Differential Activation of Cyclophosphamide and Ifosfamide by Cytochromes P-450 2B and 3A in Human Liver Microsomes


Department of Biological Chemistry and Molecular Pharmacology and Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 [T. K. H. C., G. F. W., D. J. W.], and Gentest Corporation, Woburn, Massachusetts 01801 [C. L. C.]

ABSTRACT

The present study identifies the specific human cytochrome P-450 (CYP) enzymes involved in hydroxylation leading to activation of the anticancer drug cyclophosphamide and its isomeric analogue, ifosfamide. Substantial interindividual variation (4-9-fold) was observed in the microsomes, and a significant correlation was obtained between these 2 activities (r = 0.85, P < 0.001). Enzyme kinetic analyses revealed that human liver microsomal cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation are best described by a 2-component Michaelis-Menten model composed of both low \( K_m \) and high \( K_m \) P-450 4-hydroxylases. To ascertain whether one or more human P-450 enzymes are catalytically competent in activating these oxazaphosphorines, microsomal fractions prepared from a panel of human B-lymphoblastoid cell lines stably transformed with individual P-450 complementary DNAs were assayed in vitro for oxazaphosphorine activation. Expressed CYP2A6, -2B6, -2C8, and -3A4 were catalytically competent in hydroxylation of cyclophosphamide and ifosfamide. Whereas CYP2C8 and CYP2C9 have the characteristics of low \( K_m \) oxazaphosphorine 4-hydroxylases, CYP2A6, -2B6, and -3A4 are high \( K_m \) forms. In contrast, CYP1A1, -1A2, -2D6, and -2E1 did not produce detectable activities. Furthermore, growth of cultured CYP2A6- and CYP2B6-expressing B-lymphoblastoid cells, but not of CYP-negative control cells, was inhibited by cyclophosphamide and ifosfamide as a consequence of prodrug activation to cytotoxic metabolites. Experiments with P-450 form-selective chemical inhibitors and inhibitory anti-P-450 antibodies were then performed to determine the contributions of individual P-450s to the activation of these drugs in human liver microsomes. Orphenadrine (a CYP2B6 inhibitor) and anti-CYP2B IfG inhibited microsomal cyclophosphamide hydroxylation to a greater extent than ifosfamide hydroxylation, consistent with the 8-fold higher activity of complementary DNA-expressed CYP2B6 with cyclophosphamide. In contrast, troleandomycin, a selective inhibitor of CYP3A3 and -3A4, and anti-CYP3A IgG substantially inhibited microsomal ifosfamide hydroxylation but had little or no effect on microsomal cyclophosphamide hydroxylation. By contrast, the CYP2D6-selective inhibitor quinidine did not affect either microsomal activity, while anti-CYP2A antibodies had only a modest inhibitory effect. Overall, the present study establishes that liver microsomal CYP2B and CYP3A preferentially catalyze cyclophosphamide and ifosfamide hydroxylation, respectively, suggesting that liver P-450-inducing agents targeted at these enzymes might be used in cancer patients to enhance drug activation and therapeutic efficacy.

INTRODUCTION

Cyclophosphamide and ifosfamide are anticancer alkylating agent produgs that require metabolism to produce pharmacologically active, cytotoxic species (1). Studies with rat liver microsomes have established that the activation of these oxazaphosphorines is catalyzed by overlapping subsets of liver CYP enzymes. Whereas rat cytochrome P-450 forms [individual liver CYP forms are designated according to the systematic nomenclature (2)] CYP2B1, -2C6, and -2C11 are the major catalysts of cyclophosphamide 4-hydroxylation (3), these enzymes, together with one or more CYP3A enzymes, catalyze a major fraction of ifosfamide 4-hydroxylation in rat liver microsomes (4). The 4-hydroxy metabolite formed by these enzymes equilibrates with the ring-opened aldoxiphormide, which undergoes chemical decomposition to yield a mustard derivative (phosphoramide mustard or ifosphoramide mustard) and acrolein. The primary 4-hydroxy metabolite may, alternatively, be detoxified by aldehyde dehydrogenase to yield the inactive carboxyphosphamide (5). The 4-hydroxy metabolite is generally considered to be the therapeutically significant cytotoxic metabolite (1, 6).

Cyclophosphamide P-450 has been implicated in the bioactivation of cyclophosphamide in humans, primarily based on clinical pharmacokinetic drug interaction studies, which show that the elimination half-life of this drug is decreased following the administration of phenobarbital (7) or prednisone (8), agents known to induce P-450 enzyme levels in humans (9, 10). However, there is as yet no direct evidence that human cytochrome P-450 enzymes activate cyclophosphamide or its isomeric analogue, ifosfamide. Although specific rat liver cytochromes P-450 are known to activate these oxazaphosphorines (3, 4), other enzymes, such as prostaglandin H synthase, may also activate these drugs (11). It is therefore important to examine the role of individual human cytochrome P-450s in these reactions, both to establish the role that this family of enzymes plays in activation of these widely used chemotherapeutic drugs in cancer patients, and to gain insight into the basis for the large interpatient differences in the clinical pharmacokinetics and metabolism of these anticancer drugs (1, 12, 13). Moreover, since cancer patients often undergo multikid drug therapy, identification of the specific P-450 enzyme catalysts of human liver cyclophosphamide and ifosfamide metabolism would allow clinicians to predict, and thereby avoid, potential drug interactions that might compromise therapeutic efficacy. Finally, this knowledge might lead to the design of rational strategies to enhance drug activation through modulation of liver cytochrome P-450 enzymes, with the ultimate goal of increasing drug efficacy and mitigating systemic toxicity.

