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O-Dealkylation of Fluoxetine in Relation to *CYP2C19* Gene Dose and Involvement of CYP3A4 in Human Liver Microsomes

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ABSTRACT

This work evaluated the kinetic behavior of fluoxetine *O*-dealkylation in human liver microsomes from different *CYP2C19* genotypes and identified the isoenzymes of cytochrome P450 involved in this metabolic pathway. The kinetics of the ρ-trifluoromethylphenol (TFMP) formation from fluoxetine was determined in human liver microsomes from three homozygous (*wt/wt*) and three heterozygous (*wt/m1*) extensive metabolizers (EMs) and three poor metabolizers (PMs) with *m1* mutation (*m1/m1*) with respect to CYP2C19. The formation rate of TFMP was determined by gas chromatograph with electron-capture detection. The kinetics of TFMP formation was best described by the two-enzyme and single-enzyme Michaelis-Menten equation for liver microsomes from CYP2C19 EMs and PMs, respectively. The mean intrinsic clearance (V_{max}/K_m) for the high- and low-affinity component was 25.2 μl/min/nmol and 3.8 μl/min/nmol of cytochrome P450 in the homozygous EMs microsomes and 12.8 μl/min/nmol and 2.9 μl/min/nmol of cytochrome P450 in the heterozygous EMs microsomes, respectively. Omeprazole (a CYP2C19 substrate) at a high concentration and triacetyloleandomycin (a selective inhibitor of CYP3A4) substantially inhibited *O*-dealkylation of fluoxetine. Furthermore, fluoxetine *O*-dealkylation was correlated significantly with *S*-mephenytoin 4′-hydroxylation at a low substrate concentration and midazolam 1′-hydroxylation at a high substrate concentration in liver microsomes of 11 Chinese individuals, respectively. Moreover, there were obvious differences in the *O*-dealkylation of fluoxetine in liver microsomes from different *CYP2C19* genotypes and in microsomal fractions of different human-expressed lymphoblast P450s. The results demonstrated that polymorphic CYP2C19 and CYP3A4 enzymes were the major cytochrome P450 isoforms responsible for fluoxetine *O*-dealkylation, whereas CYP2C19 catalyzed the high-affinity *O*-dealkylation of fluoxetine, and its contribution to this metabolic reaction was gene dose-dependent.

It has been well known that interindividual variations in the metabolic profile of many drugs are linked to genetic polymorphism in the cytochrome P450 (CYP) responsible for their metabolisms. The consequences of the variations may be of clinical significance with respect to either efficacy or toxicity of the drug as well as drug-drug interactions. CYP2C19 is one of the CYP isoforms concerning individual and ethnic variations of drug metabolisms that have been studied most extensively in recent years. CYP2C19-medicated *S*-mephenytoin 4′-hydroxylation shows a genetically determined polymorphism that has marked interracial differences, with the PM phenotype representing 2 to 5% of the Caucasian population but 13 to 23% of the Asian population (Wilkinson et al., 1989; Alván et al., 1990; Xiao et al., 1997). Metabolisms of a number of drugs, including diazepam (Qin et al., 1999), omeprazole (Shu et al., 2000), chloroguanide (Herrlin et al., 2000), and fluoxetine (Liu et al., 2001a, 2001b) in vivo or in vitro cosegregate with the CYP2C19 polymorphism.

Fluoxetine is a potent and selective serotonin reuptake inhibitor in the central nervous system and is widely used to treat depression and obsessive-compulsive behavior (Fuller et al., 1991; Gram, 1994). Despite the extensive use of fluoxetine clinically, it is thought that as much as 50% of its metabolism is still unclear. This study is very important because either fluoxetine metabolites are often therapeutically active or they may contribute to the side-effect profile of the parent drug. In addition, metabolites may compete with other exogenous substrates for catabolic enzymes and result in marked changes in human tissues and body fluid levels of these other drugs and their metabolites (Urichuk et al., 1997; Hiemke and Härtter, 2000). Up to now, the two major routes of metabolism for fluoxetine that have been identified are *N*-demethylation to norfluoxetine and *O*-dealkylation to

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ABBREVIATIONS: CYP, cytochrome P450; TFMP, ρ-trifluoromethylphenol; DDC, diethyldithiocarbamate; EM, extensive metabolizer; PM, poor metabolizer; GC-ECD, gas chromatograph with electron-capture detection; PFBSC, pentafluorobenzenesulfonyl chloride; TAO, triacetyloleandomycin.

TFMP (Altamura et al., 1994; Urichuk et al., 1997). Fluoxetine is metabolized extensively by the hepatic cytochrome P450 enzymes, and less than 2.5% of the drug is found unchanged in the human urine. Some studies have shown that polymorphic CYP2C19 and CYP2C9 appear to be the principal human CYP isoenzymes mediating *N*-demethylation of fluoxetine (von Moltke et al., 1997; Liu et al., 2001b) and CYP3A4 may make a minor contribution to it. To the best of our knowledge, there is no report as to which CYP isoforms are responsible for the *O*-dealkylation of fluoxetine, and TFMP has never been quantitated in human liver microsomes. Furthermore, fluoxetine *O*-dealkylation has not yet been characterized in vitro with respect to the individual *CYP2C19* genotype status. Therefore, this study is of significance in that it investigates whether one or more isoenzymes of CYP are involved in the formation of TFMP from fluoxetine. In vivo and in vitro studies for fluoxetine metabolism have shown great interindividual variabilities consistent with CYP2D6 and CYP2C19 polymorphic characteristics (Hamelin et al., 1996; Liu et al., 2001a). Considering the involvement of multienzymes and the different contributions of various CYP isoforms in the *O*-dealkylation of fluoxetine, in this study we evaluated the correlation between fluoxetine *O*-dealkylase activity and *S*-mephenytoin 4′-hydroxylase and midazolam 1′-hydroxylase activity. To further determine the role of CYP2C19 in the *O*-dealkylation of fluoxetine, we assessed the relative contribution of CYP2C19 using different genotyped liver microsomes from three homozygous EMs, three heterozygous EMs, and three PMs of CYP2C19. Various selective chemical inhibitors and recombinant CYP1A2, 2C8, 2C9, 2C19, and 3A4 were also used to identify the isoforms of CYP involved in fluoxetine *O*-dealkylation.

