EFFECTS OF PHENOBARBITAL ON STEREOSELECTIVE METABOLISM OF IFOSFAMIDE IN RATS

HONG LU, JEFF J. WANG, KENNETH K. CHAN, AND DONN YOUNG

Colleges of Pharmacy and Medicine (H.L., K.K.C.) and Biostatistical Core Resource (D.Y.), Comprehensive Cancer Center, The Ohio State University, and School of Pharmacy, University of Southern California (J.J.W.)

(Received December 23, 1996; accepted December 11, 1997)

This paper is available online at http://www.dmd.org

ABSTRACT:

Plasma and urinary levels of ifosfamide (IF) enantiomers and their metabolites 2-dechloroethylifosfamide, 3-dechloroethylifosfamide, 4-hydroxyifosfamide, and isophosphoramide mustard were determined for control and phenobarbital-treated male Sprague-Dawley rats by using pseudoracemates and GC/MS and stable-isotope dilution analytical methods. For the control rats, the mean AUC for (S)-IF in plasma was greater than that for (R)-IF (R/S AUC ratio, 0.78) and the mean half-life of 41.8 min for (S)-IF was slightly longer than that of 34.3 min for (R)-IF. Phenobarbital pretreatment significantly decreased the AUC values for (R)-IF and (S)-IF, to 21 and 30% of the control values, respectively, and shortened plasma half-lives for both enantiomers [half-life for (R)-IF, 19.8 min; half-life for (S)-IF, 19.4 min]. The urinary excretion values for (R)-IF and (S)-IF were decreased to 41 and 30% of the control values, respectively. The overall amounts of the metabolites in urine were concomitantly increased. Additionally, there were significant reversals in both the R/S AUC ratio and the urinary excretion of 3-dechloroethylifosfamide. Moreover, the enantioselectivity for the generation of 4-hydroxyifosfamide and isophosphoramide mustard disappeared after phenobarbital treatment. These results strongly suggested that the 4-hydroxylation and dechloroethylation of IF enantiomers were mediated by different P450 isozymes or the same isozyme with different stereoselective selectivities.

---

IF is a structural isomer of the oxazaphosphorine CP, and both drugs are widely used in cancer chemotherapy (Dollery, 1991a,b). Like CP, IF is a prodrug that can be activated to form cytotoxic metabolites in vivo. A detailed metabolic scheme for IF was recently reported (Wang and Chan, 1995b). It was believed that the first activation step involves the oxidation of carbon-4 of the oxazaphosphorine ring by hepatic microsomal enzymes to form HOIF, which spontaneously converts to its ring-open tautomer, aldo-IF. The generated HOIF is further decomposed to IPM (the purported ultimate intracellular alkylating metabolite) and the urotoxic agent acrolein. At the same time, HOIF can also be converted to 4-keto-IF and carboxy-IF by dehydrogenase and oxidase, respectively. The hydroxylation of IF, with subsequent formation of IPM, is termed activation. Unlike CP, oxidation of the chloroethyl side chains of IF also occurs to a large extent in vivo and leads to the formation of N2D and N3D, with the release of the neurotoxic coproduct chloroacetaldehyde (Boss et al., 1991; Goren et al., 1986). It has been reported that human CYP3A4 mediates both 4-hydroxylation of the oxazaphosphorine ring and dechloroethylation of the side chains and rat CYP2B1/2, CYP2C6/11, and CYP3A are responsible for 4-hydroxylation (Ruzicka and Ruenitz, 1992; Weber and Waxman, 1993; Chang et al., 1993; Walker et al., 1994). Like CP, IF contains a chiral phosphorus atom. The two IF enantiomers have different efficacies and metabolic behaviors, and IF metabolism seems to be more stereoselective, compared with CP metabolism, in humans and rats (Boss et al., 1991; Granvil et al., 1993, 1994; Farmer, 1988; Wang and Chan, 1995a; Misiura et al., 1993; Prasad et al., 1994; Wainer et al., 1994a,b). However, no thorough study on the enantioselective metabolism of IF, with respect to its hydroxylation and N-dealkylation, has been reported. Because enzyme induction has been a widely used, classical method to characterize P450 isozymes in animals (Okey, 1990; Barry and Feely, 1990), we investigated the influence of PB on the stereoselective metabolism of IF in rats, as the first step in the elucidation of the P450 isozymes responsible for enantioselective metabolism of IF. Granvil et al. (1994) showed that PB pretreatment significantly decreased the half-lives of both IF enantiomers and reversed the enantioselective formation of N3D from IF in rats. Those data suggest that 2- and 3-dechloroethylation are catalyzed by different isozymes. However, the stereoselective activation of IF enantiomers was not investigated.

