EFFECTS OF PHENOBARBITAL ON STEREOSELECTIVE METABOLISM OF IFOSFAMIDE IN RATS

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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 dechloroethylifosfamide of IF enantiomers were mediated by different P450 isozymes or the same isozyme with different stereochemical 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 is 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 dechloroethylifosfamide of the side chains and rat CYP2B1/2, CYP2C6/11, and CYP3A are responsible for 4-hydroxylation (Ruzicka and Ruiten, 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., 1983; 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-dechloroethylifosfamide 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-dechloroethylifosfamide 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′-2H₄]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′-2H₄]IF, [1′,1′,2′-2H₄]N2D, [4,4,6,6′,1′,2′-2H₄]N3D, [2′,2′-2H₂]IPM, and [2′,2′,2′,2′-2H₄]IPM were all synthesized in this laboratory (Wang and
of IF-6, N2D-resin was obtained from Analytichem International (Harbor City, CA). IF-ylsilylimidazole were obtained from Pierce (Rockford, IL). C18 reverse-phase resin was purchased from Fisher Scientific (Pittsburgh, PA).

Immediately before use. HPLC-grade dichloromethane and methanol were thawed at 0–5°C and each was divided into two portions. One portion (100 µl) was injected to each rat once daily for 4 days; for the control group, each rat was dosed with 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min for control rats and at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min for PB-treated rats and at 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, and 240 min for PB-treated rats. 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 sampling time.

FIG. 1. Chemical structures of IF enantiomers, (R)-IF-d4, and (S)-IF-d4.

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 (CLT), mean residence time (MRT), and steady-state volume of distribution (Vd,ss) were calculated as follows,

\[
CL_T = \text{dose} / \text{AUC}_r \\
\text{MRT} = \text{AUMC} / \text{AUC} \\
V_{d,\text{ss}} = \text{MRT} \times CL_T
\]
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 parameters between 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. For comparisons of the parameters between the control and PB-treated groups, analysis of variance was used. In most cases, the significance level was set at \( p = 0.01 \).

**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 at a dose of 40 mg/kg, plasma concentration-time profiles for both IF enantiomers declined essentially monoexponentially and were thus fitted to a one-compartment model. A set of representative plasma concentration-time profiles for IF enantiomers is shown in fig. 2. Relevant pharmacokinetic parameters for each IF enantiomer are shown in table 1. As shown, \((R)-IF\) exhibited a larger decay rate constant and a shorter half-life of 34.2 min, compared with \((S)-IF\), which showed a half-life of 41.8 min \((p < 0.01)\). This difference in half-lives gave rise to differences in AUC and total clearance values for these two enantiomers \((p = 0.02)\), but with no difference in the volumes of distribution. The ratio of the AUC of \((R)-IF\) to that of \((S)-IF\) was 0.78. Approximately 13–14% of the dose was excreted as unchanged IF in urine, with an \(R/S\) ratio near unity.

After PB treatment, the disposition of \((R)-\) and \((S)-IF\) conformed to a two-compartment open model in all rats, as manifested by the curve-fitting. A set of representative plasma concentration-time profiles for the IF enantiomers is shown in fig. 3. In most cases, the measurements of the parent drug and metabolites were essentially at the sensitivity limit of the assay. Relevant pharmacokinetic parameters for each IF enantiomer are shown in table 1. The mean half-lives for both enantiomers dramatically decreased to the same value of about 20 min (one-half of their control values), with a concomitant 4–5-fold increase in total clearance. The decrease in plasma half-lives and the increase in total clearance values are mainly attributed to an increase in metabolism, consistent with the enzyme-inducing effect of PB, because the steady-state volumes of distribution for the enantiomers of IF changed only slightly after PB treatment. In fact, the urinary excretion of the unchanged drug decreased >2-fold to 5% of control for both isomers, supporting the plasma data. The estimated nonrenal clearances (total clearance minus renal clearance) increased 4.4- and 5.6-fold for \((R)-\) and \((S)-IF\), respectively. Additionally, the concentrations remained the same after PB treatment \((p > 0.1)\). 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, and 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. HOIF.** 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 enantioselectivity, 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 enantioselectivity 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 enantioselectivity 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 enantioselectivity 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
elimination half-lives of N2D also exhibited enantioselectivity (that of (R)-N2D decreased from 14.0 to 6.9% and N2D. Urinary excretion of essentially preserved. Additionally, the times to peak concentrations 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-enantiomer, compared with 167 min for the S-enantiomer, 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>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HOIF</th>
<th>PB</th>
<th>Control</th>
<th>HOIF</th>
<th>PB</th>
<th>Control</th>
<th>HOIF</th>
<th>PB</th>
<th>Control</th>
<th>HOIF</th>
<th>PB</th>
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<tr>
<td>AUC (mg·h/l)</td>
<td>68·9±11·3</td>
<td>21·0±4·4</td>
<td>19·3±3·8</td>
<td>71·0±14·4</td>
<td>25·7±5·5</td>
<td>9·4±0·2</td>
<td>96·2±24·1</td>
<td>4·2±0·7</td>
<td>3·9±0·2</td>
<td>138·0±51·9</td>
<td>41·7±12·0</td>
<td>4·1±0·1</td>
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<tr>
<td>Cmax (mg/l)</td>
<td>19·3±3·8</td>
<td>1·29</td>
<td>4·7±0·1</td>
<td>79·9±207·6</td>
<td>0·26</td>
<td>1·0</td>
<td>1·91±0·4</td>
<td>0·7</td>
<td>1·0</td>
<td>1·30±0·27</td>
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<td>t1/2 (min)</td>
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<td>1·08</td>
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<td>2·27±6·4</td>
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<td>2·27±6·4</td>
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<tr>
<td>Values are mean±SD.</td>
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**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 variety of interesting investigations, including 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 variety of interesting investigations, including 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.
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 metabolism caused by PB treatment. On the other hand, it was not possible to definitively separate formation and elimination, because we did not study the elimination characteristics of the enantiomers, 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. 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 isoforms, 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.

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