MECHANISM-BASED INHIBITION OF HUMAN LIVER MICROSOMAL CYTOCHROME P450 1A2 BY ZILEUTON, A 5-LIPOXYGENASE INHIBITOR

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ABSTRACT:

Zileuton, a 5-lipoxygenase inhibitor, was evaluated as an inhibitor of cytochrome P450 activity in human liver microsomes. In the absence of preincubation, the racemate was found to be a weak inhibitor (IC50 > 100 μM) of phenacetin O-deethylation (POD) (CYP1A2), p-acetamidophenol 6'-hydroxyl (CYP2C8), diclofenac 4'-hydroxyl (CYP2C9), (S)-mephentoin 4'-hydroxyl (CYP2C19), bufuralol 1'-hydroxyl (CYP2D6), testosterone 6β-hydroxyl (CYP3A4), chloroxazone 6-hydroxyl (CYP2E1), and bupropion hydroxyl (CYP2B6). When preincubated with NADPH-fortified human liver microsomes in the absence of substrate, zileuton (racemate) was shown to inhibit POD. The effect was NADPH-dependent, and concentration-dependent, and was characterized by a KI (maximal rate of enzyme inactivation) and apparent KM (inhibitor concentration that supports half the maximal rate of inactivation) of 0.035 min-1 and 117 μM, respectively (KI/KM ratio of 0.0003 min-1 μM-1). Preincubation-dependent inhibition of POD activity was also observed with the individual (S)-(−) and (R)-(+) -enantiomers of zileuton [(S)-(−)zileuton; KI 0.037 min-1, KM 98.2 μM, KI/KM ratio, 0.0004 min-1 μM-1; (R)-(+)zileuton; KI 0.012 min-1, KM 66.6 μM, KI/KM ratio, 0.0002 min-1 μM-1]. In addition, the inhibition of CYP1A2 was not reversed in the presence of reduced glutathione, catalase, and superoxide dismutase and was refractory to dialysis. Therefore, zileuton was characterized as a mechanism-based inhibitor of human liver microsomal CYP1A2. Mechanism-based inhibition of CYP1A2 may explain why zileuton decreases the oral clearance of antipyrine, propranolol, (R)-warfarin, and theophylline, at doses that have a minimal effect on the pharmacokinetics of (S)-warfarin, phenytoin, and terfenadine.

Zileuton (Zyflo; N-(1-benzo[b]thien-2-ylethyl)-N′-hydroxyurea), a substituted hydroxamic acid, is a potent and selective 5-lipoxygenase inhibitor that has been approved for the prevention and chronic treatment of asthma (Isreal et al., 1990; Carter et al., 1991; Bell et al., 1992; Wenzel and Kamada, 1996; Duke et al., 1999). The drug contains a single chiral center (Fig. 1), is administered as a racemic mixture of (R)-(+) - and (S)-(−)-enantiomers, and is characterized by a plasma half-life of about 4 h (Wong et al., 1995). As a result of the short half-life, zileuton has to be dosed four times a day (q.i.d.). When preincubated with NADPH-fortified human liver microsomes in the absence of substrate, zileuton (racemate) was shown to inhibit POD. The effect was NADPH-dependent, and concentration-dependent, and was characterized by a KI (maximal rate of enzyme inactivation) and apparent KM (inhibitor concentration that supports half the maximal rate of inactivation) of 0.035 min-1 and 117 μM, respectively (KI/KM ratio of 0.0003 min-1 μM-1). Preincubation-dependent inhibition of POD activity was also observed with the individual (S)-(−) - and (R)-(+) -enantiomers of zileuton [(S)-(−)zileuton; KI 0.037 min-1, KM 98.2 μM, KI/KM ratio, 0.0004 min-1 μM-1; (R)-(+)zileuton; KI 0.012 min-1, KM 66.6 μM, KI/KM ratio, 0.0002 min-1 μM-1]. In addition, the inhibition of CYP1A2 was not reversed in the presence of reduced glutathione, catalase, and superoxide dismutase and was refractory to dialysis. Therefore, zileuton was characterized as a mechanism-based inhibitor of human liver microsomal CYP1A2. Mechanism-based inhibition of CYP1A2 may explain why zileuton decreases the oral clearance of antipyrine, propranolol, (R)-warfarin, and theophylline, at doses that have a minimal effect on the pharmacokinetics of (S)-warfarin, phenytoin, and terfenadine.

Zileuton is well absorbed, and the majority of the dose (~75%) is recovered in the urine of healthy volunteers as the N-hydroxy glucuronides of both the (R)-(+) - and (S)-(−)-enantiomers (Wong et al., 1995). Although N-hydroxy glucuronidation has been confirmed with human liver microsomes, zileuton is metabolized in the presence of NADPH (Machinist et al., 1995b; Sweeny and Nellans, 1995). In fact, CYP1A2 and CYP2C9 catalyze ring hydroxyl, whereas sulfoxidation is catalyzed largely by CYP3A4. Both reactions conform to Michaelis-Menten kinetics in human liver microsomes and are described by relatively high KM values (≥ 0.2 mM).

