STEREOSELECTIVE METABOLISM OF IFOSFAMIDE BY HUMAN P-450S 3A4 AND 2B6.
FAVORABLE METABOLIC PROPERTIES OF R-ENANTIOMER

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ABSTRACT:
The anticancer prodrug ifosfamide (IFA) contains a chiral phosphorous atom and is administered clinically as a racemic mixture of R and S enantiomers. Animal model studies and clinical data indicate enantioselective differences in cytochrome P-450 (CYP) metabolism, pharmacokinetics, and therapeutic efficacy between the two enantiomers; however, the metabolism of individual IFA enantiomers has not been fully characterized. The role of CYP enzymes in the stereoselective metabolism of R-IFA and S-IFA was investigated by monitoring the formation of both 4-hydroxy (activated) and N-dechloroethyl (DCl) (inactive, neurotoxic) metabolites. In the 4-hydroxylation reaction, cDNA-expressed CYPs 3A4 and 3A5 preferentially metabolized R-IFA, whereas CYP2B6 was more active toward S-IFA. Enantioselective IFA 4-hydroxylation (R > S) was observed with six of eight human liver samples. In the N-dechloroethylation reaction, CYPs 3A4 and 2B6 both catalyzed a significantly higher intrinsic metabolic clearance \( \frac{V_{\text{max}}}{K_m} \) of S-IFA compared with R-IFA. Striking P-450 form specificity in the formation of individual DCl metabolites was evident. CYPs 3A4 and 3A5 preferentially produced (R)N2-DCl-IFA and (R)N3-DCl-IFA (derived from R-IFA and S-IFA, respectively), whereas CYP2B6 correspondingly formed (S)N3-DCl-IFA and (S)N2-DCl-IFA. In human liver microsomes, the CYP3A-specific inhibitor troleandomycin suppressed (R)N2- and (R)N3-DCl-IFA formation by \( \geq 80\% \), whereas (S)N2- and (S)N3-DCl-IFA formation were selectively inhibited (\( \geq 85\% \)) by a CYP2B6-specific monoclonal antibody. The overall extent of IFA N-dechloroethylation varied with the CYP3A4 and CYP2B6 content of each liver, but was significantly lower for R-IFA (32 \( \pm \) 13\%) than for S-IFA (62 \( \pm \) 17\%, \( n = 8; p < .001 \)) in all livers examined. R-IFA thus has more favorable liver metabolic properties than S-IFA with respect to less extensive N-dechloroethylation and more rapid 4-hydroxylation, indicating that R-IFA may have a distinct clinical advantage over racemic IFA.

Ifosfamide (IFA)\(^1\) and its structural analog cyclophosphamide (CPA) belong to a group of oxazaphosphorine alkylating agents widely used in the therapeutic management of a variety of human malignancies (Fleming, 1997; Wright, 1997). IFA and CPA are both prodrugs that require activation by liver cytochrome P-450 (CYP) enzymes for therapeutic effect (Sladek, 1994). The primary activated prodrugs that require activation by liver cytochrome P-450 (CYP) mustards. The latter species is a DNA cross-linking derivative that is responsible for the cytotoxic activity of IFA (Wright, 1997). Alternative metabolic pathways include P-450-catalyzed dechloroethylation of IFA at N2 or N3 (Fig. 1). These reactions yield equimolar amounts of the neurotoxic metabolite chloroacetaldehyde and the corresponding N-dechloroethylated (DCl) metabolite, 2-DCl-IFA or 3-DCl-IFA, both of which lack significant host toxicity and antitumor activity (Sladek, 1994). In spite of their similarities in chemical structure and mechanism of action, CPA and IFA differ significantly with respect to their pharmacokinetic and pharmacodynamic properties (Wright, 1997; Fleming, 1997). IFA exhibits greater cytotoxicity than CPA in some tumor models, produces less myelosuppression than CPA, and induces little cross-resistance (Hilgard et al., 1983; Berger et al., 1990). In early clinical trials, IFA was found to be severely urotoxic because of high concentrations of excreted acrolein, a problem that was substantially overcome with the introduction of sodium 2-mercaptoethane sulfonate, an effective uroprotective agent (Fleming, 1997). Chloroacetaldehyde is a major metabolite of IFA that contributes to urotoxicity and also the neurotoxicity associated with this drug (Goren et al., 1986; Pohl et al., 1989). In contrast to CPA, which is metabolized predominantly by ring 4-hydroxylation, with IFA as much as 50% of the administered dose is dechloroethylated by side chain oxidation (Kurowski and Wagner, 1993; Boddy et al., 1995a). The efficacy of IFA is thus determined by a delicate balance between two alternative, CYP-catalyzed metabolic pathways: a therapeutically beneficial drug...
activation/cytotoxic pathway (4-hydroxylation) and an undesirable drug deactivation/toxification pathway (N-dechloroethylation).

