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Identification and Characterization of Human Cytochrome P450 Isoforms Interacting with Pimozide[1]

ZERUESENAY DESTA, THOMAS KERBUSCH, NADIA SOUKHOVA, EMILY RICHARD, JAE-WOOK KO and DAVID A FLOCKHART

Division of Clinical Pharmacology, Department of Medicine and Pharmacology, Georgetown University Medical Center, Washington, DC

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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_{m1} and V_{max1}) and low-affinity (K_{m2} and V_{max2}) components. The ratio of V_{max1} (14 pmol/min/mg protein)/K_{m1} (0.73 μM) was 5.2 times higher than the ratio of V_{max2} (244 pmol/min/mg protein)/K_{m2} (34 μM). K_{m2} was 91 times higher than K_{m1}. 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 ($V = 2.2 \pm 0.89$ pmol/min/pmol P450) followed by CYP1A2 ($V = 0.23 \pm 0.08$ pmol/min/pmol P450), but other isoforms tested did not. The K_m 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 ($K_i = 0.75 \pm 0.98$ μM) and a moderate inhibitor of CYP3A ($K_i = 76.7 \pm 34.5$ 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.

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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 (Woosley *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

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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.

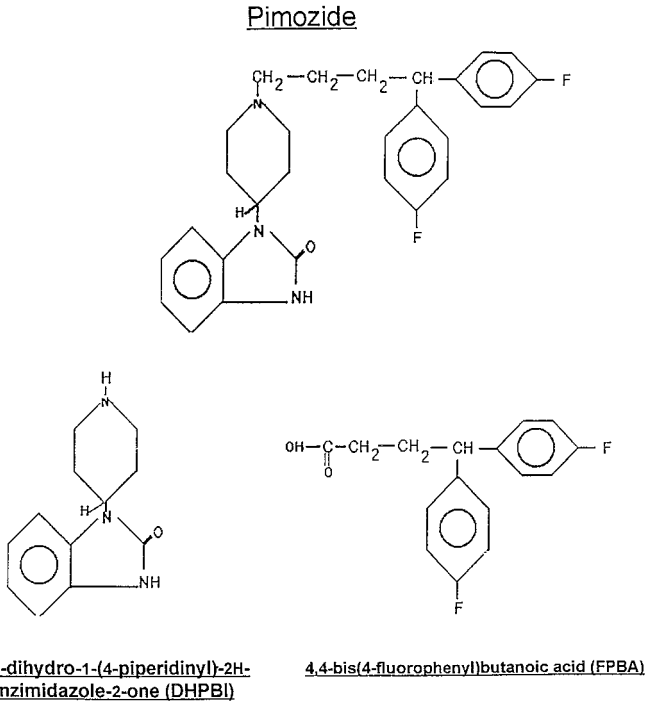

Fig. 1. Chemical structure of pimozide and N-dealkylated metabolites in humans.

FPBA. Similarly, DHPBI was reported to be a major urinary metabolite of pimozide in human volunteers (review Pinder *et al* 1976). Case reports of drug interactions with pimozide have involved drugs that interact prominently with the CYP450 system (Bertz and Granneman, 1997). These include increased neurologic adverse effects with paroxetine (Horrigan and Barnhill, 1994), adverse cardiac events and psychomimetic effects with fluoxetine (Hanssen-Grant *et al*., 1993; Ahmed *et al*., 1993) and death, probably because of cardiac arrhythmia with clarithromycin (Flockhart *et al*., 1996).

Because our preliminary *in vitro* work indicated that pimozide is metabolized by HLMs (Flockhart *et al*., 1996), we set out to identify the major metabolic routes and the specific human CYP450 isoforms that interact with pimozide using HLM preparations and recombinant CYP450 isoforms.