Zileuton also has been shown to be a weak inhibitor of human liver microsomal CYP3A4, CYP2C9, and CYP2D6 activity in vitro (Machinist et al., 1995b). As anticipated, zileuton has a minimal effect on the pharmacokinetics of drugs that are metabolized by CYP2C9 [e.g., (S)-warfarin, phenytoin, and naproxen] and CYP3A4 (e.g., terfenadine) (Awni et al., 1995b,c, 1997; Samara et al., 1995). In contrast, zileuton decreases the oral clearance of (R)-warfarin, propranolol, antipyrine, and theophylline, which indicates that it is a clinically relevant inhibitor of CYP1A2 (Awni et al., 1995c; Granneman et al., 1995; Machinist et al., 1995b; St. Peter et al., 1995). The effect of zileuton on CYP1A2 has been evaluated using precision-cut liver slices, and the compound was shown to inhibit (~60%) theophylline metabolism at a final concentration of 100 μM (Machinist et al., 1995b). However, the studies were rather limited in scope, and no attempt was made to elucidate the mechanism of inhibition or to conduct in vitro-in vivo correlations. Therefore, the aim of the present study was to further evaluate the interaction of zileuton with the human liver microsomal cytochrome P450 (P4501) system. During the
analytical or high-performance liquid chromatography grade. Zileuton was isolated from Zyflo Filmtab tablets (600 mg) by crushing them with a mortar and pestle, and partitioning the resultant white solid between water and ethyl acetate. The organic layer was filtered through a 0.45-µm membrane, then washed with water, dried over Na2SO4, and re-filtered through a second 0.45-µm membrane. The filtrate was concentrated to a white solid, which was recrystallized from acetonitrile to give zileuton as clear needles (>99% pure by reverse phase-high performance liquid chromatography with online UV detection at 210 and 254 nm). The identity of the recrystallized material was confirmed by proton NMR and LC/MS-MS. Separation of racemic zileuton using a published procedure (Thomas et al., 1992) yielded the individual (R)(+)-(+) and (S)(−)-enantiomers (>99.8% purity).

Incubations with Human Liver Microsomes. CYP1A2 (POD), CYP2B6 (bupropion hydroxylation), CYP2C8 (paclitaxel 6α-hydroxylation), CYP2C9 (diclofenac 4′-hydroxylation), CYP2C19 ([S]-mephenytoin 4′-hydroxylation), CYP2D6 (bufuralol 1′-hydroxylation), CYP2E1 (chlorozoxazone 6-hydroxylation), and CYP3A4 (testosterone 6β-hydroxylation) activity in human liver microsomes was determined using accepted P450 form marker assays (Hesse et al., 2000; Madan et al., 2002).

The incubation mixtures (0.2–0.5 ml) contained microsomal protein (0.2–0.5 mg/ml final concentration) and 100 mM potassium phosphate buffer (pH 7.4). The reaction was initiated with NADPH (1 mM) and was allowed to proceed for 10 to 30 min (37°C in a shaking water bath). In all cases, no more than 10% of the substrate was consumed. All chemical inhibitors and substrates were dissolved in 50% (v/v) acetonitrile/water. The final volume of acetonitrile in the incubation was less than 1% (v/v). Activity in the presence of solvent alone was assigned as “control” (100%). For paclitaxel 6α-hydroxylation (CYP2C8) and bupropion hydroxylation (CYP2B6), the reaction was terminated with the addition of methylene chloride (4 volumes). Baccatin and tripodeine were added as internal standards, respectively. Thereafter, the samples were vortexed and centrifuged (3,500 rpm for 10 min), and the supernatant was separated and dried under nitrogen gas. The resulting residue was dissolved in 100 µl of 40% acetonitrile, and aliquots (20 µl) were analyzed by LC/MS-MS. For the other assays, the reactions were terminated with acetonitrile (2 volumes), containing an appropriate amount of internal standard (4′-hydroxybutyranilide, flufenamic acid, phenytoin, propranolol, L-594615, and cortisone for the CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 assays, respectively). The samples were vortexed and centrifuged (3,500 rpm for 10 min), and the supernatant was separated and diluted with an equal volume of 0.05% formic acid in water. Aliquots (20 µl) were subjected to LC/MS-MS analysis.

Mechanism-based inhibition of CYP1A2, CYP2C9, and CYP3A4 was evaluated also. NADPH-activated human liver microsomes (2.0 mg/ml final concentration) were incubated at 37°C (up to 30 min) in the presence of solvent alone, or zileuton (racemic mixture or individual enantiomers), in a shaking water bath (final volume of 0.2 ml). At the appropriate times, aliquots (25 µl) of the incubate were removed and added to separate tubes containing 225 µl of fresh buffer preheated at 37°C. Substrate (phenacetin, diclofenac, and testosterone at a final concentration of 120, 100, and 250 µM, respectively) and NADPH (1.0 mM) were added, and the reaction was allowed to proceed. At the required time, the reaction was terminated as described above. Incubations carried out in the presence of solvent alone (time 0) were designated controls (100%).