In this report, we present data on the effect of PB induction on both IF enantiomers and reversed the enantioselective activation of IF enantiomers was not investigated. We have investigated the influence of PB on the stereoselective metabolism of IF in rats, as the first step in the elucidation of the P450 isozymes responsible for enantioselective metabolism of IF. Granvil et al. (1994) showed that PB pretreatment significantly decreased the half-lives of both IF enantiomers and reversed the enantioselective formation of N3D from IF in rats. Those data suggest that 2- and 3-dechloroethylation are catalyzed by different isozymes. However, the stereoselective activation of IF enantiomers was not investigated. In this report, we present data on the effect of PB induction on both 4-hydroxylation and N-dechloroethylation of IF enantiomers in control and PB-treated rats, using the pseudoracemate and GC/MS and stable-isotope dilution techniques.

Materials and Methods

Chemicals. (R)- and (S)-IF and (R)- and (S)-[6,6,2'-2H2]IF enantiomers were synthesized in this laboratory (Wang and Chan, 1996). Their chemical structures are shown in fig. 1. The metabolites HOIF, N2D, N3D, and IPM and their internal standards [4,4,5,5,6,6-2H6]IF, 4-hydroperoxy-[6,6,2'-2H2]-2'-2H2]IF, [1',1',2',2'-2H4]N2D, [4,4,6,6,1',1',2',2'-2H4]N3D, [2',2'-2H2]IPM, and [2',2'-2H2]IPM were all synthesized in this laboratory (Wang and Chan, 1996).
The internal standard [6,6,2',2',2'-'H4]-HOIF was prepared by reduction of 4-hydroperoxy-[6,6,2',2',2'-'H4]IF with sodium thiosulfate, immediately before use. HPLC-grade dichloromethane and methanol were purchased from Fisher Scientific (Pittsburgh, PA). N-Methyl-N-trimethylsilyl-trifluoroacetamide, N,O-bis(trimethylsilyl)trifluoroacetamide, and N-trimethylsilylimidazole were obtained from Pierce (Rockford, IL). C18 reverse-phase resin was obtained from Analytichem International (Harbor City, CA). IF pseudoracemate was prepared by mixing equal amounts of (R)-IF-d4 and (S)-IF or (R)-IF and (S)-IF-d4. The 1:1 composition was verified by GC/MS.

Animal Studies. Twelve male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 250–280 g each, were used for the study. The animals were divided into two groups, i.e. a six-rat control group and a six-rat PB-treated group. For the treatment group, PB (80 mg/kg) was administered by ip injection to each rat once daily for 4 days; for the control group, each rat was given 0.3 ml of normal saline solution at the same frequency. After surgical manipulation, the animal was allowed to recover for at least 1 hr before dosing. After the rat had completely regained consciousness, the appropriate IF pseudoracemate (40 mg/kg, in 0.4 ml of normal saline solution) was injected into the femoral vein cannula, followed by flushing three times with 0.9% sodium chloride solution (0.2 ml each time). Half of the control or PB-treated rats were given the pseudoracemate pair of (R)-IF-d4 and (S)-IF-d4, and half the pseudoracemate pair of the opposite configuration. Rat chow (Tekland, Indianapolis, IN) and water were available ad libitum. Blood samples (0.3 ml each) were collected, via the jugular vein cannula, at 0, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min for PB-treated rats and at 5, 15, 30, 45, 60, 75, 90, 120, 180, 240, 300, 360, and 420 min for control rats after dosing and were placed in heparinized culture tubes immersed in an ice bath. The sampling schedule for PB-treated rats was modified to increase the number of initial samples but shorten the sampling period, because the half-life of IF was anticipated to be short and, therefore, the drug levels at later times might fall below the detection limit. In examination of the initial data from several animals, this sampling strategy was found not to influence the quality of the data. Cumulative 36-hr urine samples were collected at the same time. After centrifugation at 4°C, the separated plasma and collected urine samples were frozen at −70°C until analysis.