IFA is a chiral molecule that contains an asymmetric phosphorus atom and thus exists in two enantiomeric forms, R-IFA and S-IFA. The individual DCI metabolites, 2-DCI-IFA and 3-DCI-IFA, are also chiral and exist in both R and S forms (Fig. 1). Biological preparations of IFA are racemic mixtures (50:50) of the two enantiomers (rac-IFA). Clinical and rodent model studies have reported significant differences in the stereoselective metabolism and disposition of the enantiomers, with S-IFA more extensively cleared by the N-dechloroethylation pathway than R-IFA (Wainer et al., 1994a,b; Granvil et al., 1996a). Differences in the enantiomeric composition of the 2- and 3-DCI-IFA metabolites present in urine of two rhabdomyosarcoma patients have been reported (Misiura et al., 1983), and significant interindividual differences in the stereoselectivity of urinary 3-DCI-IFA have been seen in a larger group of pelvic carcinoma patients (Granvil et al., 1996a; Wainer et al., 1996). Enantioselective excretion of IFA and its DCI metabolites was observed following a single 3-h infusion of drug in a pattern that suggests the involvement of at least two P-450 enzymes with distinct stereospecificity toward IFA DCI (Wainer et al., 1996). Support for this proposal is provided by the differential responsiveness of the individual pathways of IFA N-dechloroethylation to the liver P-450-inducing agent phenobarbital in the rat model (Granvil et al., 1994; Lu et al., 1998). Rat CYP2B and CYP3A enzymes both contribute to liver microsomal IFA N-dechloroethylation, with CYP3A enzymes playing a more significant role in the case of CPA (Yu and Waxman, 1996; Brain et al., 1998; Yu et al., 1999), however, the catalytic roles of the corresponding human P-450 enzymes in the metabolism of the individual enantiomers of IFA have not been detailed.

Distinct subsets of liver P-450 enzymes contribute to the activation and inactivation of IFA (Chang et al., 1993; Weber and Waxman, 1993; Yu and Waxman, 1996; Roy et al., 1999), raising the possibility that these alternative pathways of P-450-catalyzed IFA metabolism can be modulated in a therapeutically beneficial manner using P-450 form-specific inhibitors and inducers (Brain et al., 1998). Another strategy proposed for increasing the therapeutic index of IFA is based on the observation that R-IFA is inactivated by the same pathways as S-IFA (Weiner et al., 1995), and derives support from both human and rodent studies. Here, we present results indicating that R-IFA is more extensively N-dechloroethylated than S-IFA. The principal aims of the present study were to further investigate this proposal using human liver microsomal systems and cDNA-expressed human P-450 enzymes with the following two goals: 1) to establish the metabolic fates of R-IFA and S-IFA to better evaluate the proposed therapeutic utility of using one enantiomer compared with the other; and 2) to identify the principal human P-450 enzymes responsible for the enantioselective metabolism of R-IFA and S-IFA via both the 4-hydroxylation and the N-dechloroethylation pathways. Our findings strongly support the proposed use of R-IFA in place of rac-IFA, and further demonstrate that P-450s 3A4 and 2B6 both catalyze IFA N-dechloroethylation, but with unique stereoselectivities, enabling one to predict the contribution of each enzyme to overall human liver microsomal IFA N-dechloroethylation on the basis of the stereochirality of the individual dechloroethylated metabolites.

**Experimental Procedures**

**Materials.** Racemic IFA was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Purified R-IFA and S-IFA were obtained from Chirosciences, Ltd. (Cambridge, UK). DCI metabolites of IFA (N2-DCI-IFA and N3-DCI-IFA) and 4-hydroxy-IFA were gifts from Dr. J. Poli (ASTA Pharma AG, Bielefeld, Germany). Tro历代enomycins (TAD) were obtained from Pfizer, Inc. (Brooklyn, NY). NADPH and trofosfamide were purchased from Sigma Chemical Co. (St. Louis, MO). All other specialty chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). Anti-CYP2B6 monoclonal antibody (mAb-2B6) and Supersomes containing human P-450s expressed in a Baculovirus expression system containing NADPH P-450 reductase (OR) with or without cytochrome b$_5$ (as indicated) were obtained from Gentest Corporation (Woburn, MA). Supersomes prepared in these experiments were as follows: CYP2B6 + OR + b$_5$, P255, lot 1; CYP3A4 + OR + b$_5$, P202, lot 10; CYP3A5 + OR + b$_5$, P235, lot E43; CYP3A5 + OR, P235, lot 6.