Materials and Methods

Chemicals. Pimozide, dextromethorphan HBr, chlorzoxazone, chlorpropamide, quinidine sulfate, orphenadrine HCl, tolbutamide, diethyldithiocarbamate, troleandomycin, ketoconazole, phenacetin, acetaminophen, G-6-P, G-6-PDH, NADP and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Sulfaphenazole, furafylline, *S*-mephenytoin and 6-hydroxychlorzoxazone were obtained from Ultrafine Chemicals (Manchester, England). Levallorphan was obtained from U.S.P.C. (Rockville, MD). Dextrophan and 3-methoxymorphinan 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)butamide 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 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 $MgCl_2$, 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 $MgCl_2$ 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 Mono-chromator fluorescence detector (GTI Holding Co., Concord, MA). The separation system consisted of a stainless-steel (VYDAC) column (15 cm × 4.6 mm internal diameter) packed with 5-μm particle size (90 Å pore size) RP-C-18 (Alltech Separations Group, Hesperia, CA), a Waters Nova-Pack C_{18} guard column (4 μm, 60 Å) and a mobile phase composed of 35% acetonitrile in 50 mM NaH_2PO_4 buffer (adjusted to pH 3.0 with 1% phosphoric acid). The operating temperature was 20°C and the flow rate 1.0 ml/min. The column eluent was monitored by fluorescence with an excitation wavelength of 281 nm and an emission wavelength of 309 nm.

The peak of pimozide was noted at a retention time of 9.6 min, and another major fluorescent peak was noted at 1.9 min. The retention time of this peak was compared with reference peaks of the pimozide metabolites, DHPBI and FPBA, and corresponded to the DHPBI. Subsequent experiments were designed to characterize the human CYP450 isoforms responsible for the formation of DHPBI.

Because of limited availability of the metabolite (DHPBI), the concentrations of metabolite were measured by comparing the metabolite peak to standard curves obtained with use of known pimozide concentrations. The difference between the fluorescent activity of the metabolite and that of pimozide, determined by constructing

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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 DHPBI 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*-mephenytoin (Wrighton *et al*., 1993) and of CYP2E1 measured by the 6-hydroxylation of chlorzoxazone (Peter *et al*., 1990).

Inhibition studies. The formation rate of DHPBI 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; Bourrié *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 orphenadrine for CYP2B6 (Heyn *et al*., 1996). Pimozide was preincubated for 5 min with or without CYP450 isoform-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 preincubated 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 (Bourrié *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_i values of the inhibitor probes (Bourrié *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 DHPBI 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 (Ko *et al*., 1997), dextromethorphan *O*-demethylation for CYP2D6 (Rodrigues *et al*., 1994), dextromethorphan N-demethylation for CYP3A (Gorski *et al*., 1994) and chlorzoxazone 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 chlorzoxazone 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 DHPBI formation were performed by initial visual examination of Eadie-Hofstee plots (*V vs. V/S*) 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 (WINONLIN 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 DHPBI 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:

$$\% \text{ inhibition} = \frac{100 \times [I]}{[I] + K_i \times \left(1 + \frac{[S]}{K_m}\right)} \quad (1)$$

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

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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 (K_{m1} and V_{max1}) and low-affinity (K_{m2} and V_{max2}) 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 (V_{max1}/K_{m1}) was 5.2 times higher than that of the low-affinity component (V_{max2}/K_{m2}), and K_{m2} was 91 times higher than K_{m1}.

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).

