

## ENANTIOSELECTIVE METABOLISM AND CYTOTOXICITY OF *R*-IFOSFAMIDE AND *S*-IFOSFAMIDE BY TUMOR CELL-EXPRESSED CYTOCHROMES P450

Chong-Sheng Chen, Youssef Jounaidi, and David J. Waxman

*Division of Cell and Molecular Biology, Department of Biology, Boston University, Boston, Massachusetts*

Received March 22, 2005; accepted May 20, 2005

### ABSTRACT:

The anticancer prodrug ifosfamide (IFA) contains a chiral phosphorous atom and is administered in the clinic as a racemic mixture of *R*-IFA and *S*-IFA. Hepatic cytochrome P450 (P450) enzymes exhibit enantioselective preferences in the metabolism of *R*-IFA and *S*-IFA; however, the impact of this selectivity on P450-dependent anticancer activity is not known. Presently, the metabolism and cytotoxicity of *R*-IFA and *S*-IFA were determined in 9L gliosarcoma and Chinese hamster ovary tumor cells expressing an IFA-activating P450 enzyme and by *in vitro* steady-state kinetic analysis using cDNA-expressed P450 enzymes. Tumor cells expressing P450 enzyme CYP3A4 were the most sensitive to *R*-IFA cytotoxicity, whereas tumor cells expressing CYP2B1 or CYP2B6 were most sensitive to cyclophosphamide (CPA), an isomer of IFA. Correspondingly, CYP3A4-expressing cells and cDNA-expressed

CYP3A4 metabolized *R*-IFA to yield the active, 4-hydroxylated metabolite at a 2- to 3-fold higher rate than they metabolized *S*-IFA or CPA. CYP2B cells and cDNA-expressed CYP2B enzymes metabolized CPA almost exclusively by 4-hydroxylation, whereas *R*-IFA and *S*-IFA were substantially converted to inactive, *N*-dechloroethylated metabolites. Further investigation revealed that CYP3A1, a rat enzyme, exhibited superior kinetic properties compared with the human enzyme CYP3A4, with *R*-IFA and *S*-IFA both metabolized with high catalytic efficiency by 4-hydroxylation and with a  $K_m$  value of 200  $\mu$ M,  $\sim$ 5-fold lower than CYP3A4. Based on these kinetic parameters and metabolic profiles, *R*-IFA is expected to exert greater anticancer activity than *S*-IFA or CPA against tumors that express CYP3A enzymes, whereas tumors expressing CYP2B enzymes may be more sensitive to CPA treatment.

Ifosfamide (IFA) and its structural isomer cyclophosphamide (CPA) are clinically effective anticancer alkylating agent prodrugs used to treat a broad range of neoplasms. Both prodrugs are activated in the liver by a cytochrome P450-catalyzed 4-hydroxylation reaction. The primary 4-hydroxy metabolite exists in equilibrium with the ring-opened aldo(iso)phosphamide, which undergoes  $\beta$ -elimination to yield the therapeutically active, DNA cross-linking metabolite (iso)phosphoramidate mustard and acrolein, a reactive aldehyde (Sladek, 1988; Zalupski and Baker, 1988). An alternative, P450-catalyzed *N*-dechloroethylation reaction inactivates the parent drug and generates the neurotoxic and nephrotoxic byproduct chloroacetaldehyde (CAA). Although CPA and IFA are very similar in chemical structure and mechanism of action, they differ strikingly with respect to their pharmacokinetic and pharmacodynamic properties. CPA is metabolized predominantly by 4-hydroxylation, catalyzed by CYP2B and CYP2C enzymes, whereas IFA undergoes extensive *N*-dechloroethylation, catalyzed by CYP2B and CYP3A enzymes (Huang and Waxman, 1999; Roy et al., 1999b; Chen et al., 2004). The formation of CAA as a major byproduct of IFA *N*-dechloroethylation is associated with the neurotoxicity and urotoxicity seen in patients treated with IFA but not in the case of CPA. These side effects can be

controlled by administration of protective agents such as sodium 2-mercaptoethanesulfonate (Woodland et al., 2000).

IFA is a chiral molecule that contains an asymmetric phosphorus atom and exists in two enantiomeric forms, *R*-IFA and *S*-IFA. Studies in experimental animal models and human clinical trials indicate that the racemic IFA used in the clinic is subject to enantioselective metabolism, which may lead to distinct efficacies and toxicity profiles for each enantiomer (Boos et al., 1991; Williams and Wainer, 1999). In clinical studies, *S*-IFA was found to be metabolized more rapidly than *R*-IFA, resulting in a shorter half-life, a higher clearance, and a markedly lower recovery in urine compared with *R*-IFA (Corlett et al., 1995; Wainer et al., 1996).

IFA *N*-dechloroethylation is primarily catalyzed by two major human liver P450 enzymes. CYP2B6 metabolizes *R*-IFA and *S*-IFA to produce *S*-N3-deCl-IFA and *S*-N2-deCl-IFA, respectively, whereas CYP3A4 metabolizes *R*-IFA to produce *R*-N2-deCl-IFA and *S*-IFA to yield *R*-N3-deCl-IFA (Granvil et al., 1999; Roy et al., 1999a). CYP3A4-dependent *R*-IFA 4-hydroxylation is typically more rapid than *S*-IFA 4-hydroxylation in human liver microsomes, consistent with the high level of CYP3A4 in human liver (Roy et al., 1999b) and in agreement with pharmacokinetic studies in patients (Williams and Wainer, 1999). *R*-IFA and *S*-IFA can be purified using enantioselective chromatography, and their application to clinical chemotherapy has been studied (Wainer, 1993; Wainer and Granvil, 1993). However, characterization of *R*-IFA and *S*-IFA metabolism as it impacts the antitumor activity of each enantiomer at the cellular level has not been investigated.

Supported in part by National Institutes of Health Grant CA49248 (to D.J.W.).  
Article, publication date, and citation information can be found at  
<http://dmd.aspetjournals.org>.  
doi:10.1124/dmd.105.004788.

**ABBREVIATIONS:** IFA, ifosfamide; CPA, cyclophosphamide; P450, cytochrome P450; CAA, chloroacetaldehyde; deCl, *N*-dechloroethylation; P450R, P450 reductase; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; IRES, internal ribosome entry site; CHO, Chinese hamster ovary; MEM, minimal essential medium; HPLC, high-performance liquid chromatography.

Presently, we investigate the metabolism and cytotoxicity of *R*-IFA and *S*-IFA using tumor cells engineered to express individual CYP2B and CYP3A enzymes in combination with the flavoenzyme P450 reductase (P450R). Our findings reveal that *R*-IFA displays greater cytotoxicity than either *S*-IFA or CPA toward CYP3A4-expressing tumor cells, whereas *S*-IFA displays greater activity against CYP2B-expressing tumor cells. Moreover, *in vitro* studies using cDNA-expressed P450s identify the rat enzyme CYP3A1 as a superior catalyst of prodrug activation with both *R*-IFA and *S*-IFA as substrates, suggesting the utility of this P450 enzyme for gene-directed enzyme prodrug therapy involving IFA (Chen and Waxman, 2002). These findings are discussed with respect to the impact of tumor cell P450 expression on antitumor activity.

### Materials and Methods

**Materials.** CPA was purchased from Sigma-Aldrich (St. Louis, MO). CAA in the form of a 50% (w/v) solution in water, 3-aminophenol, semicarbazide, and fetal bovine serum (FBS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). IFA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Purified *R*-IFA and *S*-IFA were provided by Dr. Ben Skead of Cell Tech, Ltd. (Cambridge, UK). 4-OOH-CPA and 4-OOH-IFA were provided by Dr. Ulf Niemeyer (Baxter Oncology GmbH, Frankfurt, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). Supersomes containing expressed CYP2B and CYP3A enzymes, with coexpression of P450R and cytochrome *b*<sub>5</sub>, were purchased from BD Gentest Corp. (Woburn, MA). Data were obtained using the following lot numbers for each Supersome P450 preparation: 2B1, lot 5 and lot 7; 2B6, lot 8; 3A1, lot 6; 3A2, lot 6; 3A4, lot 50; 3A7, lot 1; and 3A12, lot 2.