Sample Analysis. Analysis of IF enantiomers and their metabolites was accomplished by using the methods developed in this laboratory (Wang and Chan, 1995a; Zheng et al., 1994). Briefly, plasma and urine samples were thawed at 0–5°C and each was divided into two portions. One portion (100 μl), for the analysis of IF, HOIF/aldo-IF, N2D, and N3D, was immediately placed in a culture tube containing 200 μl of 1.5 M KCN solution, pH 8, 1000 ng each of IF-d4, N2D-d4, and N3D-d4, and 2000 ng of HOIF-d4. The samples were allowed to remain at room temperature for 30 min, followed by addition of 5 ml of methylene chloride. The mixture was shaken for 15 min, and the organic phase was separated after centrifugation. After evaporation of the organic solvent to dryness under a stream of nitrogen, the residue was derivatized with 40 μl of N-methyl-N-trimethylsilyl trifluoroacetamide at 120°C for 1 hr. A 1-μl aliquot of the derivatized sample was injected into the GC/MS system.

The other portion of the plasma sample (50 μl) was used for analysis of IPM. After the addition of 1500 ng of IPM-d4, this plasma sample was placed onto a disposable Poly-prep minicolumn (Bio-Rad, Richmond, CA) containing 400 mg of C18 reverse-phase resin. The resin was washed with 1 ml of cold saline solution, followed by centrifugation at 2000g for 10 min to remove as much water as possible. The minicolumn was then eluted with 1 ml of methanol, and the methanol extract was evaporated to dryness under a stream of nitrogen. The resultant residue was derivatized with 40 μl of a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide and N-trimethylsilylimidazole (5:1, v/v) at 120°C for 40 min, and a 1-μl aliquot was analyzed by GC/MS.

GC/MS Analysis. GC/MS analysis of IF enantiomers, their metabolites, and the respective internal standards was carried out with a Finnigan IT540 ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) directly coupled to a Varian 3300/3400 gas chromatograph (Varian, Walnut Creek, CA) via a capillary splitless injector. The mixture was separated on a DB-5 fused-silica capillary column (30 m × 0.25 mm i.d.) bonded with a 0.25-μm-thick film of 5% methylsilicone (J & W Scientific, Folsom, CA). Helium was used as the carrier gas, with the head pressure set at 15 psi. The chemical ionization mode was used, with ammonia as the reagent gas. The temperatures of the injection port, transfer line, and source were set at 220, 260, and 230°C, respectively. For the analysis of IF, HOIF, N2D, N3D, IPM, and their deuterium-labeled analogs, the following GC temperature program was used. The oven temperature was initially maintained at 150°C for 2 min and then increased to 190°C at a rate of 5°C/min, followed by an increase to 250°C at a rate of 15°C/min. The final temperature was maintained for 3 min. The ions selected for monitoring and the retention times for these silylated derivatives were as follows: (S)- or (R)-IF, m/z 225 (8.62 min); (S)- or (R)-IF-d4, m/z 229 (8.56 min); IF-d4, m/z 233 (37 Cl, 8.55 min); HOIF, m/z 412 (15.25 min); IF-d4, m/z 416 (15.23 min); HOIF-d4, m/z 420 (37 Cl, 15.22 min); N2D, m/z 235 (8.58 min); N2D-d4, m/z 237 (8.57 min); N2D-d4, m/z 239 (8.56 min); N3D, m/z 235 (7.50 min); N3D-d4, m/z 239 (5.67 min); N3D-d4, m/z 243 (5.63 min); IPM, m/z 329 (9.82 min); IPM-d4, m/z 333 (37 Cl, 9.80 min); IPM-d4, m/z 337 (9.77 min).

