Identification and Characterization of Human Cytochrome P450 Isoforms Interacting with Pimozide

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Accepted for publication January 12, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Using human liver microsomes (HLMs) and recombinant human cytochrome P450 (CYP450) isoforms, we identified the major route of pimozide metabolism, the CYP450 isoforms involved, and documented the inhibitory effect of pimozide on CYP450 isoforms. Pimozide was predominantly N-dealkylated to 1,3-dihydro-1-(4-piperidinyl)-2H-benzimidazol-2-one (DHPBI). The formation rate of DHPBI showed biphasic kinetics in HLMs, which suggests the participation of at least two activities. These were characterized as high-affinity (K<sub>m1</sub> and V<sub>max1</sub>) and low-affinity (K<sub>m2</sub> and V<sub>max2</sub>) components. The ratio of V<sub>max1</sub> (14 pmol/min/mg protein)/K<sub>m1</sub> (0.73 µM) was 5.2 times higher than the ratio of V<sub>max2</sub> (244 pmol/min/mg protein)/K<sub>m2</sub> (34 µM). K<sub>m2</sub> was 91 times higher than K<sub>m1</sub>. The formation rate of DHPBI from 25 µM pimozide in nine human livers correlated significantly with the catalytic activity of CYP3A (Spearman r = 0.79, P = .028), but not with other isoforms. Potent inhibition of DHPBI formation from 10 µM pimozide was observed with ketoconazole (88%), troleandomycin (79%), furafylline (48%) and a combination of furafylline and ketoconazole (96%). Recombinant human CYP3A4 catalyzed DHPBI formation from 10 µM pimozide at the highest rate (V = 2.2 ± 0.89 pmol/min/pmol P450) followed by CYP1A2 (V = 0.23 ± 0.08 pmol/min/pmol P450), but other isoforms tested did not. The K<sub>m</sub> values derived with recombinant CYP3A4 and CYP1A2 were 5.7 µM and 36.1 µM, respectively. Pimozide itself was a potent inhibitor of CYP2D6 in HLMs when preincubated for 15 min (K<sub>i</sub> = 0.75 ± 0.98 µM) and a moderate inhibitor of CYP3A (K<sub>i</sub> = 76.7 ± 34.5 µM), with no significant effect on other isoforms tested. Our results suggest that pimozide metabolism is catalyzed mainly by CYP3A, but CYP1A2 also contributes. Pimozide metabolism is likely to be subject to interindividual variability in CYP3A and CYP1A2 expression and to drug interactions involving these isoforms. Pimozide itself may inhibit the metabolism of drugs that are substrates of CYP2D6.

Pimozide is a potent neuroleptic that has been used extensively in Europe for the treatment of schizophrenia and other psychiatric diseases (Pinder et al., 1976; Tueth and Cheong, 1993). The use of pimozide in the United States is restricted to the management of motor and phonic tics associated with Tourette's syndrome (Colvin and Tankanow, 1985). This is because of pimozide-induced prolongation of the cardiac QT interval (Fulop et al., 1987; Shapiro et al., 1989) and the risk of developing potentially fatal arrhythmia of the torsade de pointes type (Krähenbühl et al., 1995).

For a number of drugs, including pimozide, there appears to be a link between cardiac adverse events and increased plasma concentrations (Wooley et al., 1993; Antzelevitch et al., 1996; Flockhart et al., 1996). The pharmacokinetics of pimozide show wide intersubject variability (McCreadie et al., 1984; Sallee et al., 1987), which suggests that altered metabolic capacity may have important clinical consequences in terms of efficacy and safety. Data from animals suggest that pimozide undergoes extensive hepatic metabolism (Soudijn and Wijngaarden, 1969), but its human metabolism and the enzymes that are responsible are not known. An understanding of the enzymatic machinery involved is important to predict which patients might be most vulnerable to the adverse effects that result from drug interactions or from genetic polymorphism in metabolic pathways.

Indirect evidence from the literature implicates the CYP450 system in the metabolism of pimozide. Six putative metabolites of pimozide have been recovered in urine and feces in rats (Soudijn and Wijngaarden, 1969) and appear to be products of oxidative reactions. In humans, pimozide apparently undergoes oxidative N-dealkylation that cleaves the molecule in two halves (fig. 1). In a study involving three schizophrenic patients (Baro et al., 1972), ~30% of the administered pimozide dose was recovered in urine and feces as

ABBREVIATIONS: CYP450, cytochrome P450; FPBA, 4,4-bis(4-fluorophenyl)butanoic acid; DHPBI, 1,3-dihydro-1-(4-piperidinyl)-2H-benzimidazol-2-one; HLMs, human liver microsomes; HPLC, high-performance liquid chromatography; G-6-P, glucose 6-phosphate; G-6-PDH, glucose 6-phosphate dehydrogenase; NADP, β-nicotinamide adenine dinucleotide phosphate; EDTA, disodium salt of ethylenediaminetetraacetic acid.
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were medically unsuitable for liver transplantation and frozen at −80°C within 3 hr of cross-clamp time. The characteristics of liver donors, procedure for preparation of microsomal fractions and their CYP450 contents have been described previously (Harris et al., 1994). The microsomal pellets were resuspended in a reaction buffer (0.1 M Na+ and K+ phosphate, 1.0 mM EDTA, 5.0 mM MgCl₂, pH 7.4) to a protein concentration of 10 mg/ml (stock) and were kept at −80°C until used. Protein concentrations were determined with the method described by Pollard et al. (1978). Baculovirus-insect cell expressed human CYP450s 1A2, 3A4, 2D6, 2C19, 2B6 and 2E1 (with reductase) were purchased from Gentest Corporation (Woburn, MA) and stored at −80°C. Protein concentrations and CYP450 contents were supplied by the manufacturer.

