependent apoptotic pathway ***(70)***.

Together, these initial findings established the feasibility of pursuing a *CYP*-based gene therapy that utilizes established anticancer prodrugs. Several important details remained to be determined, including the optimal method for delivery of the *CYP* transgene into tumors, the route, vehicle, and schedule for delivery of the prodrug to achieve optimal efficacy and minimal toxicity, and whether the therapeutic effect could be further enhanced by combination with other treatment strategies.

5. The Bystander Effect

The original reports describing GCV/*HSV-TK*-based cancer gene therapy included an in vitro experiment that described a "bystander" cytotoxic effect. In this experiment, HSV-TK-expressing tumor cells and nonexpressing tumor cells were cocultivated in the presence of the prodrug GCV. As expected, HSV-TK-expressing cells died following GCV treatment, but, unexpectedly, the cocultivated HSV-TK nonexpressing cells were also susceptible to GCV cytotoxicity. This effect was not mediated by conditioned medium from the HSV-TK-expressing cells; rather, it required direct contact between the two cell populations ***(26,27)***. There was a dose–effect relationship whereby greater toxicity was imparted to naïve cells in proportion to the fraction of HSV-TK-expressing cells found in the coculture. Further in vitro characterization of this effect suggested that it was mediated by passage of phosphorylated GCV metabolites across cellular gap junctions, although endocytosis of HSV-TK-expressing apoptotic bodies by naïve tumor cells was also invoked as a mechanism for the bystander toxicity. To further complicate matters, in vivo bystander toxicity was also shown to be the result of immunologic crossreactive responses ***(28–30)***. Similar bystander responses have been described for other prodrug-activating gene therapies ***(31,32)***. Such bystander responses are highly significant because they may greatly amplify the cytotoxic response to include cells well beyond the cells that are initially transduced with the therapeutic, prodrug-activating gene. This is an important feature, given that an effective, anticancer strategy must kill every tumor cell within the population, and for the forseeable future, gene transfer paradigms are unlikely to reach every cell within a neoplasm.

Characterization of the CPA/*CYP2B1* gene transfer strategy with regard to its potential for bystander cytotoxicity revealed an important difference in comparison to HSV-TK: Conditioned medium from CPA-treated cells that stably expressed CYP2B1 was cytotoxic to naive tumor cells, consistent with the presence in the medium of a diffusible, activated CPA metabolite, such as 4-OH-CPA ***(24,33)***. This was shown to occur both with rat C6 and 9L glioma cell lines as well as human U87 and Gli36 glioma cells. The presence of 4-OH-CPA in conditioned medium was confirmed by a semicarbazide trapping fluorometric method ***(4,58)*** and, more recently, by a gas chromatographic–mass spectrometric technique (GC-MS) ***(34)***. The production of diffusible metabolites with CPA/*CYP2B1* gene therapy is a feature that is intrinsic to 4-OH-CPA and results in a bystander effect that, unlike that of activated GCV, is independent of gap-junctional communication or apoptotic cell body endocytosis (*see* **Fig. 3**). More recently, other prodrug-activating gene therapies have been described whose bystander effect can be mediated by conditioned medium and are thus similar to the CPA/*CYP2B1* gene therapy paradigm ***(35,36)***.