**Microsomes.** Microsomes containing cDNA-expressed human P-450s were from Baculovirus-infected insect cells (Supersomes) engineered to express individual human P-450 cDNAs and human NADPH P-450 reductase. Some of the Supersomes also contained cytochrome b$_5$ as indicated in the figures and tables. Supersomes generally exhibited specific P-450 catalytic activities severalfold higher than the average human liver microsomal sample when calculated on a per milligram protein basis. Negative controls corresponding to Supersomes, which do not contain expressed P-450s, were run in parallel in each enzymatic incubation. A panel of 17 human liver microsomes, designated HLS2 to HLS36 (Roy et al., 1999), was prepared as described (Waxman et al., 1988) using livers obtained from organ donors. Inhibition studies used TAO for selective inhibition of CYP3A enzymes and mAb-2B6 (Yang et al., 1998) for selective inhibition of CYP2B6 at concentrations and under conditions shown to be maximally inhibitory (Roy et al., 1999). Microsomal protein concentrations were determined by the Bradford method with BSA as standard. P-450 form-diagnostic activities for 10 human liver microsomal P-450s were previously assayed in this same panel of livers as reported (Roy et al., 1999), however, the catalytic roles of the corresponding human P-450 enzymes in the metabolism of the individual enantiomers of IFA have not been detailed.

**IFA 4-Hydroxylation Assay.** Microsomal IFA 4-hydroxylase activity was assayed by monitoring formation of acrolein using an HPLC method with fluorescence detection (Bohnenstengel et al., 1997), with minor modifications (Huang and Waxman, 1999). Microsomal incubations were carried out under conditions where product formation was linear with respect to time and substrate consumption was <20%, as verified by analysis of residual substrate by gas chromatography (see below). Microsomal assays contained either 1 mM R-IFA or 1 mM S-IFA or, in some cases, 2 mM rac-IFA substrate, as indicated. These concentrations can be compared with peak plasma levels of 0.4 mM IFA.
IFN-α-induced IFN-β mRNA expression in cells treated with 10 ng/ml IFN-α for 24 hours was analyzed using reverse transcription-PCR.

**Results**

The results showed a significant increase in IFN-β mRNA expression compared to control cells (p < 0.05). The same trend was observed in experiments performed in triplicate, confirming the reproducibility of the findings.

**Discussion**

The upregulation of IFN-β expression under IFN-α stimulation highlights the importance of considering IFN-α-induced responses in the context of antiviral therapy. Further studies are needed to understand the molecular mechanisms underlying this response and to explore potential therapeutic strategies.

**Conclusions**

The study provides evidence for the potential of IFN-α to upregulate IFN-β expression, which may have implications for the treatment of viral infections.

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**References**


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**Figure 2**

![Activation (4-hydroxylation) of R-IFA (1 mM), S-IFA (1 mM), and rac-IFA (2 mM) catalyzed by CYPs 2B6, 3A4, and 3A5.](image-url)
liver microsomal IFA 4-hydroxylation (Roy et al., 1999), exhibited a stereochemical selectivity in favor of S-IFA 4-hydroxylation (S > R, ~4:1), similar to CYP2B6 (data not shown).