Data Analysis. Regression analysis and pharmacokinetic model fitting were accomplished using the PCNONLIN program (Statistical Consultants, Lexington, KY) on an IBM personal computer. A weighting factor of 1/C2 was used for most of the fitting. An appropriate compartment model was selected based on the Akaike Information Criterion and the smallest values for both the SE and the weighted sum of squares. No preconceived bias was used in the model selection. The pharmacokinetic parameters total drug clearance (CL T ), mean residence time (MRT), and steady-state volume of distribution (V d,ss ) were calculated as follows,

\[ CL_T = \frac{\text{dose}}{\text{AUC}_T} \]

\[ \text{MRT} = \frac{\text{AUMC}}{\text{AUC}} \]

\[ V_{d,ss} = \text{MRT} \times CL_T \]
where dose is the dose of the parent drug, AUC is the AUC of the parent drug, and AUMC is the area under the first-moment curve. Statistical analysis was performed by using analysis of variance, the Wilcoxon signed-ranks test, and the paired t test. For comparisons of the R- and S-isomers for the control and PB-treated groups, both the Wilcoxon signed-ranks test and the paired t test were used. Either negative or positive rank was used where appropriate.

### Results

**Analysis of (R)- and (S)-IF Enantiomers and Their Metabolites.**

Assay characteristics for IF enantiomers and their metabolites were described previously (Wang and Chan, 1995a; Zheng et al., 1994). The assay was validated before the current study. Good linear relationships were found in the range of 50–2000 ng/ml in plasma, with a routine detection limit of 50 ng/ml for all compounds analyzed. The within-run coefficients of variation at 500 ng/ml, with six replicate determinations, for IF, HOIF/aldo-IF, N2D, N3D, and IPM were found to be 5.8, 2.9, 4.7, 2.9, and 6.0%, respectively. The between-run precisions of the assays for IF, HOIF/aldo-IF, N2D, N3D, and IPM were 12.2, 8.1, 10.3, 7.2, and 10.5%, respectively. The extraction recoveries for IF, HOIF/aldo-IF, N2D, N3D, and IPM at 500 ng/ml were 94.1, 52.5, 70.1, 70.4, and 95.0%, respectively.

**Pharmacokinetics of IF Enantiomers in Control and PB-Treated Rats.**

After iv administration to control rats of IF pseudoracemate (40 mg/kg) to a rat without PB pretreatment, the disposition of (R)- and (S)-IF fitted to a two-compartment open model in all rats, as manifested by the curve-fitting. After PB treatment, this enantioselectivity was essentially lost, so that these ratios became nearly unity (table 2, all p > 0.05). In comparisons of the stereochemical changes before and after PB treatment, there was a statistically significant loss of stereoselectivity in elimination half-lives, AUC values, total clearance values after PB treatment. The composition of the urinary IF showed a slight enrichment in the R-isomer, with an R/S ratio of 1.29 after PB treatment.

**Metabolite Profiles for IF Enantiomers and Their Kinetic Parameters in Control and PB-Treated Rats.**

Sets of representative plasma concentration-time profiles for HOIF enantiomers in control and PB-treated rats are shown in figs. 2 and 3, respectively. As shown in the control rats, plasma levels of HOIF generated from (R)- and (S)-IF peaked early and declined essentially in parallel with those of the respective parent drugs, consistent with metabolite kinetic principles (Chan, 1982). The relevant pharmacokinetic parameters and statistical data are shown in table 2. (R)-HOIF exhibited a slightly but statistically significantly different half-life, compared with the S-isomer. The AUC values, peak concentrations, and urinary excretion values showed strong stereoselectivity, with R/S ratios of 1.70, 1.77, and 1.85, respectively (table 2, all p < 0.01). Thus, these data indicated strong substrate stereoselectivity for hydroxylation.

However, after PB treatment, this stereoselectivity was essentially lost, so that these R/S ratios for all relevant pharmacokinetic parameters became nearly unity (table 2, all p > 0.01). There was a significant decrease in plasma half-lives for these metabolites, consistent with the changes in half-lives of the parent compounds. Urinary excretion of (R)-HOIF decreased from 2.12 to 1.30%, with only marginal significance (p = 0.04), but the urinary excretion of (S)-HOIF remained essentially unchanged after PB treatment. The changes in overall production of these hydroxylated metabolites were also reflected in the changes in the AUC values.