**9L Cell Lines Coexpressing P450 and P450R cDNAs.** The retroviral plasmid pBabe-puro, which encodes a puromycin resistance gene, was used to clone CYP2B1 and CYP2B6 cDNAs, linked via an IRES sequence to human P450R cDNA, to give a bicistronic CYP2B-IRES-P450R expression cassette as described (Jounaidi and Waxman, 2004). The CYP2B6 cDNA used in these studies (GenBank accession no. M29874) corresponds to the wild-type (*CYP2B6\**) allele, i.e., Arg22/Lys139/Gln172/Ser259/Lys262/Arg487 (Lang et al., 2001). Transfection of the ecotropic packaging cell line Bosc 23 with the retroviral plasmids pBabe-CYP2B1-IRES-P450R-puro and pBabe-CYP2B6-IRES-P450R-puro, harvesting of retroviral supernatants, and infection of rat 9L gliosarcoma cells were carried out as described (Jounaidi et al., 1998), with the following modifications. Bosc 23 cells were plated at  $2.5 \times 10^6$  cells in a 60-mm dish. Fresh culture medium (4 ml) containing chloroquine (25  $\mu$ M) was added to the cells 16 h later. The cells were cotransfected 1 h later with 5  $\mu$ g of retroviral plasmid DNA and 2.5  $\mu$ g of the helper plasmid pKat (Finer et al., 1994) using 9  $\mu$ l of the cationic liposome Fugene 6 (Roche Diagnostics, Indianapolis, IN) in a total volume of 40  $\mu$ l of DMEM without FBS. Pools of 9L cells resistant to 2.5  $\mu$ g/ml puromycin and expressing P450R in combination with CYP2B1 (9L/2B1-P450R cells) or CYP2B6 (9L/2B6-P450R cells) were selected beginning 48 h later over a 2- to 3-day period (Jounaidi and Waxman, 2004). 9L cells infected with pBabe-pyro retrovirus, without a P450-IRES-P450R insert, are termed 9L/pBABE cells and were used as a control. The tumor cell line CHO, engineered to express human P450R alone (CHO/P450R cells) or P450R together with CYP3A4 (CHO/3A4-P450R cells), was that used previously (Lu and Waxman, 2005) and was kindly provided by Dr. Thomas H. Friedberg, Biomedical Research Centre, University of Dundee, Dundee, UK (clonal cell line DHR/3A4) (Ding et al., 1997). The 9L and CHO tumor cells used in this study exhibit similar intrinsic sensitivities to activated CPA and IFA (see Table 1). Moreover, similar levels of P450 are expressed in the 9L/2B-P450R cells (Jounaidi et al., 1998) and in the CHO/3A4-P450R cells (Ding et al., 1997).

**Cytotoxicity Assays.** The chemosensitivity of P450-expressing 9L and CHO cells was determined using a 4-day growth inhibition assay. Cells were plated in triplicate at 4000 cells per well of a 48-well plate. After 18 to 24 h of growth, cells were treated for 4 days with *R*-IFA, *S*-IFA, or CPA at concentrations indicated in each experiment. The same 4-day growth inhibition assay was used to evaluate the intrinsic sensitivity of the cells to CAA, a

TABLE 1

*Cytotoxicity of anticancer prodrugs and their metabolites toward P450-expressing 9L and CHO cells*

Data are mean values based on two independent sets of 4-day cytotoxicity assays for each cell line. EC<sub>50</sub> values were calculated using the sigmoidal dose-response (variable slope) method of GraphPad Prism software.

	EC <sub>50</sub>				
	9L/pBabe	CHO/P450R	9L/2B1-P450R	9L/2B6-P450R	CHO/3A4-P450R
	$\mu$ M				
CPA	N.D. <sup>a</sup>	N.D.	54.8	105.4	88.7
<i>R</i> -IFA	N.D.	N.D.	294.8	220.1	57.9
<i>S</i> -IFA	N.D.	N.D.	91.9	110.6	125.6
4OOH-CPA	3.0	3.2	— <sup>b</sup>	—	—
4OOH-IFA	5.1	5.0	—	—	—
CAA	3.2	2.5	—	—	—

<sup>a</sup> N.D., not detectable, indicating <20% toxicity observed at the highest drug concentration tested.

<sup>b</sup> —, not investigated.

byproduct of IFA and CPA *N*-dechloroethylation, and to the 4-OOH derivatives of IFA and CPA, which decompose in aqueous medium to yield 4-OH-IFA and 4-OH-CPA, respectively. Cells were stained using a crystal violet/alcohol extraction assay and quantified. Briefly, culture dishes were washed once with phosphate-buffered saline, stained with 1.25 g of crystal violet in 500 ml containing 50 ml of 37% formaldehyde and 450 ml of methanol, and then washed with tap water. A<sub>595</sub> values were determined using a microtiter plate reader. Background absorbance measured for wells containing culture medium alone was subtracted from each value. Data are presented as mean cell number relative to drug-free controls, with 100% growth inhibition corresponding to 0 net A<sub>595</sub> and 0% growth inhibition corresponding to the A<sub>595</sub> of the drug-free control cells.

**Assay for 4-OH-CPA, 4-OH-IFA, and CAA Released into Culture Medium.** 9L and CHO cell lines were seeded in 12-well plates in duplicate at  $1 \times 10^5$  cells/well in 2 ml of DMEM containing 10% FBS (9L cells) or in 2 ml of MEM containing 10% dialyzed FBS (CHO cells). Drugs were added to the cells in 2 ml of DMEM (9L cells) or MEM (CHO cells) without FBS about 20 h later. Cells were cultured for an additional 4 h, at which time duplicate aliquots (200  $\mu$ l) of culture medium were removed. One aliquot was immediately derivatized to 1-N<sup>6</sup>-ethenoadenosine by treatment with 20  $\mu$ l of 250 mM adenosine and 30  $\mu$ l of 2 M sodium acetate solution (pH 4), and then heated at 80°C for 2.5 h (CAA determination) (Huang and Waxman, 1999). The other aliquot was immediately derivatized to 7-hydroxyquinoline by treatment for 25 min with 100  $\mu$ l of freshly prepared fluorescent reagent (60 mg of 3-aminophenol and 60 mg of hydroxylamine-HCl in 10 ml of 1 N HCl) at 90°C (Bohnstengel et al., 1997). In some cases, the derivatized samples were stored at -20°C in the dark prior to HPLC analysis.