**N-Dechloroethylation.** The stereospecificity of IFA N-dechloroethylation was investigated using cDNA-expressed CYPs 3A4, 3A5, and 2B6 (Fig. 3). These three CYPs were examined because in aggregate they catalyze >90% of human liver microsomal IFA N-dechloroethylation (Huang et al., in press; Walker et al., 1994). N-dechloroethylation of R-IFA yields R2-DCl-IFA and S3-DCl-IFA, whereas S-IFA can generate R3-DCl-IFA and S2-DCl-IFA. rac-IFA can potentially be metabolized to a mixture of all four DCl metabolites, shown at the bottom of Fig. 1. These isomeric IFA metabolites were analyzed by enantioselective gas chromatography. As shown in Fig. 3A, S-IFA was N-dechloroethylated by CYP3A4 at a higher overall rate than R-IFA. Moreover, CYP3A4 primarily N-dechloroethylated S-IFA to yield R3-DCl-IFA, whereas S2-DCl-IFA was a minor metabolite (Fig. 3A). A very similar metabolic profile was obtained for CYP3A5, where R3-DCl-IFA (derived from S-IFA) was essentially the sole DCl metabolite (Fig. 3B). In contrast, CYP2B6 N-dechloroethylated R-IFA and S-IFA at more similar rates, but exhibited a strong selectivity for formation of S3-DCl-IFA and S2-DCl-IFA, derived from R-IFA and S-IFA, respectively (Fig. 3C). Similar patterns were obtained when using rac-IFA as substrate (Fig. 3, right side).

Apparent $K_m$ and $V_{max}$ values for these N-dechloroethylation reactions were determined separately for each enantiomer, following which $V_{max}/K_m$ (intrinsinc clearance) values were calculated from Michaelis-Menten double reciprocal plots (Table 1). Analysis of the data using either Eadie-Hofstee or Hanes plots gave very similar results, with no evidence for multiphasic kinetics (data not shown). $K_m$ values ranged from 0.5 to 2.3 mM in the various N-dechloroethylation reactions, and are similar to those measured for the corresponding 4-hydroxylation reactions catalyzed by these same two CYPs (Roy et al., 1999). These low millimolar $K_m$ values are typical of many P-450 drug substrates and are in the range of peak plasma IFA concentrations in patients treated with IFA as bolus injection at 4 g/m² (Lokiec et al., 1996). For CYP3A4 and with R-IFA as substrate, the rank order of enzyme efficiency, expressed in the form of the $V_{max}/K_m$ ratio, was R2-DCl-IFA > S3-DCl-IFA; whereas with S-IFA as substrate, the order was: R3-DCl-IFA > S2-DCl-IFA (~15-fold). By contrast, for CYP2B6 and with R-IFA as substrate, the rank order of $V_{max}/K_m$ was S3-DCl-IFA > R2-DCl-IFA; whereas with S-IFA as substrate, the order was S2-DCl-IFA > R3-DCl-IFA (~5-fold). With both enzymes, greater positional selectivity in the N-dechloroethylation reaction was seen with S-IFA, where CYP3A4 preferentially metabolized the ring chloroethyl group to give the N3 metabolite, whereas CYP2B6 metabolized the exocyclic chloroethyl group to give the N2 metabolite.

**Stereoselectivity of IFA Metabolism by Human Liver Microsomes. 4-Hydroxylation.** Analysis of the R-IFA and S-IFA 4-hydroxylase activities of a panel of eight human liver microsomes revealed significant interindividual differences both in the activity and the stereochemochemical specificity of the reactions (Fig. 4). For two of the liver samples, HLS2 and HLS9, the sum of the 4-hydroxylation activities measured individually at 1 mM R-IFA and 1 mM S-IFA was similar to the activity obtained at 2 mM rac-IFA. By contrast, with the other liver samples, the 4-hydroxylation activity with rac-IFA was significantly lower than would be predicted based on the sum of the activities obtained with R-IFA and S-IFA individually, a finding that is characteristic of substrate saturation. Thus, when present as a racemic mixture, R-IFA and S-IFA do not behave as independent substrates, but have the potential for inhibitory interactions at the liver microsomal P-450’s active site in a manner similar to that seen with the cDNA-expressed enzymes (cf., Fig. 2).
N-Dechloroethylation. A panel of 17 human liver microsomes was assayed for N-dechloroethylation of \( R \)-IFA and \( S \)-IFA. Data for eight of the individual human liver microsomes, which encompass the range of CYP2B6 and CYP3A4 marker activities seen in the full panel (Table 2 and data not shown), are presented in Fig. 5. Large interindividual differences were noted in the formation of specific positional- and stereoisomers of DCl-IFA. \( R \)-2-DCl-IFA was the exception, because its levels were relatively constant across the liver panel. The overall pattern was characterized by a high level of \( R \)-3 metabolite, a medium level of \( S \)-2 and \( S \)-3 metabolites, and a low level of \( R \)-2 metabolite (Fig. 5). This pattern mirrors the relative abundance of these four IFA metabolites in cancer patients (Granvil et al., 1996a).

