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ORIGINAL INVESTIGATION

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Human cytochromes mediating *N*-demethylation of fluoxetine in vitro

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Abstract Biotransformation of the selective serotonin reuptake inhibitor antidepressant, fluoxetine, to its principal metabolite, norfluoxetine, was evaluated in human liver microsomes and in microsomes from transfected cell lines expressing pure human cytochromes. In human liver microsomes, formation of norfluoxetine from *R,S*-fluoxetine was consistent with Michaelis-Menten kinetics (mean K_m = 33 μM), with evidence of substrate inhibition at high substrate concentrations in a number of cases. The reaction was minimally inhibited by coincubation with chemical probes inhibitory for P450-2D6 (quinidine), -1A2 (furafylline, α-naphthoflavone), and -2E1 (diethyldithiocarbamate). Substantial inhibition was produced by coincubation with sulfaphenazole (K_i = 2.8 μM), an inhibitory probe for P450-2C9, and by ketoconazole (K_i = 2.5 μM) and fluvoxamine (K_i = 5.2 μM). However, ketoconazole, relatively specific for P450-3A isoforms only at low concentrations, reduced norfluoxetine formation by only 20% at 1 μM, and triacetyloleandomycin (≥ 5 μM) reduced the velocity by only 20–25%. Microsomes from cDNA-transfected human lymphoblastoid cells containing human P450-2C9 produced substantial quantities of norfluoxetine when incubated with 100 μM fluoxetine. Smaller amounts of product were produced by P450-2C19 and -2D6, but no product was produced by P450-1A2, -2E1, or 3A4. Cytochrome P450-2C9 appears to be the principal human cytochrome mediating fluoxetine *N*-demethylation. P450-2C19 and -3A may make a further small contribution, but P450-2D6 is unlikely to make an important contribution.

Key words Fluoxetine · *N*-Demethylation · Cytochromes · Microsomes · Human

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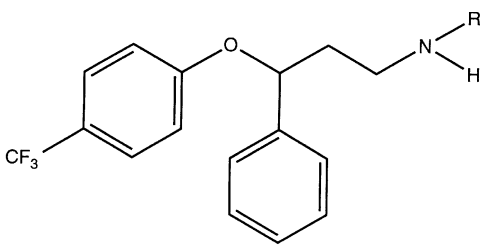
Introduction

The selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine is extensively prescribed in clinical practice throughout the world (Sommi et al. 1987; Song et al. 1993; Gram 1994; Wong et al. 1995). Biotransformation of fluoxetine proceeds by *N*-demethylation to yield norfluoxetine (Fig. 1), a pharmacologically active metabolite having an elimination half-life considerably longer than that of the parent compound (deVane 1992; van Harten 1993; Altamura et al. 1994). As a consequence of their kinetic properties, both fluoxetine and norfluoxetine accumulate extensively during chronic treatment with fluoxetine in humans.

The capacity of fluoxetine and its principal metabolite, norfluoxetine, to inhibit the activity of human



R=CH_3 Fluoxetine
R=H Norfluoxetine

Fig. 1 Structure of fluoxetine and its principal metabolite, norfluoxetine

drug-metabolizing cytochrome P450 enzymes is documented in considerable experimental and clinical literature (Ciraulo and Shader 1990; Ketter et al. 1995; Greenblatt et al. 1996; Harvey and Preskorn 1996). However, the cytochrome or cytochromes mediating *N*-demethylation of fluoxetine itself are not established. The present study evaluated the kinetic features of fluoxetine *N*-demethylation by human liver microsomes in vitro, as well as inhibition of this process by a series of chemical probes. Also studied was biotransformation of fluoxetine by microsomes containing individual specific human cytochromes as expressed by cDNA-transfected human lymphoblastoid cells.

Materials and methods

In vitro incubation procedures

Liver samples from human donors with no known liver disease were provided by the International Institute for the Advancement of Medicine, Exton, Pa., or the Liver Tissue Procurement and Distribution System, University of Minnesota, Minneapolis, Minn. Microsomes were prepared by ultracentrifugation; microsomal pellets were suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −80°C until use. Microsomes containing individual human cytochromes expressed by cDNA-transfected human lymphoblastoid cells (Gentest, Woburn, Mass., USA) were similarly stored at −80°C until use. Chemical reagents and drug entities were purchased from commercial sources or kindly provided by their pharmaceutical manufacturers.

