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Identification of human cytochrome P450 enzymes involved in the major metabolic pathway of fluvoxamine

M. MIURA[1] & T. OHKUBO[2]

[1]*Department of Pharmacy, Akita University Hospital, Akita, Japan and* [2]*Clinical Research Center, Hirosaki University Hospital, Hirosaki, Japan*

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Abstract

The metabolism of fluvoxamine to fluvoxamino acid is known to involve a two-step oxidation process via an alcohol intermediate, fluvoxamino alcohol. The present study was carried out to identify the cytochrome P450 (CYP) enzyme(s) involved in the metabolism of fluvoxamine to fluvoxamino alcohol using human liver microsomes and cDNA-expressed human CYP enzymes. The mean K_m and V_{max} values for the formation of fluvoxamino alcohol from fluvoxamine in human liver microsomes were 76.3 μM and 37.5 pmol min^{-1} mg^{-1} protein, respectively. The formation of fluvoxamino alcohol from fluvoxamine in pooled human liver microsomes was significantly inhibited by quinidine, a relatively specific CYP2D6 inhibitor, with a K_i value of 2.2 μM, whereas other several relatively specific CYP inhibitors did not inhibit the formation of fluvoxamino alcohol. In addition, only CYP2D6 of several cDNA-expressed human CYP enzymes examined showed substantial activity for the formation of fluvoxamino alcohol. Furthermore, the formation of fluvoxamino acid from fluvoxamino alcohol is potently inhibited by 4-methylpyrazole in human liver cytosol. These data suggest that CYP2D6 is the only enzyme predominantly responsible for the first-step oxidation of fluvoxamine to fluvoxamino alcohol, and alcohol dehydrogenase is involved in the second-step oxidation of fluvoxamino alcohol to the corresponding carbolic acid.

Keywords: *Fluvoxamine, cytochrome P450 (CYP) 2D6, fluvoxamino alcohol, fluvoxamino acid, alcohol dehydrogenase*

Introduction

Fluvoxamine (5-methoxy-1-[4-(trifluoromethyl)-phenyl]-1-pentanone-*O*-(2-aminoethyl)-oxime) (Figure 1) is a selective serotonin re-uptake inhibitor (SSRI) widely used in the treatment of major depression and other affective disorders (Benfield and Ward 1986;

Correspondence: T. Ohkubo, Clinical Research Center, Hirosaki University Hospital, 53 Hon-cho, Hirosaki 036-8563, Japan. Tel: 81-172-33-5295. Fax: 81-172-39-5295. E-mail: ok1231@mail.cc.hirosaki-u.ac.jp

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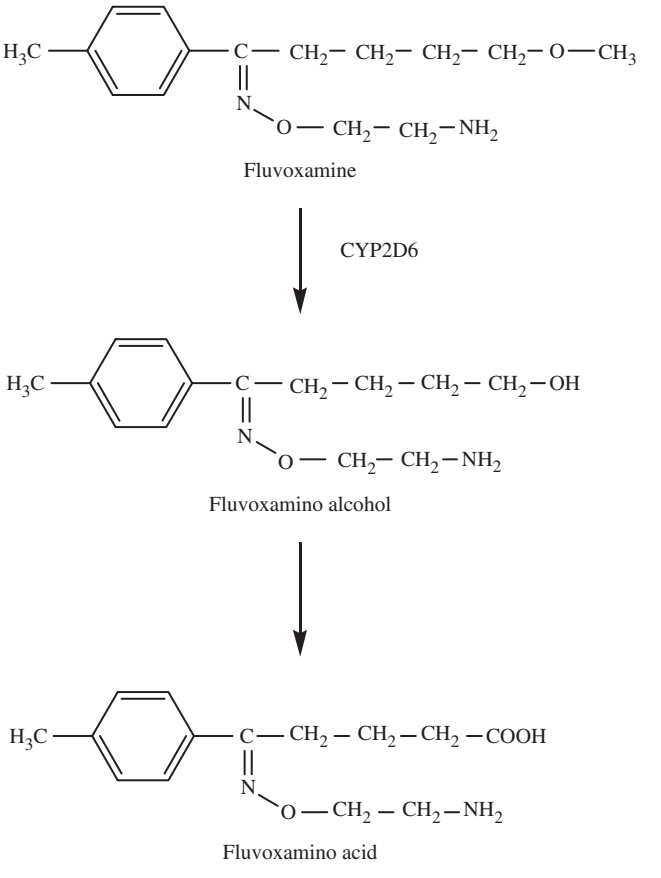

Figure 1. Metabolism of fluvoxamine to its principal metabolites: fluvoxamino alcohol and fluvoxamino acid.