Among the livers, the CYP2B6-rich liver HLS2 demonstrated the highest rate of formation of \( S \)-2-DCI-IFA and \( S \)-3-DCI-IFA, whereas liver HLS9, which expresses a particularly high level of CYP3A4 activity (Table 2) displayed the highest rate of \( R \)-3-DCI-IFA formation (Fig. 5). High amounts of \( R \)-3-DCI-IFA were also formed by HLS36, despite its low level of CYP3A-dependent (Waxman et al., 1988) testosterone 6\( \beta \)-hydroxylase activity, indicating the possible participation of other P-450s in this reaction in some livers.

Examination of the relationship between the rates of formation of each of the DCI metabolites of IFA and the CYP3A-dependent testosterone 6\( \beta \)-hydroxylase activity of each individual liver (\( n = 17 \)) revealed that liver microsomal CYP3A activity correlated with the formation of \( R \)-3-DCI-IFA (Fig. 6A). A much weaker correlation between CYP3A activity and formation of \( R \)-2-DCI-IFA, the minor metabolite of CYP3A4 (cf., Fig. 3 and Table 1) was observed (\( r = 0.39, \ p < .2; \) data not shown). In contrast, the rates of formation of \( S \)-2-DCI-IFA (Fig. 6C) and \( S \)-3-DCI-IFA (\( r = 0.81, \ p < .001; \) data not shown) correlated with the CYP2B6-dependent 7-ethoxy-4-trifluoromethyl coumarin (7-EFC) O-deethylation activity (Code et al., 1997) of the human liver microsome panel. There was no correlation between rates of formation of the two CYP3A-associated metabolites, \( R \)-2 and \( R \)-3 DCI-IFA, with the rates of formation of the two CYP2B6-characteristic metabolites, \( S \)-2 and \( S \)-3 DCI-IFA (data not shown). In contrast, \( R \)-2 and \( R \)-3 DCI-IFA correlated with each other (Fig. 6B), as did the rates of formation of \( S \)-2 and \( S \)-3 DCI-IFA (Fig. 6D). These latter findings mirror the correlations reported by Wainer and associates (Wainer et al., 1996) for the corresponding urinary metabolites in cancer patients, supporting the relevance of the in vitro liver microsomal enzyme system for prediction of metabolic profiles in patients. Of note, the single outlier point on the \( S \)-2-DCI versus \( S \)-3-DCI corre-
lation curve (Fig. 6D; circled data point; liver HLS9), was not an outlier on the CYP2B6 activity versus S3-DCl curve (Fig. 6C), indicating that this liver exhibits a S2-DCl activity that is lower than expected based on its CYP2B6 content. This may reflect the presence in liver HLS9 of a very high level of CYP3A4 (Table 2), which may compete with CYP2B6 for the rate-limiting microsomal NADPH P-450 reductase by actively metabolizing S-IFA to yield R3-DCl-IFA at the expense of S2-DCl-IFA.

Inhibition of Stereospecific IFA N-Dechloroethylation in Human Liver Microsomes. P-450 form-specific chemical inhibitors and inhibitory antibodies can be used to establish the contribution of individual P-450 enzymes to the metabolism of specific drugs in human liver microsomes. Monoclonal antibody immunoinhibitory to CYP2B6 (mAb-2B6) (Yang et al., 1998) and the CYP3A4-specific inhibitor TAO (Chang et al., 1994; Hickman et al., 1998) were employed to further establish the roles played by these two enzymes in the generation of DCl-IFA enantiomers in human liver microsomes.

In liver HLS2, saturating levels of mAb-2B6 (50 μg/100 μg microsomal protein) inhibited by 85% the formation of S3-DCl-IFA from R-IFA and completely blocked formation of S2-DCl-IFA from S-IFA (Fig. 7A, arrows). A similar inhibitory pattern was seen in a second liver sample, HLS9 (Fig. 7B). A high selectivity of the antibody was apparent, insofar as formation of the R3-DCl metabolite from S-IFA was not inhibited in either liver. In contrast, the CYP3A4 inhibitor TAO (50 μM) substantially blocked formation of R2-DCl-IFA and R3-DCl-IFA from R-IFA and S-IFA, respectively (>80–90% inhibition) in both human liver microsomes (Fig. 7, C and D, arrows). Formation of the S3 and S2 DCl-IFA metabolites was inhibited by TAO to a much lesser extent (<10–20%), consistent with the dominant role of CYP2B6 in these latter microsomal reactions indicated by the antibody inhibitions.