**Incubation conditions.** To define optimal conditions for incubation and HPLC analysis, pimozide (1–100 μM) was incubated with HLMs for 0 to 150 min across a range of microsomal protein concentrations (0.1–1 mg protein/ml). An incubation time of 30 min, human microsomal protein concentration of 1 mg/ml and pimozide concentration of 10 μM represented linear conditions and was used in the subsequent experiments unless otherwise stated. In all experiments, pimozide was dissolved and diluted serially in ethanol, and then the alcohol was removed by evaporating to dryness under reduced pressure in 1.5-ml microfuge tubes with a Speedvac SC110 model RH40–12 (Savant Instruments Inc., Farmingdale, NY). Pimozide was reconstituted in sodium monobasic phosphate buffer (pH, 7.4) and prewarmed with NADPH-generating system (13 mM NADP, 33 mM G-6-P, 33 mM MgCl₂ and 0.4 U/ml G-6-PDH) for 5 min at 37°C. Reactions were initiated by adding either 25 μl of microsomes (10 mg protein/ml) or 25 μl of recombinant human CYP450 isoforms (diluted to 250–500 pmol P450/ml with buffer; pH 7.4) and were incubated in a final incubation volume of 250 μl for 30 min at 37°C. Reactions were terminated by placing the incubation tubes on ice, immediately adding 200 μl ice-cold acetonitrile and vortex-mixing. The samples were then centrifuged at 14,000 rpm for 5 min in an Eppendorf model 5415C centrifuge (Brinkman Instruments, Westbury, NY). Aliquots of supernatant (20 μl) were injected into the HPLC system. Control incubations for each experiment were carried out without substrate, without NADPH-generating system, without microsomes (bovine serum albumin was used instead) or without inhibitors.

**Assay of pimozide and its metabolites.** An HPLC method with fluorescent detection, which was developed recently in our laboratory for pimozide assay in human plasma (Kerbusch et al., 1997), was modified to measure pimozide and fluorescent metabolites in microsomal incubates. Aliquots (20 μl) of the supernatants of the centrifuged incubates were injected into the HPLC. The HPLC system consisted of a Waters Assoc. model 600 dual-piston pump (Milford, MA), a Waters Assoc. model 717 auto-sampler and FD-300 Dual Fluorescent Detector, which was developed recently in our laboratory, with 0.4 U/ml G-6-PDH) for 5 min at 37°C. Reactions were terminated by placing the incubation tubes on ice, immediately adding 200 μl ice-cold acetonitrile and vortex-mixing. The samples were then centrifuged at 14,000 rpm for 5 min in an Eppendorf model 5415C centrifuge (Brinkman Instruments, Westbury, NY). Aliquots of supernatant (20 μl) were injected into the HPLC system. Control incubations for each experiment were carried out without substrate, without NADPH-generating system, without microsomes (bovine serum albumin was used instead) or without inhibitors.

**Materials and Methods**

**Chemicals.** Pimozide, dextromethorphan HBr, chlorzoxazone, chlorpropamide, quinidine sulfate, orphenadrine HCl, tolbutamide, diethylthiocarbamate, trolenadomycin, ketocanazole, phenacetin, acetaminophen, G-6-P, G-6-PDH, NADP and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfaphenazole, furafylidine, S-mephenytoin and 6-hydroxychlorzoxazone were obtained from Ultrafine Chemicals (Manchester, England). Levalorphanol was obtained from U.S.P.C. (Rockville, MD). Dextrophan and 3-methoxy-morphinans were purchased from Hoffman-La Roche Inc. (Nutley, NJ). Omeprazole was a generous gift from Dr. Tommy Anderson (Clinical Pharmacology, Astra Hässle AB, Mölndal, Sweden). N-(4-Hydroxyphenyl)butamidine was kindly provided by Dr. John Strong (Division of Clinical Pharmacology, Center for Drug Evaluation and Research, United States Food and Drug Administration, Rockville, MD). Putative pimozide synthetic metabolites, FPBA and DHPBI, were generously supplied by Dr. Karel Lavrijsen of the Janssen Research Foundation (Beerse, Belgium).

Human liver microsomes and recombinant CYP450 isoforms. The microsomes used were prepared from human livers that...
standard curves from direct injections of known equimolar concentrations (5, 10, 15, 25 and 100 μM each) of synthetic reference metabolite and pimozide, was less than 5.1% at any concentration (ratio, 0.98 ± 0.04; range, 0.95–1.04). At 10 and 20 μM pimozide, the interday coefficient of variance of the method was less than 10% and 2.5%, respectively, and the intraday coefficient of variance was less than 5.1% and 2.2%, respectively.