Incubation mixtures contained 50 mM phosphate buffer, 5 mM Mg^{2+}, 0.5 mM $NADP^{+}$, and an isocitrate/isocitric dehydrogenase regenerating system (von Moltke et al. 1994 a,b, 1995a, 1996 a,b,c,d; Schmider et al. 1995, 1996). Varying quantities of *R,S*-fluoxetine in methanol solution, to yield final incubate concentrations ranging from 0 to 250 μM, were added to a series of incubation tubes. The solvent was evaporated to dryness at 40°C under conditions of mild vacuum. Incubations were also performed with co-addition of three concentrations of several potential metabolic inhibitors (ketoconazole, quinidine, sulfaphenazole, and fluvoxamine), each at four different substrate concentrations. Reactions were initiated by addition of microsomal protein (up to 1.0 mg/ml). After 40 min at 37°C, reactions were stopped by cooling on ice and addition of 100 μl acetonitrile. Desipramine was added as internal standard, the incubation mixture was centrifuged, and the supernatant transferred to an autosampling vial for HPLC analysis. The mobile phase consisted of 35% acetonitrile, and 65% 50 mM phosphate buffer; the flow rate was 1.5 ml/min. The analytical column was stainless steel, 30 cm × 3.9 mm, containing reverse-phase C-18 microBondapak (Waters Associates, Milford, Mass., USA). Column effluent was monitored by ultraviolet absorbance at 226 nm (Fig. 2). Concentrations of norfluoxetine in reaction mixtures were determined based on calibration curves constructed from a series of standards containing varying known amounts of norfluoxetine together with internal standard. The rate of formation of norfluoxetine was linear with respect to time (through 50 min) and protein concentration (through 2.0 mg/ml). Reaction velocities were calculated in units of nanomol norfluoxetine formed per minute per mg microsomal protein.

Further studies were performed using fixed concentrations of *R,S*-fluoxetine (100 μM). In one series, varying concentrations of a number of possible inhibitors (ketoconazole, quinidine, sulfaphenazole, α-naphthoflavone, and fluvoxamine) were co-added. Reactions were initiated by addition of human microsomal protein, and mixtures were processed as described above. Inhibition studies of triacetyloleandomycin (TAO), furafylline, and diethyldithiocarbamate (DDC) were similarly performed, except that inhibitors were preincubated with microsomal protein and cofactors for 20 min and reactions were initiated by addition of fluoxetine. In a second series of studies, the same fixed concentration (100 μM) of fluoxetine was incubated with microsomes containing individual human cytochromes P450-1A2, 2C9, 2C19, 2D6, 2E1, and 3A4 at a concentration of 1 mg microsomal protein per ml incubation mixture. No inhibitors were used in these studies.

In three liver samples, separate incubations were performed (without inhibitors) using varying concentrations of the *R*- and *S*-isomers of fluoxetine (kindly provided by Sepracor, Marlboro, Mass., USA).