Evaluation of Metabolic Properties of R-IFA versus S-IFA. In view of the proposal, based on pharmacokinetic studies, that the metabolic properties of R-IFA may be superior to those of S-IFA (Wainer et al., 1995), we compared R-IFA and S-IFA with respect to their total 4-hydroxylase and N-dechloroethylase activities (sum of all four DCl-IFA metabolites) catalyzed by CYPs 2B6, 3A4, and 3A5 and eight individual human liver microsomal samples (Table 3). With all three expressed enzymes and with five of the eight livers, S-IFA was more actively metabolized than R-IFA [Table 3; sum (4OH+DCl)]. With cDNA-expressed CYP2B6, both R-IFA and S-IFA were metabolized predominantly by N-dechloroethylation, with little 4-hydroxylation (4OH/DCl ratio, ~1). By contrast, with CYP3A4 and R-IFA as substrate, N-dechloroethylation corresponded to only 32% of total IFA metabolism (4-hydroxy-IFA + N-DC1-IFA) compared with 84% of the total metabolism with S-IFA as substrate (Table 3, last column). An even more striking difference was seen with CYP3A5 (17% of total metabolism via N-dechloroethylation for R-IFA versus 92% for S-IFA). Moreover, in the panel of liver microsomes, the proportion of substrate channeled through the N-dechloroethylation pathway was significantly lower with R-IFA (31.8 ± 12.6% metabolism via N-dechloroethylation) than with S-IFA (61.8 ± 16.7% N-dechloroethylation) (mean ± S.D., n = 8 livers; p < .001). This reduced N-dechloroethylation of R-IFA was achieved without a
decrease in 4-hydroxylation activity compared with S-IFA as substrate (Table 3; total DC1-IFA versus 4-OH-IFA columns). This difference is also reflected in the 4-hydroxylation to N-dechloroethylation product ratio, which was $3\text{–}4$-fold higher for R-IFA than S-IFA in almost all the liver samples ($4\text{-}OH/DCl = 2.66 \pm 1.49$ versus $0.76 \pm 0.56$ for R and S IFA, respectively (mean $\pm$ S.D., $n = 8$ livers, $p < .001$).

Comparison of the rates of liver microsomal N-dechloroethylation of S-IFA versus R-IFA revealed a decrease in the extent to which S-IFA is N-dechloroethylated in incubations of rac-IFA compared with incubations containing S-IFA alone (data not shown). This resulted in a consistently higher $S/R$ N-dechloroethylation ratio ($S/R$ DC1 = $3.1 \pm 1.3$, mean $\pm$ S.D., $n = 17$) when the data was obtained from separate incubations of the two enantiomeric substrates (1 mM each) compared with incubations with rac-IFA (2 mM) ($S/R$ DC1 = $1.8 \pm 0.6$). Thus, R-IFA (1 mM), or a microsomal metabolite of R-IFA, can apparently inhibit S-IFA N-dechloroethylation.

**Discussion**

IFA metabolism is increasingly recognized as a key determinant of this drug’s antitumor activity, adverse side effects, and potential for emergence of chemoresistance. Large differences in the activity and level of expression of IFA metabolizing enzymes, specifically CYPs (Chang et al., 1993, 1997a; Walker et al., 1994), glutathione and glutathione S-transferase (Dirven et al., 1995), and aldehyde dehydrogenase (Bunting and Townsend, 1996), can in large part account for the significant interpatient differences in the clinical pharmacokinetics and biotransformation of IFA. In spite of the superior antitumor activity of IFA compared with its isomer CPA with some human cancers (Wheeler et al., 1986; de Kraker and Voute, 1989), the clinical efficacy and safety of IFA can be severely compromised due to extensive N-dechloroethylation yielding chloroacetaldehyde, believed to be responsible for several adverse side effects observed with IFA chemotherapy, including cerebral neuropathy (Boddy et al., 1995a). IFA N-dechloroethylation is both regioselective and stereospecific and gives rise to the inactive metabolites 2-DCl-IFA and 3-DCl-IFA, respectively derived from dechloroethylation of the exocyclic (N2) and ring nitrogen atoms (N3), each of which exists as a pair of enantiomers (Fig. 1). Substantial stereospecificity in IFA efficacy, toxicity, disposition, and metabolism has been described (Farmer, 1988; Wainer et al., 1994a,b; Prasad et al., 1994; Granvil et al., 1996a). The present study identifies the human liver P-450 enzymes responsible for the stereospecific formation of IFA’s DC1 metabolites and provides a metabolic basis for the wide variation in patient toxicities and responses to IFA. Moreover, the comparisons of the metabolic activity of R-IFA versus S-IFA presented here provide strong support for the proposed clinical use of R-IFA in place of the racemic drug currently in use.

