ABSTRACT

Cyclophosphamide (CPA) and ifosfamide (IFA) are widely used anti-
cancer prodrugs that are bioactivated in the liver by specific cytochrome
P450 enzymes (CYPs). The therapeutic activity of these anticancer agents
may be compromised by a low therapeutic index that is, in part, due to the
systemic distribution of activated drug metabolites. Here, recombinant
retroviruses were used to deliver six different CPA- or IFA-metabolizing
human CYP genes to 9L gliosarcoma cells: 2B6, 2C8, 2C9, 2C18 (Met305
and Thr333 alleles), 2C19, and 3A4. Intratumoral cytochrome P450 expres-
sion conferred substantial sensitivity to CPA cytotoxicity, with the most
dramatic effects seen with CYP2B6. Strong CPA chemosensitivity was also
seen following transduction of CYP2C18-Met, despite a very low level of
CYP protein expression (>60-fold lower than that of 2B6). In contrast to
CPA, the cytotoxicity of IFA was greatest toward tumor cells trans-
duced with CYP3A4, followed by CYPs 2B6 and 2C18-Met. A substantial
further increase in chemosensitivity was achieved upon transduction of
2B6 or 2C18-Met-expressing tumor cells with P450 reductase, which
provided for more efficient intratumoral prodrug activation and cytotox-
icity at lower drug concentrations. With 2B6- plus P450 reductase-trans-
duced tumor cells, CPA but not IFA conferred a strong cell contact-
dependent bystander cytotoxic effect on non-P450-expressing 9L cells.
CPA treatment of tumors that were transduced with 2B6 or 2C18-Met
plus P450 reductase and were grown s.c. in immunodeficient
mice resulted in a large enhancement of the liver P450-dependent antitu-
or effect seen with control 9L tumors, with no apparent increase in host
toxicity (growth delay of >25-50 days in P450-expressing tumors versus
~5-6 days without P450). CYP2B6 plus P450 reductase and CYP2C18-Met
plus P450 reductase thus appear to be excellent gene combinations for use
with CPA in P450/prodrug activation-based cancer gene therapy.

INTRODUCTION

Several cancer chemotherapeutic agents, including CPA,3 IFA,
procarbazine, and dacarbazine, are prodrugs that undergo liver P450-
catalyzed metabolism to yield active, chemotherapeutic metabolites
(1, 2). The administration of anticancer prodrugs may provide advan-
tages associated with improved chemical stability and slow release of
activated drug metabolites in situ. However, because the metabolic
activation of classic cancer chemotherapeutic prodrugs largely occurs in
the liver, it leads to uncontrolled release and systemic distribution of
cytoxic drug metabolites that kill sensitive host cells, in addition to
the tumor cell targets. Recently, gene therapy methods have been
introduced to address this problem by delivering prodrug activation
genes to malignant cells, which thereby acquire the capacity for
intratumoral prodrug activation (3–5). One widely studied model uses
the antiviral drug ganciclovir (6) in combination with herpes simplex
virus thymidine kinase, which phosphorylates the produg and gener-
ates nucleoside analogues that induce DNA chain termination and
cell death in actively dividing cells (7, 8). Similarly, the bacterial gene
cytosine deaminase can be used as a “suicide gene” that confers on
tumor cells the ability to activate the antifungal compound 5-fluoro-
cytosine to 5-fluorouracil, a potent anticancer agent (9, 10).

Current gene therapy technologies are limited by their inability to
deliver prodrug activation or other therapeutic genes to a population
of tumor cells with 100% efficiency. The effectiveness of this cancer
gene therapy strategy can be greatly enhanced, however, by using
drugs that exhibit a strong “bystander effect” (5). Bystander cytotox-
icity results when active drug metabolites diffuse or are otherwise
transferred from their site of generation within a transduced tumor cell
to a neighboring, naive tumor cell. Ideally, the bystander effect leads
to significant tumor regression even when a minority of tumor cells is
transduced with the prodrug activation gene (e.g., Refs. 11 and 12).
Bystander cytotoxic responses may also be mediated through the
immune system, following its stimulation by interleukins and other
cytokines secreted by tumor cells undergoing apoptosis (13). Al-
though the ganciclovir/herpes simplex virus thymidine kinase
and 5-fluorocytosine/cytosine deaminase combinations have shown good
activity in preclinical models, and clinical trials are underway (14–16),
their ultimate effectiveness may be limited by the cell cycle
dependence of their cytotoxicity and by the fact that the bystander
cytotoxic effect requires direct cell-cell contact through gap junctional
intercellular communication to transfer the active drug metabolite in
the case of ganciclovir (17). Furthermore, the nonmammalian nature of
these two enzymes could elicit immunological responses that
further limit their effectiveness in cases in which repeated gene
delivery and drug treatments are required. Finally, ganciclovir was
introduced originally for the treatment of herpes infections (6), and
consequently, its ultimate therapeutic utility for cancer treatment is
uncertain.

The oxazaphosphorine prodrugs CPA and IFA, widely used in the
treatment of human cancers (18), are bioactivated in the liver via a
4-hydroxylation reaction catalyzed by certain P450 enzymes, with
electron input from the flavoenzyme NADPH P450 reductase (19).
The resultant 4-hydroxy metabolites undergo spontaneous ß-elimina-
tion to yield acrolein and a bifunctional DNA alkylator, phosphor-
amide mustard from 4-hydroxy-CPA, and isophosphoramidemustard from
4-hydroxy-IFA. These electrophilic mustards are active in a cell
cycle-independent manner and covalently cross-link genomic DNA,
leading to cell death via an apoptotic mechanism (20, 21). In the case of
IFA, an alternate, P450-catalyzed metabolic pathway deactivates the
drug through side chain oxidation, yielding the inactive metabo-
lites 2-dechloroethyl-IFA and 3-dechloroethyl-IFA and the neurotoxic
metabolite chloroacetaldehyde (22). Although IFA is an isomer of
CPA, it is activated by a distinct subset of P450 enzymes, both in
rodent models and in humans (23, 24), and it exhibits a unique
spectrum of antitumor activity, host toxicity, and drug resistance
(25–27). Intratumoral expression of the rat CYP gene 2B1 (28), the
protein product of which activates CPA and IFA by 4-hydroxylation
(19, 23), has shown promise in preclinical studies as an effective
strategy to enhance both the selectivity and the cytotoxicity of these drugs in a variety of human and rodent tumor models (Refs. 29–32; for review see Ref. 33). It is anticipated that the clinical development of this gene therapy strategy will benefit from the use of human CYP genes involved in oxazaphosphorine activation (24, 34, 35), which avoids complications associated with immune responses elicited by the expression of CYP genes from heterologous species. This study was undertaken, therefore, to evaluate the efficacy and potential utility of human P450 genes for CPA- and IFA-based cancer gene therapy. Recombinant retroviruses based on Moloney murine leukemia virus (36) and packaged in the ecotropic packaging cell line Bosc 23 (37) are presently generated and used to establish 9L gliosarcoma cell lines that stably express six individual human CYP genes belonging to the CYP 2B, 2C, and 3A families (28). The impact of human CYP gene transfer, both alone and in combination with P450 reductase gene transfer (38), on the responsiveness to oxazaphosphorine therapy was evaluated both in vitro and in an in vivo tumor model system.