Standard curves were generated using CAA (0–5  $\mu$ M) and 4-OOH-CPA (0–50  $\mu$ M). Standards were incubated for 2 h with  $1 \times 10^5$  P450-deficient 9L or CHO cells in 2 ml of DMEM (pH 7.2)/well of a 12-well plate to correct for time- and cell-dependent degradation of the metabolites. The 2-h time point corresponds to the midpoint of the standard cell culture metabolite formation assay (4 h; see below) and serves as an estimate of the average time during which metabolites formed over the course of the 4-h incubation period are subject to time-dependent and/or cell-dependent degradation in culture medium. The recovery of acrolein following a 2-h incubation of 4-OOH-CPA in culture medium was  $76 \pm 6\%$  ( $n = 2$ ) (incubation with 9L/pBabe cells) and  $89 \pm 10\%$  ( $n = 4$ ) (incubation without cells). The recovery of CAA following a 2-h incubation with and without 9L/pBabe cells was  $63 \pm 15\%$  ( $n = 3$ ) and  $95 \pm 8\%$  ( $n = 3$ ), respectively, indicating cell-dependent degradation of CAA. The accumulation of 4-OH-IFA in culture medium of 9L/2B1-P450R cells was linear with incubation times up to 8 h, with a slope of 0.151 nmol/ml/h per  $1 \times 10^5$  cells seeded/well. CAA accumulation in the culture medium was linear for ~2 h (0.164 nmol/ml/h per  $1 \times 10^5$  cells seeded/well), after which the rate of accumulation slowed down, consistent with the 9L cell-dependent metabolism of CAA noted above. The yields of 4-OH-IFA and CAA increased linearly with 9L/2B1-P450R cell number, with slopes of  $1.37 \pm 0.03$  (4-OH-IFA) and  $0.97 \pm 0.03$  (CAA) nmol of metabolite/ml of medium/ $1 \times 10^5$  cells seeded/well. Crystal violet absorbance values (A<sub>595</sub>) were linearly correlated with cell

number from  $0.5 \times 10^5$  to  $6 \times 10^5$  9L cells/2 ml of culture medium per well of a 12-well plate. The limit of detection in culture medium (twice the background value) was  $0.15 \mu\text{M}$  for CAA and  $0.5 \mu\text{M}$  for acrolein.

**Metabolism by cDNA-Expressed P450s.** Supersomes containing baculovirus-expressed P450s (4 pmol of P450) were added to  $75 \mu\text{l}$  of 100 mM KPi buffer pH 7.4 containing various concentrations of *R*-IFA, *S*-IFA, or CPA (50  $\mu\text{M}$  to 10 mM, final substrate concentration). Assay mixtures minus NADPH were preincubated for 4 min at  $37^\circ\text{C}$ . Each reaction was initiated by addition of NADPH to 1 mM (final volume of 100  $\mu\text{l}$ , containing 75 mM KPi buffer, pH 7.34, 0.75 mM EDTA, and 1 mM NADPH). Reactions were incubated for 15 min at  $37^\circ\text{C}$  and then processed and derivatized for HPLC analysis (conversion of CAA to 1- $\text{N}^6$ -ethenoadenosine and conversion of acrolein to 7-hydroxyquinoline) (Chen et al., 2004).

**HPLC.** Analysis of CPA and IFA metabolites was carried out using a Luna  $5\text{-}\mu\text{m}$  C18 (2) HPLC column,  $150 \times 4.6$  mm (Phenomenex, Torrance, CA) (Chen et al., 2004). 7-Hydroxyquinoline was eluted with 17% methanol in 0.33% phosphoric acid at a flow rate of 1 ml/min ( $t_{\text{elution}} = 3.2$  min) and was detected by fluorescence excitation at 350 nm and emission at 515 nm. 1- $\text{N}^6$ -Ethenoadenosine was eluted with 17% methanol in water ( $t_{\text{elution}} = 7\text{--}8$  min for samples incubated with cells) or 13% methanol in water ( $t_{\text{elution}} = 8.7\text{--}10$  min for Supersomes incubations) at a flow rate of 1 ml/min and was detected by fluorescence excitation at 270 nm and emission at 411 nm. Quantification was based on integrated peak areas calculated using Millennium<sup>32</sup> software (Waters, Inc., Milford, MA).

**Data Analysis.** Data were managed using Microsoft Excel. Data based on metabolism in cell culture were corrected by subtraction of the higher of two measured background activities (culture medium from cells incubated without drugs, or from drugs incubated in medium without cells) and then converted to units of metabolite produced (nanomoles of 7-hydroxyquinoline or nanomoles of 1- $\text{N}^6$ -ethenoadenosine) per  $A_{595}$  (crystal violet staining). Data obtained from in vitro metabolism assays using Supersomes were corrected by subtracting the highest of three independent background activity values: incubation without NADPH, incubation without drug, and incubation without Supersomes. Data were converted to units of nanomoles of metabolite produced per minute per nanomole of P450. Enzyme kinetic data were analyzed using Enzyme Kinetics v 0.44 software (Trinity Software, Inc., Boone, NC). Kinetic parameters ( $K_m$  and  $V_{\text{max}}$ ) for each individual experiment, typically based on  $n = 7$  substrate concentrations, were determined by the Eadie-Hofstee method. Mean values  $\pm$  S.D. ( $n = 3$ ) or mean values  $\pm$  half the range ( $n = 2$ ) were determined based on the indicated number of independent sets of assays.  $\text{EC}_{50}$  values, based on sigmoidal dose-response analysis using a variable slope, were determined using GraphPad Prism software (GraphPad Software, San Diego, CA).

## Results

**Metabolism of *R*-IFA, *S*-IFA, and CPA by P450-Expressing Cell Lines.** The metabolism of *R*-IFA, *S*-IFA, and CPA was assayed in five cell lines: 9L gliosarcoma cells expressing either rat CYP2B1 (9L/2B1-P450R cells) or human CYP2B6 (9L/2B6-P450R cells) in combination with P450R, the P450-deficient control cell line 9L/pBabe, CHO cells expressing human CYP3A4 with P450R (CHO/3A4-P450R cells), and a control cell line expressing P450R reductase alone (CHO/P450R cells). Cells were incubated with each prodrug substrate at two concentrations (200  $\mu\text{M}$  and 2 mM), and metabolites released into the culture medium were analyzed and quantified by HPLC. All three prodrugs were metabolized by the P450-expressing cell lines but not by the P450-deficient control cells (Fig. 1). CPA was primarily converted to the active, 4-OH metabolite by both CYP2B-expressing cell lines ( $\geq 98\%$  of total metabolism) (Fig. 1A). However, this pathway corresponded to only 24 to 44% of CPA metabolism in the case of CYP3A4 cells, where *N*-dechloroethylation (drug inactivation) to yield CAA was the major metabolic reaction (56–76% of total metabolism) (Fig. 1B). With *R*-IFA and *S*-IFA as substrates, *N*-dechloroethylation was the major metabolic pathway for both CYP2B cell lines (up to 74% of total metabolism by 9L/2B1-P450R cells and up to 94% of total metabolism by 9L/2B6-P450R cells). The

