ROLE OF CYP2B6 AND CYP3A4 IN THE IN VITRO N-DECHLOROETHYLATION OF (R)- AND (S)-IFOSFAMIDE IN HUMAN LIVER MICROSOMES

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ABSTRACT:
The central nervous system toxicity of ifosfamide (IFF), a chiral antineoplastic agent, is thought to be dependent on its N-dechloroethylation by hepatic cytochrome P-450 (CYP) enzymes. The purpose of this study was to identify the human CYPs responsible for IFF-N-dechloroethylation and their corresponding regio- and enantioselectivities. IFF exists in two enantiomeric forms, (R)- and (S)-IFF, which can be dechloroethylated at either the N2 or N3 positions, producing the corresponding (R,S)-2-dechloroethyl-IFF [(R,S)-2-DCE-IFF] and (R,S)-3-dechloroethyl-IFF [(R,S)-3-DCE-IFF]. The results of the present study suggest that the production of (R)-2-DCE-IFF and (S)-3-DCE-IFF from (S)-IFF is catalyzed by different CYPs as is the production of (S)-2-DCE-IFF and (R)-3-DCE-IFF from (R)-IFF. In vitro studies with a bank of human liver microsomes revealed that the sample-to-sample variation in the production of (S)-3-DCE-IFF from (R)-IFF and (S)-2-DCE-IFF from (S)-IFF was highly correlated with the levels of (S)-mephenytoin N-demethylation (CYP2B6), whereas (R)-2-DCE-IFF production from (R)-IFF and (R)-3-DCE-IFF production from (S)-IFF were both correlated with the activity of testosterone 6β-hydroxylation (CYP3A4/5). Experiments with cDNA-expressed P-450 and antibody and chemical inhibition studies supported the conclusion that the formation of (S)-3-DCE-IFF and (S)-2-DCE-IFF is catalyzed primarily by CYP2B6, whereas (R)-2-DCE-IFF and (R)-3-DCE-IFF are primarily the result of CYP3A4/5 activity.

Ifosfamide (IFF, Fig. 1) is an extensively utilized anticancer agent whose clinical use has been questioned recently on the basis of adverse side effects, such as renal toxicity and neurotoxicity (Kamen et al., 1995). Neurotoxicity is perhaps the key barrier to the safe and effective use of IFF and has been observed with high-dose (Lewis and Meanwell, 1990), multiple-dose (Lewis et al., 1990), and oral (Lind et al., 1990) treatment regimes. IFF is a prodrug whose pharmacological activity results from its biotransformation to active metabolites. The antitumor effect of IFF is dependent on hydroxylation at the C4 position on the oxazaphosphorine ring followed by rearrangement to isophosphoramide mustard, a DNA cross-linking agent (Brade et al., 1985). The enzymes responsible for IFF activation have been identified as CYP3A, CYP2B1/2, and CYP2C6/11 in rats (Weber and Ingold-Prelog chemical nomenclature system.

In addition to C4-hydroxylation of the oxazaphosphorine ring, IFF is metabolized by β-oxidation of exocyclic chloroethyl alkyl moieties. This transformation takes place at either the N2 or N3 position on the oxazaphosphorine ring, producing N-2-dechloroethyl-IFF (2-DCE-IFF) or N-3-dechloroethyl-IFF (3-DCE-IFF) and chloroacetaldehyde as shown in Fig. 1 (Brade et al., 1985). The chloroacetaldehyde produced by this pathway has been proposed as the underlying cause of IFF-induced neurotoxicity (Goren et al., 1989). However, this was questioned recently, and a link between neurotoxicity toxicity and a 3-DCE-IFF metabolite has been suggested (Wainer et al., 1994a).

Although neurotoxicity is treatment-limiting, few reports have addressed the source of the DCE-IFF metabolites. One study demonstrated that hepatic microsomes from rats were capable of catalyzing IFF N-dechloroethylation and that this activity was induced by phenobarbital pretreatment and inhibited by SKF 525A and tamoxifen (Ruzicka and Ruenitz, 1992). A second in vitro study with human liver microsomes identified CYP3A4 as the key enzyme involved in the N-dechloroethylation of IFF (Walker et al., 1994).