MATERIALS AND METHODS

Chemicals. CPA and IFA were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). The chemically activated derivatives of CPA and IFA, 4-hydroxyperoxycPA and 4-hydroperoxy-IFA, were obtained from Nova Pharmaceutical Corp. (Bethesda, MD). Puromycin hydrochloride was purchased from Sigma Chemical Co., and hygromycin was from Aldrich.

Construction of Recombinant Retroviruses. cDNAs encoding three human CYP 2C enzymes (39), CYP2C8 (clone 7b), CYP2C18-Met388 allele (clone 6b; GenBank accession no. HUMC2C18), CYP2C18-Thr388 allele (clone 29c; GenBank accession no. HUMCYP2C18), and CYP2C19 (clone 11a; GenBank accession no. HUMCYP2C19), each cloned in the EcoRI site of pBluescript-SK+™, were provided by Dr. J. Goldstein (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The human CYP cDNAs 2C9 (plasmid 217; Cys344 variant, cloned in the EcoRI site of pUV1; GenBank accession no. HUMCYP2C9A), 2B6 (plasmid 328b; cloned in the EcoRI site of pUC9; GenBank accession no. HUMCYP2B2), and 2A4 (plasmid 359; cloned in the EcoRI site of pGEM7zf+; GenBank accession no. HUMCYPNOA) were obtained from Dr. F. Gonzalez (National Cancer Institute, Bethesda, MD). Rat P450 reductase cDNA was provided by Dr. G. Gil (University of Massachusetts, Worcester, MA). Retroviral vectors of the pBabe series (36), which encode genes that confer resistance to either pBabe (pBabe-hygro) or hygromycin (pBabe-hygro) and are transcribed from an internal SV40 early promoter, were obtained from Dr. B. Speigelman (Dana-Farber Cancer Institute, Boston, MA). P450 cDNAs were cloned into the pBabe-hygro multiple cloning site, which provides for transcription of the P450 gene from the retroviral long terminal repeat promoter. P450 reductase cDNA was subcloned into pBabe-hygro cut with EcoRI-Sall. In the final retroviral constructs, each of the human CYP cDNAs retained the following lengths of 5′- and 3′-untranslated region sequences, respectively: 2C8, 78 and 359 bp; 2C9, 11 and 369 bp; 2C18-Met388, 43 and 341 bp; 2C18-Thr388, 200 and 333 bp; 2C19, 6 and 268 bp; 3A4, 30 and 457 bp; and 2B6, 7 and 1563 bp.

Construction of Stable 9L Gliosarcoma Cell Lines by Retroviral Infection. The ecotropic packaging cell line Bosc 23 (Ref. 37; 2.5 × 10⁶ cells in a 60-mm dish), obtained from Dr. J. Aster (Brigham and Women’s Hospital, Boston, MA), was cultured in 3 ml of DMEM containing 10% heat-inactivated fetal bovine serum, 584 g/liter L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Prior to transfection, the cells were changed to fresh medium containing 25 µM chloroquine. Three h later, 24 ptg of each pBabe-based plasmid was added to the medium containing 25 µM chloroquine. Three h later, the mixture was added immediately to the cells and left for 5 h before the medium was changed. The total supernatant (3 ml) containing released viral particles was removed 48 h later and used to infect 9L rat gliosarcoma cells (0.5 × 10⁶ cells in a 100-mm dish) in the presence of 4 jig/ml polybrene. Three h later, the medium was adjusted to 10 ml by addition of fresh DMEM. The cells were trypsinized 48 h later and split into four 100-mm dishes. Selection for puromycin- or hygromycin-resistant 9L cells was carried out with 2 µg/ml puromycin and/or 250 µg/ml hygromycin for 2–3 days. Drug-resistant cells were propagated and then evaluated for CYP or P450 reductase enzyme activities and protein expression (see below). Coexpression of P450 enzymes and P450 reductase was achieved by infecting cells with retrovirus encoding a P450 gene, puromycin selection of a 9L/P450 pool of cells, clonal selection of cells that have elevated P450 levels (see below), followed by infection of the clonal 9L/P450 cell line with retrovirus encoding P450 reductase. Hygromycin selection was then carried out for 2 days to obtain pools of 9L/P450 cells that overexpress P450 reductase.

Clonal Selection of 9L/P450 Cell Lines. Cells from each pool of puromycin-resistant, P450-expressing 9L cells were trypsinized and diluted to about one cell per 50 µl and then plated at calculated densities of one, three, and five cells per well in a 96-well tissue culture plate. Wells containing single colonies were identified when the colonies were nearly confluent (~15 days later) using a light microscope; the cells were then trypsinized and split into two wells of a 48-well tissue culture plate. One well of each clone was untreated and kept as a control, and the second well was treated with either 2 µM CPA for 9L/2B6, 9L/2C8, 9L/2C9, 9L/2C18, and 9L/2C19 cells or with 2 µM IFA for 9L/J3A4 cells. Clones that exhibited an enhanced sensitivity to CPA or IFA toxicity were typically detected by day 2 or day 3 of drug treatment, i.e., ~2 days earlier than seen for the others; these clones were then propagated and further evaluated for P450 protein levels and enzyme activities. The yield of clones showing enhanced drug sensitivity was 27 of 36 for 9L/2B6, 20 of 34 for 9L/3A4, and 4–5 of 34 for the others. In the case of CYP3A4, each of the isolated clones grew more slowly than the original 9L/J3A4 pool or the 9L/IFA or 9L/PbBe controls. Slow-growing clones were occasionally seen for some of the 9L/2C isolates, but these were not selected or evaluated further.