The present study establishes that several human liver P-450s can activate these oxazaphosphorine anticancer drugs, including CYP2B2, which preferentially catalyzes cyclophosphamide activation, CYP3A, which is important for ifosfamide hydroxylation, and two CYP2C enzymes, which may contribute to some of the interindividual differences that characterize the clinical pharmacokinetics and metabolism of these anticancer drugs.

MATERIALS AND METHODS

Chemicals. Cyclophosphamide and ifosfamide were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). 4-Hydroperoxyifosfamide was a gift from Dr. J. Pohl (ASTA Pharma, Inc.).

The abbreviations used are: CYP, cytochrome P-450; TAO, tricetylethylamomycin; cDNA, complementary DNA.
Bielefeld, Germany). Orphenadrine HCl, quinidine anhydrous, and coumarin were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxycoumarin was from Enzyme Systems Products (Dublin, CA) and TA0 was kindly provided by Pfizer, Inc. (Brooklyn, NY).

**cDNA-expressed Human P-450s.** The cDNA-expressing human lymphoblast cell lines were derivatives of the AHH-1 TK- cell line (14), which also were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Ethoxycoumarin (7-demethyloxy-4-trifluoro-methylcoumarin) was from Enzyme Systems Products (Bielefeld, Germany). Orphenadrine HCl, quinidine anhydrous, and coumarin were from Aldrich Chemical Co. (Milwaukee, WI), CYP2A6 was 2A3/I-Iol (15) and contained a microsomal P-450 content of 60 pmol P-450/mg protein as measured spectrophotometrically. The cell line expressing CYP2B6 was isogenic to the CYP2A6 cell line except that it contained the CYP2B6 cDNA (1.8-kilobase EcoRI/Smal fragment) (16) and a microsomal P-450 content of 55 pmol P-450/mg protein. Cell lines were maintained as described, and microsomes were prepared from human lymphoblasts expressing CYP1A1 (25 pmol P-450/mg protein), CYP1A2 (38 pmol P-450/mg protein), CYP2C8, CYP2E1, and CYP3A4 as detailed elsewhere (17). The P-450 contents for cells expressing CYP2C8 and CYP2E1 were too low to be detected spectrophotometrically, while those for CYP3A4 was not measurable because of the rapid conversion of this CYP to P-420 in the presence of dithionite. CYP2C9- and CYP2D6-containing microsomes were prepared from HepG2 cells infected with CYP2C9- and CYP2D6-expressing recombinant vaccinia virus particles, respectively (18), and were kindly provided by Dr. F. J. Gonzalez (National Cancer Institute, Bethesda, MD). The P-450 contents measured for comparable preparations are in the range of 10-20 pmol P-450/mg total cell lystate protein (19).

**Antiproliferation Assay.** Human B-lymphoblastoid cells were diluted to 1 x 10^5 cells/ml and aliquoted into replicate 25-cm^2 tissue culture flasks (10 ml/culture). Cyclophosphamide or ifosfamide was dissolved in culture media, filter sterilized, and then added to the culture. Cultures were incubated for 3 days at 37°C, during which time the cell number in control cultures increased about 10-fold. The cell concentration was then determined by electronic particle counting. Relative growth was calculated by dividing the cell number for the drug-treated culture by the cell number for parallel cultures without drug treatment. The lower limit for relative growth was about 0.1 (the initial cell concentration divided by the final cell concentration). B-lymphoblastoid cells transformed with vector alone and expressing only a low basal level of cytochrome P-450 (primarily CYP1A1) were included in each experiment as a control for oxazaphosphorine-insensitive cells.

**Anti-P-450 Antibodies.** P-450 subfamily-specific rabbit polyclonal anti-rat CYP2B, anti-rat CYP2C (20), and anti-rat CYP3A antibodies were prepared as outlined previously (21). The cross-reactivity of these antibodies with human P-450s was assessed by Western blot analysis using individual cDNA-expressed human P-450s. Thus, our preparation of anti-CYP2B antibodies cross-reacted strongly with CYP2B6, was very weakly reactive with CYP2A6, and did not recognize CYP2C8, CYP2E1, or CYP3A4. The anti-CYP2D2 antibodies cross-reacted strongly with both CYP2C8 and CYP2C9 and only slightly with CYP2A6 but did not react with CYP1A2, CYP2E1, or CYP3A4. The anti-CYP3A antibodies cross-reacted strongly with CYP3A2, CYP3A4, and CYP3A5, but not CYP2B6. Polyclonal sheep anti-banbo CYP2A IgG, prepared as described elsewhere (22), was generously provided by Dr. P. Maurel (Institut de la Santé et de la Recherche Médicale, Montpellier, France). Our Western blot analyses revealed that this anti-CYP2A antibody preparation recognizes CYP2A6, but not CYP1A2, CYP2B, CYP2C9, or CYP3A4.

