CYP3A4 IS THE MAJOR CYP ISOFORM MEDIATING THE IN VITRO HYDROXYLATION AND DEMETHYLATION OF FLUNITRAZEPAM

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ABSTRACT:

The kinetics of flunitrazepam (FNTZ) N-demethylation to desmethylflunitrazepam (DM FNTZ), and 3-hydroxylation to 3-hydroxyflunitrazepam (3-OH FNTZ), were studied in human liver microsomes and in microsomes containing heterologously expressed individual human CYPs. FNTZ was N-demethylated by cDNA-expressed CYP2A6 (Km = 1921 μM), CYP2B6 (Km = 101 μM), CYP2C9 (Km = 50 μM), CYP2C19 (Km = 60 μM), and CYP3A4 (Km = 155 μM), and 3-hydroxylated by cDNA-expressed CYP2A6 (Km = 298 μM) and CYP3A4 (Km = 286 μM). The 3-hydroxylation pathway was predominant in liver microsomes, accounting for more than 80% of intrinsic clearance compared with the N-demethylation pathway. After adjusting for estimated relative abundance, CYP3A accounted for the majority of intrinsic clearance via both pathways. This finding was supported by chemical inhibition studies in human liver microsomes. Formation of 3-OH FNTZ was reduced to 10% or less of control values by ketoconazole (IC50 = 0.11 μM) and ritonavir (IC50 = 0.041 μM). Formation of DM FNTZ was inhibited to 40% of control velocity by 2.5 μM ketoconazole and to 30% of control by 2.5 μM ritonavir. Neither 3-OH FNTZ nor DM FNTZ formation was inhibited to less than 85% of control activity by α-naphthoflavone (CYP1A2), sulfaphenazole (CYP2C9), omeprazole (CYP2C19), or quinidine (CYP2D6). Thus, CYP-dependent FNTZ biotransformation, like that of many benzodiazepine derivatives, is mediated mainly by CYP3A. Clinical interactions of FNTZ with CYP3A inhibitors can be anticipated.

Flunitrazepam (FNTZ) is a 7-nitrobenzodiazepine having a high affinity for the benzodiazepine receptor (Mattila and Larni, 1980). In 1- to 2-mg doses, it is used to treat insomnia and as a preoperative sedative (Mattila and Larni, 1980). Like other benzodiazepines, FNTZ induces muscle relaxation, drowsiness, and slowed motor skills (Woods and Winger, 1997). FNTZ has also received attention as a drug of abuse (Saum and Inciardi, 1997; Woods and Winger, 1997). Flunitrazepam (FNTZ) is a 7-nitrobenzodiazepine having a high affinity for the benzodiazepine receptor (Mattila and Larni, 1980). In 1- to 2-mg doses, it is used to treat insomnia and as a preoperative sedative (Mattila and Larni, 1980). Like other benzodiazepines, FNTZ induces muscle relaxation, drowsiness, and slowed motor skills (Woods and Winger, 1997). FNTZ has also received attention as a drug of abuse (Saum and Inciardi, 1997; Woods and Winger, 1997; Simmons and Cupp, 1998).

Three main in vivo metabolites of FNTZ are 7-aminoflunitrazepam (7A FNTZ), desmethylflunitrazepam (DM FNTZ), and 3-hydroxyflunitrazepam (3-OH FNTZ) (Fig. 1) (Woods and Winger, 1997). It has been suggested that DM FNTZ is active (Berthault et al., 1996), and the main urinary metabolite that is also found in plasma, 7A FNTZ, can have anesthetic activity in animals (Kortilla and Linnoila, 1976). Receptor binding studies indicate that FNTZ and DM FNTZ have similar benzodiazepine receptor affinity, whereas 7A FNTZ has an order of magnitude lower affinity (Haefely et al., 1985). The binding affinity and pharmacologic activity of 3-OH FNTZ are not established.