Western Blot Analysis. Microsomes were prepared from near confluent 100-mm dishes of each P450-expressing 9L cell line. Cells were washed with ice-cold 50 mM KPi, 1 mM EDTA (pH 7.4) and then scraped into 2 ml of the same buffer and sonicated for a sufficient time to induce cell lysis (~20 s) in 15 ml Corex tubes. The homogenate was centrifuged at 12,000 rpm and 4°C for 20 min, and the resultant supernatant was then ultracentrifuged for 60 min at 45,000 rpm and 4°C. The microsomal pellet was resuspended by sonication in 50 mM KPi, 1 mM EDTA, and 20% glycerol (pH 7.4). Microsomal protein (60 µg/well) was electrophoresed through a 10% SDS-polyacrylamide gel, then transferred to nitrocellulose, and further probed with rabbit polyclonal anti-CYP2B6, anti-CYP3A4, and anti-CYP2B2 antibodies prepared against short COOH-terminal synthetic peptides (40), which were kindly provided by Dr. R. Edwards (Royal Postgraduate Medical School, London, United Kingdom). Lymphoblast-expressed CYP2B6 and CYP3A4 (Gentest Corp., Woburn, MA) and yeast-expressed CYPs 2C8, 2C9, 2C18, and 2C19 (obtained from Dr. J. Goldstein; Ref. 35) were electrophoresed at 0.2–1.2 pmol P450/well and used as standards to quantify P450 levels in the 9L cells.

BROD and P450 Reductase Assays. BROD activity was assayed using 70 µM of microsomal protein in 1 ml containing 0.1 M KP, (pH 7.4), 0.1 mM EDTA, and 4 µM 7-benzoxylresorufin (Molecular Probes, Inc., Junction City, OR). Reactions were started by adding 1 mM NADPH (final concentration), and the release of the fluorescent metabolite resorufin was measured over an 8-min time period at 20–25°C using a Shimadzu RF-1501 fluorescence spectrophotometer. Fluorescence was read at 550 nm (excitation) and 586 nm (emission). Activity values were quantitated using resorufin standard. P450 reductase activity was assayed in 0.3 µM KP, buffer (pH 7.7) using 20 µg microsomal protein by monitoring the NADPH-dependent reduction of cytochrome C at 550 nm at 30°C (E = 21 mm−1 cm−1). Under these assay conditions, 9L cell microsomes exhibited a P450 reductase activity of 50–60 nmol cytochrome C reduced/min/mg, corresponding to a P450 reductase specific content of 16–19 pmol of P450 reductase protein/mg, based on a reductase specific activity of 40 μmol/min/mg and a P450 reductase Mₜ of 78,000 (41).

RT-PCR Analysis of CYP2C18 Expression. Total RNA from 9L/P450 or 9L/PbBe cells prepared from confluent 100-mm dishes (42) was treated with RQI DNase (RNase free; Promega). Five µg of treated RNA was then heated at 70°C for 10 min in 11 µl of DEPC-treated H₂O containing 10 pmol of a reverse transcriptase primer. Two µl of 10× RT buffer (Promega), 2 µl of 0.1
Tumor cells were grown in culture to ~75% confluency, harvested by trypsin, and on the right flank with 4 X 10^6 of either 9L/2B6/reductase or 2C18-Thr385, two allelic variants of CYP2C18 that display differences in their oxazaphosphorine metabolism activities (35). The retroviral approach to stable transfection used in these studies is rapid and yields a pool of cells containing many thousands of independent clones, each of which expresses the P450 gene at a random integration site. In each case, >60–70% of the infected 9L tumor cells acquired resistance to puromycin, indicating a high efficiency for retroviral gene delivery. P450 protein expression was readily detectable in the pools of CYP2B6 and 2C9 cells, as shown by Western blot analysis of isolated cellular microsomes (Fig. 1A and data not shown). In the other cases,

RESULTS

Retroviral Expression of Human P450s in 9L Gliosarcoma Cells. 9L cells were infected with pBabe-based retroviral particles (36) produced in the packaging cell line Bosc 23 (37) and engineered to code for each of six human P450 genes: CYPs 2B6, 2C8, 2C9, 2C18, 2C19, and 3A4. The corresponding P450 proteins have each been shown to activate CPA or IFA in a heterologous cDNA expression system (24, 35). Included in these studies are 2C18-Met385 and 2C18-Thr385, two allelic variants of CYP2C18 that display differences in their oxazaphosphorine metabolism activities (35). The retroviral approach to stable transfection used in these studies is rapid and yields a pool of cells containing many thousands of independent clones, each of which expresses the P450 gene at a random integration site. In each case, >60–70% of the infected 9L tumor cells acquired resistance to puromycin, indicating a high efficiency for retroviral gene delivery. P450 protein expression was readily detectable in the pools of CYP2B6 and 2C9 cells, as shown by Western blot analysis of isolated cellular microsomes (Fig. 1A and data not shown). In the other cases,