Experimental Procedures

Materials. Fluoxetine hydrochloride was supplied by Sigma/RBI (Natick, MA). TFMP and pentafluorobenzenesulfonyl chloride (PFBSC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 2,4-Dichlorophenol (internal standard) was supplied by Shanghai Chemical Center (Shanghai, China). Omeprazole was a generous gift from Astra Hässle AB (Mölndal, Sweden). Coumarin, quinidine, triacetyloleandomycin (TAO), diethyldithiocarbamate (DDC), $NADP^+$, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO). Sulfaphenazole was a gift from CibaGeigy Ltd. (Basel, Switzerland), and furafylline was kindly donated by Dr. W. Pfleiderer (Universität Konstanz, Wurzburg, Germany). Recombinant CYP1A2, 2C8, 2C9, 2C19, and 3A4A were purchased from Gentest (Woburn, MA). Acetonitrile of high-performance liquid chromatography grade was purchased from Tedia Company Inc. (Fairfield, OH). All other chemicals were of analytic reagent grade.

Human Liver Microsomes. Adult human liver tissues were collected from renal transplant donors and patients undergoing partial hepatectomy in our liver bank. The collection and use of human liver tissues in this study were approved by the Ethics Committee of Xiang-Ya School of Medicine, Central South University. Candidate patients for liver sample collection were those who did not suffer from acute or chronic hepatitis or cirrhosis and took no medications known to induce or inhibit CYP activity. Portions of surgical liver "waste tissues" distant from disease-affected regions and appearing visually normal was collected. The collection approaches of liver tissue and its morphologic and biochemical characterization were described elsewhere (von Bahr et al., 1980). Microsomes were prepared by differential centrifugation (von Bahr et al., 1980) and stored at −80°C ready for use. Microsomal protein concentration was determined by the method of Lowry et al. (1951).

Liver donors were genotyped for *CYP2C19* from whole blood or liver tissues according to the method of de Morais et al. (1995). Of the 34 liver donors, 18 liver microsomes were genotyped as homozygous EMs (*wt/wt*), 13 heterozygous EMs (*wt/m1*), and three PMs with the *m1* mutation (*m1/m1*). No *m2* allele was found.

Fluoxetine Metabolism in Vitro and GC-ECD Analysis. Fluoxetine metabolism in vitro was used in 0.1 M potassium phosphate buffer (pH 7.4) containing 1.0 mg/ml liver microsomal protein, reduced NADP (NADPH)-generating system, and various concentrations of fluoxetine with or without inhibitors in a final volume of 500 μl. The enzyme reaction was initiated by adding 20 μl of various concentrations of substrate and NADPH-generating system consisting of 1 mM NADP, 10 mM glucose 6-phosphate, 2 IU/ml glucose-6-phosphate dehydrogenase, and 10 mM $MgCl_2$. After the incubation at 37°C in a shaking water bath for 45 min, the reaction was stopped by cooling on ice and the addition of 100 μl of acetonitrile. Preliminary experiments showed that the formation of TFMP was linear to both incubation time over 60 min and microsomal protein concentration (0.5–2 mg/ml) at 37°C. Accordingly, the incubation time of 45 min and the microsomal protein concentration of 1 mg/ml were chosen in the present study.

After the termination of the reaction, a fixed amount of internal standard (30.0 μM 2,4-dichlorophenol in methanol) was added to the incubation mixture to assay fluoxetine and TFMP, and the solution was shaken thoroughly for 30 s. Following the procedure established for derivatization with PFBSC as described by Urichuk et al. (1997), the samples were then basified by adding excess potassium bicarbonate (400 mg) and briefly vortex-mixed. Next, 4.5 ml of ethyl acetate containing acetonitrile (10%, v/v) and the derivatizing reagent PFBSC (0.1%, v/v) was added to each sample. The samples were then shaken vigorously for 20 min in a YKH-II liquid rapid shaker (Jiangxi, China) and centrifuged for 10 min (2000*g*). The upper organic layer was retained and transferred to another clean glass tube. The samples were evaporated until eventually dry under a gentle stream of nitrogen at 37°C. The residue was dissolved in 200 μl of methanol and a 2-μl aliquot was used for GC-ECD analysis. The samples were analyzed using a chromatographic system consisting of a Hewlett-Packard 5890 GC (Palo Alto, CA) equipped with electron-capture detector and a HP-5 Capillary Column (crosslinked 5% PH NE Siloxane, 15-m × 0.53-mm ×1.5-μm film thickness). The carrier gas and make-up gas were ultra-pure nitrogen at a flow rate of 10 and 100 ml/min, respectively. Retention times of TFMP, internal standard, and fluoxetine were 3.1, 4.5, and 10.1 min, respectively. The lower limit of detection for both TFMP and fluoxetine was 0.01 and 0.02 nmol, respectively, and the coefficient of variation for intra- and interday reproducibility ranged from 5.8 to 10.7%.