**N2D.** Sets of representative plasma concentration-time profiles for N2D enantiomers in control and PB-treated rats are shown in figs. 2 and 3, respectively. As shown, after reaching peak values, the levels of the N2D isomers declined monoexponentially, with half-lives significantly longer than those of the parent compounds. Overall N2D production also showed strong stereoselectivity in the control rats, with statistically different AUC, peak concentration, metabolite AUC/parent drug AUC, and urinary excretion values (table 2, all p < 0.01). The respective R/S ratios were 0.29, 0.38, 0.37, and 0.33, indicating a strong preference for the S-configuration. After PB treatment, this stereoselectivity became even more pronounced, with respective ratios of 0.19, 0.25, 0.17, and 0.21. These data indicated that the stereopreference for (S)-IF in the overall production of N2D was

---

**Fig. 2. Representative set of plasma concentration-time profiles for (R)-IF (○), (S)-IF (□), (R)-HOIF (●), (S)-HOIF (▲), (R)-N2D (△), (S)-N2D (▽), (R)-N3D (●), (S)-N3D (▲), (R)-IPM (●), and (S)-IPM (▲) after iv administration of IF pseudoracemate (40 mg/kg) to a rat without PB pretreatment.**

![Graph showing plasma concentration-time profiles for IF enantiomers and metabolites.](image-url)
The elimination half-lives of N2D also exhibited enantioselectivity (that of S-N2D. Urinary excretion of S-N2D decreased from 14.0 to 6.9% and treatment, suggesting a possible increase in subsequent metabolism.

Essentially preserved. Additionally, the times to peak concentrations and half-lives of the N2D isomers were greatly shortened after PB treatment, suggesting a possible increase in subsequent metabolism. This is supported by a general decrease in the urinary excretion of N2D. Urinary excretion of (S)-N2D decreased from 14.0 to 6.9% and that of (R)-N2D decreased from 4.5 to 1.5% in PB-treated rats. The elimination half-lives of N2D also exhibited enantioselectivity (p = 0.01) with and without PB treatment, with the R-isomer being eliminated faster.

N3D. Sets of representative plasma concentration-time profiles for N3D enantiomers in control and PB-treated rats are shown in figs. 2 and 3, respectively. After a brief rise, N3D plasma levels declined monoeXponentially, with longer half-lives (half-life of the R-enantiomer, 196 min; half-life of the S-enantiomer, 167 min), compared with the parent drugs. Unlike N2D, N3D did not exhibit highly significant enantioselectivity in most of the relevant pharmacokinetic parameters, although a trend in favor of the R-enantiomer existed, yielding R/S ratios of 1.41, 1.27, and 1.42 for AUC, peak concentration, and urinary excretion values, respectively (table 2). Because (R)-N3D is generated from (S)-IF, the overall production of N3D also showed slight S-enantioselectivity, similar to that of N2D. The terminal decay rate constants, however, exhibited highly statistically significant differences, giving a smaller value or slightly longer half-life of 196 min for the R-isomer, compared with 167 min for the S-isomer, again suggesting enantioselectivity for the subsequent disposition of N3D.

After PB treatment, the half-lives of (R)- and (S)-N3D were shortened significantly, to 52.7 and 65.8 min, respectively, values that are not statistically significantly different from each other. The enantioselectivity of N3D formation was dramatically reversed, and the R/S ratios for AUC, peak concentration, and urinary excretion values became 0.159, 0.224, and 0.187, respectively. Additionally, the metabolite AUC/parent drug AUC ratio of 0.173 showed a reversal from the control value of 1.09. The differences in these values before and after PB treatment are highly significant (table 2, all p > 0.01). Thus, PB treatment caused a drastic change in the enantiopreference for (R)-IF for N3D formation, in view of the insignificant difference in elimination half-lives. These results imply that either different isozymes and/or multiple isozymes are involved in catalyzing N2D and N3D formation.