Fig. 1. Western blot analysis of CYP protein levels in 9L/P450 cell lines. Shown are Western blots of 9L/wt and 9L/P450 cell microsomes (60 μg/lane) probed with antibody to P450 2B6 (A) or P450 2C (B). Shown in A, Lane 3, are 9L microsomes prepared from the original pool of 2B6 transduced cells, prior to selection of the 2B6 clone shown in Lane 2. cDNA-expressed P450 2B6 (A, Lanes 4 and 5; lymphoblast expression) and P450 2C standards (B, Lanes 1, 2, 9, and 10; yeast expression) were analyzed in parallel at the indicated normalized to units of cells/well. Twenty-four h later, 2 mM CPA or 2 mM IFA was added to the cells weighing 26-31 g were given s.c. tumor cell injections to form solid tumors.
the level of P450 protein expression in the pool of puromycin-resistant cells was low (2C8 and 2C19) or not detectable (CYP3A4 and both CYP2C18 alleles). Clonal selection was therefore carried out to obtain individual 9L/P450 clones with higher levels of P450 protein expression. Clones showing enhanced sensitivity to CPA or IFA (see “Materials and Methods”) were propagated and analyzed by Western blotting. In each case, the 9L/P450 clones selected on the basis of increased sensitivity to CPA or IFA had a higher specific P450 protein content (Fig. 1 and data not shown) and higher P450 enzyme activity (Fig. 2). The microsomal P450 content of the selected clones, determined by Western blotting using lymphoblast-expressed or yeast-expressed human P450s as standards, is presented in Table 1. The highest levels of expression were observed with 9L/2B6 (20–25 pmol of P450/mg of microsomal protein) and 9L/2C9 (10–15 pmol/mg) cells, whereas 2C18-Met was the lowest (<0.3 pmol/mg). 9L/2C8, 9L/2C18-Thr, 9L/2C19, and 9L/3A4 cells exhibited intermediate levels of P450 expression (0.5–3 pmol/mg). Expression of both 2C18 transcripts was confirmed by RT-PCR (data not shown). These expressed P450 protein levels can be compared to an endogenous P450 reductase protein level of ~16–19 pmol of P450 reductase protein/mg of 9L microsomal protein (see “Materials and Methods”).

**BROD Activity of the Tumor Cell-expressed P450 Genes.** P450-dependent BROD activity, which is catalyzed by many human P450 enzymes at various rates, was used to monitor the enzymatic activity of the transfected P450 genes. Fig. 2 shows that all of the P450-expressing cell lines exhibited higher BROD activity than parental (wild-type) 9L cells (9L/wt) or 9L/pBabe controls. Moreover, the isolated 9L/P450 clones (Fig. 2, □) each expressed higher BROD activity than the original 9L/P450 retroviral pools (Fig. 2, ■). 9L/P450 clonal cell lines chosen for further study (Fig. 2, arrows) were selected on the basis of their level of P450 expression (Western blotting and BROD activity) and their sensitivity to CPA or IFA.

**Cytotoxicity of CPA and IFA toward P450-expressing 9L Tumor Cells: Growth Inhibition Assays.** To evaluate the impact of retroviral P450 transduction on 9L chemosensitivity, cells were cultured in the presence of various concentrations of CPA or IFA, and cytotoxicity was evaluated by a growth inhibition assay scored 4 days later. 9L/wt and 9L/pBabe cell lines, used as P450-negative controls, were insensitive to millimolar concentrations of both CPA (Fig. 3A) and IFA (Fig. 3C). By contrast, all of the P450-expressing 9L cells showed a concentration-dependent growth inhibition by CPA. 9L/2B6 cells were the most susceptible to CPA cytotoxicity (~95% growth inhibition at 1 mM CPA), consistent with the high catalytic activity of CYP2B6 with respect to CPA activation (24) and the comparatively high level of CYP2B6 protein expression achieved using this retroviral expression system (Table 1). Each of the other tumor cell lines, except 9L/2C8, also showed significant acquired drug sensitivity: at 1 mM CPA, ~60% growth inhibition was observed for 9L/2C9 and 9L/2C18-Thr cells, 75–85% inhibition was obtained for 9L/2C19 and 9L/2C18-Met (Fig. 3B), and ~90% growth inhibition was observed for 9L/3A4 (Fig. 3A). The cytotoxicity of CPA toward 9L/2C18-Met cells is especially remarkable, given the very low level of CYP2C18-Met protein expression in these tumor cells (Table 1). This effect is likely due to the low \( K_m \) and high catalytic efficiency CYP2C18-Met for CPA (35). In contrast to the moderate to high sensitivity of many of the 9L/P450 cell lines to CPA, only three of the 9L/CYP cell lines showed significant sensitivity toward IFA when tested at concentrations up to 1 mM. 9L/3A4 cells were, by far, the most sensitive to IFA, although significant IFA growth inhibition was also achieved in 9L/2B6 and 9L/2C18-Met cells (Fig. 3, C and D).

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**Table 1** P450 protein content in 9L/P450 clonal cell lines

<table>
<thead>
<tr>
<th>9L/P450 clonal cell line</th>
<th>Clone no.</th>
<th>Specific P450 content (pmol of P450/mg of microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B6</td>
<td>1</td>
<td>20–25</td>
</tr>
<tr>
<td>2C8</td>
<td>2</td>
<td>0.5–1</td>
</tr>
<tr>
<td>2C9</td>
<td>1</td>
<td>0–15</td>
</tr>
<tr>
<td>2C18-Met</td>
<td>3</td>
<td>0.3–0.5</td>
</tr>
<tr>
<td>2C18-Thr</td>
<td>2</td>
<td>0.5–1</td>
</tr>
<tr>
<td>2C19</td>
<td>2</td>
<td>1.5–3</td>
</tr>
<tr>
<td>3A4</td>
<td>3</td>
<td>1–2</td>
</tr>
</tbody>
</table>

* Individual P450 clones were selected from pools of puromycin-resistant 9L/P450 cells on the basis of their enhanced sensitivity to CPA (or to IFA, in the case of 9L/3A4), as described in “Materials and Methods.” Microsomes prepared from the indicated clones were analyzed on Western blots (as in Fig. 1) for P450 protein content in comparison to a standard curve based on 0.2–1 pmol of the corresponding cDNA-expressed P450 protein standard using either a lymphoblast cDNA-expression system (CYPs 2B6 and 3A4; Gentest Corp.) or a yeast expression system (35). Data are shown as a range of values from two or three separate experiments.

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4 P. Roy, L. J. Yu, C. L. Crespi, and D. J. Waxman, unpublished data.
CYP2B6-BASED CANCER GENE THERAPY

Fig. 3. Growth inhibition assay to assess chemosensitization to CPA (A and B) and IFA (C and D) in 9L/P450 cells. Cells were seeded at 500 cells/well in 48-well plates and were treated with the indicated concentrations of CPA or IFA for 4 days. Control cells for each cell line received no drug treatment. Relative cell number at the end of the experiment was determined by crystal violet staining as described under "Materials and Methods." Data points, means (n = 3) based on the crystal violet absorbance in drug-treated plates as a percent of the corresponding drug-free controls; bars, SD. Data for the P450-deficient control cell lines (9L/wt and 9L/pBabe) shown in A and C are the same as shown in B and D.