However, both of these studies, as well as those concerning the 4-hydroxylation pathway, have ignored a key aspect of IFF, namely, that this agent is a chiral molecule. IFF contains an asymmetrically substituted phosphorus atom and exists in two enantiomeric forms, (R)-IFF and (S)-IFF. In clinical practice, IFF is administered as a racemic mixture, i.e., a 50:50 mixture of the two enantiomers.

The 2- and 3-DCE-IFF metabolites are also chiral and exist as R- and S-enantiomers (Fig. 1). The N2-dechloroethylation of (R)- and (S)-IFF produces (R)-2-DCE-IFF and (S)-2-DCE-IFF, respectively. However, the N3-dechloroethylation of (R)- and (S)-IFF yields (S)-3-DCE-IFF and (R)-3-DCE-IFF, respectively. This apparent inversion in stereochemical configuration is an artifact produced by the Cahn-Ingold-Prelog chemical nomenclature system.

In both humans and rats, the pharmacokinetics and metabolism of IFF are enantioselective where (S)-IFF is more extensively cleared by the N-dechloroethylation pathway than (R)-IFF (Misiura et al., 1983;
Boos et al., 1990; Crom et al., 1991; Prasad et al., 1994; Corlett et al., 1995; Granvil et al., 1996; Wainer et al., 1996). This difference may be clinically relevant because there appears to be a relationship between (S)-IFF N3-dechloroethylation [as determined the urinary excretion of (R)-3-DCE-IFF] and the occurrence of IFF-induced neurotoxicity (Wainer et al., 1994a).

A recent study also has identified two distinct urinary excretion patterns (Wainer et al., 1996). In 11 patients, the cumulative excretion of (R)-3-DCE-IFF [from (S)-IFF] was significantly correlated with (R)-2-DCE-IFF [from (R)-IFF] and the cumulative excretion of (S)-3-DCE-IFF [from (R)-IFF] was significantly correlated with that of (S)-2-DCE-IFF [from (S)-IFF]. These results suggest that formation of (R)-2-DCE-IFF and (R)-3-DCE-IFF is catalyzed by the same cytochrome P-450 (CYP) isoform and that formation of (S)-2-DCE-IFF and (S)-3-DCE-IFF is catalyzed by a second CYP isoform. A similar relationship has been observed in rats (Granvil et al., 1994; Lu et al., 1998). In these studies, pretreatment of the rats with phenobarbital selectively induced the formation of (R)-2-DCE-IFF and (R)-3-DCE-IFF.

The identification of the CYP isoforms responsible for IFF N-dechloroethylation would assist greatly in the effective clinical management of the drug, especially because this pathway is associated with treatment-limiting neurotoxicity. Thus, this information will aid in the prediction of metabolic drug interactions and their effects on IFF efficacy and toxicity. In addition, the establishment of the region- and enantioselectivities of the enzymes could help determine whether single-enantiomer IFF would be a better clinical agent than the racemate.

Materials and Methods

Chemicals. (R,S)-IFF was purchased from Bristol-Myers Canada (Belleville, Ontario), and individual IFF enantiomers were prepared by enantioselective HPLC using a previously described method (Masurel and Wainer, 1989). The purity of each enantiomer was greater than 99.5%. Microsomes prepared from a cell line expressing a recombinant human P-450 enzyme were obtained from Gentest Corporation (Woburn, MA). These microsomes were prepared from the human lymphoblastoid cell line, AHH-1 (originally derived from RPMI 1788 cell line), that was transfected with cDNA encoding a human CYP enzyme. Sulfaphenazole was obtained from Ciba-Giegy Ltd. (Basel, Switzerland). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Human liver microsomes used in this study have been described elsewhere (Pearce et al., 1996).

Antibodies against CYP2B and CYP3A. The antibodies against rat CYP2B1 and CYP3A1 enzymes were raised in male New Zealand White rabbits as described by Thomas et al. (1979, 1981). The purification and immunosorbption of these antibodies were carried out by previously described methods (Dutton and Parkinson, 1989; Halvorson et al., 1990).