Experiments using cDNA-expressed enzymes revealed higher IFA 4-hydroxylase activity associated with CYP3A4 as compared with
CYP2B6 (Fig. 2), in agreement with an earlier report (Chang et al., 1993) and a more recent comprehensive study (Roy et al., 1999) showing that CYP3A4 is a major IFA 4-hydroxylase in human liver microsomes. Moreover, R-IFA was found to be activated by CYP3A4 at a much higher rate than by CYP2B6 (Fig. 2). CYP3A5, which is expressed in human liver at a severalfold lower level than CYP3A4, at a much higher rate than by CYP2B6 (Fig. 2). CYP3A5, which is expressed in human liver at a severalfold lower level than CYP3A4, also exhibited a strong preference for 4-hydroxylation of R-IFA compared with S-IFA. In contrast, CYP2B6 activated S-IFA at a much higher rate than R-IFA, although with the cDNA-expressed enzyme preparations used in this study, S-IFA was still activated by CYP3A4 at a higher absolute rate than by CYP2B6. These findings indicate that the P-450 enzyme contributions to IFA 4-hydroxylation in human liver tissue may differ for the P-450 enzyme contributions to IFA 4-hydroxylation in human liver tissues (Shimada et al., 1994), the rate of hydroxylation of N-chloroethyl group (N2) in the case of S-IFA, whereas a second P-450 enzyme (2B6) exhibits the opposite specificity: ring dechloroethylation of R-IFA and exocyclic dechloroethylation of S-IFA (cf., Fig. 1). The specificities seen for the cDNA-expressed CYPs also characterize these enzymes in human liver tissue, as is evident from correlation studies (Fig. 6) and from liver microsomal studies using CYP3A4 and CYP2B6-selective inhibitors (Fig. 7). These findings are consistent with an earlier report, where the urinary levels of R-2-DCI-IFA and R3-DCI-IFA were closely correlated in a group of 11 adult cancer patients treated with IFA, as were those of S2-DCI-IFA and S3-DCI-IFA (Wainer et al., 1996a). The observation that the CYP2B6-derived DCI metabolites of IFA, S3-DCI-IFA, and S2-DCI-IFA, comprise a significant fraction of the total DCI metabolites of IFA, both in human liver microsomes (Fig. 5) and in cancer patients (Granvil et al., 1996a) indicates that CYP2B6 makes a significant contribution to IFA N-dechloroethylation (up to 50% in several livers). This finding is inconsistent with an earlier report suggesting that CYP3A enzymes catalyze >80% of this human liver microsomal activity (Walker et al., 1994).

Kinetic analysis of the stereoselectivity of IFA N-dechloroethylation by CYPs 3A4 and 2B6 (Table 1) revealed the highest $V_{max}$ and $V_{max}/K_m$ values for formation of R3-DCI-IFA by CYP3A4, followed by S2- and S3-DCI-IFA by CYP2B6. A similar pattern of DCI metabolites excreted into urine was observed in IFA-treated cancer patients (Wainer et al., 1996) and in human liver microsomal incubations (Fig. 5), where R3-DCI-IFA was the major metabolite, followed by the S2 and S3 metabolites, with R2-DCI-IFA generally being a minor product. Moreover, the total amount of DCI metabolites formed from S-IFA in a panel of 17 human liver microsomes was found to be ~3-fold greater than that from R-IFA. This observation is
in agreement with the more extensive clearance by N-dechloroethyl-
lation that is seen for S-IFA in clinical studies (Boos et al., 1991; Wainer et al., 1996).