Wilde et al. 1993). Fluvoxamine is extensively metabolized in the liver and the major metabolite present in the urine is fluvoxamino acid (4-carboxy-1-[4-(trifluoromethyl)-phenyl]-1-butane-*O*-(2-aminoethyl)-oxime) (Figure 1) (Perucca et al. 1994). Previous *in vivo* studies have shown that fluvoxamine is eliminated predominantly by oxidation through cytochrome P450 (CYP) (Spigset et al. 1995; 1997, 2001, Carrillo et al. 1996; Hartter et al. 2000; Christensen et al. 2002). Although the identity of the CYP involved in the oxidation of fluvoxamine is not conclusively known, there is evidence that the CYP2D6 is of major importance. Spigset et al. have reported that the metabolic clearance (Cl_m) for fluvoxamino acid was 78% lower in CYP2D6 poor metabolizers (PMs) than in CYP2D6 extensive metabolizers (EMs) after intake of a single oral dose of 50 mg fluvoxamine (211 ± 143 vs. 969 ± 704 ml min^{-1}, respectively) (Spigset et al. 2001). And Hartter et al. have reported that the area under concentration–time curve (AUC) of fluvoxamine in CYP2D6 PM (**4/*6*) was higher than in CYP2D6 EM (**1/*1*) (612 vs. 150–231 μg h ml^{-1}) (Hartter et al. 2000). On the other hand, CYP1A2 was also shown to catalyse fluvoxamine metabolism (Spigset et al. 1999; 2001). Up to now, the enzymes involved in fluvoxamine metabolism have not yet been fully clarified. The metabolism of fluvoxamine

to fluvoxamino acid is known to involve a two-step oxidation process via an alcohol intermediate, fluvoxamino alcohol (Ruijten et al. 1984). The purpose of the current study was to identify the CYP enzymes involved in formation of the major metabolite of fluvoxamine, fluvoxamino alcohol, by using human liver microsomes and cDNA-expressed human CYP enzymes.

Materials and methods

Reagents and chemicals

Fluvoxamine maleate was donated by Meiji Seika Kaisha Ltd (Tokyo, Japan). Fluvoxamino alcohol and fluvoxamino acid were synthesized in the authors' laboratory (Ohkubo et al. 2003; Miura and Ohkubo 2006). The chemical purities of fluvoxamine, fluvoxamino acid and fluvoxamino alcohol were more than 98 (data were provided by Meiji Seika Kaisha), 98 and 97%, respectively (Ohkubo et al. 2003; Miura and Ohkubo, 2006). Moperone was kindly donated by the Yamanouchi Pharmaceutical (Tokyo, Japan). The Oasis HLB extraction cartridge was purchased from Waters Co. (Milford, MA, USA). All solvents used were of high-performance liquid chromatography (HPLC) grade (Wako Pure Chemical Industries, Osaka, Japan), and all other reagents and chemicals were purchased from Wako Chemical Industries or Nacalai Tesque (Kyoto, Japan). Furafylline, sulfaphenazole, quinidine and sodium diethyldithiocarbamate (DDC) were purchased from ULTRAFINE Chemicals Ltd (Manchester, UK), and α-naphtflavone, omeprazole, erythromycin and 4-methylpyrazole were purchased from Sigma Chemical Co. (St Louis, MO, USA). Itraconazole was donated from Yansen Pharmaceutical Co. (Beerse, Belgium). The cofactors (nicotinamide adenine dinucleotide phosphate [NADP], glucose 6-phosphate dehydrogenase, and glucose 6-phosphate) were purchased from Sigma. Human liver microsomes (pooled, HH31, HH35, HH40, HH48, HH54, HH61 HH69, HH86, HH89, HH91 and HH108), human liver cytosol and human cytochrome P450 (CYP) enzymes plus reductase microsomes (CYP1A2, 2A6, 2B6, 2C9*1, 2C19, 2D6*1, 3A4 and 2E1) were purchased from GENTEST Corporation (Woburn, MA, USA). These microsomal preparations were prepared from the human B-lymphoblastoid cell line. The data for bufuralol 1′-hydroxylase activities (pmol min^{-1} mg^{-1} protein) in individual human liver microsomes were provided by GENTEST.