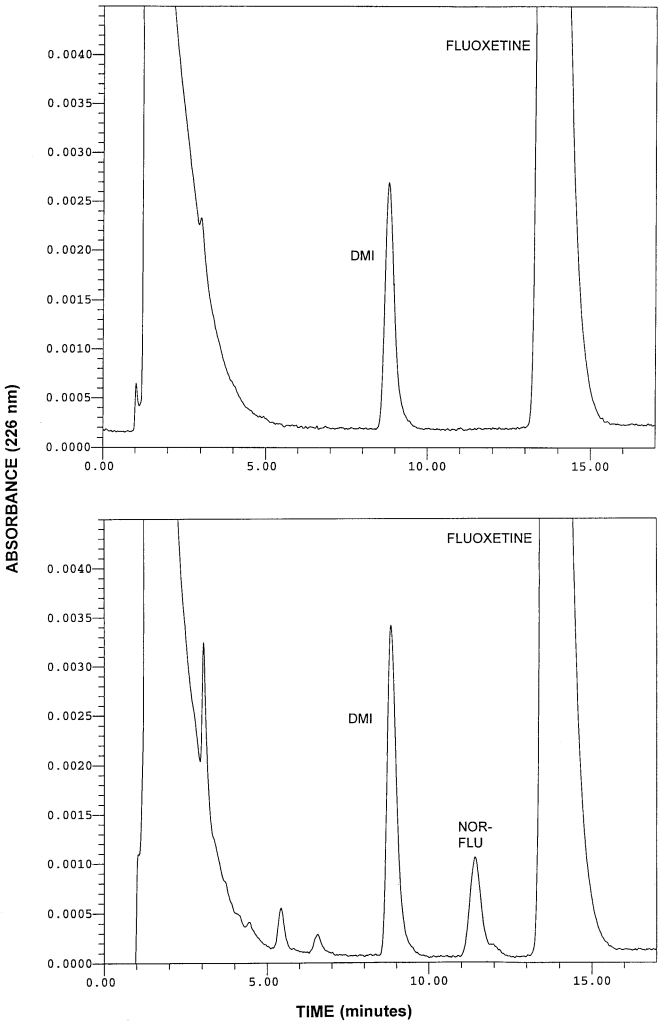


Fig. 2 *Above*: high-performance liquid chromatographic tracing of a control incubation of the substrate (*R,S*-fluoxetine) and cofactors without microsomal protein. The internal standard desipramine (*DMI*) has been added. *Below*: Incubation containing substrate, cofactors, and microsomal protein from a representative human liver. Norfluoxetine (*NOR-FLU*) is the product

Data analysis

When plots of reaction velocity versus substrate concentration in the absence of inhibitor were consistent with single-enzyme Michaelis-Menten kinetics, the following equation was fitted to

the data points using derivative-free nonlinear least squares regression:

$$V = \frac{V_{max} \cdot S}{S + K_m} \quad \text{(Eq. 1)}$$

where V is the velocity of formation of norfluoxetine corresponding to S, the concentration of substrate (fluoxetine). Iterated variables were V_{max}, the maximum reaction velocity, and K_m, the substrate concentration corresponding to 50% of V_{max}.

When plots of V versus S indicated diminishing reaction velocities at high substrate concentrations, the following equation was fitted to data points:

$$V = \frac{V_{max} \cdot S}{K_m + S\left(1 + \frac{S}{K_s}\right)} \quad \text{(Eq. 2)}$$

This is consistent with Michaelis-Menten kinetics and uncompetitive substrate inhibition (Segel 1975; Schmider et al. 1996; von Moltke et al. 1996d). V, S, V_{max}, and K_m have the same meaning as in Eq. 1; K_s is the substrate inhibition constant.

When norfluoxetine formation in the absence of inhibitor was consistent with Eq. 1, formation rates with coaddition of inhibitors were analyzed by nonlinear regression using the following equation, consistent with Michaelis-Menten kinetics and competitive inhibition:

$$V = \frac{V_{max} \cdot S}{S + K_m\left(1 + \frac{I}{K_i}\right)} \quad \text{(Eq. 3)}$$

where I is the concentration of inhibitor and K_i is the competitive inhibition constant. When control data were consistent with Eq. 2, norfluoxetine formation rates with coaddition of inhibitors were analyzed using the following equation, consistent with uncompetitive substrate inhibition and competitive exogenous inhibition (von Moltke et al. 1996d):

$$V = \frac{V_{max} \cdot S}{K_m\left(1 + \frac{I}{K_i}\right) + S\left(1 + \frac{S}{K_s}\right)} \quad \text{(Eq. 4)}$$

For studies of fluoxetine fixed at 100 μM, reaction velocities at varying concentrations of each inhibitor were expressed as a percentage of the control velocity with no inhibitor present. For studies of individual human cytochromes from cDNA transfected cells, relative rates of norfluoxetine formation were compared after normalization for molar quantities of cytochrome present.