Coller et al. (1998) reported that only DM FNTZ and 3-OH FNTZ are formed in vitro by the cytochrome P450 (CYP) enzyme system, and that CYP2C19, CYP3A4, and CYP1A2 may be involved in the formation of these metabolites, depending on the solvent (acetonitrile or dimethylformamide) used in the incubation mixture (Coller et al., 1999). The outcome of in vitro metabolic studies may be influenced by the addition of a solvent to the incubation medium, since various solvents may inhibit or activate CYPs (Cotreau-Bibbo et al., 1996; Chauret et al., 1998; Hickman et al., 1998; Busby et al., 1999; Tang et al., 2000). The objective of our study was to investigate the in vitro metabolism of FNTZ to DM FNTZ and 3-OH FNTZ in human liver microsomes and heterologously expressed human CYPs without using a solvent in the incubation buffer. We aimed to identify and kinetically characterize the CYP isoforms responsible for the N-demethylation and 3-hydroxylation of FNTZ. Our results indicate that CYP3A4 is the primary enzyme mediating DM FNTZ and 3-OH FNTZ formation.

Experimental Procedures

Materials. FNTZ was purchased from Research Biochemicals International (Natick, MA). DM FNTZ and 3-OH FNTZ were kindly provided by Hoffmann-La Roche (Nutley, NJ). 7A FNTZ was obtained from Janssen Research Foundation (Beerse, Belgium). Ritonavir was extracted from its commercial dosage form via methanol; based on comparison with a pure standard, extraction recovery was verified to be complete. The cofactors...
Fig. 1. The chemical structures of FNTZ and its main in vivo metabolites: 3-hydroxyflunitrazepam, 7-aminoflunitrazepam, and desmethylflunitrazepam.

Fig. 2. A representative HPLC chromatogram of in vitro biotransformation of FNTZ. If present, 7A FNTZ would have a retention time of 13 min. DM, desmethylflunitrazepam; 3OH, 3-hydroxyflunitrazepam; IS, internal standard (phenacetin).
 Liver samples, obtained from the International Institute for the Advancement of Medicine (Exton, PA) or the Liver Tissue Procurement and Distribution Service (University of Minnesota, Minneapolis, MN), were from 13 different transplant donors with no known liver disease, and all were CYP2C19 extensive metabolizers. The donor population (median age: 27 years; range: 3–50 years) was unselected with respect to sex, race, smoking habits, and

**Fig. 3.** FNTZ biotransformation by a representative human liver sample.

A, velocity of formation of DM FNTZ and 3-OH FNTZ versus the concentration of FNTZ for one representative liver (HLM4). (Velocity units = pmol of metabolite formed/min/mg of protein). Symbols are experimental data points, representing an average of duplicate incubations. Lines are fitted functions described by a Hill equation (eq. 2). See Table 1 for kinetic parameters. B, Eadie-Hofstee plots for velocity (formation of DM FNTZ or 3-OH FNTZ) versus the velocity/concentration of FNTZ for the same liver sample (HLM4). Symbols are experimental data points, representing an average of duplicate incubations. HLM, human liver microsomes.

NADP⁺, (±)-isocitric acid, MgCl₂, and isocitrate dehydrogenase were purchased from Sigma.

Liver samples, obtained from the International Institute for the Advancement of Medicine (Exton, PA) or the Liver Tissue Procurement and Distribution Service (University of Minnesota, Minneapolis, MN), were from 13 different transplant donors with no known liver disease, and all were CYP2C19 extensive metabolizers. The donor population (median age: 27 years; range: 3–50 years) was unselected with respect to sex, race, smoking habits, and
alcohol consumption (Venkatkrishnan et al., 1998a). The tissue was partitioned and kept at −80°C until the time of microsomal preparation as described previously (von Molkite et al., 1993, 1994).

Microsomes from cDNA-transfected human lymphoblastoid cells expressing CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, -3A4, or transfected with an expression vector without cDNA as a control (Crespi, 1995), or CYP2C19 Supersomes (baculovirus insect cell-expressed CYP) were purchased from GEN-TEST Corporation (Woburn, MA). The transfected microsomes were aliquoted, stored at −80°C, and thawed on ice before use. Microsomal protein concentrations and CYP contents were provided by the manufacturer.