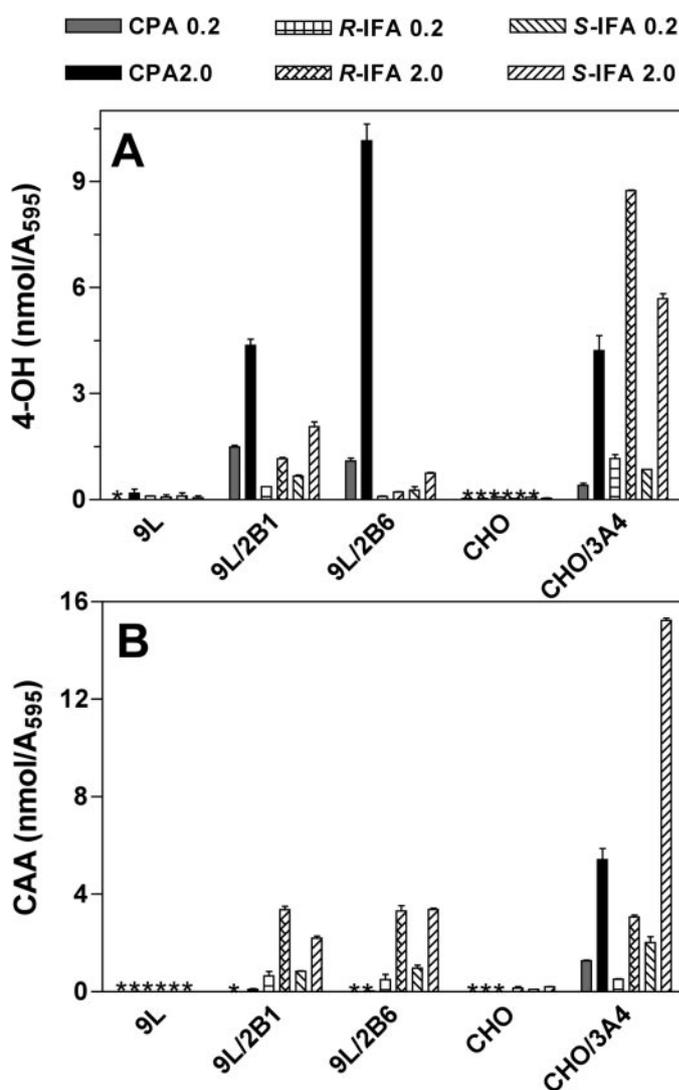


FIG. 1. Metabolism of CPA, *R*-IFA, and *S*-IFA by 9L/2B1-P450R, 9L/2B6-P450R, and CHO/3A4-P450R cells by 4-hydroxylation (A) and by *N*-dechloroethylation (B). Cell lines expressing the indicated P450s in combination with P450R were cultured in 12-well plates and incubated for 4 h with each prodrug substrate at 0.2 or 2.0 mM, followed by metabolite derivatization and HPLC analysis. Data were quantified by comparison to standard curves prepared using metabolite standards (4-OH-CPA or CAA) incubated with P450-deficient cells, as described under *Materials and Methods*. Data shown are rates of metabolite production over a 4-h period, expressed as nanomoles of metabolite per well, normalized for the cell density of each culture well ( $A_{595}$ ; crystal violet staining intensity) (mean values  $\pm$  half the range for duplicate determinations). 9L, 9L/pBabe cells; CHO, CHO/P450R cells; \*, metabolite is below limit of detection.

CYP3A4 cell line showed clear enantiomeric selectivity, with up to 74% of *R*-IFA metabolism proceeding via 4-hydroxylation, as compared with 27% in the case of *S*-IFA. Moreover, the rate of *R*-IFA 4-hydroxylation by CHO/3A4-P450R cells was 1.5-fold higher than *S*-IFA 4-hydroxylation at 2 mM substrate (Fig. 1A), whereas the rate of *N*-dechloroethylation was 80% lower for *R*-IFA than for *S*-IFA (Fig. 1B). Overall, the rates of IFA 4-hydroxylation were much higher in the CYP3A4 cells than in either CYP2B cell line.

**Cytotoxicity of *R*-IFA, *S*-IFA, and CPA.** The cytotoxicity of each prodrug was evaluated in each of the P450-expressing cell lines using a 4-day growth inhibition assay (Fig. 2). None of the prodrugs exerted significant cytotoxicity toward the control cell lines 9L/pBabe and CHO/P450R ("control" curves; Fig. 2), consistent with the inactivity of the parent prodrugs and the absence of oxazaphosphorine 4-hy-

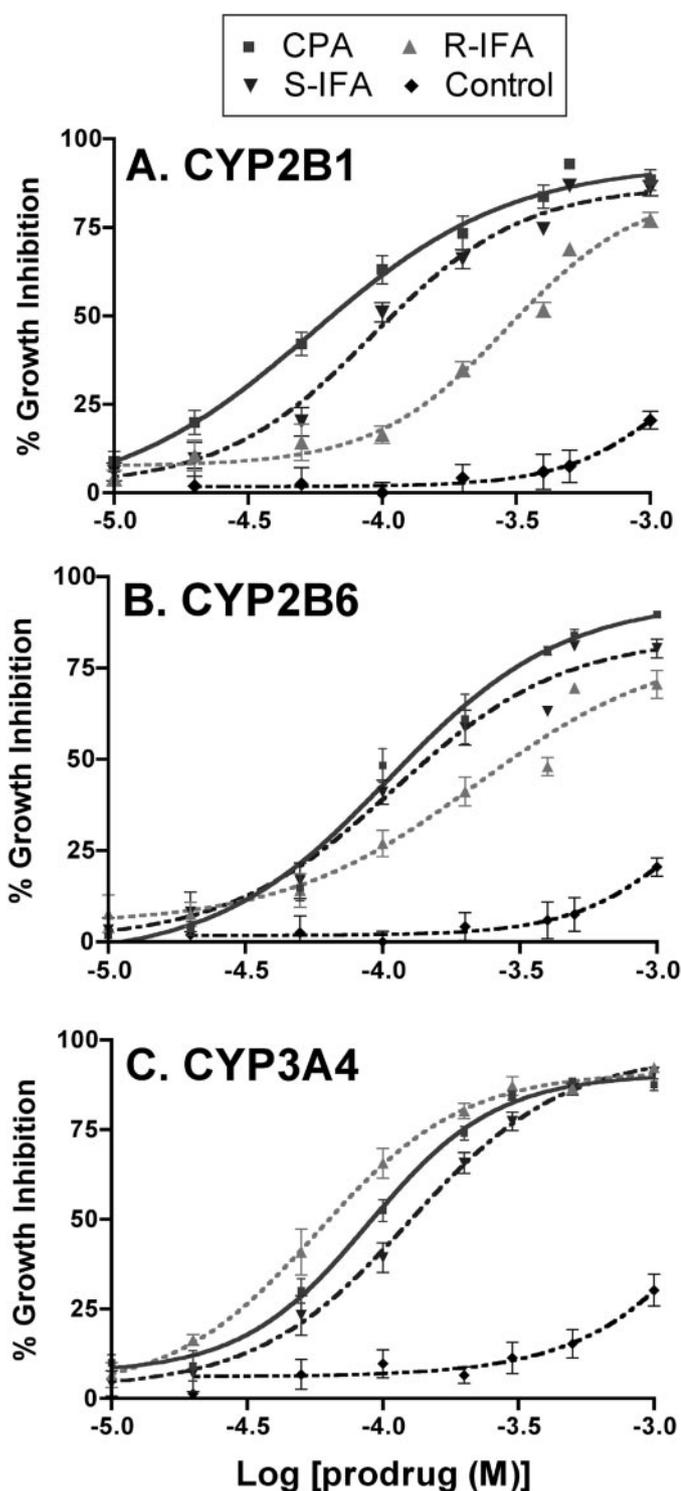


Fig. 2. Growth inhibitory effects of CPA, *R*-IFA, and *S*-IFA toward P450-expressing cell lines. The cytotoxicity of each prodrug was assayed in a 4-day growth inhibition assay carried out using 9L/2B1-P450R cells (A), 9L/2B6-P450R cells (B), and CHO/3A4-P450R cells (C) in comparison to a P450-deficient control cell line (control, 9L/pBabe for panels A and B and CHO/P450R for panel C; control data presented are mean  $\pm$  S.D. values for  $n = 3$  separate incubations carried out with each prodrug). Cells were seeded in 48-well plates at 4000 cells/well and treated with increasing concentrations of each prodrug (0 to 1 mM,  $n = 8$  concentrations, each done in triplicate) for 4 days as described under *Materials and Methods*. Data presented are mean  $\pm$  half-the-range values, with the growth inhibition of the drug-free controls set to 0 (duplicate experiments with  $n = 3$  replicates each).

TABLE 2

Steady-state kinetic analysis: CPA, *R*-IFA, and *S*-IFA metabolism by cDNA-expressed P450s 2B1, 2B6, and 3A4

Data shown are mean  $\pm$  S.D. values based on  $n = 3$  independent determinations.