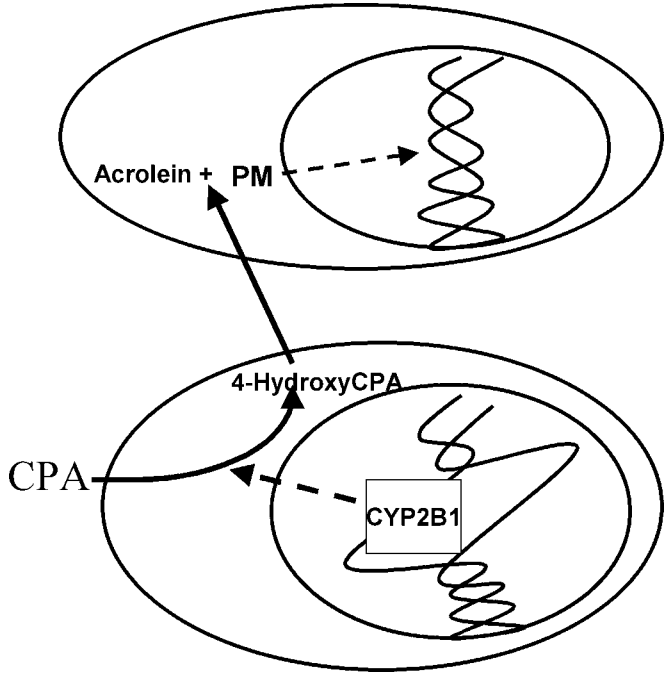

Fig. 3. The "bystander" effect with CPA/*CYP2B1* gene therapy is attributable to the diffusion of active CPA metabolites (presumably 4-hydroxycyclophosphamide) from the "converting" tumor cell into other tumor cells. In the latter, decomposition of 4-hydroxyCPA gives rise to the active metabolites (acrolein and PM). The latter possesses DNA alkylating properties that lead to observed cytotoxic effects.

6. *CYP* Gene Delivery: The Replicating Virus Strategy

Clinical trials of cancer gene therapy most commonly involve inoculation of a replication-defective vector (viral or nonviral) into the neoplastic mass. Typically, analysis of the inoculated tumor tissue reveals a pattern of gene expression that is localized along the needle tract, with little evidence for diffusion of the vector from the site of injection ***(23,37)***. For example, stereotactic injection of either a lacZ-expressing adenoviral vector or a retroviral vector into a malignant human glioma resulted in infection of a minority of tumor cells located less than 1 cm from the needle tract ***(38)***. Extremely low tumor transduction rates in cells primarily located around the needle injection tract have also been observed in a clinical trial involving stereotactic injection of HSV-TK-expressing retroviral vector producer cells into recurrent human malignant gliomas ***(23)***. These findings prompted the development of methods to improve the anatomic extent of tumor transduction by a gene therapy vector.

One approach to accomplish this objective involves the use of a replication conditional, oncolytic virus (OV) to deliver the therapeutic gene into the tumor. OVs are genetically altered viruses with deletions that restrict viral replication in normal cells

but permit replication in tumor cells ***(39,71)***. Additional approaches that can provide for tumor-selective replication include the use of tumor-specific promoters to drive viral replication ***(40)*** and alteration of the viral tropism to infect only desired target cells ***(41)***. The process of viral replication and expression of viral genes is toxic to the host cell and leads to its rapid demise. Additional inflammatory changes within the tumor can also contribute to the virus' anticancer action.

Oncolytic viruses can be engineered to deliver therapeutic genes, including prodrug-activating genes, thus providing for multimodal mechanisms of tumor cytotoxicity ***(42)***. Because the time frame of viral replication can typically last from 12 to 24 h (depending on the virus employed), expression of the therapeutic gene within an infected tumor cell may be of short duration. However, the continuous process of viral replication and subsequent propagation within the tumor can render this expression temporally and anatomically extensive within the tumor mass ***(43)***. In addition, if expression of the therapeutic gene expressed by an OV leads to formation of a diffusible factor or cytotoxic metabolite, such as 4-OH-CPA, then even a brief period of production within the infected cell could confer a significant antitumor effect.

A large number of OVs have been developed, based on herpes simple virus (HSV), adenovirus, vaccinia virus, reovirus, and others ***(39,71)***. HSV-based OVs have been engineered to possess genetic alterations in the viral gene locus *(UL39)* that encodes the viral ICP6 protein and/or the ICP34.5 protein. The former alteration restricts viral replication to cells with elevated levels of deoxynucleoside triphosphate pools, a product of cellular ribonucleotide reductase (RR) function ***(44,45)***. Because *RR* gene transcription is strictly regulated by the p16/pRB tumor suppressor pathway, replication of HSV OVs with defective ICP6 function is likely to target defects in this particular tumor suppressor pathway. In contrast, it remains unclear how viral defects in ICP34.5 function are associated with selective replication in tumors. At least two different HSV OVs have entered into clinical trials and several more are likely to begin clinical testing ***(46,47)***.