LC/MS-MS Analysis. Chromatography was conducted on a PerkinElmer high-performance liquid chromatography system, equipped with a series 200 liquid chromatography pump and autosampler, interfaced to a Sciex API 2000 tandem mass spectrometer with an atmospheric pressure chemical ionization ion source operating in the positive or negative (chlorozoxazone assay only) ion mode (PerkinElmerSciex Instruments, Boston, MA). For the POD assay, separations were carried out on an Aquasep column (2.0 × 50 mm, 5 µm; ES Industries, West Berlin, NJ). Mobile phase A consisted of 0.05% formic acid in water and mobile phase B of 0.05% formic acid in acetonitrile (constant flow rate of 1.5 ml/min). The composition of mobile phase B was increased from 5% to 100%. Both product (acetaminophen; m/z 151.9 and 110.1) and internal standard (4′-hydroxybutyranilide; m/z 180.2 and 71.0) were resolved.

Analyses of the paclitaxel and mephenytoin incubates were carried out using Zorbx 300 Extend C18 and Zorbx SB-Aq columns (4.6 × 50 mm, 5 µm; MAC-MOD Analytical Inc., Chadds Ford, PA), respectively. Mobile
phase A consisted of 0.05% formic acid in 10% aqueous methanol and mobile phase B of 0.5% formic acid in water/acetonitrile (10:90, v/v). The composition of mobile phase B was increased from 20 to 80% (acetonitrile) and 0 to 50% (methanol) at a flow rate of 1.5 ml/min. In all cases, the metabolites (4'-hydroxymephenytoin, m/z 281.1, 186.1; 6'-hydroxydiclofenac, m/z 312 and 231.1; 1'-hydroxybufuralol, m/z 278.1 and 186.1; 6'-hydroxytestosterone, m/z 305.2 and 269; and hydroxybupropion, m/z 256.2 and 184.2) and internal standard (flufenamic acid, m/z 279.2 and 208.2, respectively) were separated and yielded characteristic m/z ratios. For the chlorozoxazone assay, separations were carried out using a Synergi Polar-RP Aqueous column (4.6 × 50 mm, 5 μm; Phenomenex, Torrance, CA). Mobile phase A consisted of water (90%) and methanol (10%), whereas mobile phase B contained water (10%) and acetonitrile (90%); both mobile phases contained 0.05% formic acid. The composition of mobile phase B was increased from 5% to 40% at a flow rate of 1.5 ml/min. In all cases, the metabolites (4'-hydroxydiclofenac, m/z 312 and 231.1; 1'-hydroxybufuralol, m/z 278.1 and 186.1; 6'-hydroxytestosterone, m/z 305.2 and 269; and hydroxybupropion, m/z 256.2 and 184.2) and internal standard (flufenamic acid, m/z 279.2 and 208.2, respectively) were separated and yielded characteristic m/z ratios. For the chlorozoxazone assay, separations were carried out using a Synergi Polar-RP column (4.6 × 50 mm, 5 μm; Phenomenex). Mobile phase A consisted of 0.05% formic acid in water while mobile phase B contained 0.05% formic acid in acetonitrile. The composition of mobile phase B was increased from 20% to 80% at a flow rate of 1.5 ml/min. 6'-Hydroxychlorozoxazone (m/z 181.1 and 120.1) and L-594615 (m/z 202.2 and 165.9) were resolved.

**Data Analysis.** The observed rates of CYP1A2 inactivation (k_{inact}) were calculated from the initial slopes of the linear regression lines of semilogarithmic plots (remaining POD activity versus preincubation time). The reciprocal of k_{inact} thus obtained was plotted against the reciprocal of the zileuton concentration (Madan et al., 2002). This double reciprocal plot was used to generate estimates of k_{inact} (y-intercept) and K_{I} (negative reciprocal of the x-intercept).

**Simulations.** The in vitro data describing the mechanism-based inhibition of CYP1A2 by zileuton were used to rationalize previously observed clinical drug interactions. For mechanism-based inhibitors, one can attempt to evaluate their effect on the oral pharmacokinetics of other drugs using eq. 1 below (Mayhew et al., 2000; Jones and Hall, 2002).

\[
\frac{\text{AUC}_{(\text{po})}}{\text{AUC}_{(\text{pS})}} = \frac{1}{\left(\frac{1 + \frac{f_{a}}{f_{m,CYP1A2}} \cdot \frac{C_{\text{inact}}}{K_{I}}}{\left(1 - \frac{f_{a}}{f_{m,CYP1A2}} \cdot \frac{C_{\text{inact}}}{K_{I}}\right)} + 1 - \left(\frac{f_{a}}{f_{m,CYP1A2}} \cdot \frac{C_{\text{inact}}}{K_{I}}\right)\right)}
\]

In effect, one can relate changes in substrate AUC [AUC_{(po)}/AUC_{(pS)}] to the fraction of the dose metabolized by the inhibited enzyme (i.e., the product of f_a and f_m,CYP1A2). eq. 1 is similar to that reported for reversible inhibitors, except that the term 1 + [I]/K_I is substituted for 1 + [I]/K_{I} (Ito et al., 1998; Jones and Hall, 2002). K_I and K_{inact} are the parameters describing the inhibition of the enzyme, and K_{I} represents the rate constant for the degradation of the enzyme in the absence of inhibitor. Estimates of the concentration of the inhibitor at the enzyme ( [I]_u ) are based on free plasma concentrations (concentrations of total inhibitor drug in plasma corrected for f_a).