Incubations with Human Liver Microsomes. Incubation mixtures contained 50 mM potassium phosphate buffer (pH 7.5), 400 μg ml⁻¹ β-NADPH, 0.32 μl ml⁻¹ isocitrate dehydrogenase, 475 μg ml⁻¹ MgCl₂, 1 mg ml⁻¹ isocitrate, and substrate with or without inhibitor. Solutions of FNTZ, DM FNTZ, 3-OH FNTZ, and the inhibitors were prepared in methanol. The drug in solvent was added to an incubation tube, and the solvent was evaporated to dryness in a 40°C vacuum oven before addition of cofactors.

Substrate, inhibitors, incubation buffer, and cofactors were warmed to 37°C, and the reactions were initiated by the addition of microsomal protein. Final incubation volumes were 0.25 ml, with a human liver microsomal protein concentration of 0.25 mg ml⁻¹. Incubations were performed in a shaking water bath for 20 min at 37°C and then stopped by the addition of 100 μl of acetonitrile and cooling on ice. Phenacetin was used as an internal standard, and the mixture was spun at 16,000g for 10 min. Supernatants were injected into the HPLC. All incubations with human liver microsomes were done in duplicate using four different human livers (HLM1–4).

Full kinetic curves were generated for DM FNTZ and 3-OH FNTZ formation with 0 to 950 μM FNTZ. An inhibition screen was performed by co-incubating inhibitor [α-naphthoflavone, 1 μM (Newton et al., 1995); sulfaphenazole, 10 μM (Ono et al., 1996); omeprazole, 10 μM (Ko et al., 1997); quinidine, 5 μM, (Newton et al., 1995); and ketoconazole, 1 μM (Bouriri et al., 1996)] with 100 μM FNTZ. Inhibition of 3-OH FNTZ and DM FNTZ formation was studied with varying concentrations of ketoconazole or ritonavir (0–2.5 μM) using a fixed concentration (100 μM) of FNTZ.

**Incubations with cDNA-Expressed Microsomes and CYP2C19 Supersomes.** A screen was performed by incubating microsomes from human lymphoblastoid cells expressing CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, -3A4, cells containing only vector, or CYP2C19 Supersomes with 95 or 950 μM FNTZ. These incubations were performed as described above with a protein concentration of 1 mg ml⁻¹ and with minimal agitation as recommended by the supplier. Incubations using cDNA-expressed CYPs were done in single, due to a limited supply of microsomes. The incubation of FNTZ with CYP2C19 Supersomes was performed using 20 pmol of CYP2C19. Reactions were stopped and products were assayed in incubates using methods identical to those used with human liver microsomal protein. Metabolite formation rates at 95 μM FNTZ were normalized to pmol of CYP and expressed as a ratio relative to the rate for CYP3A4. Full kinetic curves for DM FNTZ formation were generated with CYP2A6, -2B6, -2C9, -2C19, and -3A4, and kinetic curves for 3-OH FNTZ formation were generated with CYP2A6 and -3A4, at a concentration range of 0 to 950 μM FNTZ.

**Analysis of DM FNTZ and 3-OH FNTZ by HPLC.** Concentrations of DM FNTZ or 3-OH FNTZ were determined by HPLC. Ultraviolet detection at 250 nm was used to analyze samples from the chemical inhibition screen and samples from the IC₅₀ curves with inhibition by ketoconazole (Waters Associates, Milford, MA). Since DM FNTZ had greater extinction at 215 than at 250 nm, HPLC with ultraviolet detection at 215 nm was used to analyze samples to generate kinetic curves with human liver microsomes, IC₅₀ curves with inhibition by ritonavir, and kinetic curves using cDNA-expressed CYPs. A 3.9-× 150-mm Waters Spherisorb column was used to separate samples for the kinetic curves using human liver microsomes and the IC₅₀ curves of inhibition by ritonavir. A 150-× 4.6-mm Altech (Deerfield, IL) cyanobonded Waters Spherisorb column was used for separation of samples from the chemical inhibitor screen, the IC₅₀ curves for inhibition by ketoconazole, and the kinetic curves from the cDNA-expressed CYPs because this column gave better resolution and sharper peaks. A flow rate of 1.3 ml min⁻¹ was used for all analyses. The mobile phase consisted of 96% 10 mM KH₂PO₄ and 4% acetonitrile. Standard curves were prepared by adding incubation buffer and internal standard to different amounts of DM FNTZ (0–800 ng) and 3-OH FNTZ (0–720 ng) to yield a final volume of 0.25 ml. Chromatograms were analyzed by measuring peak heights, using the internal standard method. A sample chromatogram is shown in Fig. 2. The limit of detection of DM FNTZ and of 3-OH FNTZ was 10 ng per sample.