The other P450-expressing cell lines were insensitive to IFA under these conditions (Fig. 3D).

P450 Reductase Transduction Further Enhances Cytotoxic Responses. We next investigated whether the endogenous level of P450 reductase in 9L tumor cells is sufficient to maximally support CPA and IFA activation catalyzed by the transduced human P450 genes. P450 reductase activities measured in microsomes prepared from the 9L/P450 cell lines were, in general, not substantially different from 9L/wt and 9L/pBabe controls (Fig. 4). An ~5-fold increase in P450 reductase activity was, however, obtained following retroviral transduction of the 9L/2B6 and 9L/2C18-Met cells with P450 reductase (Fig. 4). This overexpression of P450 reductase markedly increased the cytotoxicity of CPA toward the 9L/2B6 and 9L/2C18-Met cells, particularly at lower drug concentrations (e.g., growth of 2B6-expressing cells decreased from 75% to 28% of control at 0.2 mM CPA upon coexpression of P450 reductase; Fig. 5A). Similar effects of P450 reductase overexpression were observed with IFA as substrate (Fig. 5B). These effects were achieved, although endogenous P450 reductase levels in these cells (16–19 pmol of P450 reductase/mg of microsomal protein; see "Materials and Methods") are comparable to (9L/2B6 cells) or in vast molar excess of (9L/2C18-Met cells) the expressed P450 protein levels (compare Table 1). In control experiments, transduction of P450 reductase transduction alone, in the absence of P450 (Fig. 4; 9L/pBabe/Red), did not confer CPA or IFA cytotoxicity (Fig. 5). Thus, P450 reductase gene transfer enhances therapeutic activity by increasing tumor cell cytotoxicity at a given drug concentration. It is also apparent from these studies that 9L growth inhibition required higher concentrations of IFA than CPA for both CYP2B6 and CYP2C18-Met (Fig. 5, B versus A). This is, in part, a reflection of the greater intrinsic sensitivity of 9L tumor cells to activated CPA, as compared to activated IFA, as determined in cytotoxicity assays using the corresponding chemically activated 4-hydroperoxy compounds (Fig. 6). In this light, the substantially greater sensitivity of 9L/3A4 cells to IFA compared to CPA (Fig. 3, C versus A) must, indeed, reflect the higher rate of
prodrug activation catalyzed by CYP3A4 with IFA compared to CPA (24).

Metabolic Activation of CPA and IFA Enhanced by P450 Reductase Transduction. To investigate whether the P450 reductase-enhanced cytotoxicity of CPA and IFA toward 9L/2B6 and 9L/2C18-Met tumor cells is due to increased prodrug activation, we measured the levels of P450-generated 4-hydroxy metabolites in culture supernatants from each cell line (Fig. 7). P450 reductase gene transfer stimulated a significant increase in cytotoxic metabolites accumulating in the culture medium, both for CYP2B6 and for CYP2C18-Met cells. In contrast, transduction of P450 reductase in 9L parental cells did not stimulate prodrug activation. These studies also showed that, in 9L/3A4 cells, IFA 4-hydroxylation was greater than CPA 4-hydroxylation, consistent with their preferential sensitivity to IFA (Fig. 3). Moreover, 9L/2C8 cells, which are insensitive to IFA (Fig. 3D), showed IFA metabolite levels just above the 9L/pBabe background (Fig. 7). Some quantitative discrepancies were observed, however. These include the similar level of activated CPA and IFA metabolites formed in 9L/2B6/reductase culture medium (Fig. 7) versus the higher level of CPA compared to IFA activation catalyzed by cDNA-expressed CYP2B6 (24), and the similar levels of activated metabolites formed by 9L/2B6/reductase compared to 9L/2C18-Met/reductase cells (Fig. 7) versus the greater drug sensitivity of the 2B6-expressing cells (Figs. 3 and 5). These discrepancies may relate to the relative insensitivity of the fluorescence metabolite assay and to the chemical instability of the activated CPA and IFA metabolites during the 24-h tissue culture incubation in the studies described in Fig. 7. Finally, the greater intrinsic sensitivity of 9L tumor cells to activated CPA (Fig. 6) is likely to account for the greater sensitivity of the 2C18-Met-expressing cells to CPA compared to IFA (Fig. 5), despite their formation of activated CPA and IFA at similar levels (Fig. 7).

Bystander Killing Effect. CPA treatment of tumor cells transduced with rat P450 gene CYP2B1 is associated with a significant bystander cytotoxic effect, whereby neighboring tumor cells that do not express the P450 drug susceptibility gene also become sensitized to the prodrug (29, 31). Because 9L/2B6/reductase cells are themselves more readily killed by CPA and IFA than 9L/2B6 cells (Fig. 5), we investigated whether bystander tumor cells are also more chemosensitive when cultured with P450 reductase-transduced 9L/2B6 cells. Bystander cytotoxicity was evaluated by using cell culture inserts to physically separate the prodrug-activating 9L/2B6/reductase (or 9L/
2B6) tumor cells from the P450-deficient bystander 9L/pBabe cells. Fig. 8A demonstrates that 9L/2B6/reductase cells exposed to CPA confer a ~4-fold stronger bystander killing of 9L/pBabe cells than do 9L/2B6 cells, as judged from the 9L/P450:9L/pBabe cell ratio required to effect 50% bystander cytotoxicity. Follow-up experiments demonstrated that IFA does not display the strong bystander cytotoxicity seen with CPA: CPA treatment of 9L/2B6/reductase cells results in ~40% bystander toxicity toward non-P450 tumor cells when the P450:non-P450 cell ratio is ~0.5:1 and ≥80% toxicity toward the non-P450 cells as the cell ratio is increased to ≥3 (Fig. 8B). In contrast, IFA exerted only a modest bystander cytotoxicity to the non-P450-expressing pBabe controls (up to ~20% bystander cytotoxicity at cell ratios up to 3:1 and a maximum of ~50% bystander cytotoxicity at a cell ratio of 6:1; Fig. 8B). This observation, together with the greater intrinsic potency of activated CPA compared to activated IFA in the 9L gliosarcoma model (Fig. 6), demonstrates that CPA is a superior choice compared to IFA for use in conjunction with CYP2B6 plus P450 reductase gene therapy.