P450	Prodrug	4-Hydroxylation		<i>N</i> -Dechloroethylation	
		$K_m$ mM	$V_{max}$ mol/min/mol P450	$K_m$ mM	$V_{max}$ mol/min/mol P450
2B1	CPA	0.40 $\pm$ 0.06	73.5 $\pm$ 7.5	0.55 $\pm$ 0.13	3.0 $\pm$ 0.7
	<i>R</i> -IFA	0.54 $\pm$ 0.10	19.0 $\pm$ 1.5	0.70 $\pm$ 0.22	43.6 $\pm$ 14.5
	<i>S</i> -IFA	0.56 $\pm$ 0.06	32.2 $\pm$ 1.4	0.69 $\pm$ 0.04	27.2 $\pm$ 6.4
2B6	CPA	1.33 $\pm$ 0.04	123.2 $\pm$ 5.5	N.D.	0
	<i>R</i> -IFA	2.24 $\pm$ 1.15	1.98 $\pm$ 0.6	1.42 $\pm$ 0.38	62.3 $\pm$ 3.8
	<i>S</i> -IFA	1.27 $\pm$ 0.24	17.1 $\pm$ 2.8	1.24 $\pm$ 0.20	58.3 $\pm$ 4.2
3A4	CPA	1.13 $\pm$ 0.22	31.9 $\pm$ 4.9	1.61 $\pm$ 0.22	94.2 $\pm$ 9.1
	<i>R</i> -IFA	0.98 $\pm$ 1.14	63.7 $\pm$ 7.2	1.10 $\pm$ 0.27	26.0 $\pm$ 5.7
	<i>S</i> -IFA	0.99 $\pm$ 0.24	25.0 $\pm$ 2.4	1.50 $\pm$ 0.30	125 $\pm$ 23

N.D., not determined due to low activity.

droxylase activity in the P450-deficient cells. By contrast, all three prodrugs induced dose-dependent killing of the three P450-expressing cell lines. The rank order of cytotoxicity based on  $EC_{50}$  values (Table 1) was CPA > *S*-IFA > *R*-IFA for the CYP2B1 and CYP2B6 cells (Fig. 2, A and B), and *R*-IFA > CPA > *S*-IFA for the CYP3A4 cells (Fig. 2C). Comparison of the activity profiles of each prodrug revealed that CPA was most cytotoxic (lowest  $EC_{50}$  value) when activated by CYP2B1 cells, whereas *R*-IFA was most active toward CYP3A4 cells. These findings are consistent with the high CPA 4-hydroxylase activity of the CYP2B1 and CYP2B6 cells and by the high *R*-IFA 4-hydroxylase activity of the CYP3A4 cells (Fig. 1). By contrast, the CYP3A4 cells primarily metabolized *S*-IFA and CPA by *N*-dechloroethylation (Fig. 1). Together, these findings indicate that *R*-IFA is likely to have a better activity profile than *S*-IFA or CPA when used to treat tumors that express CYP3A4, whereas *S*-IFA is likely to be more active than *R*-IFA against tumors that express CYP2B6.

We next examined the intrinsic sensitivity of 9L cells and CHO cells to the reactive metabolites CAA, 4-OH-CPA, and 4-OH-IFA, with the latter two compounds delivered to the cells as their 4-OOH derivatives (Table 1). 9L/pBabe and CHO/P450R cells both displayed high intrinsic sensitivity to 4-OH-CPA ( $EC_{50} \sim 3 \mu M$ ) and 4-OH-IFA ( $EC_{50} \sim 5 \mu M$ ). Moreover, CAA was at least as cytotoxic as 4-OH-IFA ( $EC_{50} \sim 2.5\text{--}3.2 \mu M$ ), suggesting that this reactive aldehyde may contribute to the cytotoxicity of IFA seen in P450 2B6 cells (Fig. 2B), which have very low IFA 4-hydroxylase activity (Fig. 1A).

**Kinetic Analysis of CPA and IFA Metabolism by cDNA-Expressed P450 Enzymes.** Next, we characterized the metabolic profile of the three oxazaphosphorine prodrugs using individual P450 enzymes expressed in a Baculovirus expression system ("Supersomes"). Steady-state kinetic analysis (Table 2; Fig. 3) confirmed that 4-hydroxylation was the predominant pathway of CPA metabolism catalyzed by both CYP2B enzymes. The alternative *N*-dechloroethylation pathway represented only  $\sim 4\%$  of CPA metabolism in the case of CYP2B1 and was undetectable with CYP2B6 (Fig. 3, B and C). In contrast, *R*-IFA and *S*-IFA were metabolized by CYP2B1 by both pathways, with 4-hydroxylation to *N*-dechloroethylation product ratios of  $\sim 1:2.2$  for *R*-IFA and  $1:0.8$  for *S*-IFA at  $V_{max}$ . In the case of CYP2B6, however, IFA 4-hydroxylation was substantially reduced, in particular, in the case of the *R*-enantiomer ( $\sim 3\%$  of total metabolism). Overall, CYP2B1 metabolized all three prodrugs with an apparent  $K_m$  value that was 2- to 4-fold lower than that of CYP2B6 for both metabolic pathways (Table 2). CYP2B1 and CYP2B6 were both substantially more active catalysts of CPA 4-hydroxylation than CYP3A4 (Fig. 3A and Table 2).

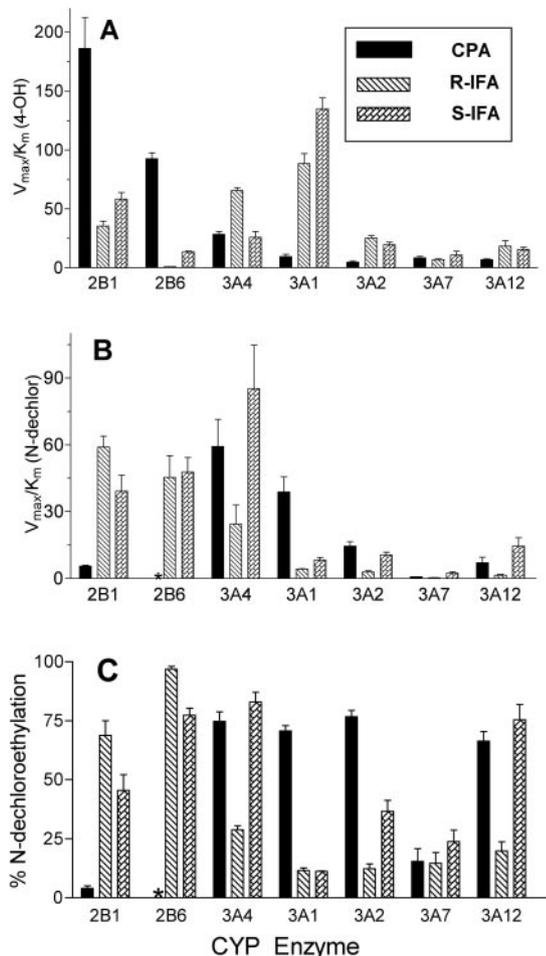


FIG. 3. Catalytic efficiencies for metabolism of CPA, *R*-IFA, and *S*-IFA by CYP2B and CYP3A enzymes and percent metabolism via *N*-dechloroethylation. Data shown are based on the steady-state kinetic analyses determined for the indicated seven cDNA-expressed P450 enzymes and summarized in Tables 2 and 3. Data shown are  $V_{max}/K_m$  values determined by Eadie-Hofstee analysis (mean  $\pm$  half-the-range for  $n = 2-3$  independent sets of determinations) for 4-hydroxylation (A) and *N*-dechloroethylation (B) of each prodrug substrate.  $V_{max}/K_m$  values are in units of mol of product/min/mol of P450/mM. C, percentage of total metabolism proceeding via *N*-dechloroethylation pathway, calculated based on the  $V_{max}$  values for each enzyme, and the metabolic profiles shown in Tables 2 and 3. \*, metabolite is below the limit of detection.