Analytical procedures

The concentrations of fluvoxamine and its metabolites were measured by the reverse-phase HPLC method (Miura and Ohkubo 2006). The apparatus consisted of a Waters HPLC system equipped with a model 510 chromatography pump (Waters) and a Waters 486 ultraviolet detector. The wavelength was set at 254 nm. Test samples were introduced using a Waters 712 WISP autosampler with an effective volume of 50 µl. The HPLC column used was a Grand-pak C4-5 (150 × 4.6 mm i.d.; Owani, Aomori, Japan); the mobile phase, which consisted of 0.5% KH_2PO_4 (pH 2.5)–acetonitrile (75:25, v/v), was degassed in an ultrasonic bath before use. Before mixing with acetonitrile, the pH of the 0.5% KH_2PO_4 was adjusted with 50% phosphoric acid, and it was degassed ultrasonically. A flow rate of 1.0 ml min^{-1} was used at an ambient temperature of 27°C. The lower limits of quantification of this assay for fluvoxamino alcohol and fluvoxamino acid were 82.2 and 78.6 nM, respectively.

The mean recoveries of these compounds were more than 92.8%, with a coefficient of variation less than 5.8%.

Microsomal and expressed CYP incubation conditions

Incubations were carried out in duplicate with the reconstituted human liver microsomes in 5-ml test tubes, on three separate occasions ($n = 3$), using a shaking water bath at 37°C. A typical incubation mixture consisted of a cofactor solution (100 μl), microsomal preparation (50 μl, 0.5 mg of microsomal protein), and substrate (5 μl, 0–346 μM) in a total volume of 0.2 ml. The cofactor solution consisted of $NADP^+$ (1.3 mM), glucose 6-phosphate (3.3 mM), glucose 6-phosphate dehydrogenase (0.4 units) and magnesium chloride (3.3 mM) in sodium phosphate buffer (0.1 M, pH 7.4). The metabolic reaction was initiated by the addition of the cofactor solution and terminated by immersing in an ice bath and the addition of 1.0 ml of 1.0 M sodium chloride. Before the extraction, moperone (50 ng) in methanol (10 μl) was added as an internal standard to the incubation mixture. The reactions with pooled human liver microsomes were carried out for 30 min. Microsomal enzyme was inactivated by heat treatment in control incubations. On the other hand, the reactions with CYP enzymes expressed in a cell line were carried out for 60 min. Controls for human CYP enzymes were expressed in a cell line containing control microsomes (i.e. a cell line transfected with a control vector).

Inhibition studies

Eight different relatively selective inhibitors were used in order to define the role of individual CYP enzymes involved in fluvoxamine metabolism. The CYP inhibitors used were α-naphtflavone (50 μM) and furafylline (50 μM) for CYP1A2, sulfaphenazole (50 μM) for CYP2C9, omeprazole (50 μM) for CYP2C19, quinidine (5.0 μM) for CYP2D6, DDC (50 μM) for CYP2A6 and CYP2E1, and itraconazole (10 μM) and erythromycin (50 μM) for CYP3A4. The inhibitors were pre-incubated with human liver microsomes and the β-nicotinamide adenine dinucleotide phosphate (NADPH)-generating system for 5 min at 37°C under the same conditions described above before the reaction was started by addition of fluvoxamine, which was at a fixed concentration of 11.5 μM. Furthermore, inhibition of fluvoxamino alcohol formation was studied with three concentrations of quinidine (1.0, 5.0 and 10 μM) using a varying concentration of fluvoxamine. After incubation for 30 min at 37°C, reactions were terminated as described above.

Cytosol incubation conditions

Fluvoxamino alcohol was incubated with human liver cytosol (50 μl, 1.0 mg protein) in sodium phosphate buffer (0.1 M, pH 7.4) containing NAD (7.5 mM). Incubations were carried out in duplicate in 5-ml test tubes, on three separate occasions ($n = 3$), using a shaking water bath for 30 min at 37°C. 4-Methylpyrazole (10, 50, 100 250 and 1000 μM) was pre-incubated with human liver cytosol and NAD for 5 min at 37°C before the reaction was started by addition of fluvoxamino alcohol (8.2 μM). After incubation for 30 min at 37°C, reactions were terminated as described above.