**Data Analysis.** All reaction velocities were based on a 20-min incubation, which falls within the linear time period of metabolite formation. Rates were expressed in picomoles per minute per milligram of protein (in studies using human liver microsomes) or picomoles per minute per picomole CYP (in studies using heterologously expressed CYPs). The kinetics of the biotrans-

### TABLE 1

*Kinetics of FNTZ biotransformation in human liver microsomes*

<table>
<thead>
<tr>
<th>Liver</th>
<th>DM FNTZ Formation</th>
<th>3-OH FNTZ Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vₘₐₓ</td>
<td>Kₘₒ</td>
</tr>
<tr>
<td>HLM1</td>
<td>308.7</td>
<td>148.8</td>
</tr>
<tr>
<td>HLM2</td>
<td>307.2</td>
<td>288.0</td>
</tr>
<tr>
<td>HLM3</td>
<td>371.7</td>
<td>222.9</td>
</tr>
<tr>
<td>HLM4</td>
<td>927.8</td>
<td>170.8</td>
</tr>
<tr>
<td>Mean*</td>
<td>478.9</td>
<td>207.6</td>
</tr>
<tr>
<td>S.E.</td>
<td>150.4</td>
<td>31.0</td>
</tr>
</tbody>
</table>

HLM, human liver microsomes.

*Vₘₐₓ in units of pmol min⁻¹ (mg protein)⁻¹.

Kₘₒ in units of μM.

Aᵢ, Hill coefficient.

Vₘₐₓ/Kₘₒ, approximate intrinsic clearance, in units of μl min⁻¹ (mg protein)⁻¹.

Mean, arithmetic mean of four different human livers (HLM1–4).

S.E., standard error of four different human livers (HLM1–4).

### TABLE 2

*Relative rates of DM FNTZ formation by cDNA-expressed CYPs and CYP2C19 Supersomes*

<table>
<thead>
<tr>
<th>CYP</th>
<th>Relative Rate/pmol CYP*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95 μM FNTZ</td>
</tr>
<tr>
<td>Vector</td>
<td>N.D.</td>
</tr>
<tr>
<td>A2</td>
<td>0.11</td>
</tr>
<tr>
<td>2A6</td>
<td>0.08</td>
</tr>
<tr>
<td>B2c</td>
<td>0.20</td>
</tr>
<tr>
<td>C28</td>
<td>N.D.</td>
</tr>
<tr>
<td>C29</td>
<td>0.47</td>
</tr>
<tr>
<td>C29*</td>
<td>0.81</td>
</tr>
<tr>
<td>C219 (Supersomes)</td>
<td>0.87</td>
</tr>
<tr>
<td>D6</td>
<td>N.D.</td>
</tr>
<tr>
<td>E2</td>
<td>N.D.</td>
</tr>
<tr>
<td>A4</td>
<td>1.00</td>
</tr>
</tbody>
</table>

N.D., no DM FNTZ detected.

*Relative rate/pmol CYP = normalized to rate of CYP3A4.

*DM FNTZ formation detected, metabolite formation rate could not be determined since vector CYP protein content is minimal and amounts are not available.
where \( K \) equals 50% of the maximal reaction velocity (\( V_{\text{max}} \)), and \( V \) is the reaction velocity. A, velocity of formation of DM FNTZ by microsomes from lymphoblastoid cells expressing CYP2A6, -2B6, -2C9, -2C19, and -3A4. (Velocity units = pmol of DM FNTZ formed/min/pmol of CYP.) B, velocity of formation of 3-OH FNTZ by microsomes from lymphoblastoid cells expressing CYP2A6 and CYP3A4. (Velocity units = pmol 3-OH FNTZ formed/min/pmol CYP.) See Table 3 for kinetic parameters. Symbols are experimental data points from single incubations.