**Human Liver Microsomes.** Human liver specimens obtained from organ donors after clinical death were kindly provided by Dr. A. Radominska (University of Arkansas for Medical Sciences, Little Rock, AR). Microsomes were prepared from individual liver samples, designated HLS2-HLS22, using methods described previously (23). Microsomal protein concentration was determined by the Bradford method with bovine serum albumin as standard. CYP2A6, CYP2B6, CYP2C8, and -3A4 proteins were detected in human liver microsomes using P-450 subfamily-specific polyclonal antibodies (see above) by Western blot analysis using a Tween 20 blocking protocol (24).

**Enzyme Assays.** Cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation were determined fluorometrically (25) as detailed elsewhere (26). Each incubation tube contained 100 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 5 mM semicarbazide HCl, 0.25 mM or 2 mM cyclophosphamide or ifosfamide (unless stated otherwise), 1 mM NADPH, and either 100 µg human liver microsomal protein or 500 µg microsomal protein prepared from P-450 cDNA-transformed human B-lymphoblastoid cells in a total volume of 200 µl. 7-Ethoxycoumarin O-deethylation activity was determined as described (27), but with minor modifications. Briefly, each incubation tube contained 100 mM potassium phosphate (pH 7.4), 20% glycerol, 0.1 mM EDTA, 1 mM 7-ethoxycoumarin, 20-40 µg microsomal protein from P-450 cDNA-transfected human B-lymphoblastoid, and 1 mM NADPH, in a total volume of 200 µl. After a 30-min incubation at 37°C, the reaction was terminated by 25 µl ice-cold 2 mM HCl. The sample was extracted with 450 µl chloroform and then 300 µl of the organic phase were back-extracted with 1 ml of 30 mM sodium borate (pH 9.2). The amount of 7-hydroxycoumarin formed was determined fluorometrically (370 nm excitation wavelength, 450 nm emission wavelength) in comparison to authentic 7-hydroxycoumarin standard. Coumarin 7-hydroxylase activity was determined by the same method using 1 mM coumarin as substrate.

**Chemical and Antibody Inhibition Experiments.** TAO dissolved in methanol was added to individual assay tubes. The solvent was evaporated under a gentle stream of nitrogen, and the residue then reconstituted with assay buffer prior to addition of the other reaction components. Complete assay mixtures (including TAO and NADPH, but without the P-450 substrate) were preincubated at 37°C for 30 min. Cyclophosphamide or ifosfamide hydroxylation was then initiated by addition of the oxazaphosphorine substrate together with another aliquot of NADPH. Experiments with the other chemical inhibitors were performed without this preincubation step. In immunoinhibition experiments, complete assay mixtures (minus NADPH) were preincubated with the indicated amount of each antibody at room temperature for 30 min prior to the addition of NADPH to initiate enzyme reaction. Control experiments were performed in parallel, using rabbit IgG fractions purified as described (28).

**RESULTS**

**Kinetic Analysis of Human Liver Microsomal Cyclophosphamide 4-Hydroxylation and Ifosfamide 4-Hydroxylation.** Steady-state enzyme kinetic studies were performed using 2 individual human liver microsomal samples, HLS8 and HLS9, at substrate concentrations ranging from 0.125 to 10 mM. Computer curve-fitting analysis indicated that the kinetics of cyclophosphamide 4-hydroxylation and ifosfamide 4-hydroxylation in human liver microsomes are best described by a 2-component Michaelis-Menten enzyme kinetic model. The appropriate model was chosen on the basis of how well the experimental data were fitted by each equation as judged by the reduced chi^2 statistic. In addition, Eadie-Hofstee and Lineweaver-Burk plots were generated to confirm the qualitative results obtained by the computer curve-fitting technique. The reported values of the apparent K_m and V_max were obtained using ENZFITTER.

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Increased the Pearson correlation coefficient to \( r = 0.92 \) (\( P < 0.001 \)).

A significant correlation between these 2 enzyme activities was also obtained when assays were carried out at 2 mM substrate (\( r = 0.83 \), \( P < 0.001 \), \( n = 15 \), including sample HLS2).