Metabolite extraction method

Each mixture was applied to an Oasis HLB extraction cartridge that had been previously activated with methanol and water (1.0 ml of each) (Miura and Ohkubo 2006). The cartridge was then washed with 40% methanol in water (1.0 ml), and then eluted with 100% methanol (1.0 ml). The eluate was evaporated to dryness in vacuum at 60°C by a rotary evaporator (Iwaki, Tokyo, Japan). The residue was dissolved in 50 µl of methanol and 50 µl of mobile phase, and each aliquot (50 µl) of the solution was injected into the HPLC apparatus.

Data analysis

The kinetics of the fluvoxamine biotransformation by human liver microsomes and cDNA-expressed CYP enzymes were described by the Michaelis–Menten equation for a one-enzyme system using a non-linear least-squares regression program, MULTI (Yamaoka et al. 1981), and apparent K_m, V_{max} and enzyme inhibition constant (K_i) values were estimated. Correlations of metabolite formation velocities with enzyme activities were calculated using Stat View software, version 5.0 (SAS Institute, Cary, NC, USA).

Results

The kinetics for the formation of fluvoxamino alcohol from fluvoxamine by human liver microsomes were consistent with the Michaelis–Menten model (Figure 2A) and the Eadie–Hofstee plot for the formation of fluvoxamino alcohol from fluvoxamine is shown in Figure 2B. The K_m and V_{max} values for the formation of fluvoxamino alcohol by pooled human liver microsomes were 76.3 ± 5.4 µM and 37.5 ± 2.1 pmol min^{-1} mg protein^{-1}, respectively. Consequently, the intrinsic clearance (V_{max}/K_m) value for fluvoxamino alcohol was 0.49 ± 0.06 µl min^{-1} mg protein^{-1}.

The effects of specific CYP inhibitors on the formation of fluvoxamino alcohol in human liver microsomes are shown in Table I. α-Naphtflavone (50 µM, CYP1A2 inhibitor), furafylline (50 µM, CYP1A2 inhibitor), sulfaphenazole (50 µM, CYP2C9 inhibitor),

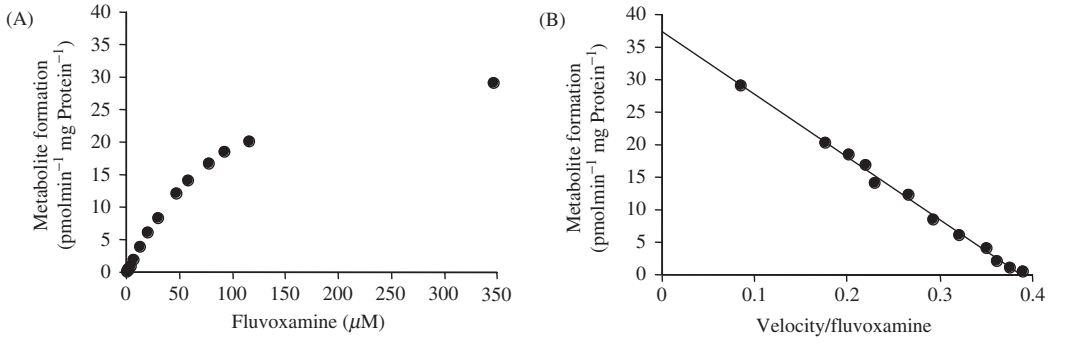

Figure 2. (A) Velocity *vs*. substrate concentration plots for fluvoxamino alcohol formation from fluvoxamine by human liver microsomes. (B) Eadie–Hofstee plots for velocity *vs*. the velocity/concentration of fluvoxamine in the same liver sample. Symbols are experimental data points representing an average of triplicate incubations.

Table I. Effects of various CYP inhibitors on the formation of fluvoxamino alcohol from fluvoxamine in human liver microsomes.

Inhibitor	μM	CYP targeted	Activity (% of control)
None	–	–	100.0
SKF-525A	50	Non-specific	39.9
Cimitidine	50	Non-specific	84.2
α-Naphtflavone	50	CYP1A2	99.9
Furafyline	50	CYP1A2	110.7
Sulphaphenazole	50	CYP2C9	107
Omeprazole	50	CYP2C19	97.4
DDC	50	CYP2E1	97.4
Quinidine	5	CYP2D6	44.5
Itraconazole	10	CYP3A4	97.1
Erythromycin	50	CYP3A4	104.4

Incubations were carried out at a fluvoxamine concentration of 11.5 μM. Results are the mean values obtained from three incubations.