With CPA as substrate, CYP3A4 displayed the highest  $V_{max}$  value for *N*-dechloroethylation,  $\sim 30$ -fold greater than that of P450 2B1 (Table 2). By contrast, CYP3A4 metabolized *R*-IFA primarily by 4-hydroxylation, with *R*-IFA *N*-dechloroethylation corresponding to only 29% of total metabolism at  $V_{max}$ . Moreover, the  $V_{max}$  and catalytic efficiency ( $V_{max}/K_m$ ) of *R*-IFA 4-hydroxylation catalyzed by CYP3A4 were 2- to 2.5-fold higher than that of CPA and *S*-IFA (Table 2 and Fig. 3A). Thus, with *R*-IFA as substrate, CYP3A4 exhibited the best kinetic profile of all three P450 enzymes, namely, high *R*-IFA 4-hydroxylase activity coupled with relatively low *N*-dechloroethylation activity, in agreement with the cell culture data presented above.

**CYP3A1 Is a High-Efficiency, Low  $K_m$  Catalyst of *R*-IFA and *S*-IFA 4-Hydroxylation.** Tumor cells may be sensitized to anticancer prodrugs, such as IFA, by introducing a prodrug-activating P450 gene into the tumor, which thereby acquires the capacity for localized prodrug activation (Chen and Waxman, 2002). Thus, CYP3A4 can be used to sensitize P450-deficient tumor cells to the cytotoxic activity of IFA (Jounaidi et al., 1998). Presently, we considered the possibility that other CYP3A enzymes might metabolize one of the enantiomers

of IFA with improved activity or catalytic specificity compared with CYP3A4. Table 3 and Fig. 3 present the steady-state kinetic parameters determined for metabolism of CPA, *R*-IFA, and *S*-IFA by four other cDNA-expressed CYP3A enzymes: rat CYP3A1, rat CYP3A2, human CYP3A7, and dog CYP3A12. All four CYP3A enzymes preferentially metabolized *R*-IFA by the 4-hydroxylation pathway to an even greater extent than seen with CYP3A4 (up to 90% of total metabolism; Fig. 3C). CPA was preferentially metabolized by *N*-dechloroethylation by all of the CYP3A enzymes except CYP3A7, which was primarily a 4-hydroxylase with CPA and with both enantiomers of IFA. However, the overall catalytic efficiency of CYP3A7 for oxazaphosphorine activation was greatly reduced by this enzyme's low intrinsic catalytic activity ( $V_{max} \sim 10-28\%$  that of CYP3A4; Table 3 versus Table 2). CYP3A12 preferentially metabolized *S*-IFA via *N*-dechloroethylation, as seen with CYP3A4, whereas CYP3A1, CYP3A2, and CYP3A7 metabolized *S*-IFA preferentially by 4-hydroxylation (Fig. 3C). Finally, CYP3A1 displayed  $K_m$  values for *R*- and *S*-IFA 4-hydroxylation of  $\sim 200 \mu\text{M}$  (Table 3),  $\sim 4$ - to 5-fold lower than the corresponding  $K_m$  values of CYP3A4 (Table 2). The high catalytic efficiency of CYP3A1 for *R*-IFA and *S*-IFA 4-hydroxylation ( $V_{max}/K_m = 88-135 \text{ mol/min/mol of P450}/\mu\text{M}$ ) compared with six other P450 enzymes (Fig. 3A), coupled with CYP3A1's low  $K_m$  value and its low relative rate of *N*-dechloroethylation (Fig. 3C), evidence the superior catalytic properties of this enzyme for IFA activation.

## Discussion

The anticancer prodrugs CPA and IFA are actively metabolized in the liver, which is a major determinant of the pharmacokinetics of prodrug activation and the rate of drug inactivation and elimination from the body. More recent studies have shown that tumor cells also express drug-metabolizing P450 enzymes, including CYP2B6 (Nakajima et al., 1996; Standop et al., 2003) and CYP3A4 (Huang et al., 1996; Murray et al., 1999; Schmidt et al., 2004). The levels of tumor cell P450 are low but may nevertheless be an important factor in the metabolism and activity of these anticancer agents. Tumor cell-expressed P450 enzymes may potentially contribute to drug inactivation leading to drug resistance, in the case of anticancer drugs than are inactivated by P450 metabolism and, conversely, may enhance anti-tumor activity in the case of P450 prodrugs, as demonstrated for CPA and IFA in cultured tumor cells and in tumor xenograft models *in vivo* (Chen and Waxman, 1995). The studies presented here investigate the metabolism and cytotoxicity of *R*-IFA, *S*-IFA, and CPA in tumor cells engineered to express individual CYP2B and CYP3A enzymes with the goal of identifying P450 prodrug-P450 enzyme combinations that are associated with strong cytotoxic responses. Our findings reveal that tumor cells that express CYP3A4 are most sensitive to the cytotoxicity of *R*-IFA, whereas tumor cells that express CYP2B enzymes (rat CYP2B1 or human CYP2B6) are most sensitive to CPA. Moreover, the combination of *R*-IFA treatment with CYP3A4 expression was associated with favorable partitioning of IFA metabolism along the therapeutically important 4-hydroxylation (prodrug activation) pathway at the expense of the alternative, *N*-dechloroethylation pathway, which inactivates the prodrug and yields the by-product CAA, which has been linked to undesirable host toxicities, notably neurotoxicity and urotoxicity. Together, these findings suggest that improvement in the effectiveness of oxazaphosphorine therapy may be achieved by individualizing drug treatment based on the P450 expression profile of each patient's tumor, e.g., as assessed by needle biopsy.

Studies using cDNA-expressed enzymes revealed that CPA 4-hydroxylation was most actively catalyzed by CYP2B1 and CYP2B6,

TABLE 3

Steady-state kinetics analysis: CPA, R-IFA, and S-IFA metabolism by cDNA-expressed P450s 3A1, 3A2, 3A7, and 3A12

Data based on two independent determinations (mean values  $\pm$  half-the-range).

P450	Drug	4-Hydroxylation		N-Dechloroethylation	
		$K_m$	$V_{max}$	$K_m$	$V_{max}$
		mM	mol/min/mol P450	mM	mol/min/mol P450
3A1	CPA	1.15 $\pm$ 0.28	10.5 $\pm$ 0.5	0.69 $\pm$ 0.24	25.5 $\pm$ 2.7
	R-IFA	0.22 $\pm$ 0.04	19.3 $\pm$ 0.5	0.61 $\pm$ 0.16	2.5 $\pm$ 0.5
	S-IFA	0.19 $\pm$ 0.03	23.1 $\pm$ 2.2	0.36 $\pm$ 0.10	2.9 $\pm$ 0.2
3A2	CPA	1.82 $\pm$ 0.34	8.5 $\pm$ 0.7	1.97 $\pm$ 0.12	28.4 $\pm$ 3.8
	R-IFA	0.95 $\pm$ 0.12	23.9 $\pm$ 0.4	1.19 $\pm$ 0.09	3.4 $\pm$ 0.9
	S-IFA	1.10 $\pm$ 0.13	21.2 $\pm$ 0.8	1.17 $\pm$ 0.06	12.5 $\pm$ 4.2
3A7	CPA	1.04 $\pm$ 0.01	8.8 $\pm$ 1.8	2.03 $\pm$ 0.74	1.6 $\pm$ 0.6
	R-IFA	1.05 $\pm$ 0.05	6.0 $\pm$ 2.3	3.41 $\pm$ 1.88	1.1 $\pm$ 0.3
	S-IFA	0.42 $\pm$ 0.02	4.4 $\pm$ 1.8	0.57 $\pm$ 0.22	1.3 $\pm$ 0.4
3A12	CPA	3.42 $\pm$ 0.37	24.2 $\pm$ 4.8	6.78 $\pm$ 0.70	55.8 $\pm$ 18.6
	R-IFA	1.83 $\pm$ 0.21	32.5 $\pm$ 8.9	5.78 $\pm$ 1.33	8.5 $\pm$ 5.0
	S-IFA	0.92 $\pm$ 0.08	14.3 $\pm$ 1.4	2.89 $\pm$ 0.84	49.4 $\pm$ 19.7