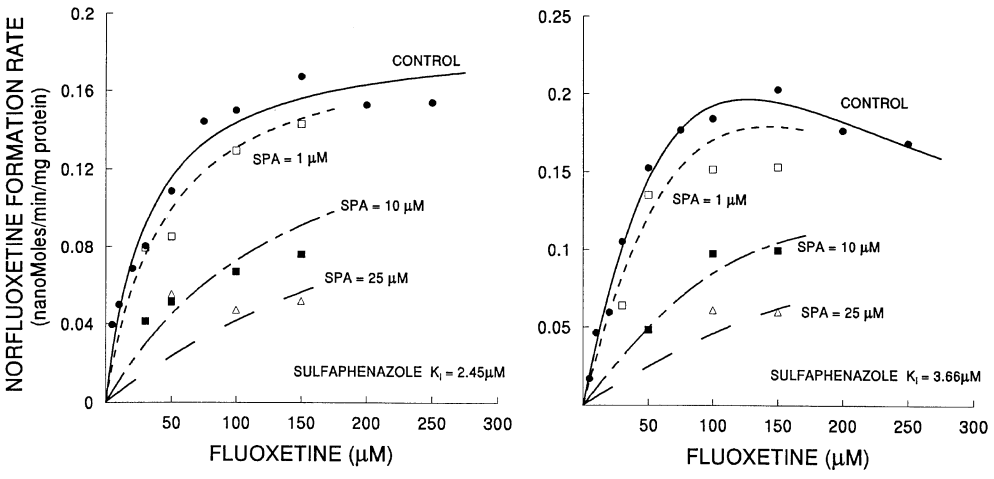
Results

Formation of norfluoxetine from *R*,*S*-fluoxetine was consistent with Eq. 1 in three incubation trials, and Eq. 2 in five others (Fig. 3). The overall mean (± SE) K_m value was 33(± 7) μM, while K_s averaged 556 (± 109) μM for the trials consistent with Eq. 2. When *R*- and *S*- fluoxetine were incubated separately, the K_m value for the *R*-isomer (mean: 33 μM) was very close to the K_m value for the racemic mixture, and was always lower than for the *S*-isomer (mean: 121 μM), indicating a higher apparent enzyme affinity for *R*-fluoxetine.

Both sulfaphenazole and ketoconazole, inhibitory chemical probes for cytochromes P450-2C9 and -3A, respectively (Baldwin et al. 1995), produced substantial inhibition of norfluoxetine formation. Calculation of competitive inhibition K_i values, using either Eqs. 3 or 4 as appropriate, yielded a mean K_i of 2.8(± 0.4) μM (n = 3) for sulfaphenazole (Fig. 3), 2.5 (± 0.5) μM for ketoconazole (n = 8), and 5.2(± 1.5) μM for fluvoxamine (n = 3). Quinidine, an inhibitory probe for P450-2D6, was a weak inhibitor, with a mean K_i of 94(± 20) μM (n = 5).

Studies of norfluoxetine formation at fixed concentrations of fluoxetine confirmed that quinidine was a very weak inhibitor, reducing reaction velocities by no more than 25% compared to control even at quinidine concentrations of 100 μM (Fig. 4). Furafylline and

Fig. 3 Rates of norfluoxetine formation in relation to concentrations of the substrate, *R*,*S*-fluoxetine. *Closed circles* represent control incubations, without coaddition of inhibitor. *Solid lines* were determined by nonlinear regression analysis, based on Eq. 1 (*left*) or Eq. 2 (*right*). Also shown are reaction velocities with coaddition of sulfaphenazole (SPA) at concentrations of 1, 10, or 25 μM. *Dashed lines* and K_i values for sulfaphenazole were determined by nonlinear regression analysis using functions consistent with Eq. 3 (*left*) or Eq. 4 (*right*)