The substrate concentration-velocity relationship for a hepatic drug biotransformation process catalyzed by multiple CYPs can in principle be described as a linear combination of velocity functions for each of the CYP isoforms \( v_i(s) \) weighted for the relative abundance of the respective isoforms \( (A_i) \) in hepatic tissue:

\[
V(s) = \sum_{i=1}^{n} A_i v_i(s). \tag{4}
\]

The relative contribution of each isoform can then be determined as follows:

\[
f_j(\%) = \frac{\sum_{i=1}^{n} A_i v_i(s)}{\sum_{i=1}^{n} A_i} \times 100. \tag{5}
\]

As reviewed previously (Crespi, 1995; Venkatakrishnan et al., 1998b), RAFs are determined for specific CYP isoforms by comparing the rate of an isoform-specific index reaction at saturating substrate concentrations in human liver microsomal preparations (that is, \( V_{\text{max}} \) for liver microsomes) with the rate of the same reaction catalyzed by the specific cDNA-expressed CYP isoform measured under identical conditions (that is, \( V_{\text{max}} \) for cDNA expressed enzyme):

\[
\text{RAFisoform} = \frac{V_{\text{max}}\text{ for isoform-specific reaction in human liver microsomes}}{V_{\text{max}}\text{ for isoform-specific reaction by cDNA-expressed isoform}}. \tag{6}
\]

All RAF estimates were determined for a panel of 12 human livers, and the median value was used in simulation analyses. For determination of CYP2B6, -2C9, -2C19 and CYP3A RAFs, bupropion hydroxylation (Hesse et al., 2000), flurbiprofen 4'-hydroxylation (Tracy et al., 1996), S-mephentoin 4'-hydroxylation (Venkatakrishnan et al., 1998b), and triazolam 1-hydroxylation (von Moltke et al., 1996) were used as index reactions, respectively. Standard nonlinear regression-based enzyme kinetic methods were used in the determination of \( V_{\text{max}} \) values for S-mephentoin 4'-hydroxylation both for human liver microsomes and lymphoblast-expressed CYP2C19. RAF estimates were then calculated using eq. 6. The determination of CYP2C19 RAFs in this panel of human livers has been described previously (Venkatakrishnan et al., 1998b).

### Table 3

**Kinetics of FNTZ biotransformation in cDNA-expressed enzymes**

<table>
<thead>
<tr>
<th>CYP</th>
<th>DM FNTZ Formation</th>
<th>3-OH FNTZ Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} )</td>
<td>( K_m )</td>
</tr>
<tr>
<td>2A6</td>
<td>1.00</td>
<td>1920.5</td>
</tr>
<tr>
<td>2B6*</td>
<td>0.27</td>
<td>100.7</td>
</tr>
<tr>
<td>2C9</td>
<td>0.38</td>
<td>50.2</td>
</tr>
<tr>
<td>2C19</td>
<td>0.83</td>
<td>59.6</td>
</tr>
<tr>
<td>3A4</td>
<td>1.54</td>
<td>154.6</td>
</tr>
</tbody>
</table>

* \( V_{\text{max}} \) in units of pmol min\(^{-1}\) (pmol CYP\(^{-1}\)).
* \( K_m \) in units of \( \mu \)M.
* Hill coefficient.
* \( V_{\text{max}}/K_m \) approximate intrinsic clearance, in units of nl min\(^{-1}\) (pmol CYP\(^{-1}\)).
* 2B6, fit to eq. 3, \( z = 0.0002 \).

### Fig. 4

**FNTZ biotransformation by expressed CYPs.**

A, velocity of formation of DM FNTZ by microsomes from lymphoblastoid cells expressing CYP2A6, -2B6, -2C9, -2C19, and -3A4. (Velocity units = pmol of DM FNTZ formed/min/pmol of CYP.) B, velocity of formation of 3-OH FNTZ by microsomes from lymphoblastoid cells expressing CYP2A6 and CYP3A4. (Velocity units = pmol 3-OH FNTZ formed/min/pmol CYP.)
Bupropion, flurbiprofen, and triazolam were used at saturating substrate concentrations of 250, 100, and 500 \( \text{mM} \), respectively. 