**Role of Individual cDNA-expressed Human Cytochrome P-450 Enzymes in Cyclophosphamide and Ifosphamide Activation.** To identify the P-450 enzymes that are catalytically competent in activating these alkylating anticancer agents, we examined a panel of human B-lymphoblastoid cell lines that were stably transformed with individual cytochrome P-450 cDNAs (17). The expressed P-450s were assayed in membrane fractions prepared from each of the cell lines after growth in culture. Enzymatic activity was verified by assaying the isolated microsomes for 7-ethoxy-4-trifluoromethyl-coumarin O-deethylation, a reaction known to be catalyzed by the human cytochrome P-450 enzymes (18). As judged by this assay, the expressed human P-450s were all enzymatically active (Table 2). With cyclophosphamide and ifosphamide as substrates, CYP2A6, -2B6, -2C8, -2C9, and -3A4 were catalytically active; whereas CYP1A1, -1A2, and -2E1 did not yield measurable product. Comparison of the activation rates at high and low oxazaphosphorine concentrations suggested that CYP2C8 and CYP2C9 are low Km enzymes, while CYP2A6, -2B6, and -3A4 are high Km oxazaphosphorine 4-hydroxylases. CYP2C8 exhibited similar activity with cyclophosphamide and ifosphamide at the lower substrate concentration, in agreement with the similar \( V_{\text{max}} \) values exhibited by the 2 drug substrates in human liver microsomes (Table 1). In contrast, CYP2C9 was 2-fold more active in hydroxylating cyclophosphamide (The Table). The high Km enzymes, CYP2A6 and -2B6, were 3- and 8-fold more active, respectively, in metabolizing cyclophosphamide, whereas CYP3A4 was 2-fold more active with ifosphamide as substrate. The role of CYP2B6 and CYP2A6 in the activation of these drugs was further supported by monitoring the effects of P-450 enzyme expression on the cytotoxicity of cyclophosphamide and ifosphamide toward the cultured lymphoblasts. As shown in Fig. 2, growth of lymphoblastoid cells expressing CYP2B6 or CYP2A6 was inhibited in a concentration-dependent manner in cells treated with cyclophosphamide (Fig. 2, A and B). The CYP2A6- and CYP2B6-expressing cells were similarly sensitive to growth inhibition by ifosphamide (Fig. 2C), even though these P-450s both activate ifosphamide in isolated microsomes at a severalfold lower rate than cyclophosphamide (Table 2). Much less inhibition was observed in the non-P-450-expressing parental control cells, a finding that is consistent with the requirement of P-450 metabolism to convert these oxazaphosphorines to cytotoxic metabolites. The P-450 metabolism-dependent nature of this cytotoxicity was confirmed in experiments using the CYP2B6 substrate 7-ethoxy-4-trifluoromethyl-coumarin (50 \( \mu \)M) and the CYP2A6 substrate coumarin (50 \( \mu \)M), which partially inhibited (2B6) or fully blocked (2A6) the cytotoxicity of cyclophosphamide (Fig. 2, A and B) and ifosphamide (data not shown) toward these cells. Thus, multiple cytochromes P-450 are catalytically competent in activating these oxazaphosphorines.

**Contributions of Individual P-450 Enzymes to Cyclophosphamide and Ifosphamide 4-Hydroxylation in Human Liver Microsomes.** The above studies demonstrate that cytochromes P-450 2A6, -2B6, -2C8, -2C9, and -3A4 are able to activate cyclophosphamide and ifosphamide. However, these data alone do not establish which of these enzymes make the dominant contribution to drug activation in human liver, where the individual P-450s are present at varying levelslective chemical inhibitors and P-450 subfamily-specific antips-450 antibodies.

### Table 1 Michaelis-Menten parameters of cyclophosphamide and ifosphamide hydroxylation reactions catalyzed by human liver microsomes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Liver microsomes</th>
<th>Specific activity ( b ) (pmol/min/mg)</th>
<th>Low Km component</th>
<th>High Km component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( K_m ) (mM)</td>
<td>( V_{\text{max}} ) (pmol/min/mg)</td>
<td>( K_m ) (mM)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>HLS8</td>
<td>0.34</td>
<td>0.031</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>HLS9</td>
<td>0.78</td>
<td>0.133</td>
<td>0.54</td>
</tr>
<tr>
<td>Ifosphamide</td>
<td>HLS8</td>
<td>0.30</td>
<td>0.007</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>HLS9</td>
<td>0.62</td>
<td>0.086</td>
<td>0.50</td>
</tr>
</tbody>
</table>

*a* Enzyme activity was determined at substrate concentrations ranging from 0.125 to 10 mM. Apparent \( K_m \) and \( V_{\text{max}} \) values were generated by the computer program ENZFITTER.

*b* Specific activities determined at 0.25 mM substrate concentration.
Recent studies from this laboratory\(^4\) have shown that the anti-Parkinson drug orphenadrine, which selectively inhibits rat CYP2B1-catalyzed enzyme activities (29), is a selective inhibitor of cDNA-expressed human P-450 2B6 at a concentration of 0.3 mM. Therefore, we used this agent to probe the role of CYP2B6 in the liver microsomal oxazaphosphorine metabolism. Orphenadrine inhibited both cyclophosphamide 4-hydroxylase and ifosfamide 4-hydroxylase activities in microsomal samples HLS2 and HLS9 (Fig. 4, A and B), although the extent of inhibition was greater for cyclophosphamide (40–47% inhibition) than for ifosfamide (21–28% inhibition) (Table 3). In microsomal sample HLS8, orphenadrine did not affect either activity (Fig. 4C). Western blot analysis revealed that the level of an immunoreactive CYP2B protein, which was indistinguishable from cDNA-expressed CYP2B6 in its electrophoretic mobility, was substantially lower in sample HLS8 than in HLS2 or HLS9 (Fig. 3A). These results suggest that a human liver CYP2B enzyme, likely CYP2B6, can be a significant contributor to liver microsomal cyclophosphamide activation, whereas it is less important in microsomal ifosfamide activation.

The macrolide antibiotic TAO is a useful diagnostic inhibitor of CYP3A-catalyzed steroid and drug oxidation in human liver microsomes (23, 30, 31). TAO was therefore used to probe whether CYP3A enzymes contribute significantly to oxazaphosphorine activation in human liver microsomes. As shown in Fig. 5 and Table 3, TAO significantly inhibited ifosfamide 4-hydroxylation activity in microsomal sample HLS9 (57% inhibition at 2 mM substrate), but it had a more modest effect (up to 20–25% inhibition) in liver samples HLS2 and HLS8. This differential inhibition by TAO (livers HLS9 > HLS2 > HLS8) is consistent with the relative CYP3A protein content of these microsomal samples, as revealed by Western blotting (Fig. 3C). By contrast, TAO did not substantially inhibit cyclophosphamide 4-hydroxylation (Fig. 5). This differential effect of TAO on ifosfamide versus cyclophosphamide 4-hydroxylation is consistent with the higher activity of cDNA-expressed CYP3A4 with ifosfamide (Table 2) and further supports the conclusion that CYP3A4, and perhaps related human CYP3A enzymes, can play a role in microsomal ifosfamide activation.