Kinetics for TFMP Formation. Ten concentrations of fluoxetine (1–200 μM) were used to characterize the kinetics of fluoxetine *O*-dealkylation by liver microsomes from different *CYP2C19* genotypes. Several enzyme kinetic equations proposed by Schmider et al. (1996) were used to fit the untransformed kinetic data (Figperfect, version 5.0; Soft Corporation, Durham, NC). The most appropriate model was obtained based on the dispersion of residuals and whether an F-test showed a significant reduction ($P < 0.05$) in the residual sum of squares. The next two equations best described the kinetics of fluoxetine *O*-dealkylation by EM (*wt/wt* and *wt/m1*) microsomes and PM (*m1/m1*) microsomes, respectively.

$$V = V_{max1} \times S/(K_{m1} + S) + V_{max2} \times S/(K_{m2} + S) \quad (1)$$

$$V = V_{max} \times S/(K_m + S) \quad (2)$$

Inhibition Studies. The inhibitory effect of various selective inhibitors was examined in four EM microsomes at a substrate concentration of 100 μM fluoxetine. The selective inhibitors used were 200 μM coumarin (CYP2A6 substrate), 10 μM quinidine

(CYP2D6 inhibitor), 20 μM DDC (CYP2E1 inhibitors), 50 μM TAO (CYP3A4 inhibitor), 25 μM furafylline (CYP1A2 inhibitor), 20 μM sulfaphenazole (CYP2C9 inhibitor), and 100 μM omeprazole (CYP2C19 substrate) (Andersson et al., 1994; Newton et al., 1995; Ko et al., 1997; Eagling et al., 1998). All inhibitors were dissolved in methanol except for DDC in distilled water. Because methanol has an inhibitory effect on CYP activity, solutions were dried before incubation. Furafylline, TAO, and DDC were preincubated with liver microsomal preparations and the NADPH generating at 37°C for 15 min before substrate was added.

To assess the contributions of CYP2C19 and the inhibitory effect of TAO in the *O*-dealkylation of fluoxetine, 50 μM TAO and three different substrate concentrations (5, 25, and 100 μM) were incubated in three homozygous EMs, three heterozygous EMs, and three PMs of CYP2C19, respectively.

To investigate the inhibitory potency of TAO and omeprazole, a range of concentrations of omeprazole (0–200 μM) and TAO (0–50 μM) were incubated with fluoxetine in four EM microsomal preparations (2 *wt*/*wt* and 2 *wt*/*m1*) at a low (5 μM) or a high (100 μM) substrate concentration.

Metabolism of Fluoxetine by cDNA-Expressed P450s. Recombinant CYP1A2, 2C8, 2C9, 2C19, and 3A4 were further used to assess the roles of CYP2C and CYP3A4 in the metabolism of fluoxetine. Fifty picomoles of human lymphoblast-expressed CYP1A2, 2C8, 2C9, 2C19, and 3A4 were coincubated with an NADPH-generating system, respectively, and the reaction was initiated by addition of 20 μl of substrate and incubated for 45 min. The reaction was stopped finally by cooling on ice and the addition of 100 μl of acetonitrile.

Correlation Studies. To further determine whether CYP2C19 and CYP3A4 are major CYP enzymes responsible for fluoxetine *O*-dealkylation, three substrate concentrations (5, 25, and 100 μM) of fluoxetine and 11 liver microsomes from EMs of CYP2C19 were used. The activities of fluoxetine *O*-dealkylation were correlated with the activities of 250 μM *S*-mephenytoin 4′-hydroxylation and 100 μM midazolam 1′-hydroxylation. The rate of formation of 4′-hydroxymephenytoin and 1′-hydroxymidazolam was determined using high-performance liquid chromatography as described by Xie et al. (1995) and by Carrillo et al. (1998), respectively.

Data Analysis. Duplicate incubations were used throughout the study. Data were analyzed by the paired and unpaired Student's *t* test and a one-way analysis of variance. The correlations between fluoxetine *O*-dealkylation in different liver microsomal preparations and *S*-mephenytoin 4′-hydroxylation and midazolam 1′-hydroxylation were determined by least-squares linear regression (SPSS for Windows 8.0; SPSS, Chicago, IL). A P value of < 0.05 was considered to be the minimum level of significance.

Results

Kinetics for TFMP Formation. The kinetics of TFMP formation was studied in liver microsomes from nine subjects (three *wt*/*wt*, three *wt*/*m1*, and three *m1*/*m1*). After iteratively fitting the different enzyme kinetic models proposed by Schmider et al. (1996) to the untransformed data of each subject, the kinetics of TFMP formation in the six EMs (three *wt*/*wt* and three *wt*/*m1*) microsomes followed the two-enzyme Michaelis-Menten model (eq. 1), whereas the kinetics in the three PMs microsomes was best described by the single-enzyme Michaelis-Menten model (eq. 2). The kinetic parameters for fluoxetine *O*-dealkylation in EMs and PMs are shown in Table 1. The substrate versus velocity plots and the Eadie-Hofstee plots for TFMP formation showed a difference in kinetic behavior between the EM microsomes and the PM microsomes (Fig. 1). In PMs microsomes, the high-affinity component of fluoxetine *O*-dealkylation was absent. Compared with EMs, the formation of TFMP in PM liver microsomes was significantly slower, especially at a low substrate concentration (data not shown). Furthermore, the concavity of Eadie-Hofstee plots in the homozygous EMs (Fig. 1A) showed the involvement of at least two CYP enzymes in the reaction, which was more apparent for the homozygous EMs than for the heterozygous EMs. Mean apparent K_m values for the high- and low-affinity components were 4.6 and 60.2 μM in the homozygous EM liver microsomes and 9.1 and 70.9 μM in the heterozygous EM liver microsomes, and V_{max} values were 116 and 224 pmol/min/nmol of P450 protein in the homozygous EM microsomes and 113 and 191 pmol/min/nmol of P450 protein in the heterozygous EM microsomes. The mean intrinsic clearances (V_{max1}/K_{m1}) of the high-affinity component was 6.6 times that (V_{max2}/K_{m2}) of the low-affinity component in the homozygous EM microsomes and 4.4 times in the heterozygous EM microsomes.