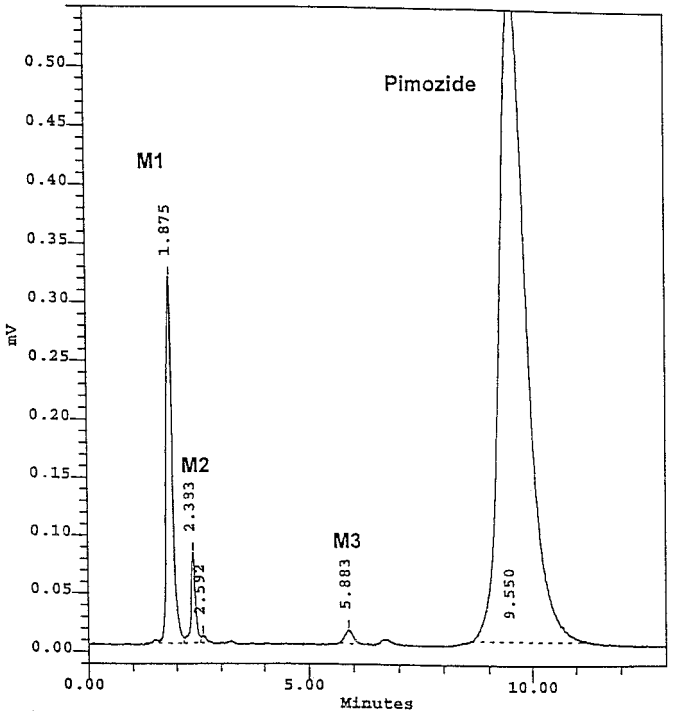

Fig. 2. Representative HPLC chromatogram of pimozide and its fluorescent metabolites from *in vitro* incubations with HLMs. Chromatographic fluorescent peaks and retention times (RTs) were as follows: M1 (DHPBI) (RT = 1.9 min); two unidentified fluorescent metabolites, M2 (RT = 2.4 min) and M3 (RT = 5.9 min); and pimozide (RT = 9.9 min). Direct injection of reference DHPBI had the same RT (1.9 min) as the major fluorescent peak from microsomal incubates.

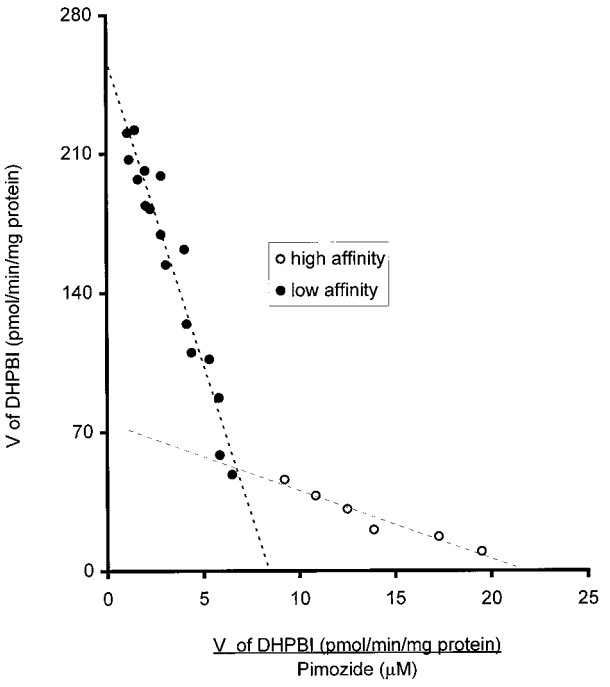

Fig. 3. Eadie-Hofstee plot for the formation of DHPBI in HLMs. Pimozide (0.5–200 μM) was incubated with HLMs and the formation of DHPBI was characterized by high-affinity (open circles) and low-affinity (filled circles) activities. Points represent duplicates of pooled livers (HL4, HL8 and HL16). The estimates from initial visual examination from the dotted lines were used as initial estimates for a nonlinear regression analysis (table 1).

To further probe the CYP450 isoforms 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 (K_i ± 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

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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.