**Determination of $K_{m}$ and $V_{max}$ for pimozide metabolism in HLMs.** Kinetic parameters for the formation of DHBP1 were obtained by incubating pimozide (0.5–200 μM) with HLM preparations (or recombinant human CYP450 isoforms) and an NADPH-generating system. Because our initial data implicated two CYP450 activities, their relative contribution was determined by incubating pimozide (0.5–200 μM) either in the presence of 1 μM ketoconazole [a specific inhibitor of CYP3A (Baldwin et al., 1995)] or 10 μM furafylline [a specific inhibitor of CYP1A2 (Sesardic et al., 1990)] in HLMs (HL4, HL8 and HL16). An appropriate model for each function was selected to calculate kinetic parameters (see “Data Analysis”).

**Correlation experiments.** Pimozide (25 μM) was incubated with nine different HLMs (HL2-HL9 and HL16) to test the correlation of pimozide N-dealkylation with the activity of CYP1A2 measured by the O-deethylation of phenacetin (Tassaneeyakul et al., 1993), of CYP2D6 measured by the O-demethylation of dextromethorphan (Rodrigues et al., 1994), of CYP3A measured by the oxidation of felodipine (Harris et al., 1994), of CYP2C19 measured by the 4-hydroxylation of S-mephentoyin (Wrighton et al., 1993) and of CYP2E1 measured by the 6-hydroxylation of chlorozaxone (Peter et al., 1990).

**Inhibition studies.** The formation rate of DHBP1 from 10 μM pimozide was evaluated in the absence (control) and presence of the following known isoform-specific inhibitors: ketoconazole and troleandomycin for CYP3A (Baldwin et al., 1995; Bourrie et al., 1996), quinidine for CYP2D6 (Broly et al., 1989), furafylline for CYP1A2 (Sesardic et al., 1990), diethyldithiocarbamate for CYP2E1 (Guengerich et al., 1991), sulfaphenazole for CYP2C9 (Baldwin et al., 1995), omeprazole for CYP2C19 (Ko et al., 1997) and ophenadrine for CYP2B6 (Heyn et al., 1996). Pimozide was preincubated for 5 min with or without CYP450 isofom-specific inhibitor and with the NADPH-generating system. HLMs were added to initiate the reaction and incubated for 30 min at 37°C in a final incubation volume of 250 μL. Troleandomycin is a mechanism-based inhibitor of CYP3A (Newton et al., 1995), and therefore, it was first preincubuted in the presence of the NADPH-generating system and HLMs at 37°C for 15 min and the reaction initiated by addition of substrate (pimozide). Furafylline is both a competitive (Bourrie et al., 1996) and a mechanism-based selective inhibitor of CYP1A2 (Sesardic et al., 1990). Because the degree of inhibition was similar with or without preincubation, the latter protocol was used. All isoform-specific inhibitors were studied at two concentrations chosen to be selective for the respective CYP450 isoforms on the basis of published $K_{m}$ values of the inhibitor probes (Bourrie et al., 1996; Newton et al., 1995). Inhibitors were dissolved in water where appropriate or in suitable organic solvents (ethanol, methanol or dimethyl sulfoxide) and then serially diluted with water to contain <0.1% of solvents in final volume. Rates of DHBP1 formation were compared with those of controls in which the inhibitor was replaced with buffer or an appropriate concentration of vehicle. Exact inhibition constants ($K_{i}$) were determined from Dixon plots obtained by incubating 1 to 50 μM pimozide with ketoconazole (0, 0.01, 0.1, 0.25 and 0.5 μM), furafylline (0, 1, 5, 10 and 20 μM) and omeprazole (0, 1, 10, 20 and 50 μM) in HLMs.

**Recombinant human CYP450 isoforms.** To test which specific CYP450 isoforms are responsible for pimozide N-dealkylation, 10 μM pimozide was incubated with 25 μL of recombinant human CYP450s 3A4, 1A2, 2C19, 2D6, 2B6 and 2E1 (250–500 pmol P450/mL in sodium monobasic phosphate buffer, pH 7.4). All other conditions of incubation remained the same as those for the experiments with HLMs. Omeprazole inhibited pimozide metabolism in whole HLMs, but recombinant human CYP2C19 did not catalyze pimozide N-dealkylation. Because omeprazole and its sulfone metabolite also inhibit CYP3A at high concentrations ($K_{i}$ = 25–44 μM) (Vanden-Branden et al., 1996), we incubated 10 μM pimozide with omeprazole (10 and 20 μM) and recombinant human CYP3A4 to test whether any observed inhibitory effect of omeprazole was mediated via this isoform.