To test the utility of OVs for delivering a therapeutic *CYP* gene, a HSV-based OV that expresses *CYP2B1* was engineered. *CYP2B1* cDNA was subcloned into the UL39 (ICP6) locus of HSV under transcriptional control of the endogenous ICP6 promoter ***(48)***. Expression of *CYP2B1* in cell lines infected with this novel OV, designated as rRp450, was confirmed by Western blot analysis and by GC-MS assays of 4-hydroxy-CPA production in infected cell supernatants ***(34)***. A schedule of four inoculations of rRp450, given every other day for 1 wk with a single injection of CPA between the first two doses, was employed and shown to be more effective in controlling ectopic growth of human tumor xenografts than either therapy applied alone.

One interesting and important finding relates to the effects of the activated CPA metabolite 4-OH-CPA. When rRp450 titers were determined in infected cells exposed to CPA, minimal effects on viral titers were found both in vitro and in vivo ***(48)***. This indicates that activated CPA displays anticancer activity but minimal antiviral effects. In contrast, activated GCV exhibits both antiviral and anticancer effects. Thus, in the presence of GCV, replication of rRp450 is completely inhibited. This reflects the fact that rRp450, like all other HSVs, expresses the *HSV-TK* gene and is an important point to be considered in the context of cancer therapies utilizing OVs. The combination of

rRp450 with CPA for tumor therapy is expected to provide for continuous viral replication within the tumor mass, with anticancer effects resulting not only from the OV's direct cytotoxic effects but also from the tumor cytotoxicity of activated CPA. By contrast, when rRp450 and GCV are used in combination, viral replication in strongly inhibited, such that the observed anticancer effects are provided primarily mediated by the activated GCV ***(42)***.

An additional and relevant consideration concerns the immunosuppressive properties of CPA. In the context of a replicating OV and of the humoral and cellular immune responses against it, transient immunosuppression provided by activated CPA favors continuous viral replication and anticancer effects in vivo, particularly when the OV is delivered intravascularly ***(49)***. Suppression of innate antiviral responses, such as the classical and lectin pathways of complement activation as well as the production of antiviral cytokines, is a likely mechanism responsible for the observed effects of CPA on OV treatment ***(50,72,73)***.

In summary, CPA/*CYP2B1* gene delivery to tumors can be carried out in an effective manner by combination with OV therapy of tumors. The combination of 4-OH-CPA-mediated toxicity toward tumor cells with minimal effects on viral replication and the suppression of antiviral immune responses results in enhanced anticancer responses associasted with intratumoral OV replication.

7. Synergy With Other Gene Therapies

Other improvements in CPA/*CYP2B1* gene therapy may derive from combinations of prodrug activation strategies ***(51)***. Application of traditional parameters of drug-interaction studies to prodrug-activation gene therapies has recently been investigated. Ganciclovir/HSV-TK can be combined with CPA/*CYP2B1* gene therapy and the occurence of pharmacologic synergy or antagonism has been ascertained ***(42)***. Using stably transfected 9L rat gliosarcoma cells, the method of Chou–Talalay ***(52)*** as well as the classical isobologram method can be applied to exclude the possibility of antagonism that may be deleterious to anticancer effects and to demonstrate pharmacologic synergy between the two gene therapies. The observed synergy can be further validated by mechanistic proposals and studies. GCV's metabolites are nucleoside analogs, whereas CPA's metabolites are alkylating agents. Reports that other nucleoside analogs are pharmacologically synergistic in their anticancer action with other alkylating agents ***(53,54)*** leads to the hypothesis that the observed synergy arises at the level of DNA repair: After DNA chain alkylation by the CPA metabolite phosphoramide mustard, single nucleotide excision repair mediated by DNA polymerases δ and ε ensues. Activated GCV is thought to primarily affect these polymerases ***(55)***. Therefore, inhibition of alkylated DNA nucleotide excision repair by GCV may represent the mechanism of observed synergy. To test this hypothesis, tumor cells were treated with CPA/*CYP2B1* gene therapy and the amount of alkylated DNA then assayed as a function of time in the presence or absence of GCV/HSV-TK gene therapy. Following CPA/*CYP2B1* gene therapy, there was a time-dependent decrease in alkylated DNA, suggesting a fairly rapid nucleotide excision repair. However, in the presence of GCV/HSV-TK gene therapy, this repair was significantly retarded, suggesting a mechanistic explanation for the observed pharmacologic synergy (*see* **Fig. 4**).