Quinidine, a potent inhibitor of CYP2D6 (Ki < 0.1 μM) (32, 33), did not affect liver microsomal cyclophosphamide 4-hydroxylation or ifosfamide 4-hydroxylation at concentrations up to 100 μM (data not shown), suggesting that CYP2D6 does not activate these drugs in human liver.

**Effects of Inhibitory Anti-P-450 Antibodies.** The contribution of individual P-450s to liver microsomal oxazaphosphorine activation was further evaluated by the use of P-450 subfamily-specific anti-P-450 antibodies. Anti-CYP2B IgG inhibited cyclophosphamide 4-hydroxylation (40% inhibition), whereas it had little or no effect on ifosfamide 4-hydroxylation (Fig. 6A; Table 3), consistent with the differential inhibition of these activities by orphenadrine in the same microsomal preparation (HLS9) (Fig. 4B). In contrast, anti-CYP3A IgG inhibited ifosfamide 4-hydroxylase activity (63% inhibition at 2 mM ifosfamide), but it had little or no effect on microsomal cyclophosphamide 4-hydroxylation (Fig. 6B). This finding is similar to the differential effect of TAO on these activities in the same microsomal sample (HLS9) (Fig. 5B). When tested in a liver sample (HLS2) that had a high CYP2A protein content (Fig. 5B) and a high CYP2A6-dependent coumarin 7-hydroxylase activity (data not shown), anti-CYP2A IgG inhibited both cyclophosphamide 4-hydroxylase and ifosfamide 4-hydroxylase activities, but only by ~20% (Fig. 6C). In control experiments carried out in the same microsomal sample, these antibodies were shown to extensively inhibit coumarin 7-hydroxylation (Fig. 6C, dashed line). Together, these results indicate that CYP2B6 contributes substantially to liver microsomal cyclophosphamide activation, whereas CYP3A enzymes are important for microsomal ifosfamide activation, and CYP2A6 contributes to a lesser extent to the metabolism of both drugs. Antibody inhibition experiments to probe for the contribution of human CYP2C enzymes to liver oxazaphosphorine activation could not be carried out since all of the heterologous anti-CYP2C IgG preparations available to us (anti-rabbit CYP 2C3, and anti-rat CYP 2C6, 2C11, 2C12, and 2C13) were either

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\(^4\) T. K. H. Chang et al., manuscript in preparation.
Fig. 3. Immunodetection of CYP2B, CYP2A, CYP3A, and CYP2C in human liver microsomes. Shown are Western blots of human liver microsome samples HLS2 (Lane 3), HLS8 (Lane 4), and HLS9 (Lane 5) probed with polyclonal anti-rat CYP2B (A), anti-baboon CYP2A (B), anti-rat CYP3A (C), or anti-rat CYP2C antibodies (D). A, of the 2 bands stained in human liver microsomes, the lower band exhibits the same electrophoretic mobility as cDNA-expressed CYP2B6 (Lanes 3 and 5 versus Lanes 2 and 6). B, the major stained band found in liver microsomes has nearly the same electrophoretic mobility as cDNA-expressed CYP2A6 (Lanes 3-5 versus Lanes 2 and 6). C, an intensely stained band found in the microsomes has the same electrophoretic mobility as cDNA-expressed CYP3A4 (Lanes 3-5 versus Lanes 2 and 6). Sample HLS9 (Lane 5) contained 3 additional unidentified bands of lower molecular weight. D, 2 intensely stained bands are detected in liver microsomes (Lanes 3-5). The top band exhibits an electrophoretic mobility indistinguishable from that of cDNA-expressed CYP2C9 (Lane 2), whereas the lower band has the same mobility as expressed CYP2C8 (Lane 6). Microsomes isolated from human B-lymphoblastoid cells transfected with the vector only were included as negative control (Lane 1). Each lane was loaded with 15 μg protein.

noninhibitory to the cDNA-expressed human CYP2C enzymes or cross-inhibitory to CYP2A6 and/or CYP2B6.

DISCUSSION

The present report directly establishes that cytochrome P-450 enzymes are the major catalysts of cyclophosphamide and ifosphamide activation in human liver and that a subset of these enzymes carries out a major fraction of drug activation with these anticancer drug substrates. Improvements in cyclophosphamide and ifosphamide therapeutic efficacy through modulation of liver drug activation may therefore require clinical strategies that focus on select P-450 enzymes.