As described previously (Mayhew et al., 2000; Jones and Hall, 2002), the derivation of eq. 1 is based on a number of simplifying assumptions. For example, the substrate (victim drug) is well absorbed and the inhibitor has no effect on absorption. The substrate exhibits linear pharmacokinetics and is metabolized (first order) in the liver only. In addition, the hepatic clearance of the substrate is adequately described by the well stirred model and the degradation of the enzyme is a first order process. Furthermore, the inhibitor has no effect on the rate of synthesis of the enzyme. Therefore, with some underlying assumptions (Mayhew et al., 2000; Jones and Hall, 2002), it is possible to use the in vitro data (K_{inact}/K_{I}) and simulate changes in substrate AUC over a range of f_a \cdot f_{m,CYP1A2} values (e.g., 0.2–0.95).

For the effect of zileuton on CYP1A2, the in vitro K_{I} and K_{inact} data were used in the simulations, in addition to estimates of K_{I} (Correa, 1991; Ito et al., 1998; Mayhew et al., 2000). The steady-state concentrations of zileuton in plasma (C_{max} and C_{ave}) were corrected for binding (f_a = 0.069) to human plasma proteins in vitro (Awwi et al., 1995a; Machinist et al., 1995a). No attempt was made to estimate the concentration of zileuton in the liver or in the portal vein following an oral dose (Ito et al., 1998). The simulated changes in AUC were then compared with the known AUC changes observed with (R)-warfarin, theophylline, propranolol, and antipyrine (Awwi et al., 1995c; Grimmnan et al., 1995b; Still et al., 1995). Estimates of f_a for each of these drugs were based on the reported fraction of the dose recovered in human excreta as unchanged parent drug, P450 and non-P450 metabolites (Walle et al., 1985; Troger and Meyer, 1995; Engel et al., 1996; Kaminsky and Zhang, 1996; Yao et al., 2001). Values for f_a,CYP1A2 were based on published in vivo P450 reaction phenotyping data (Masubuchi et al., 1994; Ha et al., 1995; Yoshimoto et al., 1995; Zhang and Kaminsky, 1995; Engel et al., 1996; Kaminsky and Zhang, 1996; Sharer and Wrighton, 1996; Tjia et al., 1996; Johnson et al., 2000).

**Results.**

**Inhibition of P450 Activities.** Zileuton was evaluated as a reversible inhibitor of P450 activity in pooled human liver microsomes (Table 1). Activities selective for different P450 forms (CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2B6, CYP2E1, and CYP3A4) were measured, and zileuton was found to be a weak inhibitor (IC_{50} > 100 μM). Negligible inhibition of CYP2D6, CYP2C9, CYP1A2, CYP2E1, and CYP2C8 activity (<20%) was observed at the highest concentration of zileuton tested (100 μM). Approximately 40% inhibition of CYP3A4, CYP2B6, and CYP2C19 activity was observed at this concentration. By comparison, fluvoxamine, quercetin, sulfaphenazole, (R)-N-3-benzylphenobarbital, quindine, ketoconazole, 4-methylpyrazole, and N-(α-methylbenzyl)-1-aminobenzotriazole were potent inhibitors (IC_{50} < 5.0 μM) of CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP2E1, and CYP2B6 activity, respectively (Table 1).

**Mechanism-Based Inhibition of CYP1A2.** When preincubated with NADPH-fortified human liver microsomes in the absence of substrate, zileuton was shown to inhibit the O-deethylation of phenacetin. The effect was NADPH-, time-, and concentration-dependent (Fig. 2A). Upon further study, it was determined that the kinetics of inhibition were described by a maximal rate (k_{inact}) of 0.035 min^{-1} and an apparent K_{I} of 117 μM (Fig. 2B). In contrast to POD activity, no preincubation-dependent inhibition of diclofenac 4'-hydroxylase and testosterone 6β-hydroxylase activity was observed. Under the same conditions, tiencillic acid (10 μM) and L-754394 (1.0 μM) served as potent mechanism-based inhibitors of the activities (>90% inhibition), respectively (data not shown). Therefore, the data suggested that zileuton did not behave as a mechanism-based inhibitor of CYP2C9 and CYP3A4.