This combined gene therapy regimen has been applied in the context of rRp450, the tumor-selective HSV oncolytic virus discussed earlier. Antitumor effects both in vivo and in vitro were significantly enhanced by the combination of GCV/*HSV-TK* with CPA/*CYP2B1*, as evaluated in rat 9L gliosarcoma cells infected with the replicating rRp450. However, one note of caution must be provided. Activated GCV will destroy rRp450 and its ability to replicate; thus, the beneficial effects of combining GCV/HSV-TK, the replicating virus, and CPA/*CYP2B1* may not be seen in all tumors and in all tumor cell lines. The interplay between replicating HSV and GCV activation will depend on the extent of gap-junction formation between tumor cells and the replicative ability of the virus. When virus replicates well and/or gap-junction formation is low, GCV will primarily antagonize the oncolytic effect. However, when conditions do not favor viral replication and/or when gap-junction formation is prominent, GCV activation combined with OV treatment will result in a more prominent anticancer effect than either treatment alone.

Further confirmation of the potentially beneficial interactions between prodrug-activation strategies involving alkylating agents and nucleoside analogs has been provided in studies using encapsulated cells engineered to express both *CYP2B1* and cytosine deaminase *(CD)* ***(56)***. The latter enzyme converts the prodrug 5-fluorocytosine (5-FC) into the anti-cancer drug 5-fluorouracil (5-FU). After cell encapsulation and implantation into TS/A murine adenocarcinoma or GR murine mammary carcinoma tumors, treatment with IFA in combination with 5-FC resulted in more potent anticancer effects than treatment with either prodrug alone. By contrast, when parental, nontransduced cells were used, the combination of prodrugs was not as effective as either prodrug used alone. Independent confirmation of the enhanced anticancer effect of these two forms of prodrug-activating gene therapy was thus obtained.

8. Delivery of CPA Via Polymers

In order to maximize local cytotoxicity and minimize systemic side effects, local tumoral activation of CPA by transferred *CYP* cDNAs might also benefit from local delivery of the prodrug. Recently, intratumoral application of a polymer impregnated with the chemotherapeutic agent BCNU was approved by the U.S. Food and Drug Administration (FDA) for local treatment of malignant gliomas ***(57)***. Based on the same concept, local application of a polymeric formulation of CPA was tested to ascertain whether increased intraneoplastic concentrations of the prodrug's active metabolites when compared to controls could be observed after tumor inoculation with rRp450 ***(34)***. The results obtained demonstrated that there was a 10-fold increase in peak levels of activated CPA metabolites in rRp450-inoculated tumors treated with polymeric CPA compared to rRp450-inoculated tumors treated with systemic CPA. This translated to AUC (area under the curve) values of approx 800 μg/mg/h for the former compared to only approx 3 μg/mg/h for the latter. When systemic blood levels of CPA metabolites were measured for each method of prodrug delivery, no differences in AUC values were seen (approx 3 μg/mL/h). Thus, when compared to systemic CPA delivery, polymer-based local delivery of CPA into a tumor that expresses