**Inhibition of CYP450 by pimozide.** The inhibitory effect of pimozide on the activities of common drug-metabolizing CYP450 isoforms was tested in HLMs (HL4, HL8 and HL16) with substrate reaction probes selective for each isoform. Pimozide (1–50 μM) was incubated with HLMs, NADPH-generating system and substrate reaction probes with incubation conditions specific to each isoform. The reaction probes used were: phenacetin O-deethylation for CYP1A2 (Tassaneeyakul et al., 1993), tolbutamide 4-methylhydroxylation for CYP2C9 (Relling et al., 1990), omeprazole hydroxylation for CYP2C19 (Ro et al., 1997), dextromethorphan O-demethylation for CYP2D6 (Rodrigues et al., 1994), dextromethorphan N-demethylation for CYP3A (Gorski et al., 1994) and chlorozaxone 6-hydroxylation for CYP2E1 (Peter et al., 1990). The assays for the activities of CYP2D6, CYP3A, CYP1A2 and CYP2E1 are used routinely in our laboratory and have been described in detail elsewhere (Ko et al., 1997). Dextromethorphan, tolbutamide, phenacetin and chlorozaxone concentrations in the final incubation were 2.5 to 75 μM, 5 to 50 μM, 20 to 100 μM and 5 to 40 μM, respectively. A method for omeprazole assay in human plasma (Balian et al., 1995) was modified to assay omeprazole and its 5’-hydroxy metabolite in HLMs. The formation of each metabolite was quantified by comparing the ratio of the area under the curve of the metabolite to the area under the curve of each internal standard with an appropriate standard curve. Apparent $K_{m}$ and $V_{max}$ values of the isoform-specific substrate probes were reported by Ko et al. (1997) for each human liver preparation used.

**Data analysis.** Kinetic analysis of the DHBP1 formation were performed by initial visual examination of Eadie-Hofstee plots (V vs. 1/V) to determine whether one or two enzymes were involved. The estimates for kinetic parameters from this analysis were used as initial estimates for nonlinear least-square regression analysis (WINNONLIN Version 1.0, Apex, NC) for apparent $K_{m}$ and $V_{max}$ values. An appropriate single- or two-site model was selected for each data set on the basis of the dispersion of residuals and standard errors of the parameter estimates (kinetic parameters are given with standard error). Correlation coefficients between DHBP1 formation and the activities of CYP450 isoforms in different livers were determined by nonparametric regression analysis (Spearman’s rank correlation test) with GraphPad Prism software (Version 2.01, San Diego, CA), and a P value less than .05 was considered statistically significant. The inhibition (%) of pimozide N-dealkylation by CYP450 isoform-specific inhibitors and of CYP450 substrate probes by pimozide was obtained by comparing the inhibited activity with control. Mechanisms of inhibition and estimates of inhibitory constants ($K_{i}$ values) were determined from Dixon plots. For certain inhibitors, approximate $K_{i}$ values were calculated assuming competitive inhibition with the following equation:

$$\text{% inhibition} = \frac{100 \times [I]}{[I] + K_{i} \times \left(1 + \frac{[S]}{K_{m}}\right)}$$

$I$ represents inhibitor concentration, $K_{i}$ inhibitory constant, $S$ substrate concentration and $K_{m}$ substrate concentration at half of the maximum velocity ($V_{max}$) of the reaction.

**Results.** A typical HPLC chromatogram of pimozide and its metabolites is demonstrated in figure 2. A fluorescent metabolite peak was formed that depended on the NADPH-generating
system, duration of incubation and microsomal protein and substrate concentrations (not shown). The retention time of this analyte (1.9 min) when compared with peaks of two putative synthetic metabolites of pimozide, DHPBI and FPBA (fig. 1), was identical with that of DHPBI (fig. 2). Pimozide was eluted at 9.9 min. Two other minor peaks (retention times, 2.4 and 5.9 min) were also noted (fig. 2), which might represent primary or secondary metabolites. Because these "metabolites" were formed in small amounts at high concentrations of pimozide (≥50 μM), and because no reference synthetic standards for these metabolites were available, no attempt was made to characterize them further. The HPLC chromatograms of blank incubate or of pimozide with inhibitors tested did not interfere with the separation of pimozide and its metabolite.

The formation rate of DHPBI from pimozide (0.5–200 μM) in HLMs exhibited biphasic kinetic behavior in Eadie-Hofstee plots (fig. 3), which suggested the involvement of at least two enzymatic activities that were best described by high-affinity (Km1 and Vmax1) and low-affinity (Km2 and Vmax2) components. The kinetic parameters from duplicate incubations of pooled HLM (HL2, HL9 and HL16) are demonstrated in table 1. The intrinsic metabolic clearance of the high-affinity component (Vmax1/Km1) was 5.2 times higher than that of the low-affinity component (Vmax2/Km2), and Km2 was 91 times higher than Km1.

In nine HLM preparations, the formation rate of DHPBI from 25 μM pimozide showed a 10.4-fold interindividual variability (range, 15.8–163.9 pmol/min/mg protein) (table 2). There was a significant correlation between pimozide N-dealkylation and the activity of CYP3A in different human livers (Spearman r = 0.79, P = .028), as measured by felodipine oxidation. No correlation was observed with the catalytic activities of CYP2D6 (r = 0.61, P = .12), CYP1A2 (r = 0.46, P = .27), CYP2C19 (r = 0.37, P = .33) or CYP2E1 (r = 0, P = 1).