As described in Table 2, a number of attempts were made to reverse the preincubation-dependent inhibitory effect of zileuton on POD activity. The presence of GSH, catalase, and SOD had no effect, which suggested that any reactive intermediate formed could not be scavenged prior to the enzyme inactivation event, and that the inactivation process was likely confined to the enzyme and did not involve peroxidative reactions outside the active site. In addition, dialysis had a minimal effect on the inhibition. Collectively, therefore, the data indicated that zileuton was a mechanism-based inhibitor of human liver microsomal CYP1A2.

The study was extended to encompass the evaluation of the individual enantiomers of zileuton as mechanism-based inhibitors of
 effect of zileuton racemate on various P450 activities in human liver microsomes in the absence of preincubation

<table>
<thead>
<tr>
<th>P450 Form</th>
<th>Reaction</th>
<th>Test Article</th>
<th>IC50 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethyltion</td>
<td>Fluvoxamine</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel 6α-hydroxylation</td>
<td>Zileuton</td>
<td>3.5</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4’-hydroxylation</td>
<td>Sulfinaphenazole</td>
<td>0.5</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin 4’-hydroxylation</td>
<td>(R)-N-3-Benzylphenobarbital</td>
<td>0.3</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Bufuralol 1’-hydroxylation</td>
<td>Zileuton</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylation</td>
<td>Quinidine</td>
<td>0.1</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion hydroxylation</td>
<td>Zileuton</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorozuxnzone 6-hydroxylation</td>
<td>Zileuton</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

CYP1A2. Upon preincubation with human liver microsomes, both enantiomers were shown to inhibit POD activity in a time-dependent manner (Fig. 3). However, the extent of inhibition was greater in the presence of (S)-(−)-zileuton. This was reflected in the \( k_{\text{inact}} \), which was higher for the (S)-(−)-enantiomer (0.037 versus 0.012 min\(^{-1}\)). The \( K_e \) values for (S)-(−)- and (R)-(+) zileuton were comparable (98.2 and 66.6 μM, respectively).

Retrospective in Vitro-in Vivo Correlations. Having determined that zileuton behaved as a mechanism-based inhibitor of CYP1A2 in human liver microsomes, an attempt was made to relate the in vitro data (\( K_e \) and \( k_{\text{inact}} \) for the racemate) to the pharmacokinetics of zileuton. In turn, the effect of zileuton on the pharmacokinetics (plasma AUC) of CYP1A2 substrates was simulated, assuming that the degradation of CYP1A2 was a first order process (\( k_e \) ranging from 0.00026 to 0.0012 min\(^{-1}\); half-life of 10–44 h).

Use of \( C_{\text{max}} \) (19 μM) and \( C_{\text{ave}} \) (9.0 μM) values for total zileuton in plasma (Awni et al., 1995a) resulted in overestimates of AUC changes (data not shown). After correction for binding of zileuton (\( I_f = 0.069 \)) to human plasma proteins (Machinist et al., 1995a), more reasonable estimates of AUC ratios were obtained (Fig. 4). For compounds undergoing extensive metabolism by CYP1A2, where the majority of the dose (>70%) is metabolized by the enzyme (\( f_m \cdot f_{\text{in,CYP1A2}} > 0.7 \)), zileuton would be expected to increase the AUC 1.4- to 2.2-fold (\( k_e \cdot 0.00026 \to 0.0005 \) min\(^{-1}\)). When CYP1A2 plays less of a role in clearance (\( f_m \cdot f_{\text{in,CYP1A2}} < 0.4 \)), changes in AUC are less marked (<1.3-fold). As shown in Fig. 4, simulated indications that if the degradation of CYP1A2 in vivo were characterized by a \( k_e \) of 0.0012 min\(^{-1}\) (half-life of 10 h), then the majority of its substrates would be refractory to the effects of zileuton (<1.3-fold increase in AUC).

Discussion

Numerous clinical drug interaction studies have been performed with zileuton. Overall, it appears that the compound is a weak inhibitor of CYP2C9 and CYP3A4, as evidenced by its minimal effect on the pharmacokinetics of orally dosed phenytoin, (S)-warfarin, and terfenadine (Awni et al., 1995c; 1997; Samara et al., 1995). Zileuton also has a minimal effect on the oral clearance (~8%) decrease of naproxen (Awni et al., 1995b). This is expected, because naproxen is largely metabolized by glucuronidation (~70%), and there are no reports that zileuton is an inhibitor of UDP-glucuronosyltransferases in vivo (Rodrigues et al., 1996). At the same time, the O-demethylation of naproxen is catalyzed by CYP2C9 and CYP1A2, but the overall contribution of each enzyme to overall clearance is low (\( f_m \cdot f_{\text{in,P450}} \sim 0.15 \)).