Inhibition with Selective Chemical Inhibitors. Omeprazole (100 μM) and TAO (50 μM) caused a mean 46.9% ($P < 0.001$) and 69.8% ($P < 0.001$) reduction in the formation of TFMP. At a substrate concentration of 100 μM, 10 μM quinidine inhibited this reaction to a minor extent. However, no inhibitory effect was observed for coumarin, furafylline, DDC, and sulfaphenazole. The addition of 100 μM omepra-

TABLE 1

Kinetic parameters for fluoxetine *O*-dealkylation in human liver microsomes from three homozygous EMs, three heterozygous EMs, and three PMs of CYP2C19

K_{m1} and K_{m2} expressed as micromolar concentration, V_{max1} and V_{max2} expressed as picomoles per minute per nanomole of P450, and V_{max1}/K_{m1}, and V_{max2}/K_{m2} were expressed as microliters per minute per nanomole of P450.

Liver Samples	K_{m1}	V_{max1}	V_{max1}/K_{m1}	K_{m2}	V_{max2}	V_{max2}/K_{m2}
Homozygous EMs (genotype)						
HL-4 (*wt*/*wt*)	4.6	116	25.2	56.8	216	3.8
HL11 (*wt*/*wt*)	3.7	98	26.5	46.2	206	4.5
HL-19 (*wt*/*wt*)	5.6	134	23.9	77.5	248	3.2
Mean ± S.D.	4.6 ± 0.8	116 ± 15	25.2 ± 1.1	60.2 ± 1.29	224 ± 18	3.8 ± 0.5
Heterozygous EMs (genotype)						
HL-10 (*wt*/*m1*)	6.3	79	12.5	45.1	189	4.2
HL-22 (*wt*/*m1*)	12.6	125	9.9	63.8	166	2.6
HL-44 (*wt*/*m1*)	8.4	134	16.0	103.8	218	2.1
Mean ± S.D.	9.1 ± 2.6	113 ± 24	12.8 ± 2.5	70.9 ± 24.5	191 ± 21	2.9 ± 0.9
PMs (genotype)						
HL-24 (*m1*/*m1*)	—	—	—	40.2	124	3.1
HL-34 (*m1*/*m1*)	—	—	—	83.3	175	2.1
HL-46 (*m1*/*m1*)	—	—	—	26.2	136	5.3
Mean ± S.D.				49.9 ± 24.3	145 ± 22	3.5 ± 1.3

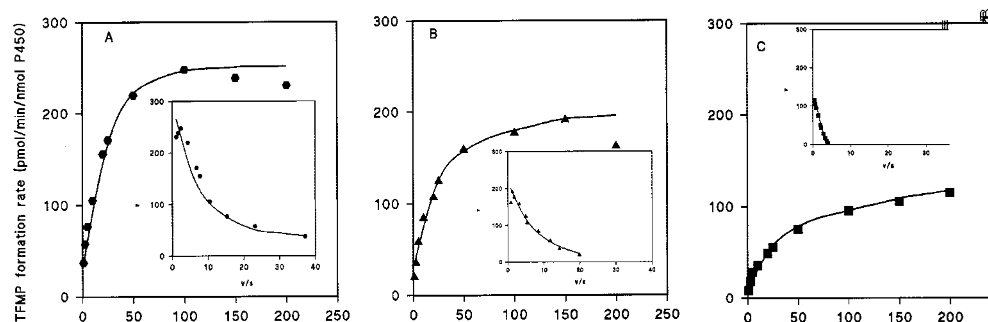

Fig. 1. Representative substrate versus velocity and Eadie-Hofstee plots for the formation of TFMP in human liver microsomes from different genotypes of CYP2C19, including homozygous EMs microsomes (A), heterozygous EMs microsomes (B), and PMs microsomes (C). The values are the means of duplicate incubations.

zole plus 50 μM TAO produced a maximum inhibition of 82.8% ($P < 0.001$) for TFMP formation (Fig. 2).

Omeprazole was a relatively weak inhibitor of the high-affinity site of TFMP formation, whereas its inhibitory effect was greater at the low (5 μM) substrate concentration than at the high (100 μM) substrate concentration (Fig. 3A). In contrast, TAO had a strong inhibitory effect on this reaction at the high (100 μM) substrate concentration than at the low (5 μM) substrate concentration (Fig. 3B).

Inhibitory Effect of TAO on TFMP Formation by Different CYP2C19 Genotyped Microsomes. Three substrate concentrations (5, 25, and 100 μM) were used to assess the inhibitory effect of 50 μM TAO on TFMP formation in the nine liver microsomes (three *wt/wt*, three *wt/m1*, and three *m1/m1*). At a low substrate concentration (5 μM), TAO had a relatively minor inhibitory effect (<47%) on TFMP formation in both the homozygous EMs microsomes and heterozygous EMs microsomes. However, the mean percentage inhibition by TAO was lower in the homozygous EM microsomes than in the heterozygous EM microsomes (35.3 versus 47.0%, $P <$ 0.05). With the increase of substrate concentration, the mean percentage inhibition of fluoxetine *O*-dealkylation increased to 47.4 and 59.3% at 25 μM fluoxetine and to 65.9 and 74.3% at 100 μM fluoxetine in the homozygous and the heterozygous microsomes, respectively. In the PM microsomes, 50 μM TAO almost abolished TFMP formation at all the three substrate concentrations (>90%) (Fig. 4).