IPM. Although IPM is an achiral molecule, its stereochemical origin can be tracked with deuterium labels. Thus, the “R” and “S” designations of IPM refer to those of the parent enantiomers. Sets of representative plasma concentration-time profiles for IPM derived from (R)- and (S)-IF in control and PB-treated rats are shown in figs. 2 and 3, respectively. As shown, no difference in the terminal half-lives for IPM formed from the two enantiomeric precursors was

| TABLE 1 |

Relevant pharmacokinetic parameters for (R)-IF and (S)-IF after iv administration of IF enantiomers (40 mg/kg) to control and PB-treated rats

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Control (N = 6)</th>
<th>R/S</th>
<th>Treated (N = 6)</th>
<th>R/S</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₀ (µM)</td>
<td>R 99.6 ± 38.0</td>
<td>1.03</td>
<td>R 116 ± 43</td>
<td></td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>α (min⁻¹)</td>
<td>R 103 ± 39.0</td>
<td>0.959</td>
<td>R 112 ± 43</td>
<td></td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>t½ (min)</td>
<td>R 0.173 ± 0.089</td>
<td></td>
<td>S 0.183 ± 0.063</td>
<td>0.945</td>
<td></td>
</tr>
<tr>
<td>β (×10⁻² min⁻¹)</td>
<td>R 2.07 ± 0.31</td>
<td>1.41</td>
<td>S 3.61 ± 0.67</td>
<td></td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>t½ (min)</td>
<td>R 1.68 ± 0.23</td>
<td>1.23</td>
<td>R 3.62 ± 0.39</td>
<td>0.991</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>R 4.19 ± 0.60</td>
<td>0.820</td>
<td>R 19.4 ± 2.1</td>
<td>1.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AUC (min · µM)</td>
<td>R 4.853 ± 1942</td>
<td></td>
<td>S 14.79 ± 556</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>CL°F (ml/min/kg)</td>
<td>R 17.9 ± 6.8</td>
<td>1.29</td>
<td>R 58.8 ± 26.6</td>
<td>1.09</td>
<td>&gt;0.01</td>
</tr>
<tr>
<td>Vₚ (litre/kg)</td>
<td>R 14.0 ± 5.3</td>
<td>0.934</td>
<td>R 63.0 ± 22.3</td>
<td>0.930</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>U (% of dose in 36-hr urine)</td>
<td>R 13.1 ± 3.7</td>
<td>0.53</td>
<td>S 5.38 ± 0.99</td>
<td></td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

R and S refer to (R)- and (S)-IF enantiomers, respectively. The differences in the R/S ratios between control and PB-treated rats were statistically significant, except for Vₑ and C₀ at p = 0.01. p values represent the significance levels for the differences in the parameters for R- or S-isomers between the control and treated groups.

Significantly different from S-isomer value.

Fig. 3. Representative set of plasma concentration-time profiles of (●)(R)- and (○)(S)-IF, (■)(R)- and (□)(S)-HOIF, (▲)(R)- and (△)(S)-N2D, (●)(R)- and (○)(S)-N3D, and (●)(R)- and (○)(S)-IPM after iv administration of IF pseudoracemate at 40 mg/kg to a rat with PB pretreatment.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>R/S</th>
<th>R/S</th>
<th>R/S</th>
<th>Control</th>
<th>R/S</th>
<th>R/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (×10^-3 m/s⁻¹)</td>
<td>18.0±3.3</td>
<td>10.9±2.6</td>
<td>3.3±0.9</td>
<td>12.4±2.9</td>
<td>9.9±2.3</td>
<td>16.0±3.1</td>
<td>10.8±2.6</td>
</tr>
<tr>
<td>t1/2 (min)</td>
<td>36.0±3.5</td>
<td>21.9±3.5</td>
<td>11.8±2.5</td>
<td>36.0±3.5</td>
<td>21.9±3.5</td>
<td>11.8±2.5</td>
<td>36.0±3.5</td>
</tr>
<tr>
<td>AUC (µM.min/mg)</td>
<td>335.0±35.0</td>
<td>234.0±24.0</td>
<td>129.0±12.9</td>
<td>335.0±35.0</td>
<td>234.0±24.0</td>
<td>129.0±12.9</td>
<td>335.0±35.0</td>
</tr>
<tr>
<td>Cmax (µM)</td>
<td>120.0±12.0</td>
<td>80.0±8.0</td>
<td>40.0±4.0</td>
<td>120.0±12.0</td>
<td>80.0±8.0</td>
<td>40.0±4.0</td>
<td>120.0±12.0</td>
</tr>
<tr>
<td>% ID dose in R/S</td>
<td>35.0±3.5</td>
<td>25.0±2.5</td>
<td>15.0±1.5</td>
<td>35.0±3.5</td>
<td>25.0±2.5</td>
<td>15.0±1.5</td>
<td>35.0±3.5</td>
</tr>
<tr>
<td>% ID dose in R/S</td>
<td>30.0±3.0</td>
<td>20.0±2.0</td>
<td>10.0±1.0</td>
<td>30.0±3.0</td>
<td>20.0±2.0</td>
<td>10.0±1.0</td>
<td>30.0±3.0</td>
</tr>
</tbody>
</table>