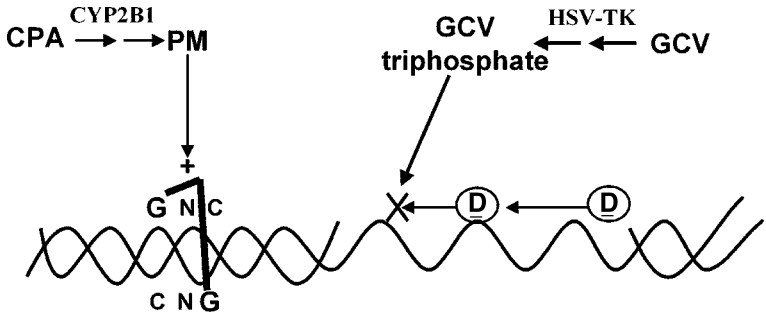

Fig. 4. Mechanism postulated to be responsible for the observed synergy between CPA/*CYP2B1* and GCV/*HSV-TK* gene therapies. The active metabolite of CPA (PM) produces inter- and intrastrand crosslinks between guanine residues within the sequence 5'-GNC-3'. Such crosslinks can be excised and repaired by DNA repair enzymes, such as DNA polymerase delta (D). However, the active metabolite of GCV (GCV triphosphate) functions as an inhibitor of DNA polymerase delta activity, thus potentiating the effects of PM.

CYP2B1 substantially increases the concentration of CPA's cytotoxic metabolites within the neoplastic mass without increasing their systemic concentration.

9. Coexpression of P450 Reductase Enhances CPA/CYP-Based Gene Therapy

The P450 system is actually comprised of two polypeptide components, the heme-containing cytochrome P450 and the flavoprotein P450 reductase (P450R). These two proteins are localized in the endoplasmic reticulum. P450R is a flavin adenine dinucleotide (FAD)- and flavin mononucleotide (FMN)-containing flavoenzyme that catalyzes the transfer of electrons required for all microsomal P450-dependent monooxygenase reactions. A total of two electrons are transferred from NADPH to P450R, first to FAD, then to FMN, and then on to the P450 hemeprotein, which utilizes these reducing equivalents to hydroxylate its substrates. Therefore, P450R and P450 need to be coexpressed for CYP2B1 or CYP2B6 to be catalytically active in the context of cancer gene therapy. Although tumor cells have been reported to express sufficient P450R to allow for expression of the transduced CYP gene's intrinsic capability to activate CPA, supplementation of cellular P450R levels by P450R gene transfer substantially enhances the cytotoxic potency of CPA/*CYP2B1* gene therapy in vitro and in vivo ***(58)***. For example, in studies of rat 9L gliosarcoma cells that were stably transfected with *CYP2B1*, *P450R*, or both cDNAs in combination, up to a 4- to 10-fold increase in P450R enzyme activity could be achieved by delivery of the P450R gene. This resulted in increased CPA conversion, as measured by formation of CPA's activated metabolites, when compared to control cells. Tumor excision studies demonstrated up to a 10-fold increase in CPA's cytotoxicity toward 9L gliosarcoma cells stably transfected with *CYP2B1* and *P450R* when compared to 9L tumor cells transfected with CYP2B1 alone.

The incorporation of P450R into the P450-based gene therapy strategy not only increases CYP2B1-mediated conversion of CPA to alkylating metabolites but also provides for the ability to convert other prodrugs into active anticancer agents ***(59)***. One such agent is tirapazamine (TPZ), a bioreductive prodrug that is particularly active against hypoxic cells. TPZ is activated by P450R, via a one-electron reduction, into a nitroxide radical that causes single- and double-strand DNA breaks. This TPZ radical can be further converted into an inactive product by a second one-electron reduction. Under normoxic conditions, the TPZ radical can be rapidly reoxidized, concomitantly producing reactive oxygen species and superoxide radicals. Because both prodrugs are metabolized by the same *CYP/P450R* gene combination, concern might exist about antagonism from CPA and TPZ competition for the same enzymatic sites. However, the addition of TPZ to CPA was found to augment the cytotoxic effects against 9L cells retrovirally transduced with both *CYP2B6* and *P450R* when treated under either normoxic or hypoxic conditions. Moreover, the in vivo anticancer effects of this prodrug combination were significant, increasing tumor growth delay from 23 d in CPA-treated mice to 35 d in mice treated with CPA plus TPZ. This benefit was partially offset by some increase in host toxicity, manifested by body weight loss, which may have been the result of drug-induced nausea. These findings demonstrate the important principle that transduction of tumor cells with *P450R* not only increases CYP-catalyzed CPA activation but also provides an additional capability for activation of bioreductive prodrugs.