Weak inhibition of CYP2C9 and CYP3A4 in vivo has been confirmed in vitro. For example, Machinist et al. (1995b) reported zileuton as a weak reversible inhibitor (IC\(_{50} > 200 \) μM) of tolbutamide hydroxylation (CYP2C9) and terfenadine oxidation (CYP3A4) in human liver microsomes. These observations have been duplicated in the present study using a larger panel of P450 form-selective substrates (Table 1). In our hands, zileuton was a weak reversible inhibitor of not only CYP2C9 and CYP3A4 (IC\(_{50} > 100 \) μM), but also CYP1A2, CYP2C8, CYP2C19, CYP2D6, CYP2E1, and CYP2B6 activity. Most of the drug interaction studies reported to date have used one of two zileuton oral dosing regimens (600 mg q.i.d. or 800 mg b.i.d.), both of which result in a plasma concentration of 20 to 40 μM (Awni et al., 1995a). Even if liver tissue levels approximate the concentration of total zileuton in plasma, this \( C_{\text{max}} \) is below the in vitro IC\(_{50} \) values (C\(_{\text{max}}/\text{IC}_{50} \leq 0.2 \)).

In contrast to drugs such as (S)-warfarin and phenytoin, zileuton has been shown to decrease the oral clearance of (R)-warfarin (15%), theophylline (49%), propranolol (42%), and antipyrine (52%) (Awni et al., 1995c; Granneman et al., 1995; Machinist et al., 1995b; St. Peter et al., 1995). All of the aforementioned drugs are known to be metabolized by CYP1A2 (Masubuchi et al., 1994; Ha et al., 1995; Yoshimoto et al., 1995; Zhang and Kaminsky, 1995; Engel et al., 1996; Kaminsky and Zhang, 1996; Sharer and Wrighton, 1996; Tjia et al., 1996; Johnson et al., 2000). However, these clinical observations cannot be reconciled with the in vitro results, although it has been reported that zileuton (100 μM) is able to inhibit (~60%) the metabolism of theophylline (12-h coincidence) in precision-cut human liver slices (Machinist et al., 1995b). In particular, zileuton is a weak reversible inhibitor of both CYP1A2 and CYP2C9 activity in human liver microsomes, yet it is only the pharmacokinetics of (R)-warfarin that are affected when warfarin is dosed as a racemic mixture (Awni
et al., 1995c). In addition, CYP1A2 plays a major role in the overall metabolism of theophylline and zileuton increases its AUC almost 2-fold (Granneman et al., 1995; Ha et al., 1995; Troger and Meyer, 1995; Zhang and Kaminsky, 1995; Tjia et al., 1996; Yao et al., 2001).

**Fig. 2.** Concentration- and time-dependent inactivation of POD activity in human liver microsomes by racemic zileuton.

A, aliquots were removed from the primary reaction mixture at the indicated time points and were assayed for residual POD activity (see Materials and Methods). Percentage of activity remaining (related to time 0 in the presence of solvent alone) was plotted in the logarithmic scale determined from a single experiment. B, the corresponding double reciprocal plot of the rates of inactivation as a function of zileuton concentration. The $k_{\text{inact}}$ (0.035 min$^{-1}$) and $K_I$ (117 μM) were obtained from the y-intercept and the negative reciprocal of the x-intercept, respectively.
which was sufficient time to completely reverse the inhibitory effects of fluvoxamine (not a

TABLE 2
Irreversibility of the inactivation of human liver microsomal POD activity by zileuton racemate

<table>
<thead>
<tr>
<th>Assay Component</th>
<th>Percentage of Control</th>
</tr>
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<tbody>
<tr>
<td>Solvent plus NADPH</td>
<td>100</td>
</tr>
<tr>
<td>Zileuton minus NADPH</td>
<td>91.6 ± 6.17</td>
</tr>
<tr>
<td>Zileuton plus NADPH</td>
<td>49.9 ± 1.32</td>
</tr>
<tr>
<td>Zileuton plus NADPH and GSH (2 mM)</td>
<td>52.4 ± 1.89</td>
</tr>
<tr>
<td>Zileuton plus NADPH and catalase (500 units)</td>
<td>50.8 ± 4.01</td>
</tr>
<tr>
<td>Zileuton plus NADPH and SOD (500 units)</td>
<td>50.7 ± 3.00</td>
</tr>
<tr>
<td>Zileuton plus NADPH and dialysis</td>
<td>61.7 ± 1.17</td>
</tr>
<tr>
<td>Furafylline (10 μM) plus NADPH</td>
<td>22.6 ± 0.94</td>
</tr>
</tbody>
</table>

This increase in AUC is comparable to that reported for more potent reversible inhibitors of CYP1A2 (K_i ≤ 47 μM) such as fluvoxamine, propafenone, and mexiletine (Lo et al., 1991; Brosen et al., 1993; Spinler et al., 1993; Rasmussen et al., 1995; Rasmussen et al., 1997; Kobayashi et al., 1998; Yao et al., 2001). Overall, zileuton appears to be a clinically relevant inhibitor of CYP1A2 despite being a relatively weak reversible inhibitor of the enzyme in vitro. This inhibition is clinically important, because warfarin and theophylline are both characterized by a relatively narrow therapeutic index, which requires patient monitoring and dose adjustment (Awni et al., 1995c; Granneman et al., 1995).