Considerable intersample variation was observed in oxazaphosphorine activation by our panel of human liver microsomes. Cyclophosphamide 4-hydroxylation and ifosphamide 4-hydroxylation varied over a 9-fold and a 4-fold range, respectively. These differences may be related to the induction status of the individual liver donors or to genetic differences relating to expression of the specific liver cytochromes P-450 involved in oxazaphosphorine activation. In the majority of human liver samples examined, ifosphamide was activated at a lower rate than cyclophosphamide. This finding in part reflects the lower intrinsic ifosphamide 4-hydroxylase activity of the high K_m P-450s that contribute to human liver microsomal oxazaphosphorine metabolism under our assay conditions (Tables 1 and 2) and is consistent with the clinical observation that an equimolar dose of ifosphamide produces less plasma alkylating activity than cyclophosphamide in cancer patients (34). The lower inherent metabolism of ifosphamide via the 4-hydroxylation pathway may, in part, be a factor for the increased efficacy of cyclophosphamide relative to ifosphamide.
The rate of cyclophosphamide hydroxylation by the CYP2B6 inhibitor orphenadrine, was related to the level of immobilereactive CYP2B, in particular CYP2B6 protein, in the individual microsomal samples. Considerable variation in CYP2B6 mRNA levels has been observed in human liver specimens (16), a finding that could be related to genetic factors, or perhaps to a variable induction of CYP2B6 in these livers by exposure to drugs and xenobiotics. CYP2B enzymes belonging to species such as rat (CYP2B1 and 2B2), rabbit (CYP2B4 and 2B5), and also primates [cynomolgus monkey liver P-450 CMLa (38)] are highly inducible by phenobarbital and other lipophilic drugs (39), suggesting that CYP2B6 may be subject to similar regulation in human liver. An important observation in the present study is that in a liver microsomal sample with a low CYP2B level (liver HLS8), this enzyme did not contribute to cyclophosphamide or ifosphamide activation as evidenced by the lack of inhibition by orphenadrine.

Although CYP2B6 was found to make a significant contribution to human liver cyclophosphamide metabolism, this enzyme has little or no effect on microsomal ifosphamide activation. CYP2B6 is, however, catalytically competent with respect to ifosphamide activation, as revealed by the CYP2B6-dependent toxification of ifosphamide to cultured lymphoblastoid cells, and by the activity of the cDNA-expressed P-450. This CYP2B6 activity was, however, ~8-fold lower with ifosphamide than with cyclophosphamide, a difference that probably accounts for the minor role of liver microsomal CYP2B6 in ifosphamide activation. Our observation that CYP2B6-expressing cultured lymphocytes are sensitive to both cyclophosphamide and ifosphamide may indicate that ifosphoramide mustard, derived from ifosphamide, is more toxic to these cells than is phosphoramide mustard. Alternatively, the continuous drug exposure/antiproliferation assay used to monitor drug cytotoxicity in this cellular system may not be linearly responsive to differences in the rates of oxazaphosphorine activation [cf., lack of major effect of liver cyclophosphamide 4-hydroxylation rate on overall drug cytotoxicity at standard drug dosages (1)].

CYP3A enzymes contribute significantly to liver microsomal ifosphamide 4-hydroxylation, as demonstrated by the catalytic activity of cDNA-expressed CYP3A4, and by the partial inhibition of this microsomal activity by TAO and by anti-CYP3A antibody. Intersample differences were observed in the extent of inhibition of microsomal ifosphamide activation by TAO and this was related to the level of CYP3A in the liver microsomal sample. Interindividual differences in human liver CYP3A levels can be attributed to environmental and genetic factors. CYP3A4 is present in all individuals and is inducible by drugs such as rifampin and dexamethasone (31) while CYP3A5 is expressed in only 10–30% of liver samples (40, 41). Although CYP3A4, and perhaps other related CYP3A enzymes, can catalyze

![Figure 4](https://example.com/fig4.png)

**Fig. 4.** Interindividual variation in the inhibition of cyclophosphamide and ifosphamide 4-hydroxylation by orphenadrine in human liver microsomes. Microsomal incubations containing the indicated concentrations of orphenadrine were performed at 0.25 mM cyclophosphamide (CPA) or ifosphamide (IFA) as described in "Materials and Methods" with microsomal samples HLS2 (A), HLS9 (B), and HLS8 (C). Control cyclophosphamide 4-hydroxylation activities for the uninhibited controls in units of nmol/min/mg protein were 1.02 (HLS2), 0.77 (HLS9), and 0.25 (HLS8). Control ifosphamide 4-hydroxylation activities were 0.42 (HLS2), 0.47 (HLS9), and 0.19 (HLS8) nmol/min/mg.

A CYP2B enzyme, likely CYP2B6, was shown to contribute substantially to the metabolism of cyclophosphamide in some, but not all, of our human liver microsomal preparations. This intersample variation, evidenced by the variable extent of inhibition of liver microsomal cyclophosphamide hydroxylation by the CYP2B6 inhibitor orphenadrine, was related to the level of immobilereactive CYP2B, in particular CYP2B6 protein, in the individual microsomal samples. Considerable variation in CYP2B6 mRNA levels has been observed in human liver specimens (16), a finding that could be related to genetic factors, or perhaps to a variable induction of CYP2B6 in these livers by exposure to drugs and xenobiotics. CYP2B enzymes belonging to species such as rat (CYP2B1 and 2B2), rabbit (CYP2B4 and 2B5), and also primates [cynomolgus monkey liver P-450 CMLa (38)] are highly inducible by phenobarbital and other lipophilic drugs (39), suggesting that CYP2B6 may be subject to similar regulation in human liver. An important observation in the present study is that in a liver microsomal sample with a low CYP2B level (liver HLS8), this enzyme did not contribute to cyclophosphamide or ifosphamide activation as evidenced by the lack of inhibition by orphenadrine.