Incubation with	Kinetics of DHPBI formation					
	K_{m1}	V_{m1}	V_{max1}/K_{m1}	K_{m2}	V_{max2}	V_{max2}/K_{m2}
Pimozide alone	0.37 ± 1.3	14 ± 12	37.5	34 ± 6.7	244 ± 11	7.2
Pimozide + ketoconazole (1 μM)	–	–		36 ± 5.4	34.7 ± 1.6	–
Pimozide + furafylline (10 μM)	0.19 ± 1.2	17.2 ± 12	89.0	58 ± 21	173 ± 14	3.0

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

Liver	Activities of CYP450s					V of DHPBI
	CYP3A	CYP2D6	CYP1A2	CYP2C19	CYP2E1	
			pmol/min/mg P			*pmol/min/mg P*
2	475	488	2400	7.1	906	163.9
3	270	0	750	0.9	ND	30.9
4	225	159	505	14.7	212	68.8
5	110	0	505	3.5	177	31.0
6	140	129	400	3.1	1118	15.8
7	350	118	450	2.3	224	77.3
8	185	229	1505	6.4	671	67.5
9	455	247	1400	23.6	471	74.7
16	ND	ND	ND	4.4	ND	141.0
Correlation (rs)	0.79	0.61	0.46	0.37	0	
P value	0.028*	0.12	0.27	0.33	1	

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

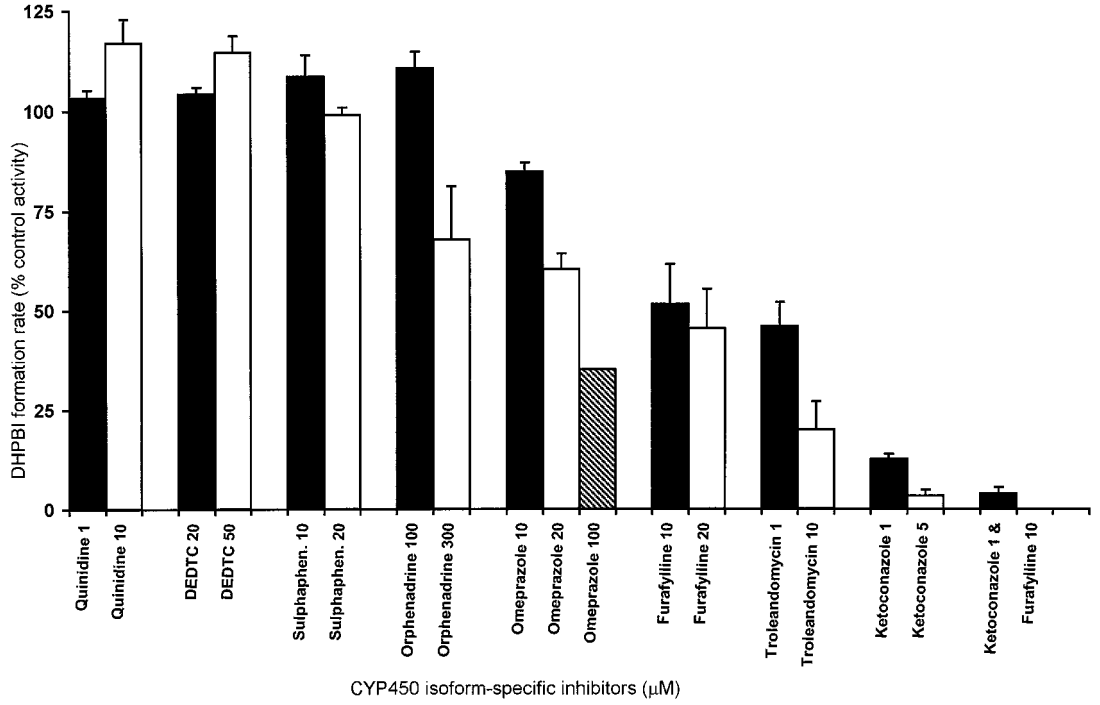

Fig. 4. Inhibition of DHPBI formation by CYP450 isoform-specific inhibitors in HLMs. Pimozide (10 μM) was incubated with at least two inhibitor concentrations in HLMs, and the combined inhibitory effect of ketoconazole (1 μM) and furafylline (10 μM) was tested. For incubation conditions, see "Materials and Methods." Results are mean ± S.D. of two independent incubations of pooled microsomes from HL2, HL9 and HL16 (n = 4 determinations). The inhibited activities were compared with uninhibited activities (controls). Abbreviations: DEDTC, diethyldithiocarbamate; Sulphaphen = sulphaphenazole