To further probe the CYP450 isofoms participating in the N-dealkylation of pimozide, 10 μM pimozide was incubated with CYP450 isoform-specific inhibitors in HLMs. As shown in figure 4, ketoconazole was the most potent inhibitor of DHPBI formation (88% at 1 μM and 96% at 5 μM) followed by troleandomycin (54% at 1 μM and 78.8% at 10 μM) and furafylline (48% at 10 μM and 55% at 20 μM). When pimozide was incubated with furafylline (10 μM) and ketoconazole (1 μM), the formation of DHPBI was inhibited almost completely (~96%). Omeprazole did not inhibit pimozide N-dealkylation at concentrations known to be selective for the CYP2C19 isoform (<10 μM) (Ko et al., 1997), but did inhibit at higher concentrations (15%, 40% and 65% inhibition at 10, 20 and 100 μM omeprazole, respectively). A high concentration of orphenadrine (300 μM) inhibited pimozide N-dealkylation by ~32%, but incubating pimozide with 100 μM orphenadrine had little effect on the formation of DHPBI. Other isoform-specific inhibitors [quinidine (CYP2D6), sulfaphenazole (CYP2C9) and diethyldithiocarbamate (CYP2E1)] did not inhibit the formation of DHPBI. In figure 5, Dixon plots from pooled HLMs (HL2, HL9 and HL16) for the inhibition of DHPBI by CYP3A, CYP1A2 and CYP2C19 isoform-specific inhibitors are demonstrated. The inhibitory constants (Ki ± S.D.) were 0.25 ± 0.08 μM for ketoconazole, 8.8 ± 1.7 μM for furafylline and 26.8 ± 4.9 μM for omeprazole. The Eadie-Hofstee plots for the formation of DHPBI
from pimozide (0.5–200 μM), selected to represent the $K_{m1}$ (high affinity) and $K_{m2}$ (low affinity), and the effect of furafylline or ketoconazole are demonstrated in figure 6. The respective kinetic parameters derived are compared with control incubations (table 1). In the presence of ketoconazole, the formation rate of DHPBI was described best by a single enzyme system model in which the high-affinity component was eliminated completely ($V_{max1}$ from 14 pmol/min/mg pro-

### TABLE 1

Estimated kinetic parameters for the formation of DHPBI from pimozide (0.5–200 μM) and inhibition by ketoconazole or furafylline in HLMs $K_{m1}$ and $V_{max1}$ refer to Michaelis-Menten parameters for the high-affinity component, whereas $K_{m2}$ and $V_{max2}$ refer to the low-affinity component.

<table>
<thead>
<tr>
<th>Incubation with</th>
<th>$K_{m1}$</th>
<th>$V_{max1}$</th>
<th>$V_{max1}/K_{m1}$</th>
<th>$K_{m2}$</th>
<th>$V_{max2}$</th>
<th>$V_{max2}/K_{m2}$</th>
</tr>
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<tbody>
<tr>
<td>Pimozide alone</td>
<td>0.37 ± 1.3</td>
<td>14 ± 12</td>
<td>37.5</td>
<td>34 ± 6.7</td>
<td>244 ± 11</td>
<td>7.2</td>
</tr>
<tr>
<td>Pimozide + ketoconazole (1 μM)</td>
<td>–</td>
<td>–</td>
<td>36 ± 5.4</td>
<td>34.7 ± 1.6</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Pimozide + furafylline (10 μM)</td>
<td>0.19 ± 1.2</td>
<td>17.2 ± 12</td>
<td>89.0</td>
<td>58 ± 21</td>
<td>173 ± 14</td>
<td>3.0</td>
</tr>
</tbody>
</table>

$K_m$ is expressed as μM and $V_{max}$ as pmol/min/mg protein. Values are mean and standard error (S.E.) of estimates of nonlinear least-square regression analysis obtained by WINNONLIN (see, “Materials and Methods”).

### TABLE 2

Correlation of DHPBI formation rate ($V$) from 25 μM pimozide with the activities of different human CYP450 isoforms

Data are averages of duplicate measurements

<table>
<thead>
<tr>
<th>Liver</th>
<th>CYP3A</th>
<th>CYP2D6</th>
<th>CYP1A2</th>
<th>CYP2C19</th>
<th>CYP2E1</th>
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</thead>
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<tr>
<td></td>
<td>pmol/min/mg</td>
<td>$P$</td>
<td>pmol/min/mg</td>
<td>$P$</td>
<td></td>
</tr>
<tr>
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<td>488</td>
<td>2400</td>
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<tr>
<td>3</td>
<td>270</td>
<td>0</td>
<td>750</td>
<td>0.9</td>
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</tr>
<tr>
<td>4</td>
<td>225</td>
<td>159</td>
<td>505</td>
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<tr>
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<td>110</td>
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<td>505</td>
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<tr>
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<td>129</td>
<td>400</td>
<td>3.1</td>
<td>1118</td>
</tr>
<tr>
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<td>118</td>
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<td>229</td>
<td>1505</td>
<td>6.4</td>
<td>671</td>
</tr>
<tr>
<td>9</td>
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<td>247</td>
<td>1400</td>
<td>23.6</td>
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<td>4.4</td>
<td>ND</td>
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<tr>
<td>Correlation (rs)</td>
<td>0.79</td>
<td>0.61</td>
<td>0.46</td>
<td>0.37</td>
<td>0</td>
</tr>
<tr>
<td>P value</td>
<td>0.028*</td>
<td>0.12</td>
<td>0.27</td>
<td>0.33</td>
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</table>

* Statistically significant (activity of CYP3A vs. $V$ of DHPBI); ND, not determined.