Metabolism of IFF Enantiomers. (R)- and (S)-IFF metabolism was carried out in 0.5-ml incubation mixtures containing potassium phosphate buffer (100 mM, pH 7.4), EDTA (1 mM), MgCl₂ (3 mM), microsomes (0.5 mg/ml protein), NADP⁺ (1 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 U/ml), and (R)- and (S)-IFF (200 μM) with or without other additions as described in Results. The reactions were initiated by the addition of an NADPH-generating system. Reactions were carried out in a shaking water bath and were stopped after 0 to 60 min by sequential addition of 0.4 ml of 5.5% ZnSO₄, 0.4 ml of saturated Ba(OH)₂, and 0.2 ml of 0.01 M HCl.

IFF enantiomers and the DCE-IFF metabolites were quantified by an enantioselective gas chromatographic method with mass spectrometric detection (GC/MS), as described previously (Granvil et al., 1993). Briefly, the supernatant from the microsomal incubations was mixed with 10 μl of 100 μg/ml trofosfamide in methanol (internal standard) and 3 ml of chloroform. The resulting mixture was mixed on a vortexer for 1 min and then centrifuged at 1000g for 15 min. The aqueous layer was discarded and an aliquot of the organic phase was evaporated to dryness. The residue was reconstituted in 100 μl of toluene, and 1 μl was analyzed.

The GC was a Varian 3400 GC equipped with a Finnigan A 200S GC autosampler operating in the splitless mode containing a capillary column (8 m × 0.25 mm I.D., 0.25-mm film thickness) coated with a chiral stationary phase based on heptakis [2,6-di-0-methyl-3-0-pentyl]-β-cyclodextrin. The conditions for the GC were: injection port temperature, 210°C; the GC column temperature was programmed from 110°C at 4°C/min to 180°C; helium pressure, 55.2 kPa. The total analysis time was 18 min. The MS was a Finnigan Mat Model Incos 50 operating in the electron-impact and selective ion-monitoring (SIM) mode. Temperatures were set as follows: ion source temperature, 180°C; transfer line temperature, 250°C; electron energy, 70 eV; and emission current, 300 mA. The detection and quantification of the analytes were accomplished by selected-ion monitoring at: m/z 211 (IFF), m/z 149 (2- and 3-DCE-IFF), m/z 273 (trofosfamide). The method was linear for IFF concentrations ranging from 0.48 to 268 nmol/ml of each IFF enantiomer and 0.15 to 101 nmol/ml for each enantiomer of 2-DCE-IFF and 3-DCE-IFF. The intraday and interday coefficients of variance for precision and accuracy were less than 8%.

Kinetic Analyses. Kinetic parameters were then determined by nonlinear regression using Grafit V.3.09b Software (Limited, London, UK). Eadie-Hofstee plots (V/S versus V) were constructed to assess whether one or two enzymes were involved in the N-dechloroethylation of (R)- and (S)-IFF.

**Fig. 1.** The proposed scheme for the stereoselective metabolism of ifosfamide.
Results

Kinetic Analyses of N-Dechloroethylation of (R)- and (S)-IFF in Human Liver Microsomes. The rate of formation of (R)-2-DCE-IFF and (S)-3-DCE-IFF from (R)-IFF and (S)-2-DCE-IFF and that of (R)-3-DCE-IFF from (S)-IFF was linear with incubation times of up to 120 min and human liver microsome protein concentration of up to 1.0 mg/ml (data not shown). Unless otherwise noted, an incubation time of 60 min and a concentration of 0.5 mg/ml microsomal protein were used to ensure initial rate conditions for all experiments.

An attempt was made to estimate the kinetic constants for the dechloroethylation of (R)- and (S)-IFF using a pool of seven human liver microsomes (0.5 mg) was incubated with various concentrations of (R)-IFF and (S)-IFF (from 10 to 1000 μM) and NADPH-generating system for 60 min at 37°C. The rates of N2- and N3-dechloroethylation of ifosfamide were measured by GC-MS as described in Materials and Methods.