with little (CYP2B1) or no (CYP2B6) *N*-dechloroethylation activity detected. The overall catalytic efficiency ( $V_{max}/K_m$ ) was highest with CYP2B1, largely due to its  $\sim$ 3-fold lower  $K_m$  for CPA, and this translated into a 2-fold increase in cytotoxicity compared with CYP2B6, as indicated by  $EC_{50}$  values (Table 1). CYP3A4 preferentially metabolized CPA by *N*-dechloroethylation ( $\sim$ 75% of total metabolism), with the catalytic efficiency for the 4-hydroxylation reaction being only 15 to 30% that of CYP2B1 and CYP2B6 (Fig. 3A). However, with *R*-IFA as substrate, CYP3A4 exhibited high 4-hydroxylase activity, coupled with a comparatively low *N*-dechloroethylation activity. CYP3A4 is a major catalyst of IFA metabolism in human liver microsomes (Chang et al., 1993; Walker et al., 1994) and the most abundant cytochrome P450 enzyme in human liver. The present findings suggest that the anticancer activity of racemic IFA, as used in the clinic, is in large part due to *R*-IFA, insofar as *S*-IFA is primarily metabolized by *N*-dechloroethylation, catalyzed by CYP3A4 and CYP2B6 (Fig. 3C). These findings support the use of *R*-IFA in place of racemic IFA or its *S*-enantiomer, as proposed by Williams and Wainer (1999) based on the more extensive 4-hydroxylation of *R*-IFA than *S*-IFA seen in pharmacokinetic studies (Wainer et al., 1994) and by the finding that the *N*-dechloroethylation of *R*-IFA is less extensive than that of *S*-IFA in incubations with human liver microsomes (Roy et al., 1999a).

CYP3A4 can be used in a P450-based enzyme prodrug therapy to sensitize tumor cells to racemic IFA and, to a lesser extent, CPA (Jounaidi et al., 1998). Analysis of *R*-IFA and *S*-IFA metabolism catalyzed by a panel of cDNA-expressed CYP3A enzymes revealed that the rat enzyme CYP3A1 exhibits superior catalytic activity compared with four other CYP3A enzymes, including CYP3A4. CYP3A1 exhibited high intrinsic 4-hydroxylase activity, minimal *N*-dechloroethylation activity (11% of total metabolism at  $V_{max}$ ), and a comparatively low  $K_m$  value compared with the other CYP3A enzymes examined, indicating that this P450 may be useful in combination with *R*-IFA or *S*-IFA in clinical trials of this P450-based cancer therapy (Lohr et al., 2003; Braybrooke et al., 2005). CYP3A12, which is expressed in dog liver, exhibited a preferential 4-hydroxylation of *R*-IFA compared with *S*-IFA; however, CYP3A12 suffered from a high  $K_m$  value and low catalytic efficiency for *R*-IFA 4-hydroxylation compared with CYP3A1. CYP3A5, like CYP3A4, also exhibits a preference for 4-hydroxylation of *R*-IFA compared with *S*-IFA (Roy et al., 1999a). Another human liver enzyme, CYP3A7, exhibited the lowest catalytic activity of all five CYP3A enzymes toward both enantiomers of IFA.

CAA, a byproduct of oxazaphosphorine metabolism, displayed

appreciable cytotoxic activity in cell culture ( $EC_{50} \sim 2.5 \mu\text{M}$ , compared with  $EC_{50}$  values of 3–5  $\mu\text{M}$  for the 4-hydroxy derivatives of CPA and IFA; Table 1), in agreement with earlier studies in various tumor cell lines (Brueggemann et al., 2002). However, CAA is not generally regarded as an active, therapeutic metabolite of IFA or CPA. CAA exhibits some antitumor activity in vivo, albeit substantially less than that of 4-OH-IFA (Borner et al., 2000). Of note, we observed that the rate of 4-hydroxylation of *R*-IFA by CYP2B6-expressing 9L gliosarcoma cells was 5-fold lower than that of 9L cells expressing CYP2B1, yet in growth inhibition assays, the CYP2B6 cells showed sensitivity to *R*-IFA comparable with that of CYP2B1 cells. This discrepancy suggests that at least a portion of the cytotoxic activity of *R*-IFA (and also *S*-IFA) is due to the formation of CAA via *N*-dechloroethylation in the case of the CYP2B6 cells.

Given the host toxicities that are widely believed to be linked to CAA exposure, decreasing *N*-dechloroethylation through the use of *R*-IFA may result in an increase in therapeutic index. Further study is required to determine whether *R*-IFA treatment in vivo leads to enhanced *R*-IFA metabolism ("autoinduction"), via increased expression of CYP3A4 (Chang et al., 1997), as has been seen with racemic IFA (Kerbusch et al., 2001). It will also be important to determine whether *R*-IFA treatment is associated with antiangiogenic activity similar to that seen when CPA is administered repeatedly in low doses using metronomic (Man et al., 2002) or antiangiogenic treatment schedules (Browder et al., 2000). This latter approach is highly effective when applied to CPA in the context of prodrug-activation gene therapy using CYP2B6 (Jounaidi and Waxman, 2001). Based on the present findings, this latter strategy may also be implemented by targeted delivery of CYP3A1 to tumors in situ in combination with *R*-IFA treatment. The low  $K_m$  value for IFA exhibited by CYP3A1 compared with CYP3A4 may provide the opportunity to shift prodrug activation from the liver to the tumor, thereby increasing the antitumor effect and potentially reducing host toxicity.

## References

- Bohnstengel F, Eichelbaum M, Golbs E, and Kroemer HK (1997) High-performance liquid chromatographic determination of acrolein as a marker for cyclophosphamide bioactivation in human liver microsomes. *J Chromatogr B Biomed Sci Appl* 692:163–168.
- Boos J, Welslau U, Ritter J, Blaschke G, and Schellong G (1991) Urinary excretion of the enantiomers of ifosfamide and its inactive metabolites in children. *Cancer Chemother Pharmacol* 28:455–460.
- Borner K, Kistro J, Brueggemann SK, Hagenah W, Peters SO, and Wagner T (2000) Metabolism of ifosfamide to chloroacetaldehyde contributes to antitumor activity in vivo. *Drug Metab Dispos* 28:573–576.
- Braybrooke JP, Slade A, Deplanque G, Harrop R, Madhusudan S, Forster MD, Gibson R, Makris A, Talbot DC, Steiner J, et al. (2005) Phase I study of MetXia-P450 gene therapy and oral cyclophosphamide for patients with advanced breast cancer or melanoma. *Clin Cancer Res* 11:1512–1520.

- Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, and Folkman J (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* **60**:1878–1886.
- Brueggemann SK, Schlenke P, Klich S, Deeken M, Peters SO, and Wagner T (2002) Stem cell toxicity of oxazaphosphorine metabolites in comparison to their antileukemic activity. *Biochem Pharmacol* **63**:1337–1341.
- Chang TK, Weber GF, Crespi CL, and Waxman DJ (1993) Differential activation of cyclophosphamide and ifosfamide by cytochromes P450 2B and 3A in human liver microsomes. *Cancer Res* **53**:5629–5637.
- Chang TK, Yu L, Maurel P, and Waxman DJ (1997) Enhanced cyclophosphamide and ifosfamide activation in primary human hepatocyte cultures: response to cytochrome P-450 inducers and autoinduction by oxazaphosphorines. *Cancer Res* **57**:1946–1954.
- Chen CS, Lin JT, Goss KA, He YA, Halpert JR, and Waxman DJ (2004) Activation of the anticancer prodrugs cyclophosphamide and ifosfamide: identification of cytochrome P450 2B enzymes and site-specific mutants with improved enzyme kinetics. *Mol Pharmacol* **65**:1278–1285.
- Chen L and Waxman DJ (1995) Intratumoral activation and enhanced chemotherapeutic effect of oxazaphosphorines following cytochrome P450 gene transfer: development of a combined chemotherapy/cancer gene therapy strategy. *Cancer Res* **55**:581–589.
- Chen L and Waxman DJ (2002) Cytochrome P450 gene-directed enzyme prodrug therapy (GDEPT) for cancer. *Curr Pharm Des* **8**:1405–1416.
- Corlett SA, Parker D, and Chrystyn H (1995) Pharmacokinetics of ifosfamide and its enantiomers following a single 1 h intravenous infusion of the racemate in patients with small cell lung carcinoma. *Br J Clin Pharmacol* **39**:452–455.
- Ding S, Yao D, Burchell B, Wolf CR, and Friedberg T (1997) High levels of recombinant CYP3A4 expression in Chinese hamster ovary cells are modulated by coexpressed human P450 reductase and hemin supplementation. *Arch Biochem Biophys* **348**:403–410.
- Finer MH, Dull TJ, Qin L, Farson D, and Roberts MR (1994) kat: a high-efficiency retroviral transduction system for primary human T lymphocytes. *Blood* **83**:43–50.
- Granvil CP, Madan A, Sharkawi M, Parkinson A, and Wainer IW (1999) Role of CYP2B6 and CYP3A4 in the in vitro N-dechloroethylation of (R)- and (S)-ifosfamide in human liver microsomes. *Drug Metab Dispos* **27**:533–541.
- Huang Z, Fasco MJ, Figue HL, Keyomarsi K, and Kaminsky LS (1996) Expression of cytochromes P450 in human breast tissue and tumors. *Drug Metab Dispos* **24**:899–905.
- Huang Z and Waxman DJ (1999) High-performance liquid chromatographic-fluorescent method to determine chloroacetaldehyde, a neurotoxic metabolite of the anticancer drug ifosfamide, in plasma and in liver microsomal incubations. *Anal Biochem* **273**:117–125.
- Jounaidi Y, Hecht JED, and Waxman DJ (1998) Retroviral transfer of human cytochrome P450 genes for oxazaphosphorine-based cancer gene therapy. *Cancer Res* **58**:4391–4401.
- Jounaidi Y and Waxman DJ (2001) Frequent, moderate-dose cyclophosphamide administration improves the efficacy of cytochrome P-450/cytochrome P-450 reductase-based cancer gene therapy. *Cancer Res* **61**:4437–4444.
- Jounaidi Y and Waxman DJ (2004) Use of replication-conditional adenovirus as a helper system to enhance delivery of P450 prodrug-activation genes for cancer therapy. *Cancer Res* **64**:292–303.
- Kerbusch T, Mathot RA, Keizer HJ, Kaijser GP, Schellens JH, and Beijnen JH (2001) Influence of dose and infusion duration on pharmacokinetics of ifosfamide and metabolites. *Drug Metab Dispos* **29**:967–975.
- Lang T, Klein K, Fischer J, Nussler AK, Neuhaus P, Hofmann U, Eichelbaum M, Schwab M, and Zanger UM (2001) Extensive genetic polymorphism in the human CYP2B6 gene with impact on expression and function in human liver. *Pharmacogenetics* **11**:399–415.
- Lohr M, Kroger J-C, Hoffmeyer A, Freund M, Hain J, Holle A, Knofel WT, Liebe S, Nizze H, Renner M, et al. (2003) Safety, feasibility and clinical benefit of localized chemotherapy using microencapsulated cells for inoperable pancreatic carcinoma in a phase I/II trial. *Cancer Ther* **1**:121–131.
- Lu H and Waxman DJ (2005) Antitumor activity of methoxymorpholinyl doxorubicin: potentiation by cytochrome P450 3A metabolism. *Mol Pharmacol* **67**:212–219.
- Man S, Bocci G, Francia G, Green SK, Jothy S, Hanahan D, Bohlen P, Hicklin DJ, Bergers G, and Kerbel RS (2002) Antitumor effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. *Cancer Res* **62**:2731–2735.
- Murray GI, McFadyen MC, Mitchell RT, Cheung YL, Kerr AC, and Melvin WT (1999) Cytochrome P450 CYP3A in human renal cell cancer. *Br J Cancer* **79**:1836–1842.
- Nakajima T, Wang RS, Nimura Y, Pin YM, He M, Vainio H, Murayama N, Aoyama T, and Iida F (1996) Expression of cytochrome P450s and glutathione S-transferases in human esophagus with squamous-cell carcinomas. *Carcinogenesis* **17**:1477–1481.
- Roy P, Tretyakov O, Wright J, and Waxman DJ (1999a) Stereoselective metabolism of ifosfamide by human P-450s 3A4 and 2B6. Favorable metabolic properties of R-enantiomer. *Drug Metab Dispos* **27**:1309–1318.
- Roy P, Yu LJ, Crespi CL, and Waxman DJ (1999b) Development of a substrate-activity based approach to identify the major human liver P450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P450 profiles. *Drug Metab Dispos* **27**:655–666.
- Schmidt R, Baumann F, Knupfer H, Brauckhoff M, Horn LC, Schonfelder M, Kohler U, and Preiss R (2004) CYP3A4, CYP2C9 and CYP2B6 expression and ifosfamide turnover in breast cancer tissue microsomes. *Br J Cancer* **90**:911–916.
- Sladek NE (1988) Metabolism of oxazaphosphorines. *Pharmacol Ther* **37**:301–355.
- Standop J, Schneider M, Ulrich A, Buchler MW, and Pour PM (2003) Differences in immunohistochemical expression of xenobiotic-metabolizing enzymes between normal pancreas, chronic pancreatitis and pancreatic cancer. *Toxicol Pathol* **31**:506–513.
- Wainer IW (1993) Stereoisomers in clinical oncology: why it is important to know what the right and left hands are doing. *Ann Oncol* **4** (Suppl 2):7–13.
- Wainer IW, Ducharme J, and Granvil CP (1996) The N-dechloroethylation of ifosfamide: using stereochemistry to obtain an accurate picture of a clinically relevant metabolic pathway. *Cancer Chemother Pharmacol* **37**:332–336.
- Wainer IW and Granvil CP (1993) Stereoselective separations of chiral anticancer drugs and their application to pharmacodynamic and pharmacokinetic studies. *Ther Drug Monit* **15**:570–575.
- Wainer IW, Granvil CP, Wang T, and Batist G (1994) Efficacy and toxicity of ifosfamide stereoisomers in an in vivo rat mammary carcinoma model. *Cancer Res* **54**:4393–4397.
- Walker D, Flinois JP, Monkman SC, Beloc C, Boddy AV, Cholerton S, Daly AK, Lind MJ, Pearson AD, Beaune PH, et al. (1994) Identification of the major human hepatic cytochrome P450 involved in activation and N-dechloroethylation of ifosfamide. *Biochem Pharmacol* **47**:1157–1163.
- Williams ML and Wainer IW (1999) Cyclophosphamide versus ifosfamide: to use ifosfamide or not to use, that is the three-dimensional question. *Curr Pharm Des* **5**:665–672.
- Woodland C, Ito S, Granvil CP, Wainer IW, Klein J, and Koren G (2000) Evidence of renal metabolism of ifosfamide to nephrotoxic metabolites. *Life Sci* **68**:109–117.
- Zalupski M and Baker LH (1988) Ifosfamide. *J Natl Cancer Inst* **80**:556–566.

---

**Address correspondence to:** Dr. David J. Waxman, Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215. E-mail: djw@bu.edu

---