**Fig. 4.** Inhibition of DHPBI formation by CYP450 isoform-specific inhibitors in HLMs. Pimozide (10 μM) was incubated with at least two inhibitor concentrations in HLMs, and the combined inhibitory effect of ketoconazole (1 μM) and furafylline (10 μM) was tested. For incubation conditions, see “Materials and Methods.” Results are mean ± S.D. of two independent incubations of pooled microsomes from HL 2, HL 9 and HL 16 (n = 4 determinations). The inhibited activities were compared with uninhibited activities (controls). Abbreviations: DEDTC, diethyldithiocarbamate; Sulphaphen = sulphaphenazole
tein in control to undetectable rates). Ketoconazole also markedly reduced the $V_{\text{max}}$ of the low-affinity component by 7-fold with no effect on $K_m^2$ (table 1). The shape of the Eadie-Hofstee plot of DHPBI formation remained biphasic in the presence of furafylline (fig. 6), which appeared to inhibit only the low-affinity component of the reaction because there was a significant reduction in $V_{\text{max}}^2$ with little effect on $K_m^1$ or $V_{\text{max}}^1$ values (table 1).

Recombinant human CYP3A4 isoform catalyzed DHPBI from 10 $\mu$M pimozide at the highest rate ($V = 2.2 \pm 0.89$ pmol DHPBI/min/pmol P450) followed by CYP1A2 ($V = 0.23 \pm 0.08$ pmol DHPBI/min/pmol P450), but not by other isoforms (CYP450s 3A4, 1A2, 2C19, 2D6, 2B6 and 2E1) (fig. 7). The enzymes responsible for the low-affinity and high-affinity components were investigated further by incubating pimozide (0.5–200 $\mu$M) with recombinant human CYP1A2 and CYP3A4. Lineweaver-Burk plots ($1/V$ vs. $1/S$) are shown in figure 8. Data from HLMs indicated the involvement of a two-affinity system. However, pimozide N-dealkylation by recombinant CYP3A4 ($V_{\text{max}} = 4.5 \pm 0.20$ pmol DHPBI/min/pmol P450; $K_m = 5.70 \pm 1.1 \mu$M) and CYP1A2 ($V_{\text{max}} = 0.52 \pm 0.06$ pmol DHPBI/min/pmol P450; $K_m = 36.1 \pm 12.9 \mu$M) was described best by a simple Michaelis-Menten function. Because omeprazole inhibited the N-dealkylation of pimozide in HLMs (fig. 4) and because recombinant CYP2C19 was not able to catalyze formation of DHPBI (fig. 7), we tested whether the omeprazole inhibition was mediated by CYP3A4. Indeed, 10 and 20 $\mu$M omeprazole inhibited recombinant CYP3A4-mediated N-dealkylation of pimozide by 9% and 23%, respectively (fig. 7). The estimated $K_i$ value (35 $\mu$M) for this inhibition was close to the $K_i$ value obtained from HLMs (26.8 $\mu$M).

To gain further insight into the mechanism of CYP450-mediated drug interactions with pimozide, we determined the ability of pimozide to inhibit CYP450 isoforms in HLMs by a specific probe reactions. Our preliminary data showed an effect of preincubation on the degree of CYP450 inhibition by pimozide. Dixon plots for the inhibition of CYP2D6 and CYP3A4 by pimozide were obtained by preincubating pimozide (5–50 $\mu$M) in the presence of 25 $\mu$M pimozide was linear for at least 40 min in both the 5- and...
15-min preincubation protocols. As depicted in figure 9, pimozide was a potent inhibitor of CYP2D6 ($K_i = 20.2 \pm 12.8$ μM and $K_i = 0.75 \pm 0.98$ μM after 5 and 15 min preincubation, respectively) and a moderate inhibitor of CYP3A ($K_i = 124 \pm 67$ μM and 76.7 ± 34.5 μM after 5 and 15 min preincubation, respectively). The inhibitory effects of pimozide on other drug-metabolizing CYP450 isoforms (CYP2C19, CYP2C9, CYP1A2 and CYP2E1) also were tested. The $K_i$ value for the inhibition of CYP2E1 by pimozide was greater than 70 μM. Pimozide was a weak inhibitor of CYP2C19 with a $K_i$ value of 82.7 μM. Pimozide did not inhibit CYP2C9 and CYP1A2. The degree of inhibition of the CYP450 isoforms that were inhibited by a range of pimozide concentrations (5–50 μM) is summarized in figure 10.

**Fig. 7.** Formation of DHPBI (pmol product/pmol P450) by recombinant human CYP450 isoforms from incubations of pimozide (10 μM) (A) and inhibition of recombinant CYP3A4 by 10 and 20 μM omeprazole (B). Data are mean ± S.D. of two independent duplicate incubations ($n = 4$ determinations).