Although CYP2B6 was found to make a significant contribution to human liver cyclophosphamide metabolism, this enzyme has little or no effect on microsomal ifosphamide activation. CYP2B6 is, however, catalytically competent with respect to ifosphamide activation, as revealed by the CYP2B6-dependent toxification of ifosphamide to cultured lymphoblastoid cells, and by the activity of the cDNA-expressed P-450. This CYP2B6 activity was, however, ~8-fold lower with ifosphamide than with cyclophosphamide, a difference that probably accounts for the minor role of liver microsomal CYP2B6 in ifosphamide activation. Our observation that CYP2B6-expressing cultured lymphocytes are sensitive to both cyclophosphamide and ifosphamide may indicate that ifosphoramide mustard, derived from ifosphamide, is more toxic to these cells than is phosphoramide mustard. Alternatively, the continuous drug exposure/antiproliferation assay used to monitor drug cytotoxicity in this cellular system may not be linearly responsive to differences in the rates of oxazaphosphorine activation [cf., lack of major effect of liver cyclophosphamide 4-hydroxylation rate on overall drug cytotoxicity at standard drug dosages (1)].

CYP3A enzymes contribute significantly to liver microsomal ifosphamide 4-hydroxylation, as demonstrated by the catalytic activity of cDNA-expressed CYP3A4, and by the partial inhibition of this microsomal activity by TAO and by anti-CYP3A antibody. Intersample differences were observed in the extent of inhibition of microsomal ifosphamide activation by TAO and this was related to the level of CYP3A in the liver microsomal sample. Interindividual differences in human liver CYP3A levels can be attributed to environmental and genetic factors. CYP3A4 is present in all individuals and is inducible by drugs such as rifampin and dexamethasone (31) while CYP3A5 is expressed in only 10–30% of liver samples (40, 41). Although CYP3A4, and perhaps other related CYP3A enzymes, can catalyze

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Cyclophosphamide</th>
<th>Ifosphamide</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 mM</td>
<td>2 mM</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>HLS9</td>
<td>(0.77)</td>
<td>(4.63)</td>
<td>(0.47)</td>
</tr>
<tr>
<td>+Orphenadrine</td>
<td>40</td>
<td>47</td>
<td>21</td>
</tr>
<tr>
<td>+Anti-CYP2B IgG</td>
<td>38</td>
<td>37</td>
<td>15</td>
</tr>
<tr>
<td>HLS9</td>
<td>(0.75)</td>
<td>(3.63)</td>
<td>(0.52)</td>
</tr>
<tr>
<td>+TAO</td>
<td>6</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>+Anti-CYP3A IgG</td>
<td>12</td>
<td>37</td>
<td>32</td>
</tr>
</tbody>
</table>

* Experiments were performed with liver microsomal sample HLS9 as described in the legends to Figs. 4, 5, and 6.

b Results are expressed as percentage of inhibition at the highest inhibitor concentration used, or as nmol product formed/min/mg microsomal protein (numbers in parentheses).

Data shown for 0.25 mM orphenadrine are from Fig. 4, while the 2 mM data for TAO and the antibody inhibition experiments are from Figs. 5 and 6. Other data are based on similar inhibition curves not shown.

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Fig. 5. Differential effect of TAO on cyclophosphamide and ifosphamide 4-hydroxylation catalyzed by human liver microsomes. Complete assay mixtures (including NADPH but without substrate) were preincubated with the indicated concentration of TAO for 30 min at 37°C prior to adding cyclophosphamide (CPA, 2 mM) or ifosphamide (IFA, 2 mM). Enzyme assays were performed as described in "Materials and Methods" with microsomal sample HLS2 (A), HLS9 (B), and HLS8 (C). Uninhibited cyclophosphamide 4-hydroxylation activities in units of nmol/min/mg protein were: 5.05 (HLS2), 3.63 (HLS9), and 0.90 (HLS8). Uninhibited ifosphamide 4-hydroxylase activities were: 1.86 (HLS2), 2.68 (HLS9), and 0.64 (HLS8) nmol/min/mg.

Liver microsomal ifosphamide activation, they make little or no contribution to microsomal cyclophosphamide hydroxylation. These results are analogous to our recent findings with rat liver microsomes, where a dexamethasone-inducible rat CYP3A accounts for the majority of ifosphamide 4-hydroxylase activity, but does not contribute to cyclophosphamide 4-hydroxylation (4).