10. Suppression of Liver P450 Prodrug Activation

Another pharmacologic approach to increasing the therapeutic index of CPA/CYP gene therapy involves the selective inhibition of hepatic metabolism of the prodrug ***(60)***. This could have the desirable effect of decreasing the systemic toxicity of activated CPA metabolites while increasing the availability of systemically delivered prodrug to tumor cells that express CYP. One approach to inhibition of hepatic but not tumor-cell-catalyzed CPA activation takes advantage of the fact that, in rat liver, the P450R gene is regulated by thyroid hormone, which is required for enzyme expression. This thyroid hormone dependence provides a means to downregulate P450R expression and enzyme activity by using antithyroid drugs such as methimazole (MMI). MMI treatment of rats significantly inhibits liver P450R expression, thereby reducing hepatic *CYP*-mediated prodrug activation. P450R expression and P450R activity in transfected rat 9L tumors is not inhibited by MMI, as expected from the fact that the *P450R* transgene is under control of a heterologous, non-thyroid-hormone-dependent promoter. This selective inhibition of hepatic CPA activation resulted in a 30% decrease in peak plasma concentration and a twofold increase in half-life of the activated CPA metabolite. Correspondingly, MMI treatment improved the anticancer effect of CPA (75 mg/kg) against subcutaneous 9L tumors transfected with *CYP2B1* and *P450R*, although this improvement was not apparent at a higher dose of CPA (100 mg/kg). An advantage of using MMI in this context is that it decreases the incidence of CPA side effects, such as body weight loss and hematuria. Inhibition of weight loss may be a consequence of the hypothyroid state induced by MMI, whereas the decrease

in hematuria may be the result of the reduction in urinary excretion of acrolein, one of CPA's metabolites, associated with MMI's inhibition of hepatic and kidney CPA metabolism.

A second pharmacologic approach to improve the balance between tumor vs hepatic CPA activation uses liver CYP enzyme-specific chemical inhibitors ***(61)***. In rats, the hepatic enzymes CYP2C6 and CYP2C11 make major contributions to the activation of CPA into its cytotoxic metabolites. Five inhibitors of CYPs were tested: metyrapone and chloramphenicol, both known inhibitors of CYP2B subfamily of P450 enzymes; DDEP (3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine) and 1-aminobenzotriazole, which inhibit CYP2C P450 enzymes; and SKF-525A, a general P450 inhibitor. In vitro, significant reductions in liver microsomal activation of CPA were observed with all 5 P450 inhibitors. Pharmacokinetic studies in rats confirmed the inhibitory action of these compounds, with 1-aminobenzotriazole causing a sevenfold increase in the half-life of the activated CPA metabolite 4-OH-CPA. However, only modest effects of these inhibitors on CPA's anticancer activity against 9L/*2B1-P450R* tumors were obtained, and no amelioration of CPA's toxic side effects were observed. Unlike the relatively selective inhibitory action of MMI against liver P450R, the chemical CYP inhibitors studied lack sufficient hepatic CYP inhibitory potency and selectivity to achieve the desired liver-specific inhibitory effect. The testing and evaluation of more specific and more potent liver CYP inhibitors could lead to improved therapeutic results, if differences between the transduced tumor CYP and the hepatic CYP catalysts of CPA activation can be exploited based on differences in P450 enzyme structure or transcriptional regulation.