**Discussion**

Although it is possible to use a chiral column to separate and quantitate enantiomers (Granvil et al., 1993, 1994; Masurel and Wainer, 1989; Corlett and Chrystyn, 1994) for studies of enantioselectivity in drug metabolism, this method cannot discriminate enantioselectivity when asymmetry is lost through metabolism. For example, HOIF equilibrates with its open-ring form, aldo-IF, leading to a loss of asymmetry. Subsequent cleavage of the side chain also gives rise to the achiral IPM. These metabolites are no longer separable on a chiral column. Therefore, information on substrate-controlled enantioselective metabolism becomes unobtainable; this information may be important in the case of multiple metabolic pathways. In contrast, use of a pseudoracemate allows the tracking of products formed from each of the enantiomers, despite a loss of symmetry. The labeled precursor generates labeled metabolites, irrespective of the asymmetry. This technique enables discrimination, by means of the labels, of enantioselective generation (substrate control). Additionally, analysis of the products formed from enantiomeric precursors in the same metabolizing system reduces the problem of interindividual variability, which occurs frequently when experiments are performed separately. A consideration for the use of pseudoracemates is that the location of the label must not be a site of metabolic attack and must not impart significant isotope effects. In our previous study (Wang and Chan, 1996), this problem was evaluated and suitable labeling sites were selected. Additionally, we showed that, in our control experiments, with an admixture of labeled and unlabeled IF of the same configuration there was a lack of isotopic preference in the metabolic studies. Moreover, we used pseudoracemates consisting of two possible permutations, and the two yielded similar results (data not shown). Similar methods were used in enantioselective metabolism studies of CP with mouse, rat, and rabbit microsomes by Cox et al. (1978).

Pseudoracemate techniques have been successfully used in a number of interesting investigations, including studies of autoinduction for the demethylation of (R)-mephénytoïn during chronic drug treatment (Kupfer et al., 1982) and enantioselective interactions of drugs in vitro (Gal et al., 1976; Kroemer et al., 1991) and in vivo (Giacomini et al., 1986). The current study did not examine the question of potential
enantiomeric interactions of IF, because of limited amounts of labeled material, and this remains to be studied.

The overall disposition of IF metabolites includes formation and elimination. For many of the enantioselective processes studied here, it was not possible to definitively separate formation and elimination, because we did not study the elimination characteristics of the enantiomers after their direct administration. However, because many of these metabolites displayed terminal plasma half-lives longer than that of the parent compound, the possible influences of these two processes could be dissected according to metabolite kinetic principles (Pang and Kwan, 1983; Chan, 1982). In most of the cases, particularly for IPM, the enantioselectivity might reside in formation, rather than elimination. However, because distinction of these processes was not rigorous, we used overall production as an apparent composite of formation and elimination.