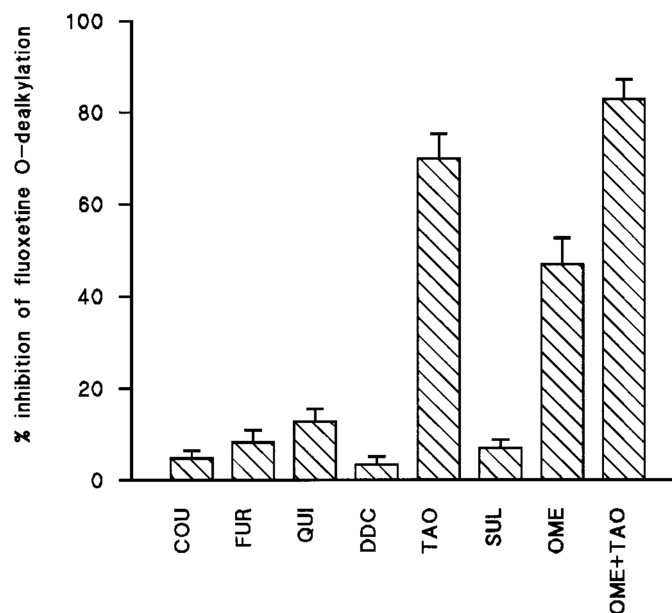

Fig. 2. Effect of selective cytochrome P450 inhibitors, 200 μM coumarin, 25 μM furafylline, 10 μM quinidine, 20 μM DDC, 50 μM TAO, 20 μM sulfaphenazole, 100 μM omeprazole, and 100 μM omeprazole plus 50 μM TAO on the formation of TFMP in human liver microsomes from four EMs (two *wt/wt*, HL-4 and HL-11; two *wt/m1*, HL-10 and HL-22) at a substrate concentration of 100 μM fluoxetine. The values are the mean inhibition percentage (±S.D.). COU, coumarin; FUR, furafylline; QUI, quinidine; DDC, diethyldithiocarbamate; SUL, sulfaphenazole; OME, omeprazole.

Correlations Studies. The microsomal activities of fluoxetine *O*-dealkylation at low (5 μM), medial (25 μM), and high (100 μM) substrate concentrations were measured for 11 liver microsomes from EMs of CYP2C19. The rate of formation of 4′-hydroxymephenytoin and 1′-hydroxymidazolam in the these liver microsomes was also determined by previously described methods of Xie et al. (1995) and Carrillo et al. (1998), which were reflected with the CYP2C19 activity and CYP3A4 activity. At a low substrate concentration of 5 μM, a good correlation ($r = 0.740$, $P < 0.01$) was found between fluoxetine *O*-dealkylation and *S*-mephenytoin 4′-hydroxylation (Fig. 5A). However, with the increase of substrate concentration, the correlation coefficient between these two metabolic reactions decreased to $r = 0.530$ ($P > 0.05$) at 25 μM fluoxetine (Fig. 5B) and to $r = 0.402$ ($P > 0.05$) at a high substrate concentration of 100 μM (Fig. 5C), indicating no significant correlation. In contrast, The formation of TFMP at high (100 μM) and medial (25 μM) substrate concentrations showed close correlation with midazolam 1′-hydroxylation ($r = 0.763$, $P < 0.01$, Fig. 6C; $r = 0.679$, $P < 0.05$, Fig. 6B; respectively) whereas no significant correlation between these two metabolic reactions was found at a low substrate concentration of 5 μM ($r = 0.424$, $P > 0.05$) (Fig. 6A).

Effects of Recombinant CYP1A2, 2C8, 2C9, 2C19, and 3A4 on Fluoxetine Metabolism. Effects of recombinant CYP1A2, 2C8, 2C9, 2C19, and 3A4 on fluoxetine metabolism were observed only at low (5 μM) and high (100 μM) substrate concentrations (Table 2). The results showed that CYP2C19 and CYP3A4 were two major cytochrome P450 isoforms responsible for fluoxetine *O*-dealkylation, and CYP1A2, CYP2C8, and CYP2C9 catalyzed this reaction to a minor extent. At a substrate concentration of 5 μM, recombinant CYP2C19 produced a maximal catalyzing activity in the *O*-dealkylation of fluoxetine, however, CYP3A4 had higher catalyzing activity in this reaction than CYP2C19 at a substrate concentration of 100 μM (Fig. 7).