During the course of the present studies, zileuton was shown to be a mechanism-based inhibitor of CYP1A2 activity in human liver microsomes. The inhibition was concentration-, time-, and NADPH-dependent and was refractory to the effect of dialysis and scavenging agents such as GSH, catalase, and SOD. Moreover, the effect was selective for CYP1A2, despite the fact that three P450s (CYP1A2, CYP2C9, and CYP3A4) are involved in metabolism (Machinist et al., 1995b). Notably, a second hydroxysteroids-containing 5-lipoxygenase inhibitor (ABT-761) is a poor CYP1A2 substrate and has been shown to have a minimal effect on the pharmacokinetics of theophylline (Machinist et al., 1998; Wong et al., 1998). Therefore, the CYP1A2-dependent ring hydroxylation of zileuton and mechanism-based inhibition are linked in some way and may involve the formation of a reactive arene oxide intermediate. Although there is no evidence for CYP1A2-catalyzed sulfonation of zileuton (Machinist et al., 1995b), it is also possible that CYP1A2 does catalyze sulfonation to a reactive benzothiope S-oxide, which gives rise to mechanism-based inhibition (Mansuy et al., 1991; Lopez-Garcia et al., 1994). At least for CYP3A4, the products of sulfonation are stable, and there is no evidence for mechanism-based inhibition of the enzyme.

In our hands, the inhibitory effect of zileuton on POD activity in human liver microsomes was amenable to kinetic analysis, yielding estimates of k_inact (0.035 min^{-1}), K_I (117 μM), and a k_inact/K_I ratio of 0.0003 min^{-1} μM^{-1}. The k_inact/K_I ratio is considerably lower than that reported for furafylline (0.01–0.09 min^{-1} μM^{-1}), which decreases the oral clearance of caffeine 10-fold (Tarrus et al., 1987; Kunze and Trager, 1993; Clarke et al., 1994; Jones and Hall, 2002). Despite a relatively low k_inact/K_I ratio, we sought to relate the in vitro inhibition parameters for zileuton to its pharmacokinetics and attempted to rationalize the observed clinical drug interactions with CYP1A2 substrates.

Efforts have been made to evaluate the mechanism-based inhibition of P450s using nonphysiology- and physiology-based models (Ito et al., 1998; Mayhew et al., 2000; Jones and Hall, 2002). Overall, it is accepted that the net effect of the inhibitor is dependent upon the k_inact/K_I ratio, k_v, and the dose of the inhibitor (concentration of the inhibitor at the enzyme active site). However, the weakest part of the models has been the estimation of k_v for the human P450s. Most of the authors have relied on in vitro human (hepatocyte) data or have made use of in vivo rodent data (Ito et al., 1998; Mayhew et al., 2000; Jones and Hall, 2002; Madan et al., 2002). As a result, in the absence of in vivo human data, the k_v values used in the models have been somewhat arbitrary.

For the sake of simplicity, the retrospective analysis of the zileuton data were based on a kinetic model (eq. 1), using a range of k_v values for CYP1A2. Correia (1991) has reported that the half-life of CYP1A2 in rat liver is about 10 h (k_v = 0.0012 min^{-1}), whereas estimates of half-life for a human P450 (e.g., CYP3A4) have ranged from 24 to 44 h (k_v = 0.0005–0.00026 min^{-1}) (Ito et al., 1998; Mayhew et al., 2000). With such estimates of k_v, it has been possible to carry out retrospective in vitro-in vivo correlations for mechanism-based inhibitors of CYP3A4 (Mayhew et al., 2000; Jones and Hall, 2002). Therefore, zileuton (racemate) was evaluated in a similar manner using the in vitro k_inact and K_I data (Fig. 2) and the plasma levels (corrected for f_m) reported in the literature (Awni et al., 1995a).

Assuming a k_v of 24 to 44 h, the simulations indicated that AUC changes are marked (up to 2-fold) when a large fraction of the dose (f_m * f_m,CYP1A2 > 0.6) is metabolized by CYP1A2 (Fig. 4, B and C). When CYP1A2 plays a relatively minor role in the overall clearance (f_m * f_m,CYP1A2 < 0.4), then the AUC increases elicited by zileuton are less pronounced (<1.3-fold). In light of the reported zileuton clinical data, the simulations employing the reported k_v value for rat liver CYP1A2 (0.0012 min^{-1}; Correia, 1991) gave relatively poor results (<1.3-fold increase in AUC) over the entire range of f_m * f_m,CYP1A2 values (Fig. 4A).