Although cDNA-expressed CYP2A6 is catalytically competent in activating cyclophosphamide and ifosphamide, CYP2A6 was found to play only a minor role in the activation of these drugs in human liver microsomes. This is most likely a reflection of the low specific content of CYP2A6 in human liver, estimated to be only 1–10% of the total spectral cytochrome P-450 in this tissue (42, 43). Substantial interindividual variation in CYP2A6-dependent coumarin 7-hydroxylase, CYP2A-immunoreactive protein (43, 44), and CYP2A6 mRNA (45) has been observed in human liver samples. The human liver sample used in our antibody inhibition studies (liver HLS2; Fig. 6C) corresponds to the sample in our panel with the highest level of CYP2A6-mediated coumarin 7-hydroxylase activity. Therefore, the 20% contribution of CYP2A6 toward microsomal oxazaphosphorine activation

Fig. 6. Effects of anti-cytochrome P-450 antibodies on cyclophosphamide and ifosphamide 4-hydroxylase in human liver microsomes. Complete assay mixture (minus NADPH) was preincubated with the indicated amount of anti-CYP2B IgG (A), anti-CYP2A IgG (B), or anti-CYP2A IgG (C) for 30 min at room temperature prior to adding NADPH to initiate cyclophosphamide (CPA, □) or ifosphamide hydroxylation (IFA, △). Incubations with control rabbit IgG were done in parallel (cyclophosphamide, ◯; ifosphamide, ▲). Enzyme assay was performed as described in "Materials and Methods" (2 mM substrate) with microsomal sample HLS9, except in the experiment with anti-CYP2A IgG (C), in which sample HLS2 was used (0.25 mM substrate). Anti-CYP2A IgG is seen to extensively inhibit coumarin 7-hydroxylation activity (CMR) (C, ---), even though it had only modest effects on cyclophosphamide and ifosphamide hydroxylation in the same microsomal sample. Uninhibited cyclophosphamide 4-hydroxylation activities in units of nmol/min/mg protein were 4.63 (HLS9) and 0.75 (HLS2). Control ifosphamide 4-hydroxylation activities were 3.00 (HLS9), 0.33 (HLS2). Control coumarin 7-hydroxylation activity was 1.19 nmol/min/mg.
K oxazaphosphorine 4-hydroxylases, in contrast to CYP2A6, 2B6, and 3A4, which have high Km forms. These low Km CYP2C enzymes are likely to be more relevant pharmacologically because the peak plasma concentration of cyclophosphamide and ifosphamide achieved in cancer patients given standard drug dosages is typically 0.1–0.7 μM (46) and 0.2–1.2 μM (47, 48), respectively. However, the high Km enzymes could also contribute significantly to oxazaphosphorine activation, particularly in induced livers, because of their substantially higher intrinsic catalytic activity (Vmax) with these drugs. The proposed participation of CYP2C8 and CYP2C9 in cyclophosphamide and ifosphamide metabolism could also explain, at least in part, the large interindividual differences in the clinical pharmacokinetics and metabolism of these drugs (1, 12, 13) since CYP2C8 and CYP2C9 and closely related human CYP2C enzymes appear to be expressed in a polymorphic fashion in human liver (49, 50).

CYP1A1 is absent or present at very low levels in human liver (51, 52), but it can be expressed in extrahepatic tissues such as lung (53, 54). In contrast, CYP1A2 is present constitutively in human liver (55, 56). However, neither CYP1A1 nor CYP1A2 was found to be capable of hydroxylating cyclophosphamide or ifosphamide. Therefore, the activation of these anticancer drugs is not likely to be influenced by modulators of CYP1A, such as cigarette smoke (56, 57) and the antitussive agent omeprazole (58, 59). Although the present study suggests that human CYP1A1 is not likely to activate cyclophosphamide or ifosphamide in lung tissues, a recent report indicates that another enzyme, prostat glandin H synthase, may activate cyclophosphamide in rodent lung (11).

CYP2D6 is a polymorphically expressed enzyme for which 5–10% of the Caucasian population exhibits a genetic deficiency associated with the debrisoquine hydroxylation poor metabolizer phenotype (60). In the present study, microsomal cyclophosphamide and ifosphamide hydroxylation were unaffected by the CYP2D6-selective inhibitor quinidine, suggesting that this enzyme does not participate in the activation of these anticancer drugs. This conclusion is consistent with the inactivity of cDNA-expressed CYP2D6 with these drug substrates (data not shown) and with the observation that neither cyclophosphamide nor ifosphamide competitively inhibits microsomal metabolism of the CYP2D6 substrate bufuralol (61). These are in vitro studies with CYP2D6 in accord with a recent clinical report that in cancer patients, the total body clearance of ifosphamide is not correlated with the debrisoquine metabolic ratio (62), which is used as an index for CYP2D6 activity in vivo (63).

Hepatic CYP2E1 levels are elevated in alcoholics and in patients treated with the antituberculosis drug isoniazid (64, 65). Since CYP2E1 was shown in the present study not to metabolize cyclophosphamide or ifosphamide, oxazaphosphorine activation in cancer patients is not likely to be altered by blood alcohol levels or by other CYP2E1-specific modulators.

In summary, human CYP2A6, -2B6, -2C8, -2C9, and -3A4 enzymes are capable of activating cyclophosphamide and ifosphamide, whereas CYP1A1, -1A2, -2D6, and -2E1 are catalytically inactive with these drug substrates. Since CYP2B and CYP3A preferentially activate cyclophosphamide and ifosphamide, respectively, in human liver microsomes, clinical strategies to improve the therapeutic efficacy of cyclophosphamide through modulation of liver cytochrome P-450 enzyme levels should therefore focus on CYP2B and, in the case of ifosphamide, CYP3A. Further studies are required to evaluate the usefulness of altering the expression of select liver cytochrome P-450 enzymes in order to modulate cyclophosphamide and ifosphamide therapy.

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Differential Activation of Cyclophosphamide and Ifosfamide by Cytochromes P-450 2B and 3A in Human Liver Microsomes


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