IC\(_{50} \) values for chemical inhibitors using a fixed concentration of substrate were determined by nonlinear regression of data to the following equation:

\[
R = 100 \left( 1 - \frac{E_{\text{max}}C^A}{(IC_{50})^A + C^A} \right)
\]

where \( R \) is the percentage of uninhibited reaction velocity that is observed at an inhibitor concentration \( C \); \( E_{\text{max}} \) is a parameter that describes the extent of maximal inhibition; \( A \) is an exponent reflecting the sigmoidicity of the equation; and IC\(_{50} \) is the apparent IC\(_{50} \) (concentration of inhibitor at which \( R \) equals 100\( (1 - 0.5 E_{\text{max}}) \)), from which the true IC\(_{50} \) is calculated using the following equation:

\[
\text{IC}_{50} = \frac{\text{IC}_{50}^*}{(2E_{\text{max}} - 1)^{\frac{1}{A}}}.
\]

Results

The kinetics of FNTZ \( N \)-demethylation and 3-hydroxylation by human liver microsomes were consistent with a Hill enzyme model (eq. 2, Fig. 3A, Table 1). Eadie-Hofstee plots for DM FNTZ and 3-OH FNTZ formation by one representative liver are shown in Fig. 3B. At a concentration of 95 \( \mu \text{M} \) FNTZ, DM FNTZ formation was detectable using microsomes from cDNA-transfected human lymphoblastoid cells expressing CYP2A6, -2B6, -2C9, -2C19, and -3A4 (Table 2), and full kinetic curves were generated (Fig. 4A, Table 3). FNTZ was 3-hydroxylated by CYP2A6 and CYP3A4 at 95 \( \mu \text{M} \), although reaction velocities with CYP2A6 were very low compared with CYP3A4 (Table 3). Full kinetic curves were generated for 3-OH FNTZ formation by CYP2A6 and CYP3A4 (Fig. 4B, Table 3). Kinetic parameters from the individual isoforms and RAF values were used to determine the relative contribution of each isofrom to the net reaction velocity of \( N \)-demethylation (Fig. 5A) and to the net reaction rate of total biotransformation (Fig. 5B). At low concentrations of FNTZ, CYP3A4 is estimated to contribute to more than 70\% of the net reaction rate for \( N \)-demethylation, close to 100\% for 3-hydroxylation, and more than 80\% of the net reaction rate.

\( \alpha \)-Naphthoflavone (1 \( \mu \text{M} \), CYP1A2 inhibitor), sulfaphenazole (10 \( \mu \text{M} \), CYP2C9 inhibitor), omeprazole (10 \( \mu \text{M} \), CYP2C19 inhibitor), and quinidine (5 \( \mu \text{M} \), CYP2D6 inhibitor) did not inhibit \( N \)-demethylation or 3-hydroxylation by more than 15\% (100 \( \mu \text{M} \) FNTZ) (Fig. 6). Ketoconazole (1 \( \mu \text{M} \), CYP3A4 inhibitor) inhibited \( N \)-demethylation and 3-hydroxylation to 40 and 11\% of control activity, respectively; the mean (S.E.) IC\(_{50} \) value for 3-hydroxylation was 0.11 \( \pm \) 0.02 \( \mu \text{M} \) (Fig. 7A, Table 4). Ritonavir, a potent CYP2C9, CYP2C19, CYP2D6, and CYP3A4 inhibitor (von Moltke et al., 1998), inhibited \( N \)-demethylation to 30\% of control activity and inhibited 3-hydroxylation almost to completion, with IC\(_{50} \) values of 0.28 \( \pm \) 0.10 and 0.041 \( \pm \) 0.016 \( \mu \text{M} \), respectively (95 \( \mu \text{M} \) FNTZ) (Fig. 7B, Table 5).