**Evaluation of Human P450-based Gene Therapy in a scid Mouse Model.** The impact of human P450 gene transfer on 9L gliosarcoma chemosensitivity was evaluated in vivo using the immunodeficient scid mouse solid tumor model (43). The scid mouse model is free from the complications associated with immunogenic responses toward 9L tumors expressing human P450 genes, and it eliminates any contributions from immunological components to bystander cytotoxicity (13). scid mice were inoculated s.c. with 9L/pBabe tumors on the left flank and with 9L/2B6/reductase tumors on the right flank. This experimental design enabled us to control for any systemic effects that the P450-expressing tumor might have on liver P450 metabolism. In the absence of drug treatment, 9L/2B6/reductase tumors grew at a somewhat slower rate than 9L/pBabe tumors, as had been observed for the cells in culture (Fig. 9, B versus A). Mice were treated with CPA given as two daily i.p. injections at 150 μg/kg body weight on days 26 and 27 after tumor implantation, at which time the tumors were well established in all of the mice. This initial round of drug treatment resulted in no growth delay for the 9L/pBabe tumors compared to saline-treated controls (Fig. 9A, ΔCPA versus +CPA). In contrast, growth of the 9L/2B6/reductase tumors in the same mice was fully blocked by CPA treatment (Fig. 9B). Administration of a second cycle of CPA treatment (~3.5 weeks after the first cycle (Fig. 9, arrows) effected a modest growth delay effect against 9L/pBabe control tumors, whereas it prolonged the strong antimetastatic effect seen in the 9L/2B6/reductase tumors until at least day 75 after tumor inoculation.

In an experiment designed to evaluate the impact of CYP2C18-Met gene therapy, CPA treatment effected a modest growth delay effect (~5–6 days) toward the 9L/pBabe control tumors, which likely is a consequence of liver P450-catalyzed drug activation (Fig. 9C). In contrast, a significant, albeit incomplete growth inhibition of the 9L/2C18-Met/reductase tumors was observed (growth delay of at least 25 days; Fig. 9D). This effect is especially striking when taken in the context of the very low level of P450 protein expression in the 9L/2C18-Met tumor cells (Table 1).

**DISCUSSION**

The primary goals of this study were: (a) to extend earlier preclinical cancer gene therapy studies based on the rat CYP2B1 gene; (b) to identify human P450 genes that serve as suitable candidates for P450-based prodrug activation/cancer gene therapy; (c) to assess the extent to which an improvement in the efficiency of intratumoral drug activation catalyzed by human P450s can be achieved by cotransfer of the P450 reductase gene; and (d) to determine whether CPA and IFA both exhibit bystander cytotoxic effects when activated within tumor cells by human P450 enzymes. Transduction of the rat gliosarcoma cell line 9L with replication-defective retroviral particles encoding each of six human P450 genes was found to chemosensitize the tumor cells to the cytotoxic effects of CPA, albeit with different apparent efficiencies (2B6 > 2C18-Met > 3A4 > others). In the case of IFA, CYP3A4 was the most effective in chemosensitizing the target tumor cells, followed by CYPs 2B6 and 2C18-Met. A substantial further improvement in chemosensitivity toward CPA and IFA was obtained by transduction of P450-expressing tumor cells with the P450 reductase gene. This led to a striking growth delay (>50 days) following CPA treatment of 2B6- plus P450 reductase-transduced tumors grown in vivo in scid mice, in which complications associated with immune rejection (44) and immunological contributions to bystander cytotoxicity (13) are avoided. A significant, albeit less dramatic, CPA growth delay (>25 days) was seen with 9L/2C18-Met/reductase tumors, despite their very low P450 expression level. The human CYP genes 2B6 and 2C18-Met are, thus, strong candidates for further preclinical evaluation for CPA-based P450 gene therapy.

The six human CYP genes investigated in this study were expressed in the transduced clonal 9L tumor cell lines at widely different protein levels (>0.3 to 20–25 pmol of P450 protein/mg of microsomal protein). Although P450 protein levels and CPA cytotoxicity were somewhat lower in the corresponding initial pools of transduced tumor cells, the rank order of P450 protein expression level was similar (2B6 > 2C9 > 2C8 ~ 2C19 > 2C18 and 3A4; data not shown). This suggests that the large differences in P450 protein levels in the isolated clonal cell lines do not primarily reflect clonal differences in the site of retroviral integration. Rather, because all six P450 genes were transcribed from the same long terminal repeat retroviral promoter, the observed differences in P450 protein expression levels are more likely to be due to differences in the stabilities of the individual P450 proteins and their mRNAs. Conceivably, some of the differences in expression could also reflect differential toxic effects that some of the P450 forms may have on the gliosarcoma recipient cell. Of note, clonal 9L cell lines selected for elevated expression of CYP3A4 consistently exhibited slower growth rates in cell culture compared to the initial pool of retrovirally infected cells (data not shown). CYP2B6 was expressed at the highest level, and correspond-
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(Table 1) and given the intrinsic lower cytotoxicity of activated IFA toward 9L tumor cells compared to activated CPA (Fig. 6). In the case of CYP3A4, the lower level of tumor cell P450 protein may, in part, reflect toxicity of the CYP3A4 gene product to the 9L cells, as suggested by the slower growth rate that was repeatedly obtained upon retroviral transduction with CYP3A4, both in retroviral pools and in individual clones, as noted above. This apparently toxicity of CYP3A4 might be circumvented by use of an inducible promoter (49, 50), thereby augmenting intratumoral expression of CYP3A4 in the context of IFA-based gene therapy. However, CYP3A4 is not likely to exhibit toxicity toward all tumor cells because slower cell growth has not been described in other mammalian CYP3A4 expression systems (51).

