HYDROXYLATION AND DEMETHYLATION OF THE TRICYCLIC ANTIDEPRESSANT NORTRIPTYLINE BY cDNA-EXPRESSED HUMAN CYTOCHROME P-450 ISOZYMES

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ABSTRACT:

The metabolism of nortriptyline was studied in vitro using cDNA-expressed human cytochrome P450 isozymes 1A2, 3A4, 2C19, and 2D6. CYP2D6 was the sole isozyme mediating hydroxylation of nortriptyline, the quantitatively most important metabolic pathway, and only (E)-10-OH-nortriptyline was formed. CYP2D6, 2C19, and 1A2, mentioned in decreasing order of significance, mediated the demethylation reaction of nortriptyline, whereas 3A4 did not participate in the metabolism of nortriptyline.

Concerning the quantitative relations, CYP2D6 exhibited a high affinity with respect to hydroxylation and demethylation (Km 0.48–0.74 μmol/l), a high hydroxylation capacity (Vmax 130 mol/hr/mol CYP) and a somewhat lower demethylation capacity (Vmax 19 mol/hr/mol CYP). The affinities of 1A2 and 2C19 were 100-fold lower (Km 54–118 μmol/l). The capacity of 1A2 was low (Vmax 6.8 mol/hr/mol CYP), whereas 2C19 had the highest demethylation capacity (Vmax 93 mol/hr/mol CYP). Taking into account the relative amounts of CYP isozymes present in the liver, about 90% of the metabolism was estimated to depend on CYP2D6, with CYP2C19 and 1A2 mediating the remaining 10%. In subjects lacking the 2D6 isozyme, CYP2C19 and 1A2 are expected to be of major importance for elimination of nortriptyline.

The metabolism of nortriptyline, one of the most frequently used tricyclic antidepressants, has been extensively studied in vitro and in vivo (1–6). In particular, the relation to the cytochrome P450 2D6 isozyme (CYP2D6) has been in focus (2–6). In vitro studies have documented that CYP2D6 mediates the main metabolic pathway, hydroxylation to (E)-10-OH-nortriptyline (4). Minor pathways are N-demethylation and 10-hydroxylation to the (Z) isomer of 10-OH-nortriptyline (fig. 1). The CYP isozymes mediating the latter reactions have not been identified. The minor pathways may be of main importance for elimination of nortriptyline in subjects lacking CYP2D6, so-called poor metabolizers, which constitute 5–10% of white subjects (6). Furthermore, patients being co-medicated with compounds that inhibit CYP2D6 may also be more dependent on the minor pathways.

To identify the isozymes that are responsible for the demethylation of nortriptyline and to clarify whether CYP2D6 is the sole isozyme catalyzing formation of (E)-10-hydroxy-nortriptyline, we performed in vitro metabolism studies based on cDNA-expressed human CYP isozymes. We focused on CYP2D6 and three isozymes known to mediate N-demethylation reactions: CYP1A2, CYP2C19, and CYP3A4 (6, 7). The latter three isozymes have been shown to mediate N-demethylation of tertiary tricyclic antidepressants such as imipramine and amitriptyline (6, 8–10). CYP2C19 is the isozyme related to the mephenytoin oxidation polymorphism (11). The relative importance of the individual isozymes was evaluated on the basis of estimated Km and Vmax values for the hydroxylation and demethylation reactions.