Fig. 2. The rate of formation of N-dechloroethylation of (R)- and (S)-IFF in relation to concentration of (R)- and (S)-IFF and the Eadie-Hofstee plots (A–D). Lines on the Eadie-Hofstee plots represent estimation of kinetic parameters for the high-affinity activity as determined at 10, 16.7, 33.3, and 125 μM substrate with the exception of B, from which no data were obtained at 10 μM substrate. A pool of seven human liver microsomes (0.5 mg) was incubated with various concentrations of (R)-IFF and (S)-IFF (from 10 to 1000 μM) and NADPH-generating system for 60 min at 37°C. The rates of N2- and N3-dechloroethylation of ifosfamide were measured by GC-MS as described in Materials and Methods.

In clinical use, the plasma concentrations of (R)-IFF and (S)-IFF after administration of (R,S)-IFF have been reported to be about 100 to 300 μM (Granvil et al., 1996). All subsequent experiments were carried out at an IFF concentration equal to 200 μM, which is also the pharmacologically relevant concentration. It is possible that, at this concentration, the enzyme reaction may involve contribution from both low and high Km enzymes.

Biotransformation of N-Dechloroethylation of (R)- and (S)-IFF in Human Liver Microsomes. All N-dechloroethylated IFF metabolites were detected in the incubates from a bank of human liver microsomes (N = 16). There was a large sample-to-sample variation
TABLE 1

Kinetic constants for the formation of dechloroethylation of (R)-IFF and (S)-IFF by human liver microsomes (high-affinity activity)

<table>
<thead>
<tr>
<th></th>
<th>$K_{\text{m}}$</th>
<th>$V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu M$</td>
<td>pmol/min/mg</td>
</tr>
<tr>
<td>From (R)-IFF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(R)-2-DCE-IFF</td>
<td>32</td>
<td>65</td>
</tr>
<tr>
<td>(S)-3-DCE-IFF</td>
<td>49</td>
<td>110</td>
</tr>
<tr>
<td>From (S)-IFF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S)-2-DCE-IFF</td>
<td>93</td>
<td>87</td>
</tr>
<tr>
<td>(R)-3-DCE-IFF</td>
<td>110</td>
<td>230</td>
</tr>
</tbody>
</table>

(R)-IFF is the source of (R)-2- and (S)-3-DCE-IFF and (S)-IFF is the source of (S)-2- and (R)-3-DCE-IFF.

A pool of seven human liver microsomes were incubated using (R)- and (S)-IFF concentrations ranging from 10 to 1000 $\mu M$ in the presence of an NADPH generating system. The rates of dechloroethylation IFF were measured by GC-MS, and $K_{\text{m}}$ and $V_{\text{max}}$ values were determined as described in Materials and Methods.

in the rate of formation of (R)-2-DCE-IFF and (S)-3-DCE-IFF from (R)-IFF (7–25 and 13–309 pmol/min/mg protein, respectively) and (S)-2-DCE-IFF and (R)-3-DCE-IFF from (S)-IFF (8–163 and 47–688 pmol/min/mg protein, respectively).

The sample-to-sample variations in the formation of (R)-2-DCE-IFF from (R)-IFF were correlated significantly with the sample-to-sample variations in the formation of (R)-3-DCE-IFF from (S)-IFF ($r^2 = 0.53$; $p < .001$). The sample-to-sample variations in the formation of (S)-3-DCE-IFF from (R)-IFF were also highly correlated with the sample-to-sample variations in the formation of (S)-2-DCE-IFF from (S)-IFF ($r^2 = 0.92$; $p < .0001$), Fig. 3.

The sample-to-sample variations in the formation of (R)-2-DCE-IFF, (S)-2-DCE-IFF, (R)-3-DCE-IFF, and (S)-3-DCE-IFF metabolites in human liver microsomes then were correlated with the marker activities of various CYPs, and the results are shown in Table 2 and Figs. 4 and 5. The production of (R)-2-DCE-IFF and (R)-3-DCE-IFF was correlated modestly with testosterone 6β-hydroxylation (Fig. 4), a reaction catalyzed by CYP3A4/5 that suggests the possible contribution of other enzymes. The formation of (S)-2-DCE-IFF and (S)-3-DCE-IFF was highly correlated with (S)-mephenytoin N-demethylation, a reaction catalyzed by CYP2B6 (Heyn et al., 1996) (Fig. 5), suggesting that this pathway is mediated primarily by CYP2B6. It should be noted that the correlation data shown in Figs. 3B, 4B, and 5 appear to be influenced by an apparent outlying data point. Therefore, correlation analyses also were performed by deleting the apparent outlying data point. The correlation coefficients, albeit lower than those reported, were highly significant even when the outlying data points were removed (data not shown). (R)-IFF and (S)-IFF metabolism was not significantly correlated with marker activities of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP4A9/11 (Table 2).