Although 4-hydroxy-CPA and 4-hydroxy-IFA yield distinct DNA-cross-links (five versus seven atoms in the cross-link, respectively), the two drugs are presumed to act via similar mechanisms in eliciting apoptosis. Nevertheless, 9L gliosarcoma cells were found to be ~3-fold more sensitive to activated CPA compared to activated IFA. This

CYP2B6 conferred on the 9L tumor cells the greatest CPA sensitivity. CYP2B6 is thus the gene of choice for CPA-based cancer gene therapy, both in terms of the apparent greater protein/mRNA stability suggested for CYP2B6 by the current study and for its high inherent catalytic activity for CPA 4-hydroxylation and its low rate of CPA deactivation by N-dechloroethylation. However, CYP2C18-Met may also be a useful candidate for CPA-based cancer gene therapy, given the high chemosensitization achieved in CYP2C18-Met-expressing cells in the context of very low expressed P450 protein levels. Higher levels of CYP2C18 protein expression may be achievable using improved retroviral or other vectors, including nonviral vectors for gene delivery presently being developed (45–48).

In contrast to the effective intratumoral activation of CPA by a broad range of P450s, intratumoral IFA activation was catalyzed by a more restricted subset of the human P450 genes investigated. As anticipated from studies using expressed P450 cDNAs and human liver microsomes (24, 35), CYP3A4 was the most potent activator of IFA, followed by CYP2B6 and CYP2C18-Met, the only CYP2C subfamily member able to sensitize 9L tumor cells toward IFA at 1 mM. Some IFA chemosensitivity was observed with the other CYP2Cs at higher IFA concentrations (data not shown). The chemosensitization to IFA conferred by CYP3A4 (Fig. 3C) is striking, given the much lower level of expression of this P450 compared to CYP2B6

9L Tumor Cell lines

![Fig. 7. Semicarbazide trapping/fluorescence assay for 4-hydroxy metabolites in cell culture. Each of the indicated 9L cell lines was seeded on a 48-well plate in duplicate at 2 × 10⁴ cells/well. Twenty-four h later, 2 mM CPA (□) or 2 mM IFA (■) was added to the cells together with 5 mM semicarbazide (final concentration) in a volume of 1 ml. The culture medium was analyzed for 4-hydroxy-CPA or 4-hydroxy-IFA 24 h after drug addition. Data shown is normalized to the total cell number in each well, as described in “Materials and Methods.” Background fluorescence observed in culture medium from 9L/Babe cell controls was subtracted from each sample.](image)

![Fig. 8. Bystander cytotoxicity of 9L/2B6/reductase and 9L/2B6 cells. A, bystander cytotoxicity of 9L/2B6/reductase cells is compared to that of 9L/Babe cells. In both cases, 9L/pBabe served as the bystander cells. 9L/pBabe cells were plated in duplicate at 10⁴ cells/well of a 6-well plate (lower culture chamber). 9L/2B6/reductase cells or 9L/2B6 cells were plated in 25-mm cell culture inserts (upper chamber) at concentrations ranging from 10⁴ to 6 × 10⁵ cells, corresponding to ratios of 0.1 to 6 relative to the bystander 9L/pBabe cells growing in the lower chamber, as shown on the X axis. Cells were incubated in a total volume of 3 ml DMEM, 10% FBS, containing 1 mM CPA, final CPA concentration. B, bystander cytotoxicity of 9L/2B6/reductase cells toward 9L/pBabe cells with CPA compared to the bystander cytotoxicity with IFA as prodrug. The experimental design was the same as in A, except that the final drug concentration was 0.67 mM. Data shown for both panels correspond to the survival of 9L/pBabe bystander cells in the lower chamber.](image)

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Fig. 9. Tumor growth delay assay in scid mice bearing 9L/pBabe, 9L/2B6/reductase and 9L/2C18-Met/reductase solid tumors. Shown are the effects of CPA treatment on growth of 9L/pBabe tumors growing on the left flank of scid mice that also bear either a 9L/2B6/reductase tumor (A) or a 9L/2C18-Met/reductase tumor (C) on the right flank. Also shown are the effects of CPA on growth of 9L/2B6/reductase (B) or 9L/2C18-Met/reductase tumors (D) in these same mice. Tumor areas were measured twice a week with Vernier calipers. Arrows (X axis), days on which CPA was given by i.p. injection at 150 mg/kg, as described under “Materials and Methods.” Data points, mean tumor areas (mm²; n = 4 tumors per treatment group); bars, SE. Mice not treated with CPA died earlier than the CPA-treated mice, as indicated by the earlier decrease in number of remaining mice from n = 4 (initial number of mice/group) to n = 3, 2, or 1. O, CPA-treated mice; □, saline controls.

differential cytotoxic effect was most evident at lower drug concentrations, which may be particularly relevant to the clinical situation. IFA is known to be activated by hepatic P450s less efficiently than CPA and is also more extensively deactivated by the N-dechloroethylation pathway (22, 25, 52). Although the N-dechloroethylation by-product chloroacetaldehyde is generally not considered to be an active, chemotherapeutic metabolite when it is generated via hepatic P450 metabolism, it may nevertheless contribute to IFA cytotoxicity when it is generated within the target tumor cell in situ, in the context of P450-based cancer gene therapy. This possibility is suggested for CYP2B6-expressing tumor cells, which were sensitized to both CPA and IFA cytotoxicity, despite the fact that CYP2B6 metabolizes IFA primarily via the N-dechloroethylation pathway to yield chloroacetaldehyde rather than by 4-hydroxylation, the major CYP2B6-catalyzed metabolic route for CPA. Important differences between CYP2B6-catalyzed CPA and IFA metabolism were also evident from coculture experiments, in which CPA could mediate a striking bystander cytotoxic effect but IFA could not. This intriguing finding may relate to the lower intrinsic cytotoxicity of 4-hydroxy-IFA compared to 4-hydroxy-CPA in this tumor cell line, coupled with the fact that CYP2B6 primarily metabolizes IFA via an N-dechloroethylation pathway that generates chloroacetaldehyde, which likely contributes to the 9L/2B6 cell’s chemosensitivity in these studies but may confer little or no bystander cytotoxicity. Because the bystander effect provides an important mechanism to enhance the effectiveness of prodrug activation-based cancer gene therapy by compensating for gene transfer efficiencies that may be well below 100% (5), IFA-based P450 gene therapy is not likely to be as effective using CYP2B6 and will require a more active catalyst of IFA 4-hydroxylation, such as CYP3A4.