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-

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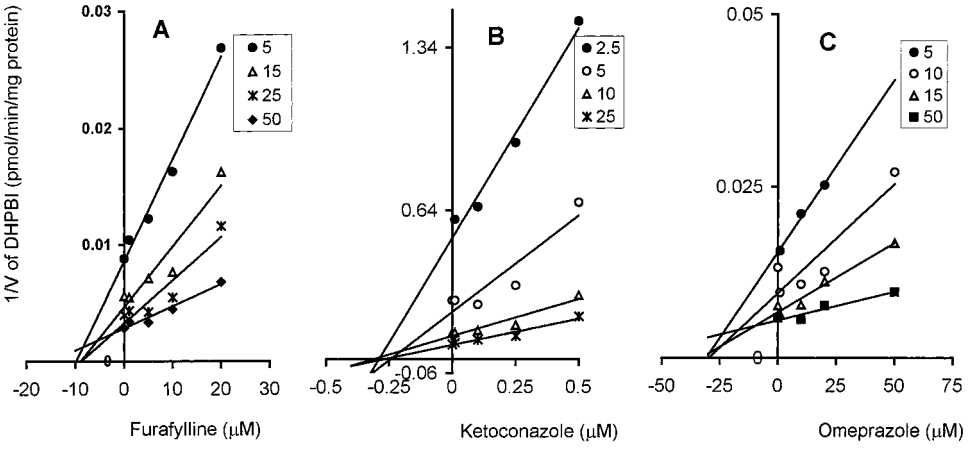

Fig. 5. Dixon plots of the inhibitory effect of furafylline (1–20 μM) (A), ketoconazole (0.01–0.5 μM) (B) and omeprazole (1–50 μM) (C) on DHPBI formation rates in incubations of pimozide (5–50 μM) with HLMs. Each point represents averages of duplicates of pooled human livers (HL2, HL9 and HL16).

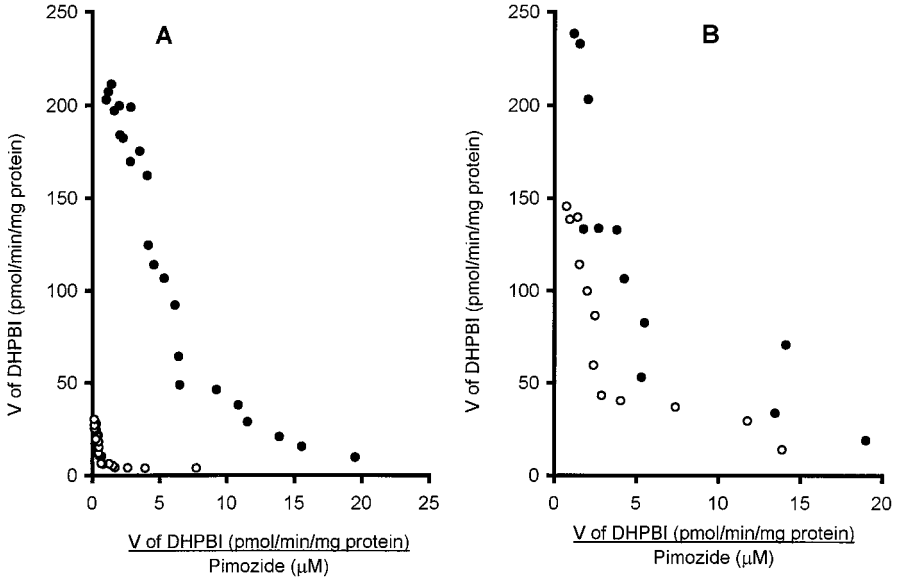

Fig. 6. Eadie-Hofstee plots for the formation of DHPBI in control (filled circles) and after coincubation with 1 μM ketoconazole (A) or 10 μM furafylline (B) (open circles) in HLMs. A range of pimozide concentrations (0.5–200 μM) are used, and each point represents averages of duplicate incubations from pooled human livers (HL4, HL8 and HL16).

tein in control to undetectable rates). Ketoconazole also markedly reduced the V_{max} of the low-affinity component by 7-fold with no effect on K_{m2} (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_{max2} with little effect on K_{m1} or V_{max1} values (table 1).