Discussion

In general, the CYP2C19 oxidation polymorphism caused impaired drug metabolism and affected as many as 20% Asians but only 3% Caucasians. We observed a biphasic-

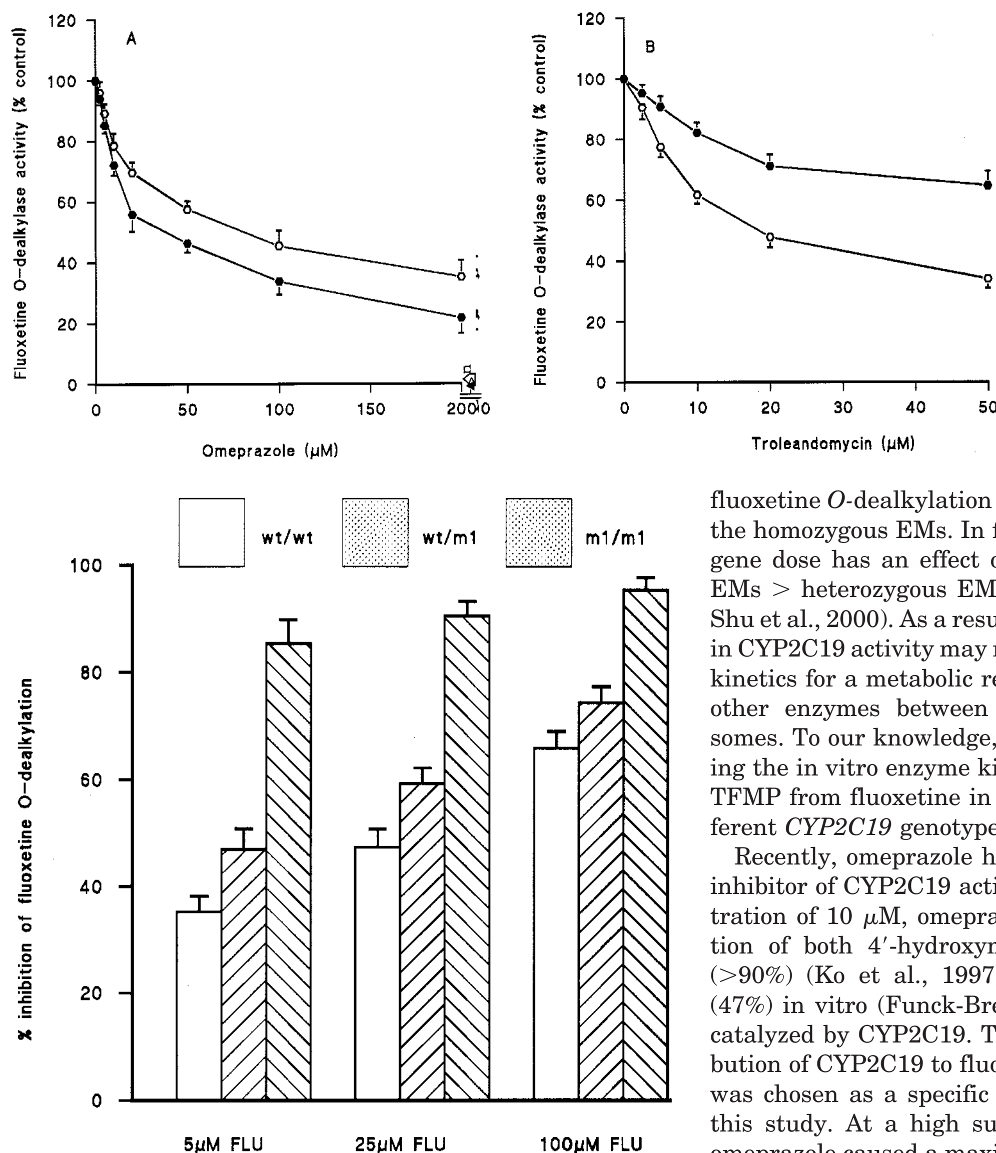

Fig. 3. Effect of omeprazole (left, A) and TAO (right, B) on the formation of TFMP in four EMs microsomes (two *wt*/*wt*, HL-4 and HL-11; two *wt*/*m1*, HL-10 and HL-22) at a low substrate concentration of 5 μM (solid circle) and at a high substrate concentration of 100 μM (open circle). The values are the mean inhibition percentage (±S.D.).

Fig. 4. Effect of 50 μM TAO on the formation of TFMP at different substrate concentrations (5, 25, and 100 μM) in liver microsomes from different *CYP2C19* genotypes (three *wt*/*wt*, three *wt*/*m1*, and three *m1*/*m1*). The values are the mean inhibition percentage (±S.D.). FLU, fluoxetine.

enzyme kinetics for the formation of TFMP from fluoxetine in EM microsomes and a monophasic-enzyme kinetics in PM microsomes. These data indicated clearly that at least two CYP isoenzymes were involved in the *O*-dealkylation of fluoxetine with high- and low-affinity components. However, fluoxetine *O*-dealkylation lacked the high-affinity component and exhibited monophasic enzyme kinetics in PM microsomes. Furthermore, the intrinsic clearance of the high-affinity component for fluoxetine *O*-dealkylation was 6.6 and 4.4 times that of the low-affinity component in the homozygous EM microsomes and the heterozygous EM microsomes. These kinetic data suggested that CYP2C19 was the major CYP enzyme contributing to fluoxetine *O*-dealkylation in vitro. Moreover, the homozygous EMs showed a more typical substrate versus concentration plot and Eadie-Hofstee plot for the two-enzyme model compared with the heterozygous EMs (Fig. 1). This was probably due to the higher activity of fluoxetine *O*-dealkylation at a low substrate concentration in the homozygous EMs. In fact, some studies have shown that gene dose has an effect on CYP2C19 activity (homozygous EMs > heterozygous EMs > PMs) (de Morais et al., 1995; Shu et al., 2000). As a result, the genotype-related differences in CYP2C19 activity may result in different apparent enzyme kinetics for a metabolic reaction mediated by CYP2C19 and other enzymes between different genotyped liver microsomes. To our knowledge, this is the first study characterizing the in vitro enzyme kinetic behavior for the formation of TFMP from fluoxetine in human liver microsomes from different *CYP2C19* genotypes.