Using existing in vitro P450 reaction phenotyping data (f_m,CYP1A2) and clinical data (f_m), it is possible to obtain estimates of f_m * f_m,CYP1A2 for both theophylline (~0.7) and (R)-warfarin (~0.4). The latter is partially metabolized by carboxyl reductase (~40%) and the remainder of the metabolism is more or less equally divided between CYP1A2 and CYP3A4 (Kaminsky and Zhang, 1996; Scordo et al., 2002). In contrast, the majority of theophylline N-demethylation and C_8 oxidation is catalyzed by CYP1A2 at clinically relevant concentrations (Ha et al., 1995; Troger and Meyer, 1995; Zhang and Kaminsky, 1995; Tija et al., 1996; Yao et al., 2001). Therefore, zileuton would be expected to increase the AUC of theophylline up to 1.8-fold and have less of an effect (~1.3-fold increase) on the AUC of (R)-warfarin (Fig. 4, B and C). In agreement, zileuton increases the AUC of theophylline and (R)-warfarin 1.9- and 1.2-fold, respectively (Awni et al., 1995c; Granneman et al., 1995). CYP1A2 can also play an important role in the overall clearance of antipyrine and propranolol (f_m * f_m,CYP1A2 as high as 0.56), and the simulations (Fig. 4C) indicate that AUC increases of up to 1.6-fold are possible (Walle et al., 1985; Masubuchi et al., 1994; Yoshimoto et al., 1995; Engel et al., 1996; Sharer and Wrighton, 1996; Johnson et al., 2000). This is in reasonable agreement with the reported effect of zileuton on the oral clearance of antipyrine (~50% decrease; ~2-fold increase in AUC) and propranolol (~42% decrease; ~1.6-fold increase in AUC) (Machinist et al., 1995b; St. Peter et al., 1995). Therefore, with some assumptions, it has been possible to use the in vitro data and reconcile it with the results of previously reported clinical drug interaction studies.

Interestingly, both enantiomers of zileuton were shown to inhibit CYP1A2 activity in human liver microsomes, although the k_inact (0.037 versus 0.012 min^{-1}) and k_inact/K_I ratio (0.0004 versus 0.0002 min^{-1} μM^{-1}) were higher for the (S)-enantiomer (Fig. 3). Because

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the concentrations of both enantiomers in plasma are similar (Wong et al., 1995), the in vitro data suggest that the (S)-enantiomer may play more of a role in vivo. However, $f_w$ is higher (0.122 versus 0.037) for the (R)-enantiomer (Machinist et al., 1995a), which could offset the difference in the $k_{\text{max}}/K_I$ ratio.

Based on the results of this study, it is concluded that zileuton is a

![Figure 3](https://example.com/figure3.png)

**Fig. 3. Concentration- and time-dependent inactivation of POD activity in human liver microsomes by the individual enantiomers of zileuton.**

Aliquots were removed from the primary reaction mixture at the indicated time points and were assayed for residual POD activity (see Materials and Methods). For (R)- (+) and (S)- (-)-zileuton, the $k_{\text{max}}$ and $K_I$ values were obtained from the y-intercept and the negative reciprocal of the x-intercept (inset), respectively (see legend to Fig. 2). $k_{\text{max}}$ and $K_I$ values for (S)-(-)-zileuton were 0.037 min$^{-1}$ and 98.2 M, respectively. $k_{\text{max}}$ and $K_I$ values for (R)-(+)-zileuton were 0.012 min$^{-1}$ and 66.6 M, respectively. In each case, time-dependent inhibition was evaluated in the presence of solvent alone (○), 10 μM zileuton (□), 25 μM zileuton (■), 50 μM zileuton (▼), 100 μM zileuton (▲), 200 μM zileuton (●).
The effect of zileuton on the pharmacokinetics of CYP1A2 substrates [AUC_{po(i)}-to-AUC_{po(c)} ratio] was simulated using eq. 1 (see Materials and Methods). In vitro $k_{inact}$ (0.035 min$^{-1}$) and $K_I$ (117 nM) data were used in the simulation, along with published values for zileuton plasma $C_{max,ss}$ (19 nM) and $C_{ave,ss}$ (9 nM) corrected for protein binding ($f_u = 0.069$). The fraction of the dose metabolized by CYP1A2 ($f_m \cdot f_m,_{CYP1A2}$) is considered for different substrates. The degradation of CYP1A2 is assumed to be a first order process, characterized by a rate constant of 0.0012 min$^{-1}$ (A), 0.0005 min$^{-1}$ (B), and 0.00026 min$^{-1}$ (C).
weak reversible inhibitor of P450 activities in human liver microsomes. However, the compound behaves as a mechanism-based inhibitor of CYP1A2 ($K_{inact}$ 0.035 min$^{-1}$, $K_i$ 117 μM). Despite metabolism by CYP1A2, CYP2C9, and CYP3A4 (Machinist et al., 1995b), the compound is not a mechanism-based inhibitor of CYP2C9 and CYP3A4. This P450 form-selective, mechanism-based inhibition may explain why zileuton elicits clinical drug interactions with CYP1A2 substrates [e.g., theophylline, (R)-warfarin, propranolol, and antipyrine] at doses that have a minimal effect on drugs metabolized by other P450s [e.g., terfenadine, phenytoin, and (S)-warfarin]. Moreover, this effect on CYP1A2 is clinically relevant, despite the enzyme playing a relatively minor role in the overall clearance of zileuton.

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References