Based on these observations, the four reactions can be divided into two groups on the basis of the CYP(s) that catalyze them. The first group (group 1) is composed of N2-dechloroethylation of (R)-IFF and N3-dechloroethylation of (S)-IFF, where the products formed are (R)-2-DCE-IFF and (R)-3-DCE-IFF. The second group (group 2) is composed of N2-dechloroethylation of (S)-IFF and N3-dechloroethylation of (R)-IFF, where the products formed are (S)-2-DCE-IFF and (S)-3-DCE-IFF. These results are consistent with urinary excretion data from 11 female cancer patients undergoing IFF therapy, where the group 1 and group 2 metabolites exhibited urinary excretion patterns that were similar within each group but distinct between the two groups (Wainer et al., 1996).

N-Dechloroethylation of (R)- and (S)-IFF by cDNA-Expressed P-450 Enzymes. (R)- and (S)-IFF (200 $\mu M$) were incubated independently with microsomes from cells transfected with cDNAs encoding CYP2B6 or CYP3A4, and the results are shown in Fig. 6. The formation of group 1 metabolites was catalyzed preferentially by

![Figure 3](image-url)

**Fig. 3. Sample-to-sample variation in the dechloroethylation of (R)- and (S)-iffosfamide by human liver microsomes.**

A bank of 16 human liver microsomes was incubated at 37 ± 1°C for 60 min with (R)- and (S)-IFF (200 $\mu M$) in the presence of NADPH-generating system. The rates of N2- and N3-dechloroethylation of ifosfamide were measured by GC-MS as described in Materials and Methods. The sample-to-sample variation in N2- and N3-dechloroethylation of (R)- and (S)-iffosfamide were correlated with each other. Data are averages of duplicate determinations; $r^2$ = regression coefficient. Statistically significant correlation values were determined by Pearson Product Moment. *$p < .05$; **$p < .001$; ***$p < .0001$. 

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- **TABLE 1**: Kinetic constants for the formation of dechloroethylation of (R)-IFF and (S)-IFF by human liver microsomes (high-affinity activity).
- **Fig. 3**: Sample-to-sample variation in the dechloroethylation of (R)- and (S)-iffosfamide by human liver microsomes. The rates of dechloroethylation IFF were measured by GC-MS, and $K_{\text{m}}$ and $V_{\text{max}}$ values were determined as described in Materials and Methods.
CYP3A4, and the formation of group 2 metabolites was catalyzed preferentially by CYP2B6. These data are consistent with those obtained from the correlation analysis of the sample-to-sample variation in IFF metabolism by human liver microsomes.

Inhibition of ((R))- and ((S))-IFF N-dechloroethylation. The effect of various chemical inhibitors and inhibitory antibodies on the N-dechloroethylation of ((R))- and ((S))-IFF was investigated in human liver microsomes. The results are shown in Figs. 7 and 8. Coumarin (50 μM), sulfaphenazole (50 μM), hexobarbital (500 μM), quinidine (10 μM), and methyl pyrazole (10 μM), which are inhibitors of 