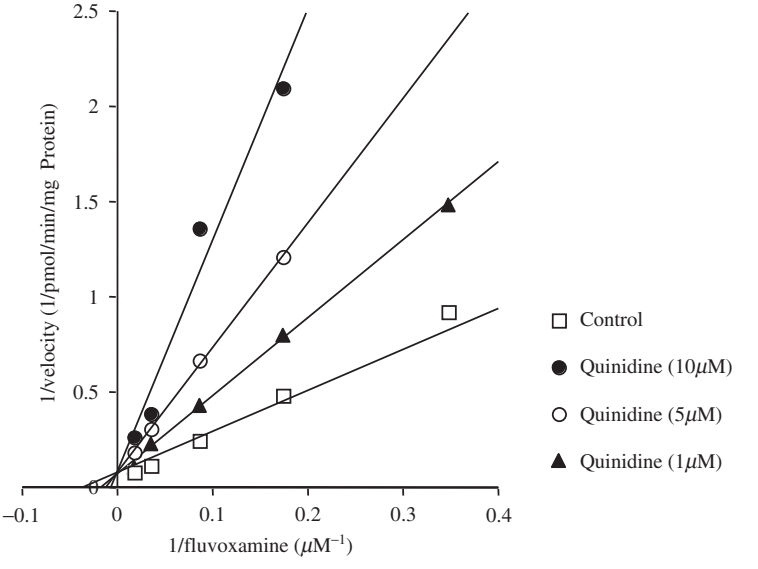

Figure 3. Inhibitory effect of quinidine on fluvoxamino alcohol formation in human liver microsomes. Lineweaver–Burk plots were obtained from a 30-min incubation of fluvoxamine with 0 (□), 1 (▲), 5 (○) and 10 (●) μM quinidine after a 5-min pre-incubation in human liver microsomes. Data are an average of triplicate experiments.

omeprazole (50 μM, CYP2C19 inhibitor), DDC (50 μM, CYP2A6 and CYP2E1 inhibitor), itraconazole (10 μM, CYP3A4 inhibitor) and erythromycin (50 μM, CYP3A4 inhibitor) did not have any effect on the formation of the alcohol metabolite at a concentration of 11.5 μM of fluvoxamine. However, quinidine (5 μM, CYP2D6 inhibitor) inhibited the formation of fluvoxamino alcohol to 44.5% of the control activity. In addition, quinidine showed dose-dependent inhibition of the metabolism of fluvoxamine (Figure 3). The K_i value of quinidine for fluvoxamino alcohol formation in human liver microsomes was 2.2 μM.

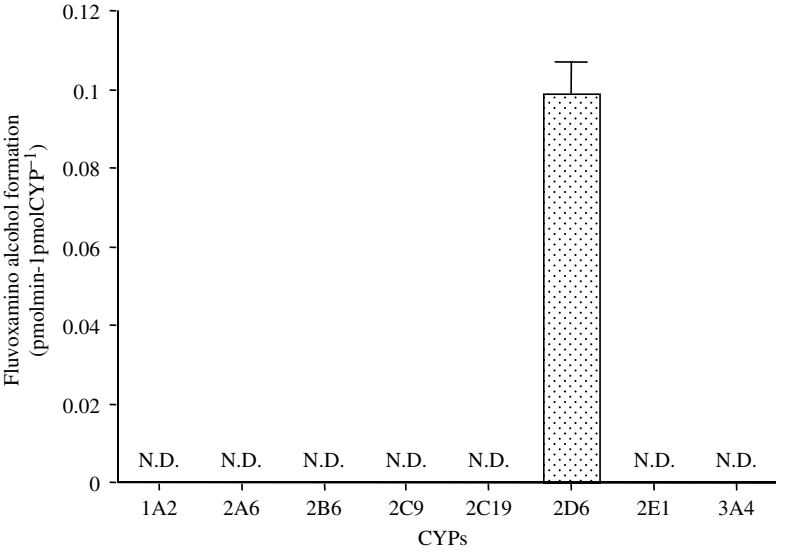

Figure 4. Fluvoxamino alcohol formation from fluvoxamine by expressed human CYP enzymes. Incubations with cDNA-expressed human CYP enzymes were carried out at a substrate concentration of 11.5 μM of fluvoxamine. Data are means ± standard deviation obtained from three experiments.