11. Antiangiogenic Scheduling of CPA

One of the critical and essential steps in tumorigenesis is the active recruitment of a neovascular supply by the growing neoplastic mass. The balance between angiogenic and antiangiogenic factors dictates the establishment not only of a tumor mass but also that of metastatic foci. Recently, Browder et al. reported a significant antiangiogenic effect associated with a novel schedule of CPA administration, involving a repeated, 6-d cycle of CPA at a dose that is lower that the commonly used intermittent high-dose CPA schedule ***(62)***. Investigation of the therapeutic effectiveness of this antiangiogenic CPA schedule in combination with CYP gene therapy revealed that 9L tumors expressing CYP2B6 and P450R, established in the flank of scid mice, could be erradicated by a 6-d repeated course of CPA (140 mg/kg) given over 11 cycles ***(63)***. The conventional CPA schedule in this tumor model, 150 mg/kg CPA given twice over a period of 24 h, did not produce tumor eradication although it did produce significant tumor growth delay. The change in schedule also ameliorated toxicity in the animals, as indicated by the minimization of body weight loss. Several weeks after cessation of CPA treatment, regrowth of four of eight 9L/2B1-P450R tumors was observed. Three of the four tumors responded to a second course of CPA treatment. This novel CPA schedule was then applied to very large tumors (8–19% of animal's body weight). Substantial regression of the large tumors was achieved, although some resistance to CPA was encountered toward the end of the schedule. Analysis of the

mechanism of resistance revealed that the 9L/2B6-P450R tumors had lost the transduced *P450R* gene and *P450R* enzyme activity as well as reduced expression of the CYP2B6 and, hence, the ability to activate CPA efficiently. Importantly, the resistant tumor cells did not acquire intrinsic cellular resistance to CPA (e.g., by overexpression of aldehyde dehydrogenase). This finding underscores the importance of continuous expression of the prodrug-activating CYP transgene, a feat that might be achievable by repeated treatment with the gene therapy vector or by use of replicating agents or self-perpetuating vector systems.

12. CYP2B1 Delivery Using Encapsulated Cells or Macrophages

In addition to the use of viral vectors for transduction of CYP cDNAs, described earlier, several cellular and other nonviral CYP delivery methods have been investigated. In one approach, feline kidney cells were transfected with a CYP2B1 cDNA expression plasmid and then encapsulated into cellulose–sulfate capsules in order to protect the cells from the host immune system ***(64)***. Capsules contained up to 10^4 cells and cell viability studies in mice showed that approx 50% of the cells were alive 4 wk after encapsulation. In vivo efficacy studies were then performed using human pancreatic tumors established in the flank of nude mice. When the tumors reached a size of 1 cm^3, 20–40 capsulates containing CYP2B1-expressing cells were implanted directly into the tumor followed by treatment with IFA. Four of 12 tumors regressed completely, and the remainding tumors showed evidence of partial responses. These encouraging results have provided a basis for a clinical trial of IFA with CYP2B1 gene therapy in pancreatic cancer patients (*see* **Subheading 15.**).

Macrophages, which can infiltrate tumors and may predominate in hypoxic regions of a tumor, could serve as a useful delivery vehicle for prodrug-activating genes, such as CYPs. In a recent study, adenoviral vectors expressing human *CYP2B6* were used to infect primary human macrophages ***(65)***. Treatment of these cells with CPA did not affect the viability of the macrophages, as assessed by mitochondrial function. The *CYP*-expressing macrophages were then incubated with tumor cells growing in culture as spheroids, which they were able to infiltrate. Subsequent treatment with CPA resulted in a decrease in spheroid tumor volume as well as a decrease in spheroid clonogenic efficiency. Similar results were obtained when a hypoxia-responsive DNA enhancer was used to regulate expression of CYP2B6 in the macrophages. Therefore, the use of intact cells to deliver CYP enzyme activity into tumors, allowing for CPA chemosensitization, provides an exciting alternative to direct injection of viral vectors. Both the extent of prodrug activation catalyzed by these cells and the distribution of these cells within tumors require further investigation and will be a critical factor in the success of this approach.