**Materials and Methods**

**Chemicals and Microsomal Preparations.** Nortriptyline, demethyl-nortriptyline, (E)- and (Z)-10-OH-nortriptyline, amitriptyline, and citalopram (internal standard) were kindly supplied by Lundbeck A/S, Copenhagen, Denmark. Caffeine and 1,7-dimethylxanthine (paraxanthine) were from Sigma Chemical Co. (St. Louis, MO). (S)-mephenytoin and 4-OH-(S)-mephenytoin were purchased from Gentest Co. (Woburn, MA). Other chemicals were of analytical grade. Microsomal preparations of cDNA-expressed human CYP1A2, 3A4, 2C19, and 2D6 were obtained from Gentest Co. The microsomal preparations were received frozen and were stored at −80°C until use.
Experimental Procedure. Enzymatic reactions were carried out in polypropylene tubes in a total volume of 300 μl at pH 7.4 (12). A nortriptiline stock solution was prepared as an 8 mM solution in 0.01N HCl and further diluted with 0.1 M phosphate buffer, pH 7.4. Fifteen μl was added as substrate. Drug and NADPH generating system were preincubated for 3 min at 37°C. The NADPH generating system consisted of 5 mM NADP, 5 mM isocitrate and 5 mM MgCl₂ with 1 U isocitrate dehydrogenase. The buffer was a 0.1 M potassium phosphate buffer with pH 7.4. The reaction was started by adding 2.5–12 pmol CYP enzyme, which had been stored on ice after thawing. After an incubation period of 15 min (CYP2D6), 60 min (2C19), or 90 min (1A2) at 37°C under air, the reaction was stopped by adding 1.0 ml ice cold 0.5 M carbonate-bicarbonate buffer pH 10.5, and the compounds were extracted shortly after. The enzymatic reactions were linear with regard to time and to the added amount of isozyme within the limits used in the present study. Enzymatic activity was expressed as mol/hr product formed per mol CYP isozyme.

Assay of Nortriptiline and its Metabolites. After the addition of 25 μl citalopram (12 μM) as an internal standard, 5 ml heptane/isoamylalcohol 98.5:1.5 (v/v) was added. After shaking for 2 min and centrifuging at 1500 g, the aqueous layer was frozen by placing the tubes in a cooling bath of dry ice and ethanol. The organic layer was decanted into new tubes and evaporated to dryness at 60°C in a gentle stream of nitrogen. The residue was dissolved in 75-μl mobile phase, of which 65 μl was injected into the HPLC apparatus. The compounds were separated by use of an acetonitrile-phosphate-triethylamine buffer (60:40:0.1), pH 6.2 as a mobile phase on a Zorbax C18, 3.5 μm 4.6 × 150 mm column, and measured by UV detection at 240 nm.

Assay of CYP Isozyme Activities. Prior to use in the nortriptiline experiments, the activities of the individual CYP isozymes were checked by established marker reactions. CYP1A2 activity was monitored by the 3-demethylation of caffeine to 1,7-dimethylxanthine (13). CYP3A4 is known to demethylate tricyclic antidepressants, and we chose to monitor activity by measurement of N-demethylation of amitriptyline to nortriptyline (9). CYP2C19 activity was assessed by the conversion of (S)-mephenytoin to 4-HO-(S)-mephenytoin (14). Finally, nortriptyline itself served to assess the CYP2D6 activity of CYP2D6 on the basis of the conversion to (E)-10-OH-nortriptyline (14). CYP1A2 catalyzed demethylation of nortriptyline. The 3A4 isozyme did not metabolize nortriptyline. The marker reaction for the 3A4 isozyme verified that the enzyme system performed satisfactorily.

Results

Initial screening of the CYP isozymes 1A2, 3A4, 2C19, and 2D6 for activity towards nortriptiline at a concentration level of 100 μmol/l showed that 1A2, 2C19, and 2D6 displayed significant demethylation activity, but only 2D6 was able to hydroxylate nortriptiline. The (E)-isomer of 10-OH-nortriptyline was exclusively formed. The 3A4 isozyme did not metabolize nortriptiline. The marker reaction for the 3A4 isozyme verified that the enzyme system performed satisfactorily.