Recently, omeprazole has been used as a potent selective inhibitor of CYP2C19 activity (Ko et al., 1997). At a concentration of 10 μM, omeprazole strongly inhibited the formation of both 4′-hydroxymephenytoin from *S*-mephenytoin (>90%) (Ko et al., 1997) and cycloguanil from proguanil (47%) in vitro (Funck-Brentano et al., 1997), two reactions catalyzed by CYP2C19. Therefore, to investigate the contribution of CYP2C19 to fluoxetine *O*-dealkylation, omeprazole was chosen as a specific selective inhibitor of CYP2C19 in this study. At a high substrate concentration of 100 μM, omeprazole caused a maximum of 46.9% reduction compared with the control value in the formation of TFMP, suggesting the involvement of CYP2C19. A selective and potent inhibitor of CYP3A4 (Pessayre et al., 1983; Xu et al., 1999), 50 μM TAO inhibited fluoxetine *O*-dealkylation by up to 69.8% at a high substrate concentration of 100 μM, indicating that CYP3A4 may also be the main enzyme involved in the *O*-dealkylation of fluoxetine.

To further assess the relative contribution of major CYP isoforms responsible for fluoxetine *O*-dealkylation in liver microsomes from Chinese individuals, inhibition experiments, heterologous expression experiments, and correlation studies were carried out. Within a range of concentrations of omeprazole (0–200 μM) and TAO (0–50 μM), we found that the inhibitory effect on TFMP formation by omeprazole was greater at a low substrate concentration (5 μM) than at a high substrate concentration (100 μM). However, TAO had a stronger inhibitory effect on this reaction at a high substrate concentration (100 μM) than at a low substrate concentration (5 μM). Furthermore, in the EM microsomes, TFMP formation was inhibited slightly by 50 μM TAO at a low substrate concentration. With the increase of substrate concentrations to 25 and 100 μM, the inhibition of TFMP formation by TAO

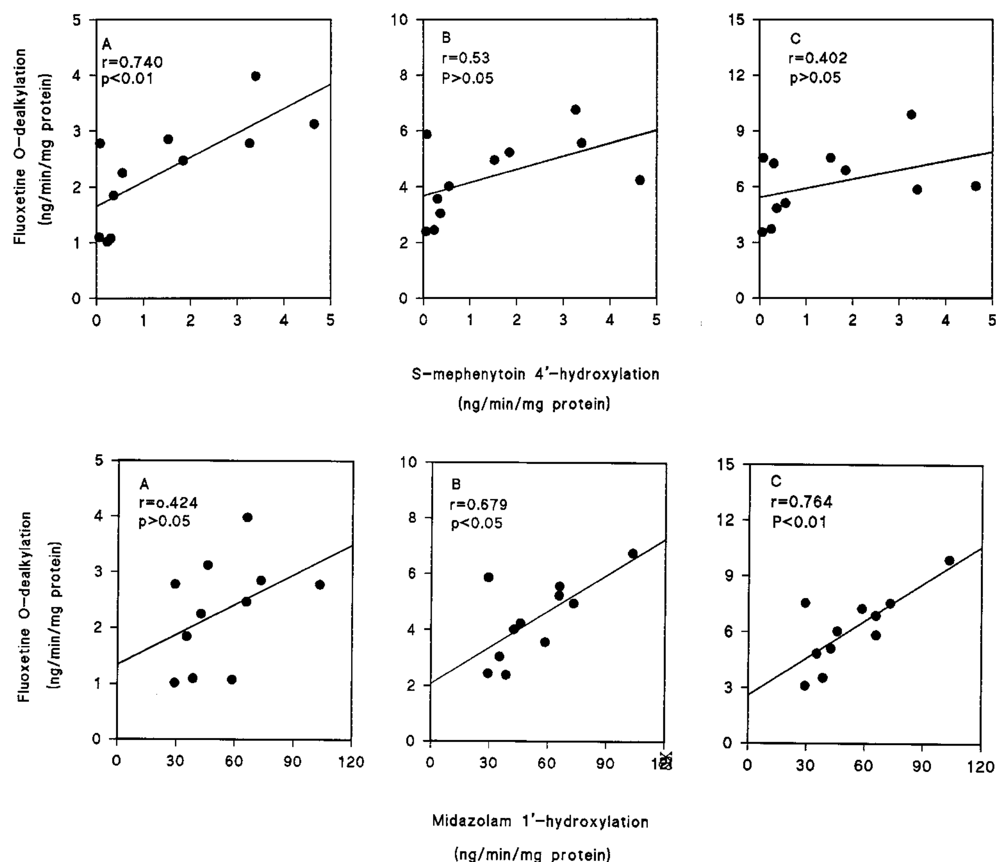

Fig. 5. Correlation between *S*-mephenytoin 4′-hydroxylation and fluoxetine *O*-dealkylation at different substrate concentrations (A, 5 μM; B, 25 μM; C, 100 μM) in the liver microsomes of 11 Chinese individuals genotyped as EMs with respect to CYP2C19.

Fig. 6. Correlation between midazolam 1′-hydroxylation and fluoxetine *O*-dealkylation at different substrate concentration (A, 5 μM; B, 25 μM; C, 100 μM) in the liver microsomes of 11 Chinese individuals genotyped as EMs of CYP2C19.