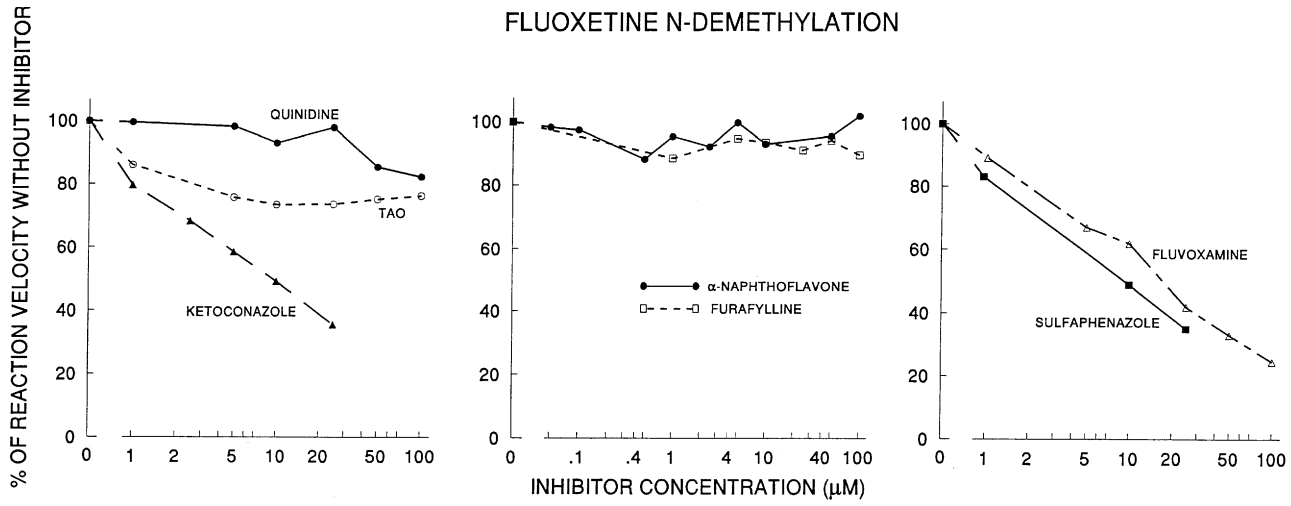


Fig. 4 Rate of formation of norfluoxetine from *R,S*-fluoxetine in relation to concentration of a number of co-added inhibitors. The fluoxetine concentration was fixed at 100 μM. Velocities with inhibitor present are expressed as a percentage of the control velocity with no inhibitor. Each point represents the mean of up to 11 determinations. *TAO* triacetyloleandomycin

α-naphthoflavone, commonly used as inhibitory chemical probes for cytochrome P450-1A2, likewise produced minimal inhibition of norfluoxetine formation, as did DDC, an inhibitory probe for P450-2E1 (data not shown). At 5 μM, TAO (P450-3A inhibitory probe) reduced the velocity of norfluoxetine formation by 23(± 7)% compared to control (n = 4), with no further inhibition at higher concentrations of TAO. Sulfaphenazole, ketoconazole, and fluvoxamine all produced concentration-dependent inhibition of norfluoxetine formation. Estimated 50% inhibitory concentrations (IC_{50}) were: 9.0 μM for sulfaphenazole, 10.0 μM for ketoconazole, and 15.0 μM for fluvoxamine.

Microsomes containing individual human cytochromes P450-2C9, -2C19, and -2D6 all produced detectable amounts of norfluoxetine when incubated with 100 μM fluoxetine. Relative normalized activities among these three were: 57% for 2C9, 34% for 2C19, and 9% for 2D6. Detectable amounts of norfluoxetine were not produced by cDNA-expressed cytochromes P450-3A4, -1A2, or -2E1.

Discussion

Formation of norfluoxetine from *R,S*-fluoxetine by human liver microsomal preparations was consistent with Michaelis-Menten kinetics, with a mean K_m of 33 μM. In five of eight incubation trials, reaction velocities diminished at higher substrate concentrations. Although the mechanism of this phenomenon is not established, the pattern has been observed in studies of α-OH-midazolam formation from midazolam (Kronbach et al. 1989; Ghosal et al. 1996; von Moltke et al. 1996d), nortriptyline formation from amitriptyline (Schmider et al. 1995, 1996), and phenytoin para-hydroxylation (Schmider et al. 1997), as well as formation of norfluoxetine from *R*- or *S*-fluoxetine (Stevens and Wrighton 1993), and is mathematically consistent with uncompetitive substrate inhibition. The appropriate model (Eq. 2) was applied in these instances, yielding a mean substrate inhibition constant of 556 μM. It is not clear why this pattern was evident in some incubation trials and not others. Separate incubations of the *R*- and *S*-isomers of fluoxetine indicated higher apparent enzyme affinity of *R*-fluoxetine, consistent with previous in vitro observations (Stevens and Wrighton 1993), and with clinical studies showing higher oral clearance of *R*-fluoxetine, compared to *S*-fluoxetine (Peyton et al. 1991). However, we were not able to evaluate the possible interaction between the *R*- and *S*- isomers of fluoxetine themselves.

Fluoxetine is reported to have nonlinear kinetics during chronic therapy in humans, with steady-state plasma concentrations of the parent drug (but not of the metabolite) increasing disproportionately with daily dosage (Bergstrom et al. 1986). Findings from the present study could in part explain these clinical observations, since reported steady-state plasma levels of fluoxetine with daily doses of 20–60 mg (approximate range: 50–450 ng/ml) translate to intrahepatic concentrations ranging from 2 to 17 μM after accounting for extensive partitioning between plasma and liver tissue (von Moltke et al. 1994a, 1996a; Greenblatt et al. 1996). This approaches the mean K_m for fluoxetine in vitro.

The possibility that cytochrome P450-2D6 may contribute importantly to the biotransformation of fluoxetine to norfluoxetine in humans has been suggested by cosegregation studies, in which apparent clearance of fluoxetine was lower in individuals phenotyped to be "poor" metabolizers via P450-2D6 as compared to "extensive" metabolizers (Hamelin et al.