Plots of velocity V as function of substrate concentration for the isozymes 1A2, 2C19, and 2D6 show the presence of saturation kinetics (fig. 2-4). The estimated Kₘ and Vₘₙₐₓ values (table 1) show that (E)-10-hydroxylation is the most effectively catalyzed pathway with a low Kₘ value (0.74 μM) and a high Vₘₙₐₓ value (130 mol/hr/mol CYP). CYP2D6 also exhibited a high affinity with regard to demethylation (Kₘ 0.48 μM), but the Vₘₙₐₓ was only about a sixth of that of the hydroxylation reaction. The affinities of CYP1A2 and 2C19 were much lower with Kₘ values more than 100 times higher than that of CYP2D6 with regard to demethylation. CYP1A2 had a low Vₘₙₐₓ value (6.8 mol/hr/mol CYP), whereas 2C19 had the highest Vₘₙₐₓ value with regard to demethylation (93.1 mol/hr/mol CYP).

The intrinsic clearance values displayed in table 1 give an impression of the relative importance of the three isozymes for the hydroxylation and/or demethylation of nortriptiline under the assumption that saturation is not present. Expressed per mol CYP isozyme, 2D6 is far the most important isozyme. The intrinsic clearance for (E)-10-hydroxylation is more than four times larger than the total intrinsic clearance for demethylation. Further, 2D6 contributes an intrinsic clearance with regard to demethylation, which is more than 50 times higher than the intrinsic clearance values mediated by the other isozymes. However, to judge the relative importance of the isozymes in vivo, one has to take into account the relative amounts of CYP
isozymes present in the liver and the possible occurrence of saturation (15). On average, the 1A2 isozyme corresponds to about 10% of the total amount of CYP isozymes. Data on the quantitative amount of 2C19 are not available, but the isozyme constitutes a fraction of the total 2C content of 20% (15, 16). Other members of the 2C group are the isoforms 2C8, 2C9, and 2C18. Somewhat arbitrarily, one may set 2C19 equal to a quarter of the total 2C content of 20%. Setting 2D6 equal to 2% then results in a relative distribution of 10:5:2 for the amounts of 1A2, 2C19, and 2D6, respectively (15, 16). Assuming a concentration ratio of about 10:1 for hepatic vs. serum concentrations, the clinically relevant range extends up to about 5 mmol/l (therapeutic serum interval: 200 – 500 nmol/l) (17, 18). Fig. 5 displays the estimated relative contribution of the isozymes for the concentration interval 0.5 to 10 mmol/l. Up to the level of 5 mmol/l, the 2D6 isozyme takes care of about 90% of the metabolism (80% corresponding to hydroxylation and 10% to demethylation), and 2C19 is responsible for the major part of the remaining activity. The 1A2 isozyme is of negligible importance. However, it is also clear that for poor metabolizers, i.e., subjects lacking the 2D6 isozyme, the demethylation activity of the 2C19 isozyme is likely to be of major importance.

### Discussion

Our *in vitro* studies on the metabolism of nortriptyline based on cDNA-expressed isozymes supported the dominating role of CYP2D6, which is well-known from earlier *in vitro* and *in vivo* studies (2–6). More specifically, earlier studies have shown that the formation of (E)-10-hydroxy-nortriptyline, the main metabolite, is correlated to the CYP2D6 activity assessed either *in vitro* or *in vivo* as the hydroxylation rate of debrisoquine (2). The use of pure cDNA-expressed isozymes allows for very specific studies, and it is of interest to note that no (Z)-isomer was formed at all. Thus, the stereospecificity of 2D6 with regard to formation of (E)/(Z)-isomers appears to be absolute. Our studies did not disclose any enzymatic formation of the (Z)-isomer at all. An earlier study showing both hepatic and intestinal formation of the (Z)-form might have pointed towards a role for the 3A4 isozyme, which is present also in the intestines, but that was not the case as shown here (4). The present study is the first to identify CYP2D6, 2C19, and 1A2 as being responsible mediators of demethylation.