### Table 2

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>CYP Isoforms</th>
<th>(R)-2-DCE-IFF CYP3A4</th>
<th>(R)-3-DCE-IFF CYP3A4</th>
<th>(S)-2-DCE-IFF CYP2B6</th>
<th>(S)-3-DCE-IFF CYP2B6</th>
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<tbody>
<tr>
<td>7-Ethoxyresorufin O-dealkylation</td>
<td>1A2</td>
<td>0.22</td>
<td>0.08</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>2A6</td>
<td>0.10</td>
<td>0.03</td>
<td>0.34</td>
<td>0.32</td>
</tr>
<tr>
<td>S-mephenytoin N-demethylation</td>
<td>2B6</td>
<td>0.35</td>
<td>0.22</td>
<td><strong>0.96</strong>*</td>
<td><strong>0.99</strong>*</td>
</tr>
<tr>
<td>Taxol 6α-hydroxylation</td>
<td>2C8</td>
<td>0.07</td>
<td>0.06</td>
<td>0.36</td>
<td>0.34</td>
</tr>
<tr>
<td>Tolbutamide methyl-hydroxylation</td>
<td>2C9</td>
<td>0.14</td>
<td>0.00</td>
<td>0.30</td>
<td>0.20</td>
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<tr>
<td>S-mephenytoin 4-hydroxylation</td>
<td>2C19</td>
<td>0.25</td>
<td>0.00</td>
<td>0.28</td>
<td>0.22</td>
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<tr>
<td>Dextromethorphan O-demethylation</td>
<td>2D6</td>
<td>0.07</td>
<td>0.00</td>
<td>0.15</td>
<td>0.14</td>
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<tr>
<td>Chlorozoxone 6-hydroxylation</td>
<td>2E1</td>
<td>0.09</td>
<td>0.04</td>
<td>0.003</td>
<td>0.01</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>3A4/5</td>
<td><strong>0.51</strong>*</td>
<td><strong>0.76</strong>*</td>
<td>0.36</td>
<td>0.34</td>
</tr>
<tr>
<td>Lauric acid 12-hydroxylation</td>
<td>4A9/11</td>
<td>0.01</td>
<td>0.04</td>
<td>0.18</td>
<td>0.08</td>
</tr>
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</table>

**Table 2. Regression analysis for the formation of dechloroethylation of ((R))- and ((S))-IFF with different CYP enzyme activities in human liver microsomes**

Each data point represents the average of duplicate determinations; \( r^2 \) = regression coefficient. Statistically significant correlation values were determined by Pearson Product Moment. \( *p < .05; **p < .001; ***p < .0001 \). The remaining correlations data between (R)-3-DCE-IFF of (S)-IFF and (R)-2-DCE-IFF of (R)-IFF and any other marker P-450 enzyme activities are summarized in Table 2.

CYP3A4, and the formation of group 2 metabolites was catalyzed preferentially by CYP2B6. These data are consistent with those obtained from the correlation analysis of the sample-to-sample variation in IFF metabolism by human liver microsome.

Inhibition of ((R))- and ((S))-IFF N-dechloroethylation. The effect of various chemical inhibitors and inhibitory antibodies on the N-dechloroethylation of ((R))- and ((S))-IFF was investigated in human liver microsomes. The results are shown in Figs. 7 and 8. Coumarin (50 μM), sulfaphenazole (50 μM), hexobarbital (500 μM), quinidine (10 μM), and methyl pyrazole (10 μM), which are inhibitors of 

### Figure 4

**Correlations between sample-to-sample variation of ((R))-2-DCE-IFF of ((R))-IFF (A) and (R)-3-DCE-IFF of (S)-IFF (B) and testosterone 6β-hydroxylation (CYP3A4/5) in microsomes from 16 human liver microsomes.** Each data point represents the average of duplicate determinations; \( r^2 \) = regression coefficient. Statistically significant correlation values were determined by Pearson Product Moment. \( *p < .05; **p < .001; ***p < .0001 \). The remaining correlations data between ((R))-3-DCE-IFF of (S)-IFF and ((R))-2-DCE-IFF of (R)-IFF and any other marker P-450 enzyme activities are summarized in Table 2.

### Figure 5

**Correlations between sample-to-sample variation of (S)-3-DCE-IFF of (R)-IFF (A) and (S)-2-DCE-IFF of (S)-IFF (B) and S-mephenytoin N-demethylation (CYP2B6) in microsomes from 16 human liver microsomes.** Each data point represents the average of duplicate determinations; \( r^2 \) = regression coefficient. Statistically significant correlation values were determined by Pearson Product Moment. \( *p < .05; **p < .001; ***p < .0001 \). The remaining correlations data between (R)-3-DCE-IFF of (S)-IFF and (R)-2-DCE-IFF of (R)-IFF and any other marker P-450 enzyme activities are summarized in Table 2.