Furthermore, to confirm the findings from the inhibition study, the catalytic activities of individual human CYP enzymes expressed in a cell line were examined. As shown in Figure 4, only recombinant CYP2D6*1 exhibited a markedly high activity toward the formation of alcohol metabolite, indicating that CYP2D6 had a high substrate specificity for fluvoxamine, whereas other CYPs (1A2, 2A6, 2B6, 2C9, 2C19, 2E1 and 3A4) were not involved (at a fluvoxamine concentration of 11.5 μM). The formation of fluvoxamino alcohol by recombinant CYP2D6 followed one-enzyme Michaelis–Menten kinetics. The estimated K_m and V_{max} values for the formation of fluvoxamino alcohol by recombinant CYP2D6*1 were 73.2 ± 0.4 μM and 0.69 ± 0.08 pmol min^{-1} $pmol^{-1}$ CYP, respectively.

The correlations between the formation rates of fluvoxamino alcohol from fluvoxamine and the activity of CYP2D6 measured by bufuralol, which are marker probes for CYP2D6, are shown in Figure 5. As shown, CYP2D6 activities were highly correlated with the biotransformation of fluvoxamino alcohol from fluvoxamine ($r = 0.985$, $p < 0.0001$, $n = 11$).

The biotransformation to fluvoxamino acid from fluvoxamino alcohol was not observed by pooled human liver microsomes and individual human CYP enzymes expressed in a cell line (CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 or 3A4) (data not shown). However, the oxidation of fluvoxamino alcohol in human liver cytosol was inhibited by 4-methylpyrazole, which is an inhibitor of alcohol dehydrogenase, in a concentration-dependent manner over the range 10–1000 μM (Figure 6).

Discussion

Fluvoxamine is mainly metabolized in human to fluvoxamino acid via fluvoxamino alcohol by oxidative demethylation of the aliphatic methoxy group (65%), whereas lesser metabolism proceeds at the primary amino group (15%) and by removal of the ethanolamine

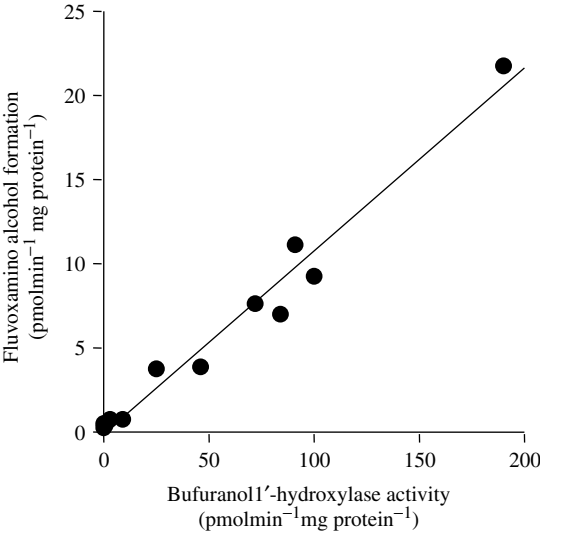

Figure 5. Correlation between the fluvoxamino alcohol formation from fluvoxamine and the bufuralol 1′-hydroxylase activity in individual human liver microsomes. Incubations with individual human liver microsomes were carried out at a substrate concentration of 11.5 μM of fluvoxamine. Symbols are experimental data points representing an average of triplicate incubations. The data of bufuralol 1′-hydroxylase activities (pmol min^{-1} mg^{-1} protein) in individual human liver microsomes are provided by GENTEST.