1996). However, Stevens and Wrighton (1993) reported that in vitro biotransformation of both *R*- and *S*-fluoxetine to norfluoxetine was incompletely correlated with immunodetectable P450-2D6, and was minimally inhibited by coincubation with quinidine or with antibodies inhibitory for 2D6. Otton et al. (1993) also reported preliminary in vitro results indicating that 2D6 does not mediate fluoxetine *N*-demethylation. The present study likewise indicated that quinidine has very little inhibitory effect on *N*-demethylation of racemic fluoxetine by human liver microsomes. Microsomes containing pure human P450-2D6 had low but measurable fluoxetine *N*-demethylation activity in vitro; this is unlikely to be of importance in vivo in view of the low abundance of P450-2D6 in human liver (Shimada et al. 1994; von Moltke et al. 1995b), and the lack of inhibition by quinidine in human liver microsomal preparations.

Important participation of P450-1A2 and 2E1 in fluoxetine *N*-demethylation is essentially excluded by the lack of inhibition by furafylline, α-naphthoflavone, and diethyldithiocarbamate (Newton et al. 1995; Bourrié et al. 1996; Ono et al. 1996), and by the lack of detectable *N*-demethylation activity by the corresponding individual cytochromes. TAO in concentrations of 5 μM and greater produced an approximately 25% reduction in the norfluoxetine formation rate. Ketoconazole was also an inhibitor of norfluoxetine formation, with a mean competitive K_i of 2.6 μM; this is considerably higher than K_i values for ketoconazole versus "pure" P450-3A substrates such as triazolam, midazolam, and alprazolam (mean $K_i < 0.1$ μM). At 1.0 μM, a concentration at which ketoconazole is a relatively specific 3A inhibitor, only approximately 20% inhibition was produced. The findings are consistent with a small contribution (20–25%) of P450-3A isoforms to fluoxetine *N*-demethylation. Although microsomes from cDNA-transfected cells expressing P450-3A4 did not produce detectable amounts of norfluoxetine, a low level of fluoxetine *N*-demethylation that is not detectable using these preparations in vitro may still produce a small net contribution to clearance in vivo due to the high abundance of P450-3A isoforms in human liver.

Cytochrome P450-2C isoforms apparently account for the major component of fluoxetine *N*-demethylation. Biotransformation by human microsomes was highly sensitive to inhibition by sulfaphenazole, and cDNA-expressed P450-2C9 was the most active relative contributor. A contribution of P450-2C19 was also evident, although the inhibitory activity of sulfaphenazole – relatively specific for 2C9 (Baldwin et al. 1995; Newton et al. 1995; Bourrié et al. 1996; Ono et al. 1996) – suggests that P450-2C9 is most important in vivo.

We evaluated fluvoxamine as a potential inhibitor of fluoxetine *N*-demethylation, since fluvoxamine has been proposed as a relatively specific index inhibitor of P450-1A2 (Brøsen et al. 1993; Jeppesen et al. 1996b). Fluvoxamine is a potent inhibitor of P450-1A2 (Brøsen et al. 1993; Jeppesen et al. 1996a,b; von Moltke et al. 1996b), but also is a moderately strong inhibitor of P450-3A isoforms (von Moltke et al. 1995a, 1997) as well as 2C9 (Schmider et al. 1997) and 2C19 (Jeppesen et al. 1996a; Xu et al. 1996). Fluvoxamine clearly inhibited fluoxetine *N*-demethylation in the present study, but this effect must be due to actions on cytochromes other than 1A2. Thus fluvoxamine is not of definitive value as an index inhibitor of P450-1A2, or of any other cytochrome.

In summary, formation of norfluoxetine from fluoxetine in humans appears to be mediated primarily by P450-2C9, with a possible contribution of -2C19 and -3A isoforms. P450-2D6 is not likely to contribute significantly. The mechanism of the association of poor 2D6 metabolizer phenotype with low clearance of fluoxetine in vivo (Hamelin et al. 1996) is not established, but the association does not by itself verify a role of P450-2D6 in fluoxetine clearance. This may reflect a chance association, or a linkage of poor 2D6 metabolizer phenotype with reduced activity or quantity of P450-2C9. Confirmation or exclusion of a role of P450-2D6 in the clearance of fluoxetine in vivo will require assessment of the effects of coadministration of a specific 2D6 inhibitor such as quinidine (Zhang et al. 1992; Schadel et al. 1995).

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