Discussion

Previous studies have not clearly established the human CYP isoforms mediating FNTZ biotransformation, although it has been suggested that CYP2C19 has an important role (Kilicarslan et al.,
Kilicarslan et al. (1998) reported that FNTZ metabolism and clinical effects appear to be influenced by the CYP2C19 isoform, since FNTZ biotransformation is inhibited by omeprazole ($K_i = 200 \mu M$), partially inhibited by $S$-mephentoin (34% inhibition), and pharmacodynamic effects of FNTZ were increased in two CYP2C19 poor metabolizers. Using microsomes from human lymphoblastoid cells expressing human CYPs and baculovirus insect cell-expressed CYP2C19 (Supersomes), we have found that several CYPs, including CYP2C19, are involved in the N-demethylation of FNTZ. These studies, as well as inhibition studies with ritonavir and ketoconazole, indicate that CYP3A4 is the major CYP involved in both the 3-hydroxylation and N-demethylation of FNTZ, with CYP2C19 having a contributory although less important role.

We did not detect 7A FNTZ formation by human liver microsomes, supporting results published by Coller et al. (1998). 7A FNTZ, the main urinary metabolite of FNTZ, accounted for 10% of the dose recovered in urine among at least 11 different metabolites (Berthault et al., 1996). This suggests that 7A FNTZ may be formed through a mechanism other than the CYP system. Coller et al. (1999) reported that CYP2C19 has a major role in the formation of both DM FNTZ and 3-OH FNTZ in vitro, as determined using cDNA-expressed human CYPs and baculovirus insect cell-expressed CYP2C19 (Supersomes). This group used a solvent, dimethylformamide or acetonitrile, in the incubation mixture to solubilize FNTZ, and the results depended on the solvent used. They found that CYP2C19 had no role in demethylation when dimethylformamide was used as the solvent, but when acetonitrile was used, CYP2C19 appeared to have a more important role than CYP3A4 in DM FNTZ formation. In addition, when acetonitrile was used as the solvent, Coller et al. (1999) found that CYP2C19 had a role in 3-OH FNTZ formation, which we did not observe. We used methanolic solutions of FNTZ and evaporated the methanol before the start of incubations such that the incubation mixture only contained phosphate buffer.

Many studies have shown that the presence of a solvent in the incubation mixture may affect the metabolite formation rate by inhibiting or accelerating formation (Cotreau-Bibbo et al., 1996; Chauret et al., 1998; Hickman et al., 1998; Busby et al., 1999). We have previously reported that polyethylene glycol and acetone had varying effects on CYP activity for different substrates (Cotreau-Bibbo et al., 1996). Busby et al. (1999) studied the effect of several solvents on the in vitro activities of cDNA-expressed human cytochrome P450s and reported that 1% acetonitrile decreased activities of CYP1A1, -2B6, -2A6, -3A4, -2C19, and -2D6, while it increased CYP2C9 activity. Hickman et al. (1998) reported that 1% acetonitrile was the least inhibitory toward CYP2C19 and -2E1, while 1% dimethylformamide was least inhibitory toward CYP1A2 and -2C9. Thus, differences between our results and those of Coller et al. (1999) may be attributable to our having no solvent in the incubation mixture.

Coller et al. (1999) reported that FNTZ was not completely soluble in phosphate buffer, and they had used solvent in the incubation mixture to increase FNTZ solubility. Insolubility in the incubation buffer alone (i.e.,

**TABLE 4**

*IC₅₀ values for the inhibition of FNTZ hydroxylation by ketoconazole*

<table>
<thead>
<tr>
<th>Liver</th>
<th>IC₅₀ Value: 3-OH FNTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM1</td>
<td>0.04 μM</td>
</tr>
<tr>
<td>HLM2</td>
<td>0.16 μM</td>
</tr>
<tr>
<td>HLM3</td>
<td>0.12 μM</td>
</tr>
<tr>
<td>HLM5</td>
<td>0.13 μM</td>
</tr>
<tr>
<td>Mean</td>
<td>0.11 μM</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.02 μM</td>
</tr>
</tbody>
</table>

HLM, human liver microsomes.

*Mean, arithmetic mean of four different human livers.