TABLE 2

The rate of formation of TFMP from fluoxetine catalyzed by human lymphoblast-expressed P450s in different substrate concentrations

P450s	TFMP Production	
	5 μmol	100 μmol
	pmol/min/pmol of P450	
CYP1A2	0.0081	0.0254
CYP2C8	0.0018	0.0086
CYP2C9	0.0104	0.0328
CYP2C19	0.0865	0.3102
CYP3A4	0.0578	0.4985

substantially increased. In particular, under different substrate concentrations, the inhibitory potency of TAO on TFMP formation in the heterozygous liver microsomes was higher than that of homozygous liver microsomes, indicating that TAO had a markedly different inhibitory effect on fluoxetine *O*-dealkylation in the different *CYP2C19* genotyped liver microsomes. Moreover, the correlation between fluoxetine *O*-dealkylation with *S*-mephenytoin 4′-hydroxylation declined with the increase of fluoxetine concentrations. However, the correlation between fluoxetine *O*-dealkylation with midazolam 1′-hydroxylation increased with the increase of substrate concentrations. These results showed that fluoxetine *O*-dealkylation had a substrate concentration-dependent contribution of CYP2C19 and CYP3A4. In addition, TFMP formation in the PM microsomes was inhibited almost completely by TAO (>90%), indicating that CYP3A4 is the principal enzyme responsible for this reaction in the PM microsomes. Finally, heterologous expression experiment further showed that recombinant CYP2C19 and CYP3A4 produced an maximum catalyzing effect on fluoxetine *O*-dealkylation at a low and a high substrate concentration, respectively. Accordingly, at therapeutically relevant substrate concentrations, e.g., at 5 μM, fluoxetine *O*-dealkylation was mediated predominantly via CYP2C19, with only a minor contribution of CYP3A4.

The fact that gene dose has an effect on drug metabolism has been reported before. Hamelin et al. (1996) reported that the metabolic ratio of fluoxetine *N*-demethylation in homozygous EMs to CYP2D6 is higher than that of heterozygous EMs. Recent studies from our laboratory have shown that the metabolism of *S*-mephenytoin (Shu et al., 2000) and diazepam (Qin et al., 1999) were gene dose-dependent. In this study, we found that at the therapeutically relevant substrate concentration of 5 μM, the mean TFMP formation of the three homozygous EM livers was significantly higher than that of the three heterozygous EM livers, indicating a gene dose effect on fluoxetine *O*-dealkylation. Thus, gene dose effect may result in differential inhibition of the affected CYP isoforms by substrate or inhibitors between different genotyped subjects, as demonstrated in our inhibition studies. In addition, recent studies conducted in our laboratory showed that the in vivo induction of CYP2C19 by rifampicin was gene dose-dependent (Feng et al., 1998). The higher proportion of heterozygous CYP2C19 EMs in Asian subjects is considered to be a cause of the differences between Caucasian and Asian subjects in the metabolism of chloroguanide (Herrlin et al., 2000) and diazepam (Qin et al., 1999). The genetic polymorphism of CYP2C19 is likely to be one of the major factors causing the interindividual and interracial differences of some drugs that are metabolized by CYP2C19.

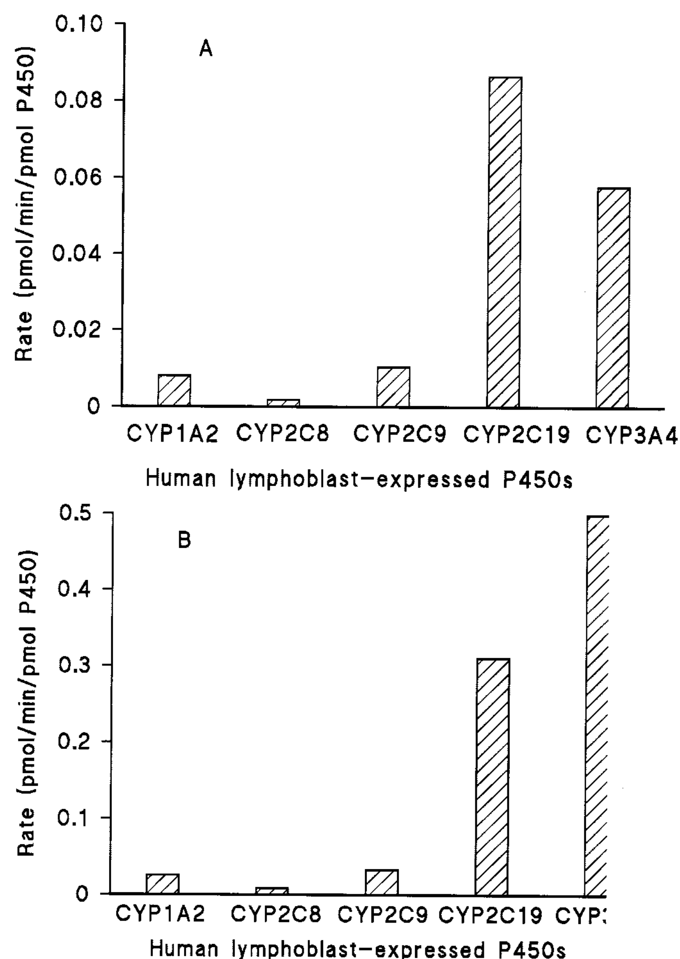

Fig. 7. The rate of formation of TFMP from fluoxetine catalyzed by human lymphoblast-expressed CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP3A4, respectively, at a low (5 μM, A) and high (100 μM, B) substrate concentrations.

In conclusion, polymorphic CYP2C19 is a high-affinity enzyme responsible for fluoxetine *O*-dealkylation in human liver microsomes. CYP3A4 is a low-affinity enzyme that contributes little to this metabolic reaction at the therapeutically relevant substrate concentration in EM microsomes but a lot to PM microsomes. The contribution of CYP2C19 to fluoxetine *O*-dealkylation is gene dose-dependent.

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