The present in vitro studies support the proposed use of R-IFA in place of rac-IFA currently in clinical use, insofar as R-IFA undergoes less extensive deactivation/neurotoxicity via N-dechloroethylation than the S-IFA enantiomer in all of the liver samples examined (Table 3), and in some cases exhibits a higher rate of 4-hydroxylation (Fig. 4). Moreover, because CYP3A4 is the most abundant P-450 associated with drug metabolism in human liver (Shimada et al., 1994) and can be induced severalfold by a number of drugs commonly prescribed together with IFA (Maurel, 1996), the use of R-IFA in place of rac-IFA may provide for an even greater therapeutic advantage. cDNA expression studies demonstrated that CYP2B6 exhibits proportionally higher R-IFA N-dechloroethylation compared with CYP3A4 (≥96% versus 32%) as well as minimal R-IFA 4-hydroxylase activity (Table 3). Hence, patients with a low liver CYP2B6 level would be expected to exhibit less extensive R-IFA N-dechloroethyl-
ation (cf., livers HLS 25, 27, 28; Table 3). In contrast, patients with a high level of hepatic CYP2B6 (e.g., HLS2; Table 1) and/or a comparatively low level of hepatic CYP3A4 are likely to exhibit more extensive R-IFA N-dechloroethylation. This was clearly evident for the CYP2B6-enriched liver HLS2, which exhibited the highest extent of R-IFA metabolized by the N-dechloroethylation pathway (53%) compared with the other livers (Table 3) and might not benefit from using R-IFA to the same extent as would a CYP2B6-deficient liver (e.g., HLS25). Nevertheless, the proportion of R-IFA subject to N-
dehydroxylase was found to be significantly lower than that of S-IFA in all livers examined, highlighting the benefit of using R-IFA. These findings further suggest that the therapeutic efficacy of IFA may be enhanced by administration of a CYP2B6-specific chemical inhibitor in combination with R-IFA with the goal of further suppressing N-dechloroethylase. Alternatively, individuals with a high CYP2B6/CYP3A4 ratio could benefit from using CPA in place of IFA, in view of the major role played by CYP2B6 in CPA activation and that of CYP3A4 in CPA N-dechloroethylation in human liver (Chang et al., 1993; Roy et al., 1999). It should be noted, however, that hepatic P-450 profiles are not fixed for a given patient, and can be induced or otherwise modulated by exposure to drugs routinely administered to cancer patients (Brain et al., 1998; Yu et al., 1999), including IFA itself (Chang et al., 1997b). Methods to phenotype individual patients for their levels of expression of individual P-450s using noninvasive methods are presently available or under development (Kivisto and Kroemer, 1997) and should find increasing use in efforts to improve drug responses by tailoring the choice of chemotherapeutic drugs to individual patients on the basis of their hepatic P-450 profiles.

After submission of this manuscript, Granvil and coworkers (Granvil et al., 1999) reported a major role for CYP3A4 in human liver micromosomal metabolism of R-IFA and S-IFA to yield R2-DCI-IFA and R3-DCI-IFA, respectively, and of CYP2B6 to yield S3-DCI-IFA and S2-DCI-IFA, respectively, in agreement with the present report. Those investigators also presented multiphasic Eadie-Hofstee plots suggestive of the presence of high-affinity (Km ≥ 10 μM), low-capacity IFA N-dechloroethylase enzyme(s) in human liver microsomes; however, the relationship of that low-capacity micromosomal activity to CYP2B6 and CYP3A4, characterized in the present study as high Km, high-capacity IFA N-dechloroethylases (Table 1), was not established in that report.

In conclusion, substantial interindividual differences in the human hepatic levels and expression of the IFA-metabolizing P-450 enzymes CYP3A4 and CYP2B6 are likely to make important contributions to the interpatient variability in the enantioselective metabolism and clinical response to IFA. Metabolic studies carried out with cDNA-expressed human P-450 enzymes and human liver microsomes strongly support the proposal that R-IFA can be used to reduce N-dechloroethylation and the associated formation of the neurotoxic metabolite chloroacetaldehyde commonly seen with rac-IFA. IFA induces several human hepatic P-450 enzymes active in IFA metabol-
ism (Chang et al., 1997b) via a process that is likely to contribute to the autoinduction pharmacokinetics that characterizes the use of IFA in the clinic (Kurowski and Wagner, 1993; Prasad et al., 1994). It will thus be of interest to investigate whether R-IFA and S-IFA exhibit differential capacities to induce human hepatic P-450s capable of metabolizing cancer chemotherapeutic drugs, such as CYP3A4, CYP2B6, and CYP2C subfamily enzymes. Finally, hepatic P-450 profiling in cancer patients may be useful for tailoring the choice of chemotherapeutic drugs before the initiation of treatment in an effort to better design drug regimens on an individualized basis.

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