Looking at kinetics, CYP2D6 is characterized by low $K_m$ and high $V_{max}$ values compared with the other isozymes. This agrees with the established role of CYP2D6 as a high affinity isozyme (6). In a study by Su *et al.* on the metabolism of imipramine by cDNA expressed CYP2D6, $K_m$ values were reported in the range 5–8 mmol/l, *i.e.* only moderately higher than the values found here (19). Assuming a

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Kinetic Parameters</th>
<th>CYP Isozymes</th>
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<tr>
<td>(E)-10-hydroxylation</td>
<td>$K_m$ (μmol/l)</td>
<td>0.74 (0.12)</td>
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<tr>
<td></td>
<td>$V_{max}$ (mol/hr/mol CYP)</td>
<td>130 (16)</td>
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<tr>
<td></td>
<td>$Cl_{int}$ (10$^{-6}$ 1 hr/mol CYP)</td>
<td>179 (6.6)</td>
</tr>
<tr>
<td>Demethylation</td>
<td>$K_m$ (μmol/l)</td>
<td>54.2 (6.7)</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$ (mol/hr/mol CYP)</td>
<td>118 (2.7)</td>
</tr>
<tr>
<td></td>
<td>$Cl_{int}$ (10$^{-6}$ 1 hr/mol CYP)</td>
<td>93.1 (2.6)</td>
</tr>
</tbody>
</table>

TABLE 1

* Mean values with SE in parentheses; $N = 3$ independent series.
hepatic-to-serum concentration ratio of about 10 implies that saturation is likely at therapeutic serum levels, as is also evident from the estimation results of fig. 5. This agrees with the fact that clinical studies have shown the occurrence of nonlinear kinetics for nortriptyline, especially for subjects exhibiting high metabolic capacity (low steady-state serum concentration-to-dose ratio) (20). The estimated ratio of about 4:1 for the intrinsic clearance of (E)-10-hydroxy-nortriptyline formation compared with demethylation agrees with clinical studies pointing towards hydroxylation as the major pathway corresponding to about 80% of total metabolism (1). Thus, although the estimated pattern of fig. 5 may be associated with some uncertainties, the general picture appears plausible. Demethylated nortriptyline has received sparse interest, but it has been shown that a major part of demethylated nortriptyline is hydroxylated farther (1). Thus, when judging the importance of demethylation in vivo, the hydroxylated demethylated metabolite should also be recognized.

The other isozymes taking part in the demethylation reaction both display higher \( K_m \) values than that of the CYP2D6 isozyme. Thus, they represent low affinity systems that may play a role at high drug concentrations with increasing saturation of 2D6, e.g. in case of intoxications, and when 2D6 is absent or inhibited. In the latter situations, demethylation plus some (Z)-10-hydroxylation is expected to constitute the major pathways. In particular, the latter situation is of relevance in clinical psychiatry where polypharmacy is a frequently occurring phenomenon. For example, many patients are co-medicated with antidepressants and neuroleptics. In this situation, 2D6 may be more or less inhibited turning the individual to a "functional poor metabolizer" (6). The role of 1A2 and 2C19 with regard to nortriptyline parallels the role of these isozymes with regard to tricyclic antidepressants, e.g. demethylation of imipramine or amitriptyline (6, 8, 10, 21, 22). Only the absence of 3A4 activity towards nortriptyline differs with the pattern observed for tertiary tricyclic antidepressants.

As regards the validity of studies based on cDNA-expressed CYP isozymes some authors have argued that studies based on human liver microsomes are more reliable (23). However, the kinetic parameters obtained here appear plausible compared to the existing literature on kinetic constants for these isozymes. With regard to CYP1A2, Jensen et al. found that the kinetic parameters of artificially expressed isozyme agreed with those of naturally expressed isozyme in hepatic microsomes (24). Thus, although differences can not be excluded, the general pattern found here and elsewhere suggests that results based on cDNA-expressed CYP isozymes are reasonable. Further, cDNA-expressed CYP studies have the advantage of offering very specific indications of isozyme-substrate relations and have been used frequently in recent years to elucidate human drug metabolism (7, 12, 13, 19, 25).

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References