**TABLE 5**

*IC₅₀ values for the inhibition of FNTZ demethylation and hydroxylation by ritonavir*

<table>
<thead>
<tr>
<th>Liver</th>
<th>DM FNTZ IC₅₀ Value</th>
<th>3-OH FNTZ IC₅₀ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLM1</td>
<td>0.16 μM</td>
<td>0.016 μM</td>
</tr>
<tr>
<td>HLM2</td>
<td>0.31 μM</td>
<td>0.022 μM</td>
</tr>
<tr>
<td>HLM3</td>
<td>0.09 μM</td>
<td>0.040 μM</td>
</tr>
<tr>
<td>HLM4</td>
<td>0.54 μM</td>
<td>0.086 μM</td>
</tr>
<tr>
<td>Mean</td>
<td>0.28 μM</td>
<td>0.041 μM</td>
</tr>
<tr>
<td>S.E.</td>
<td>0.10 μM</td>
<td>0.016 μM</td>
</tr>
</tbody>
</table>

HLM, human liver microsomes.

*Mean, arithmetic mean of four different human livers.

S.E., standard error of four different human livers.

**Fig. 7.** Percentage of control activity (N-demethylation or 3-hydroxylation) versus concentration of inhibitor ketoconazole (A) or ritonavir (B). Symbols represent the average of four different human livers. Each liver was incubated in duplicate. See Tables 4 and 5 for IC₅₀ values of inhibition by ketoconazole and ritonavir, respectively.
a purely aqueous environment) does not necessarily warrant the use of a solvent, since the presence of microsomal protein can increase the apparent solubility of the substrate (Coteau-Bibbo et al., 1996). Although nonspecific microsomal binding may be a potential source of artifact at high protein concentrations (Obach, 1997; McLure et al., 2000; Venkatkrishnan et al., 2000), we verified greater than 80% solubility of FNTZ in the incubation mixture without an organic solvent at a relatively low microsomal protein concentration of 0.25 mg ml⁻¹.

Although five CYPs appear to demethylate FNTZ, CYP3A4 has a major role in N-demethylation since CYP3A4 has relatively high abundance in human livers (Shimada et al., 1994). Formation of 3-OH FNTZ was almost completely dependent on CYP3A. The relative importance of CYP3A in FNTZ biotransformation was also established by chemical inhibition studies in liver microsomes. Ketoconazole and ritonavir significantly, although incompletely, inhibited DM FNTZ formation and produced more than 85% inhibition of 3-OH FNTZ formation. Other isoform-specific chemical inhibitors inhibited biotransformation to no less than 85% of control activity. The participation of CYP2C19 in FNTZ clearance, suggested in some clinical studies (Kilicaslan et al., 1998), appears to be relatively small. We have also observed (unpublished data) that kinetics of FNTZ biotransformation in microsomes from a CYP2C19 poor metabolizer liver sample were indistinguishable from the kinetic profile in normal metabolizer phenotype liver microsomes.

Clinical interaction of FNTZ with CYP3A inhibitors or inducers can be anticipated, especially since FNTZ appears to be a low clearance drug with a reported total clearance of 1.50 ml/min/kg (Greenblatt et al., 1982). CYP3A4 inhibitors such as ketonazole, itraconazole, or ritonavir may reduce the clearance of FNTZ, resulting in increased FNTZ plasma levels in vivo. Plasma levels of ritonavir or ketoconazole during usual clinical use generally exceed 1 to 3 μM (Como and Dismukes, 1994; Greenblatt et al., 1998; Hsu et al., 1998). Since the calculated IC₅₀ values for inhibition of 3-OH FNTZ and DM FNTZ formation by ritonavir and ketoconazole (Tables 4 and 5) are considerably lower than typical clinical plasma concentrations, inhibition of FNTZ biotransformation by the CYP3A inhibitors, ritonavir and ketoconazole, is likely to be of clinical importance. Luurila et al. (1996) reported that coadministration of erythromycin (CYP3A4 inhibitor) and FNTZ resulted in an increased AUC of FNTZ. Therefore, the coadministration of FNTZ with CYP3A4 inhibitors may pose a clinical hazard. Of particular concern is the abuse or recreational use of FNTZ by HIV-infected individuals who are concurrently taking viral protease inhibitors or non-nucleoside reverse transcriptase inhibitors, many of which are CYP3A4 inhibitors.

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References


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