(R)-IFF is the source of (R)-2- and (S)-3-DCE-IFF and (S)-IFF is the source of (S)-2- and (R)-3-DCE-IFF.

Human liver microsomes from 16 individuals were incubated with (R)- and (S)-IFF (200 μM) in the presence of an NADPH-generating system. All incubations were carried out in duplicate. The rates of dechloroethylation IFF were measured by GC-MS as described in Materials and Methods. Data are linear regression coefficients, and the values in boldface are statistically significant as determined by Pearson’s Product Moment. **\( P < .001; ***P < .0001 \).
CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1, respectively, had little or no effect on any of the pathways of ifosfamide metabolism (results not shown).

Ketoconazole, which is a potent inhibitor of CYP3A4/5 (Sheets et al., 1986), was found to have a strong inhibitory effect on the formation of group 1 metabolites at 0.2, 0.5, 2.0, and 10 μM concentrations (Fig. 7, A and B). It also markedly inhibited the formation of group 2 metabolites (mediated by CYP2B6), and high concentrations of ketoconazole produced a strong inhibition of (S)-2-DCE-IFF formation. However, high concentrations of ketoconazole produce nonselective CYP inhibition (Baldwin et al., 1995).

Orphenadrine, which is an inhibitor of both CYP2B6 and CYP3A4 (Reidy et al., 1989; Chang et al., 1993; Royer et al., 1996) and possibly CYP2C9 (Ren et al., 1997), inhibited the formation of group 2 metabolites (Fig. 7, C and D). At orphenadrine concentrations of 250, 500, and 1000 μM, the generation of (S)-2-DCE-IFF was decreased by 35, 45, and 55% whereas the formation of (S)-3-DCE-IFF was reduced by 19, 31, and 51%. The addition of 250, 500, and 1000 μM orphenadrine to the incubation also produced a 19 to 50% inhibition of (R)-3-DCE-IFF formation (Fig. 7B). These results suggest that CYP3A4/5 and CYP2B6 contribute significantly to the N-dechloroethylation of IFF by human liver microsomes.

The antibodies against CYP3A significantly inhibited (75–91%) the formation of group 1 metabolites whereas the antibodies against CYP2B predominately inhibited (40–67%) the formation of group 2 metabolites. A weak inhibitory effect of anti-CYP2B1 on (R)-2-DCE-IFF production was also observed. The data from these experiments are consistent with the hypothesis that CYP3A4 and CYP2B6 are responsible for the formation of group 1 and group 2 metabolites, respectively.

Discussion

IFF-induced neurotoxicity is a serious and poorly understood side effect that has been linked to N-dechloroethylation (Goren et al., 1989; Wainer et al., 1994a). Therefore, it is clinically important to determine the metabolic pathway(s) involved in the formation of the N-dechloroethylated metabolites of IFF. Previously reported in vitro studies carried out with rat and human liver microsomes (Weber and Waxman, 1993) have suggested that CYP3A is responsible for this metabolism. In contrast, a recent study with liver microsomes from phenobarbital-treated rats indicated that IFF N-dechloroethylation is catalyzed primarily by CYP2B1 and that this pathway was distinct from CYP3A activation of IFF via 4-hydroxylation (Yu et al., 1996). However, these metabolic studies measured the formation of chloro-
acetaldehyde. Thus, the data could not reflect the chirality of IFF nor the fact that IFF N-dechloroethylation is both enantioselective and regioselective (Fig. 1). It is difficult to obtain a clear picture of the N-dechloroethylation pathway without an understanding of the stereochemical consequences of IFF metabolism.