**Fig. 8.** Lineweaver-Burk plot for the rate of formation of DHPBI by recombinant human CYP3A4 (A) and CYP1A2 (B) from pimozide (0.5–200 μM). Points are duplicate determinations.

**Discussion**

We present here the first characterization of the human metabolism of pimozide, a neuroleptic of recognized narrow neurologic and cardiac therapeutic range, that has been marketed in the United States since 1984. These data form an important scientific basis for clinical studies designed to protect patients from lack of efficacy or life-threatening adverse effects through documentation of important metabolic pathways vulnerable to the influence of other drugs.

Our data show that pimozide oxidative N-dealkylation to DHPBI is the predominant pathway *in vitro* and provide strong evidence for the involvement of two CYP450 isoforms in this reaction, CYP3A and CYP1A2. First, Eadie-Hofstee plots constructed to test the kinetics of pimozide metabolism across a wide concentration range show biphasic kinetics consistent with at least two activities. Second, CYP3A and CYP1A2 isoform-specific chemical inhibitors were the most potent inhibitors of DHPBI formation. Third, of the recombinant isoforms tested, only recombinant human CYP3A4 and CYP1A2 were able to catalyze pimozide N-dealkylation. Fourth, the $K_m$ values derived from recombinant isoforms were consistent with $K_m$ values obtained in mixed HLMs, i.e., the $K_m$ value obtained from CYP3A4 was relatively closer to $K_m1$ than $K_m2$, whereas $K_m2$ in HLMs (34 μM) is very close to the recombinant CYP1A2 $K_m$ (36 μM). Biphasic kinetics also may be observed when two catalytic sites of a single isoform catalyze the same reaction, and this property has been described for both CYP3A4 (Ueng et al., 1997) and CYP1A2 (Sesardic et al., 1990). Our data obtained from recombinant CYP3A4 or CYP1A2 experiments in which a single activity was observed make this possibility unlikely.

Our *in vitro* data suggest that CYP3A is the main enzyme responsible for pimozide N-dealkylation and is probably the responsible isoform at therapeutically relevant pimozide concentrations. First, the rate of pimozide N-dealkylation in a variety of HLM preparations significantly correlates with the rate of felodipine oxidation, a probe activity for CYP3A (Harries et al., 1994). Second, among the chemical inhibitors tested, selective CYP3A inhibitors such as ketoconazole and troleandomycin are the most potent inhibitors of the reaction. Third, recombinant human CYP3A4 is able to catalyze the reaction in the absence of any other isoform. Fourth, the activity of the high-affinity component of pimozide N-dealkylation is abolished completely by ketoconazole, and a relatively low $K_m$ (5.7 μM) value is obtained from recombinant CYP3A4. Furthermore, the rate of reaction with CYP3A must be considered in the context of its relatively high abundance in human livers (Shimada et al., 1994). The evidence for the involvement of CYP1A2 includes the ability of furafylline to inhibit pimozide N-dealkylation and of recombinant CYP1A2 to catalyze the reaction. CYP2C19 apparently plays no appreciable role. Omeprazole is able to inhibit the formation of pimozide metabolite, but its ability to do so is consistent with its affinity for CYP3A (fig. 7; VandenBranden et al., 1996), and recombinant CYP2C19 was a poor catalyst of pimozide N-dealkylation. Orphenadrine has been proposed and used as a selective inhibitor of CYP2B6 (Heyn et al., 1996). However, other authors (Ekins et al., 1997) have shown that the currently available substrate and inhibitor probes of CYP2B6 are far from specific, which makes the
small inhibition of DHPBI formation by 300 \(\mu M\) orphenadrine difficult to interpret.

There are indications in the literature that CYP2D6 might metabolize pimozide in humans. Concomitant administration of drugs that are strong inhibitors of CYP2D6 [e.g., fluoxetine and paroxetine (Bertz and Granneman, 1997)] has been reported to increase the cardiac and neurologic adverse effects of pimozide (Horrigan and Barnhill, 1994; Ahmed et al., 1993; Hanssen-Grant et al., 1993). About 10% of patients administered pimozide have been reported to experience electrocardiographic changes (Fulop et al., 1987), and CYP2D6 is absent in 7 to 10% of the Caucasian population (May, 1994). The half-lives of pimozide have been found to vary greatly among patients (McCreadie et al., 1984; Sallee et al., 1987). Possibly because of prior exposure to neuroleptics that are inhibitors of CYP2D6 in schizophrenics, there is greater variability in schizophrenic patients than in patients with Tourette’s syndrome (Salle et al., 1987). However, our data did not indicate that CYP2D6 catalyzes the formation of DHPBI, although its role in other routes of pimozide elimination can not be ruled out.