Recombinant human CYP3A4 isoform catalyzed DHPBI from 10 μM 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 μM) 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 ($V_{max} = 4.5 \pm 0.20$ pmol DHPBI/min/pmol P450; $K_m = 5.70 \pm 1.1$ μM) and CYP1A2 ($V_{max} = 0.52 \pm 0.06$ pmol DHPBI/min/pmol P450; $K_m = 36.1 \pm 12.9$ μM) 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 μM omeprazole inhibited recombinant CYP3A4-mediated N-dealkylation of pimozide by 9% and 23%, respectively (fig. 7). The estimated K_i value (35 μM) for this inhibition was close to the K_i value obtained from HLMs (26.8 μM).

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 isoform 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 μM) with HLMs and the NADPH-generating system for 5 and 15 min. Reactions were started by adding dextromethorphan (2.5–75 μM) and then were incubated for another 30 min. The preincubation protocol was designed to test the possibility of mechanism-based inhibition. Metabolite formation (dextrophan, a marker of CYP2D6; and 3-methoxymorphinan, a marker of CYP3A) from dextromethorphan (50 μM) in the presence of 25 μM pimozide was linear for at least 40 min in both the 5- and

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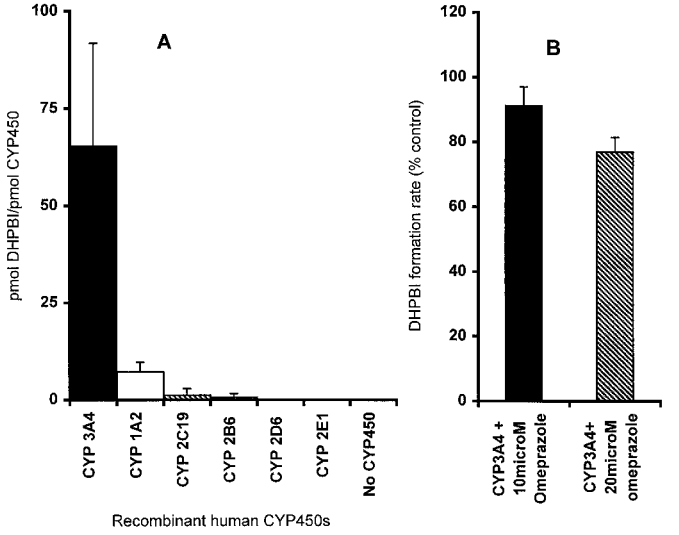

Fig. 7. Formation of DHPBI (pmol product/pmol P450) by recombinant human CYP450 isoforms from incubations of pimozide (10 μM) (A) and inhibition of recombinant CYP3A4 by 10 and 20 μM omeprazole (B). Data are mean ± S.D. of two independent duplicate incubations (n = 4 determinations).

15-min preincubation protocols. As depicted in figure 9, pimozide was a potent inhibitor of CYP2D6 (K_i = 20.2 ± 12.8 μM and K_i = 0.75 ± 0.98 μM after 5 and 15 min preincubation, respectively) and a moderate inhibitor of CYP3A (K_i = 124 ± 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.