Coexpression of rat P450 reductase with rat CYP2B1 leads to enhanced CPA and IFA chemosensitivity (38). This finding was unexpected, in view of the comparatively high level of endogenous P450 reductase in 9L gliosarcoma cells and the low absolute level of P450 protein that is expressed. Large increases in tumor cell sensitivity to CPA and IFA were also seen in this study following transduction of P450 reductase, not only with CYP2B6-expressing tumor cells but also with 9L/CYP2C18-Met cells, which have a very low expressed P450 protein level (Table 1), corresponding to a >50-fold molar excess of P450 reductase. The increased chemosensitivity of 9L/P450 cells transduced with a P450 reductase gene was paralleled by an enhanced accumulation of active metabolites for both CPA and IFA (Fig. 7) and by a substantial increase in bystander cytotoxicity (Fig. 8A). P450 reductase gene transfer in the absence of P450 expression did not confer any chemosensitivity to CPA or IFA, confirming the P450 dependence of these enhanced cytotoxic responses. Given the clear advantages of transducing tumor cells with P450 reductase in combination with P450, bioreductive prodrugs that are activated by P450 reductase under hypoxic conditions may readily be added to a combination chemotherapy/gene therapy regimen that includes a P450-activated prodrug. Examples of such bioreductive prodrugs include mitomycin C and various other quinones, nitroimida- zoles, heterocyclic N-oxides, and bioreducible DNA alkylators (53, 54). Although this strategy might seem counterintuitive, given the requirement of O₂ for P450-catalyzed monooxygenase reactions, recent studies have shown no significant loss of P450-dependent prodrug
activation in P450-transduced tumor cells grown under hypoxic conditions. This, in turn, suggests that hypoxia response elements (55) may be useful for transcriptional targeting (56) of P450 in combination with P450 reductase to solid tumors, which are characterized by a localized hypoxic environment (57).

Of the four human CYP2C gene products, CYP2C18 is the most active catalyst of CPA and IFA 4-hydroxylation (35), and in this study, CYP2C18 was the most active in chemosensitizing 9L gliosarcoma cells to both CPA and IFA. Of the two CYP2C18 alleles studied, CYP2C18-Met was particularly active, despite its very low level of expression, as noted above. Indeed, CYP2C18-Met mediated a 9L cell killing effect that is comparable to that seen in CYP3A4-expressing tumor cells, in which the P450 protein level was several-fold higher. This observation has important implications both for the potential utility of CYP2C18-Met for CPA-based cancer gene therapy and with respect to its role in CPA and IFA activation in human liver tissue. Whereas CYP3A4 is the most abundant P450 in a typical human liver sample, CYP2C18 is in much lower abundance (58, 59).

Although CYP2C18 is an active CPA and IFA 4-hydroxylase when assayed in a yeast cDNA expression system (35), the proposed direct involvement of this or other specific human CYP 2Cs in oxazaphosphorine metabolism in human liver (24, 60) cannot be clearly established, owing to the high cross-reactivity among the human P450 2C proteins and the absence of P450 2C form-specific inhibitory probes. Nevertheless, the present demonstration that all four CYP 2C members can sensitize 9L tumor cells to CPA provides further support for the proposal that, in aggregate, these enzymes contribute significantly to the metabolism of CPA in human liver tissue. Interestingly, CYP2C18-Thr, though just detectable when retrovirally expressed in 9L tumor cells, conferred sensitivity to CPA, but not to IFA up to 1 μM. The two CYP2C18 allelic variants differ at a single amino acid (Met versus Thr465) and exhibit clear kinetic differences using CPA and IFA as substrates (35), implicating residue 385 as an important determinant of the specificity of CYP2C18 toward these isomeric prodrug substrates. Clinically, this observation could be important when using IFA, in view of the much more rapid activation of IFA by CYP2C18-Met compared to CYP2C18-Thr, and given that the 2C18-Met allele occurs in ~30% of the Caucasian population, whereas the 2C18-Thr allele is more abundant, present in ~70% of the population (61).

CYP2C9 is the most abundant CYP 2C enzyme expressed in human liver (58) and can activate CPA, and at a 3-fold lower catalytic efficiency, IFA in a yeast expression system (CYP2C9-Cys444 allele; Ref. 35). The CYP2C9-selective inhibitor sulfaphenazole has been reported to slow the elimination of CPA in a subset of cancer patients (62), suggesting a role for CYP2C9 in hepatic metabolism of this drug. Consistent with these observations is our finding that CYP2C9 can sensitize 9L tumor cells to CPA, albeit with much lower efficiency than CYP2C18 and CYP2C19, particularly when the several-fold higher level 9L CYP2C9 protein expression is taken into account. Although CYP2C9 is, thus, not the gene of choice for CPA or IFA-based P450 gene therapy, this and other P450 genes (28) may prove useful when combined with other cancer chemotherapeutic prodrugs (2).

The use of retroviruses and other viral vectors for delivery of therapeutic genes to tumor cells has become increasingly feasible with recent improvements in vector design (45, 63, 64). Nonviral vectors, including cationic liposomes, DNA-protein complexes, nonviral T7 autogene vectors, and other approaches (47, 48, 65) have also undergone significant development and improvement in recent years. Further enhancement of P450/P450 reductase gene delivery may be achieved by linking these genes using an internal ribosome entry site sequence (66) to achieve their coordinate expression on a bicistronic message. Alternatively, construction of a fusion gene which encodes a catalytically active P450/P450 reductase fusion protein (67) may allow for highly efficient expression of P450 activity. Limitations associated with comparatively low retroviral titers could, in part, be overcome by the use of more powerful promoters to ensure a high level of gene expression, coupled with the use of drugs, such as CPA, that exhibit a strong bystander cytotoxic effect. Innovative strategies to achieve tumor-selective gene expression, including transcriptional targeting, cellular targeting, and selective delivery in situ of both the prodrug and the therapeutic gene have been described (68-70). Further development of CYP2B6, CYP2C18-Met, and other P450 genes for prodrug activation cancer gene therapy will be directed at the implementation of these and other approaches to enhance the enzymatic activity, tumor cell chemosensitization, and targeting specificity of P450/P450-reductase-based gene therapy.

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Youssef Jounaidi, Jodi E. D. Hecht and David J. Waxman


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