On the basis of our in vitro data, clear differences between the kinetics of pimozide metabolism by CYP3A and CYP1A2 were observed. CYP3A seems to play a major role in pimozide metabolism, whereas the contribution of CYP1A2 seems to be quite marginal. This, however, does not exclude the possibility that CYP1A2 may assume a greater role if the activity of CYP3A is very low. In view of the difficulty of extrapolating from in vitro results to the clinic, the relative in vivo involvement of the two isoforms is difficult to ascertain, because both experience highly variable expression (Shimada et al., 1994) and are amenable to induction and inhibition by a large variety of xenobiotics. Nevertheless, our data suggest two possible clinical consequences of importance to prescribing physicians.

First, we expect a greater risk of adverse effects when pimozide is coprescribed with metabolic inhibitors. These might include theazole antifungals and macrolide antibiotics that are inhibitors of CYP3A (Ketter et al., 1995), and CYP1A2 inhibitors such as fluvoxamine (Brosen, 1995) and...
quinolone antibiotics (Gillum et al., 1993). The risk of concomitant administration of CYP3A with pimozide is empha-
sized by our recent report which documented QT prolonga-
tion associated with fatal cardiac arrhythmia in patients
taking pimozide and clarithromycin (Flockhart et al., 1996).
We have documented inhibition of pimozide metabolism by
clarithromycin in vitro (Flockhart et al., 1996), consistent with
its ability to inhibit elimination of drugs metabolized by
CYP3A, such as cyclosporine A, terfenadine, carbamazepine
and midazolam (Nahata, 1996).

Second, there may be loss of pimozide effect in the presence of
metabolic inducers of CYP3A such as rifampin and car-amazepine (Ketter et al., 1995), and smokers may require
higher pimozide doses because of higher CYP1A2 activity
(Parsons and Neims, 1978). These influences may explain in
part the well-documented interindividual variability in pimo-
zone pharmacokinetics (McCreath et al., 1984; Salle et al.,
1987) and pharmacodynamics (Cohen et al., 1992). The daily
dose of pimozide varies widely in patients with Tourette’s
syndrome (2–20 mg/day), delusional disorders (2–12 mg/day)
and schizophrenia (40–80 mg/day) (Tueth and Cheong, 1993).

The inhibitory effect of pimozide on CYP2D6 that we ob-
served was potent in contrast to others (Inaba et al., 1985)
who reported a weak inhibition in vitro, a discrepancy that
may be the result of differences in study protocol. Pimozide
inhibited CYP2D6 without appearing to be an important
substrate of this isozyme. This is not surprising because
drugs such as quinidine (Ching et al., 1995), halofantrine
(Halliday et al., 1995) and methadone (Wu et al., 1993) are
also strong inhibitors of CYP2D6 without being important
substrates. Preincubation of pimozide with HLMs and an
NADPH-generating system for 15 min increased its inhibi-
tory potency for CYP2D6, which suggests a metabolism-me-
diated inhibition by pimozide, as has been described for other
agents (Ortiz de Montellano et al., 1981). This may be caused by
mechanism-based inhibition or by accumulation of an
inhibitory metabolite. Either mechanism may result in an
inhibitory effect that persists beyond the presence of the
parent drug in plasma. Pimozide is known to be concentrated
in the liver (~11-fold) relative to plasma (Pinder et al., 1976).
The K_i (<1 µM) of pimozide for the inhibition of CYP2D6 in
the present study is close to therapeutic concentrations of
pimozide in the liver in vivo, which suggests that pimozide is
likely to be a clinically important CYP2D6 inhibitor. The
inhibitory effect of pimozide on other CYP450 isoforms
(CYP2E1, CYP2C9, CYP2C19 and CYP1A2) was small even
at concentrations that are 100 times higher than therapeutic
plasma concentrations of pimozide.

We have demonstrated for the first time that pimozide is
metabolized in humans via N-dealkylation and that this me-
tabolic step is catalyzed principally by human CYP3A. We
also have provided evidence that pimozide is a strong inhib-
tor of CYP2D6. Although the use of pimozide in the United
States is small, it is a critical drug for many patients with
Tourette’s syndrome who cannot tolerate haloperidol. Recent
reports suggest that pimozide is superior to haloperidol in
controlling symptoms of Tourette’s syndrome and has less
extrapyramidal symptoms (Salle et al., 1997). In addition,
pimozide is used widely in Europe for the treatment of schizo-
phrenia and other psychiatric disorders (Opler and Feinberg,
1991; Tueth and Cheong, 1993). Pimozide reportedly is as
effective as other classical neuroleptics such as chlorproma-
azine, fluphenazine, flupenthixol, perphenazine and thiorid-
azine for the treatment of schizophrenia or superior to halo-
peridol and trifluoperazine (Pinder et al., 1976, Opler and
Feinberg, 1991; Tueth and Cheong, 1993). Identifying poten-
tial risk factors that could modulate the efficacy and toxicity
of pimozide is important to optimize the use of this otherwise
effective neuroleptic drug. The results of our study suggest
that patients may be placed at risk for therapeutic failure by
drug interactions with inducers or toxicity for inhibitors of
CYP3A4 and CYP1A2. Pimozide is a strong inhibitor of
CYP2D6 in vitro and may increase plasma levels of drugs
that are substrates of CYP2D6 (e.g., tricyclic antidepress-
ants, neuroleptics and codeine) in vivo (Bertilsson, 1995).

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