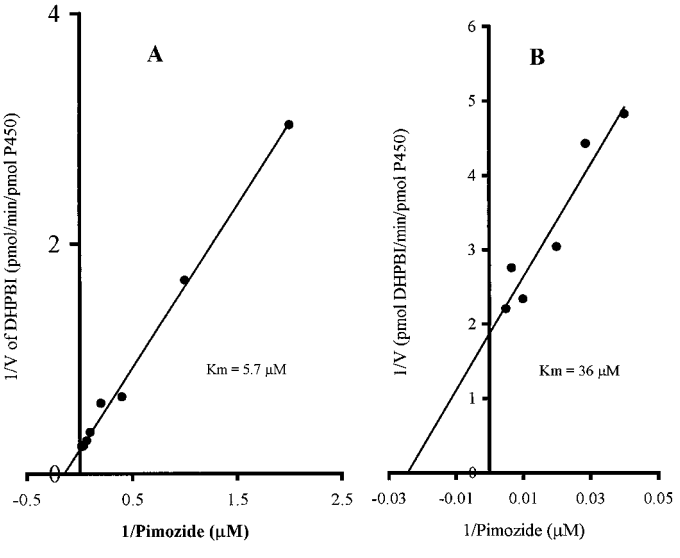

Fig. 8. Lineweaver-Burk plot for the rate of formation of DHPBI by recombinant human CYP3A4 (A) and CYP1A2 (B) from pimozide (0.5–200 μM). Points are duplicate determinations.

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 (Harris *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

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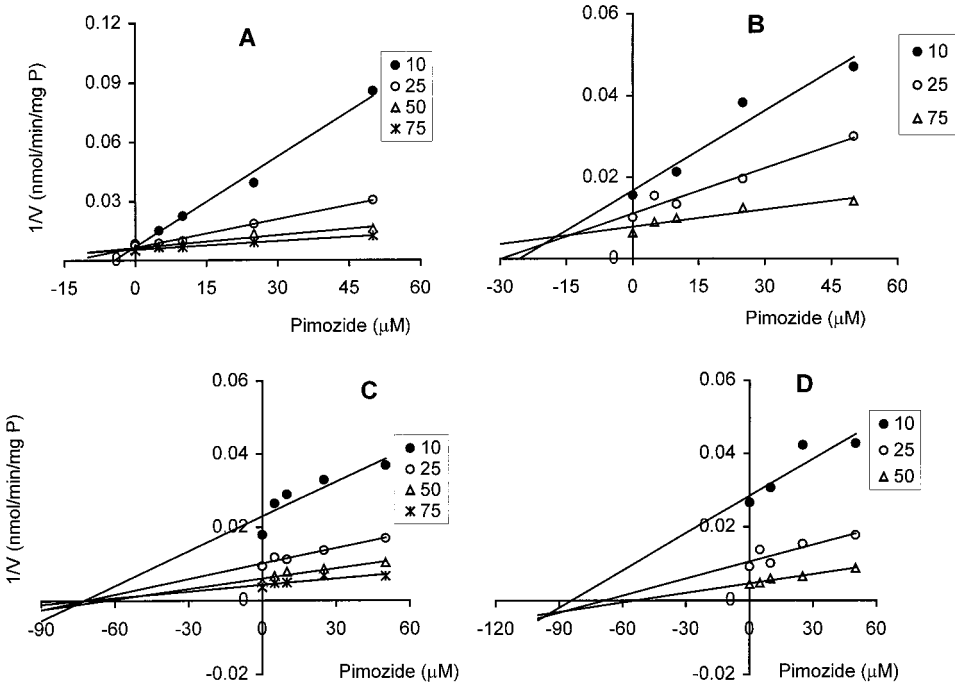

Fig. 9. Dixon plots for the inhibition of dextromethorphan *O*-demethylation (CYP2D6 marker) (A and B) and dextromethorphan N-demethylation (CYP3A marker) (C and D) by pimozide (5–50 μM) in HLM. A and C represent a preincubation of pimozide for 15 min, and B and D represent a preincubation of pimozide for 5 min. Dextromethorphan was used at concentrations of 10 to 75 μM. Data are averages of duplicates from pooled livers (HL4, HL8 and HL16).