13. Activation of 4-Ipomeanol by CYP4B1

The rabbit CYP enzyme CYP4B1 has been shown to activate the prodrugs 4-ipomeanol (4IM) and 4-aminoanthracene (4-AA) into their respective anticancer alkylating metabolites ***(66)***. In fact, 4IM was originally synthesized as a potential cancer chemotheraputic agent, but human clinical trials revealed a lack of efficacy as well as

host toxicity. This is most likely the result of inefficient activation of the prodrug by the endogenous human CYP4B enzyme, whose catalytic activity with the prodrug substrate is only 1% that of the corresponding rabbit enzyme CYP4B1. Therefore, transfer of the rabbit *CYP4B1* might provide a means to achieve increased activation of 4IM in tumors. To test this hypothesis, cultured rat 9L gliosarcoma or human U87 glioma cells were stably transfected with *CYP4B1* and then treated with 4-AA. A significant drug-dependent antiproliferative effect was observed, and fluorescence-activated cell sorter (FACS) analysis revealed that this effect was the result of apoptotic cell death. Crosslinking assays showed that DNA from treated cells was alkylated and in vivo studies confirmed a significant anticancer action for this gene therapy strategy. Similar results were observed with hepatocellular carcinoma cell lines. Therefore, 4IM or 2-AA in combination with CYP4B1 gene transfer provides a second example of CYP-mediated activation of prodrugs into their alkylating agents.

14. Prodrug Activation by Other CYPs

Recently, exploitation of other CYPs and their abilities to activate anticancer prodrugs has been reported. For instance, several human CYP2C enzymes has been shown to activate CPA ***(4)*** and CYP3A has been shown to activate IFA ***(4)*** as well as the prodrug AQ4N ***(67)***. CYP1A2 can metabolize acetaminophen into the toxic metabolite NABQI ***(68)***. This exploits a minor pathway of acetaminophen activation. Generally, the drug is eliminated in the liver by conjugation, forming sulfate or glucuronide derivatives. A minor pathway consists of drug oxidization into NABQI by CYP1A2, CYP3A4, and CYP2E1, with CYP1A2 carrying out the majority of hepatic activity. NABQI is cytotoxic presumably because of its oxidation and arylation of protein thiol groups. In fact, when Chinese hamster ovary cells, stably transfected with a CYP1A2 cDNA, were exposed to acetaminophen, decreases in cell viability were evident. A bystander effect was also observed, although it remained unclear if this effect was mediated by diffusible metabolites.

15. Clinical Trials

The preclinical studies summarized in the chapter indicate that further evaluation of the potential of CYP-based gene therapies leading to human clinical trials are warranted. In the United Kingdom, clinical trials for breast cancer, using a human *CYP2B6* cDNA delivered by intratumoral injections of a retroviral vector followed by systemic CPA treatment, are being pursued in a phase I setting. Preliminary findings in a group of 12 patients have shown a good safety record and encouraging evidence regarding efficacy (Kingsman, Oxford Biomedica, personal communication). In Germany, a separate trial of patients with pancreatic cancer treated using encapsulated cells that express rat *CYP2B1* followed by treatment with IFA has also shown evidence of safety and encouraging results regarding efficacy ***(69)***. Clearly, more conclusive proof of efficacy will have to await results from phase II/III trials.

In conclusion, the available evidence shows the versatility and potential for CYP-based prodrug-activation gene therapies. Multiple CYPs appear to be useful for the activation of different prodrugs and, even in the context of one CYP/prodrug combi-

nation, multiple variables can be modified to render treatment more effective. More extensive clinical trials appear warranted and should provide for a more conclusive test of the efficacy and utility of this treatment strategy.

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