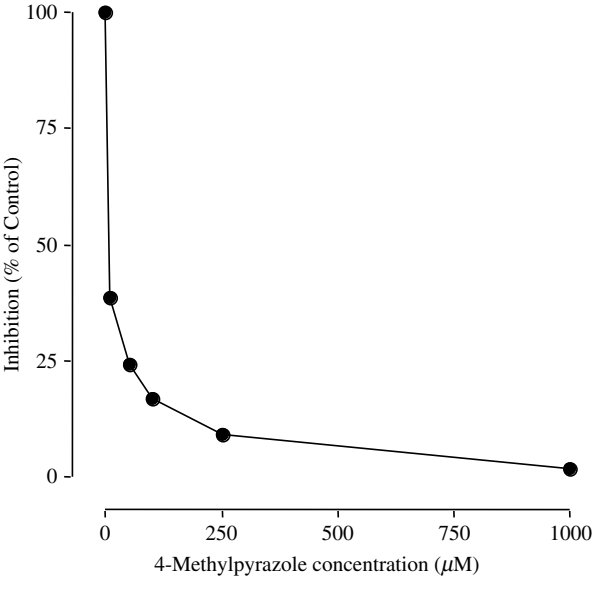

Figure 6. Inhibition of fluvoxamino alcohol formation in human liver cytosol using 4-methylpyrazole. Assays were performed at pH 7.4, 37°C with a fluvoxamino alcohol concentration of 8.2 μM and 4-methylpyrazole concentrations of 0–1000 μM. Each point represents the mean of triplicate experiments.

group (10%) (Ruijten et al. 1984; Perucca et al. 1994). The purpose of the current study was to identify the CYP enzymes involved in the major metabolic pathway to fluvoxamino alcohol from fluvoxamine and our present *in vitro* data demonstrate that CYP2D6 catalyses the metabolism of fluvoxamine to fluvoxamino alcohol.

CYP2D6 polymorphisms have been extensively studied at both the phenotype and genotype levels. Studies have shown that although there is a difference in enzyme activity between CYP2D6 PMs and EMs, there is also variation in enzyme activity among patients with CYP2D6 EMs (Droll et al. 1998). Due to the polymorphism of the CYP2D6 gene, CYP2D6 activity varies markedly between individuals with *1, *2, *10 and *17 alleles (Nakamura et al. 2002; Yu et al. 2002; Zanger et al. 2004). The biotransformation to fluvoxamino alcohol from fluvoxamine was correlated to CYP2D6 activity. Therefore, CYP2D6 PMs or individuals with a low activity of CYP2D6 should have a high plasma concentration of fluvoxamine, thereby resulting in an enhancement of the *in vivo* inhibitory effect on CYP1A2 and CYP2C19 for fluvoxamine (Sperber et al. 1991, Spina et al. 1993; Donaldsson et al. 1994; Jeppesen et al. 1996a; b, Sproule et al. 1997).

In the present study, CYP1A2 was not involved in the formation of fluvoxamino alcohol. Spigset et al. reported that the oral clearance (Cl/*F*) of fluvoxamine in CYP2D6 EMs was significantly higher in smokers than in non-smokers (4111 vs. 3452 ml min^{-1}, respectively, $p = 0.017$), whereas there was no significant difference in the metabolic clearance (Cl_m) to fluvoxamino acid formation between these two groups (1056 vs. 969 ml min^{-1}, respectively) (Spigset et al. 1997; 2001). These latter results support our *in vitro* finding that CYP1A2, which is induced by cigarette smoking, is not involved in fluvoxamino acid formation. Furthermore, Carrillo et al. reported that there were no significant differences between the mean AUC values of CYP2D6 EMs and PMs in smokers (1239 vs. 1539 nmol $h\,l^{-1}$); however, in non-smokers, AUC values of CYP2D6 PMs were significantly higher than those of CYP2D6 EMs (Carrillo et al. 1996). These data together strongly suggest that CYP1A2 is a minor enzyme involved in the metabolic pathway of fluvoxamine in humans. It is conceivable that CYP1A2 contributes to other minor metabolic pathways of fluvoxamine.

Generally, the metabolism of primary alcohols to carboxylic acids is most commonly mediated by cytosolic alcohol dehydrogenase or microsomal CYP (Mori et al. 1989; Aasmore et al. 1998). In the present study, the formation of fluvoxamino acid from fluvoxamino alcohol depends on alcohol dehydrogenase not CYP. Therefore, fluvoxamine is first metabolized by CYP2D6 to the alcohol metabolite and is further completely metabolized by a cytosolic alcohol dehydrogenase to the corresponding carboxylic acid.

In conclusion, the present *in vitro* studies indicate that only CYP2D6 catalyses the major metabolic pathway of fluvoxamine. Therefore, whenever prescribing fluvoxamine, the effects of CYP2D6 genotype on the pharmacological activities and CYP inhibitory effects of fluvoxamine must be carefully taken into consideration.

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