Plasma concentration-time profiles for the IF enantiomers in the control rats declined monoexponentially. However, after PB treatment all of these profiles changed to biexponential declines, as indicated by the best fit of the data. Several explanations for this phenomenon are possible. First, this result could suggest the existence of product-inhibition metabolism caused by PB treatment. On the other hand, conversion to a two-enzyme system as a controlling step in elimination might also be possible after PB treatment. The latter possibility is consistent with the results of Chang et al. (1993), who identified a two-component, ring-hydroxylating enzyme system for oxazaphosphorines in human liver microsomes, with high and low Km values. It has been shown that in rats PB induces enzymes of the CYP2B family, which are not significantly involved in the metabolism of IF in uninduced rats (Weber and Waxman, 1993). After PB treatment, the increase in metabolism and the loss of enantioselectivity observed in the present study suggest the involvement of other isozymes, possibly with different affinities. Other possible explanations (such as changes in tissue affinity for the enantiomers after PB treatment), although less likely, have not been entirely ruled out.

It has generally been agreed that PB induction greatly elevates the amounts of CYP2B1/2 proteins (which are undetectable in control rats) and, to a lesser extent, CYP3A1/2 proteins (Waxman and Azaroff, 1992) but decreases the activity of the CYP2C11 isozyme, which appears to be male-specific. Coupled with our present results, this suggests that in untreated rats CYP2C6 and CYP2C11 display enantioselectivity for IF, with respect to the activation pathway. However, after PB treatment the predominant P450 enzymes of the CYP2B family would be mainly responsible for hydroxylation, with altered stereoselectivity.

N-Dechloroethylation is a major elimination pathway for IF, and the byproduct chloroacetaldehyde has been implicated in the central nervous system toxicity of IF (Goren et al., 1986; Lewis and Meanwell, 1990). Based on the structure of IF, regioselectivity and enantioselectivity for this process are expected, and some results have been published (Boss et al., 1991; Misiura et al., 1983; Wainer et al., 1988, 1994a). The regioselectivity and enantioselectivity may be influenced by PB treatment. Ruzicka and Ruefert (1992) found an approximately 8-fold increase in N-dechloroethylation of IF in rat microsomes after PB treatment, but no information on the regioselectivity or enantioselectivity was provided. More recently, Yu and Waxman (1996) reported a greater increase of N-dechloroethylation, compared with 4-hydroxylation, of IF in rat microsomes after PB pretreatment; the increase was reversed after dexamethasone pretreatment. Granvil et al. (1994) showed that PB treatment greatly accelerated the elimination of IF enantiomers and altered the N-dechloroethylation of (S)-IF. (S)-IF was found to be primarily converted to (R)-N3D in control rats. However, after PB treatment this metabolite became undetectable, although the amount of (S)-N2D was greatly increased. Our results are in general agreement with the results of Granvil et al. (1994) with respect to the trend in enantioselectivity for the parent drug, despite a large difference in the administered doses [125 mg/kg in the study by Granvil et al. (1994) vs. 40 mg/kg in this study] and gender and strain differences in the animals used [female Fisher rats in the study by Granvil et al. (1994) and male Sprague-Dawley rats in our study]. However, our results showed major differences in the dechloroethylation pathways. We were able to detect (R)-N2D in both control and PB-treated rats, and the AUC value was significantly higher for (S)-N2D, similar to the results of Granvil et al. (1994). However, after PB treatment the AUC values for both (R)- and (S)-N2D decreased significantly. This difference may be the result of the aforementioned factors (dose, gender, and strain). In the case of N3D, the results were remarkably similar. In the present study, after PB treatment there was a significant reversal of stereoselectivity (R/S ratio), as manifested in both the AUC and urinary excretion of N3D, although only a minor reversal of stereoselectivity for the parent drug was observed. The present results strongly indicate that hydroxylation and dechloroethylation of IF enantiomers are catalyzed by different P450 isozymes, which have different structural or stereochemical selectivities. Recently, Granvil et al. (1996), in an abstract, suggested that CYP3A4 catalyzes the formation of (R)-N2D and (R)-N3D, whereas CYP2B1 is responsible for the formation of (S)-N2D and (S)-N3D (Waxman and Azaroff, 1992). The exact P450 isozymes responsible for the metabolism of each IF enantiomer remain to be elucidated.

References
Masreul D and Wainer IW (1989) Analytical and preparative high-performance liquid chromat-