Using a pharmacologically relevant concentration of (R)-IFF and (S)-IFF (200 μM), this report presents the first study to address the stereoselective aspects of CYP-mediated IFF N-dechloroethylation. The results indicate that in human liver microsomes, CYP3A4/5 is primarily responsible for the formation of (R)-2-DCE-IFF from (R)-IFF and of (R)-3-DCE-IFF from (S)-IFF and that CYP2B6 is primarily responsible for the formation of (S)-3-DCE-IFF from (R)-IFF and of (S)-2-DCE-IFF from (S)-IFF. The results also demonstrate a unique regioselectivity/enantioselectivity in IFF N-dechloroethylation, i.e., (R)-IFF is N-dechloroethylated at the N2 position by CYP2B6 and at the N3 position by CYP3A4/5, whereas (S)-IFF is metabolized at the N2 moiety by CYP3A4/5 and at the N3 moiety by CYP2B6. These results are in agreement with Bullock et al. (1997), which suggested that total N-dechloroethylation of (R)-IFF and (S)-IFF is enantioselective and that it is catalyzed quite differently by CYP2B6 and CYP3A4 in a concentration-dependent manner.

The multiphasic Eadie-Hofstee plots (Fig. 2) suggest the involvement of both high- and low-affinity enzymes in the biotransformation of (R)- and (S)-IFF. Although the data obtained in the present study did not produce an accurate determination of $K_m$ and $V_{max}$ for either the low- or high-affinity enzymes, it could be used to estimate the $K_m$ and $V_{max}$ values for the high-affinity enzymes (Table 1 and Fig. 2). In turn, these results can be used to estimate the relative importance of the individual metabolic pathways as (R)-3-DCE-IFF > (S)-2-DCE-IFF > (S)-3-DCE-IFF > (R)-2-DCE-IFF. Although this study cannot definitively establish this relationship, the suggested order is consistent with previously published data obtained in cancer patients wherein the relative cumulative urinary excretion was (R)-3-DCE-IFF > (S)-3-DCE-IFF ≅ (S)-2-DCE-IFF > (R)-2-DCE-IFF (Wainer et al., 1996).

The in vitro and in vivo data indicate that the CYP3A4/5-catalyzed N-dechloroethylation of (S)-IFF at the N3 position occurs to a greater extent than the CYP3A4/5-catalyzed N-dechloroethylation of (R)-IFF at the N2 position. Thus, there is a high degree of enantioselectively and regioselectively in the CYP3A4/5 catalyzed N-dechloroethylation of (R)-IFF and (S)-IFF. In contrast, the CYP2B6-mediated N-dechloroethylation of (R)- and (S)-IFF is regioselective, N3 versus N2, but proceeds with little or no enantioselectivity.

The clinical relevance of these observations can be extrapolated from the urinary excretion data. In a recent case study, one patient who experienced IFF-related neurotoxicity excreted increased amounts of (R)-3-DCE-IFF and (R)-2-DCE-IFF (Wainer et al., 1994a, 1996). This suggests that a high level of CYP3A activity was the underlying metabolic cause of the observed neurotoxicity. It should be noted that two additional patients in the study excreted increased amounts of dechloroethylated metabolites but did not experience neurotoxicity. These metabolites arose from N3-dechloroethylation of (R)-IFF and N2-dechloroethylation of (S)-IFF, which may be due to increased levels of CYP2B6. This suggests that an overall increase in IFF N-dechloroethylation was not the underlying cause of neurotox-
A pool of human liver microsomes (0.5 mg) was mixed with rabbit anti-rat CYP2B1 (1.0 mg) and rabbit anti-rat CYP3A1 (1.0 mg). The control incubations contained 1 mg of IgG obtained from preimmune rabbit serum. The above mixture was incubated for 15 min at room temperature, the tubes were chilled in ice, and then the mixture was incubated with (R) -IFF or (S) -IFF (200 μM) and an NADPH-generating system for 60 min at 37°C. The rates of N2- and N3-dechloroethylation of IFF were measured by GC-MS as described in Materials and Methods. The control rates of (R)-2-DCE-IFF and (S)-3-DCE-IFF of (R)-IFF were 36 and 105 pmol/min/mg protein and those of (S)-2-DCE-IFF and (R)-3-DCE-IFF of (S)-IFF were 43 and 185 pmol/min/mg protein. Data are averages of duplicate determinations and expressed as percentage of the respective control rates.

![Graphs showing % of control for different treatments](image)

**Fig. 8.** Effects of polyclonal antibodies against CYP2B and CYP3A on (R)-IFF and (S)-IFF N-dechloroethylation by human microsomes.

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References