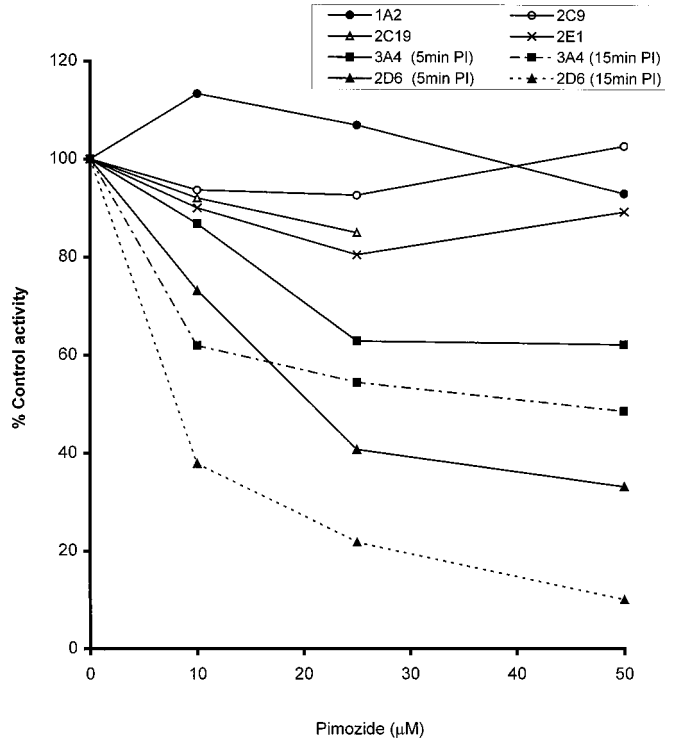

Fig. 10. Inhibition of different CYP450 isoforms by pimozide (10–50 μM) in HLMs. The activities of each isoform were measured by specific substrate reaction probes: the reaction probes were phenacetin (35 μM) for CYP1A2, tolbutamide (25 μM) for CYP2C9, omeprazole (50 μM) for CYP2C19, chlorzoxazone (10 μM) for CYP2E1 and dextromethorphan for both CYP2D6 and CYP3A. The effects of preincubation (PI) of pimozide for 5 or 15 min were tested for CYP3A and CYP2D6 isoforms. Data represent averages of duplicates and are given as percent control activities.

small inhibition of DHPBI formation by 300 μM 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 the azole antifungals and macrolide antibiotics that are inhibitors of CYP3A (Ketter *et al.*, 1995), and CYP1A2 inhibitors such as fluvoxamine (Brosen, 1995) and

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quinolone antibiotics (Gillum *et al*., 1993). The risk of concomitant administration of CYP3A with pimozide is emphasized by our recent report which documented QT prolongation 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 carbamazepine (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 pimozide pharmacokinetics (McCreadie *et al*., 1984; Sallee *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 observed 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 isoform. 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 inhibitory potency for CYP2D6, which suggests a metabolism-mediated 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 metabolic step is catalyzed principally by human CYP3A. We also have provided evidence that pimozide is a strong inhibitor 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 (Sallee *et al*., 1997). In addition, pimozide is used widely in Europe for the treatment of schizophrenia and other psychiatric disorders (Opler and Feinberg, 1991; Tueth and Cheong, 1993). Pimozide reportedly is as effective as other classical neuroleptics such as chlorpromazine, fluphenazine, flupenthixol, perphenazine and thioridazine for the treatment of schizophrenia or superior to haloperidol and trifluoperazine (Pinder *et al*., 1976, Opler and Feinberg, 1991; Tueth and Cheong, 1993). Identifying potential 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 antidepressants, neuroleptics and codeine) *in vivo* (Bertilsson, 1995).

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Send reprint requests to: David A Flockhart MD, PhD, Assistant Professor of Medicine and Pharmacology, Division of Clinical Pharmacology, Georgetown University Medical Center, 3900 Reservoir Rd, NW, Washington, DC 20007.

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