METABOLISM OF CLOZAPINE BY cDNA-EXPRESSED HUMAN CYTOCHROME P450 ENZYMES

KRISTIAN LINNET AND OLE V. OLESEN

Institute for Basic Psychiatric Research, Department of Biological Psychiatry, Psychiatric Hospital in Aarhus, Aarhus University Hospital

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ABSTRACT:

The metabolism of clozapine was studied in vitro using cDNA-expressed human cytochrome P450 (CYP) enzymes 1A2, 3A4, 2C9, 2C19, 2D6, and 2E1. CYP1A2, 3A4, 2C9, 2C19, and 2D6 were able to N-demethylate clozapine. N-Oxide formation was exclusively catalyzed by CYP3A4. CYP2E1 did not metabolize clozapine. With regard to quantitative relationships, CYP1A2, 2C9, 2C19, and 2D6 displayed Km values ranging from 13 to 25 μM, whereas CYP3A4 had a 5–10 times higher Km value. CYP2C19 and 2D6 had the highest Vmax values (149–366 mol/hr/mol CYP). Taking into account the typical relative distribution of amounts of CYP enzymes in the liver, a simulation study suggested that at therapeutic concentrations CYP2C19 and CYP3A4 each accounted for about 35% of the metabolism. At toxic concentrations, the relative importance of CYP3A4 increased.

In recent years, the relative importance of various CYP enzymes for the metabolism of psychotropic drugs has been extensively studied (1). Knowledge of involved CYP isoforms is valuable in the context of predicting and explaining pharmacokinetic drug interactions, which represents an important issue in psychiatry (1, 2). Concerning the atypical neuroleptic agent clozapine, the main metabolic pathways consist of N-demethylation and N-oxide formation (3). In vitro studies based on selective inhibition of human microsomal CYP enzymes have pointed to CYP1A2 and perhaps also 3A4 as being of importance for N-demethylation (4). Fischer et al. (5) found that CYP2D6 might play a role in the formation of metabolites other than N-demethylclozapine and the N-oxide. In vivo studies have supported a role for CYP1A2, but no association has been found for metabolizer status with regard to debrisoquine (CYP2D6) or (S)-mephenytoin (CYP2C19) (6, 7). Thus, the results of both in vitro and in vivo studies suggest that several CYP isoforms are involved in the metabolism of clozapine, but the exact pattern is at present not clarified.

With this background, we undertook in vitro metabolic studies on clozapine using cDNA-expressed human CYP enzymes. We investigated CYP enzymes known to be frequently involved in N-demethylation reactions, i.e. 1A2, 3A4, 2C9, 2C19, 2D6, and 2E1 (1, 8). Both the N-demethylation and N-oxide-forming reactions were considered. Using a sensitive analytical method, we focused on concentrations of relevance to the human therapeutic situation.

Materials and Methods

Chemicals and Microsomal Preparations. Clozapine, N-demethylclozapine, and clozapine N-oxide were generous gifts from Sandoz Ltd. (Basel, Switzerland). Amitriptyline, nortriptyline, and (E)-10-hydroxyamitriptyline were kindly supplied by Lundbeck A/S (Copenhagen, Denmark). Imipramine, used as an internal standard, was a gift from Ciba-Geigy Ltd. (Basel, Switzerland). Caffeine and 1,7-dimethylxanthine (paraxanthine) were from Sigma Chemical Co. (St. Louis, MO). (S)-Mephenytoin, 4′-hydroxyamitriptyline, 4′-hydroxydilofenac, chloroxazone, and 6-hydroxyclozapine were purchased from Gentest Co. (Woburn, MA). Other chemicals were of analytical grade. Microsomal preparations of cDNA-expressed human CYP1A2, 3A4, 2C9, 2C19, 2D6, and 2E1 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 polystyrene tubes, in a total volume of 300 μl, at pH 7.4 (9). A clozapine stock solution was prepared as a 8 mM solution in 0.01 N HCl and further diluted with 0.1 M phosphate buffer, pH 7.4; 15 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 unit of isocitrate dehydrogenase. The buffer was a 0.8 M potassium phosphate buffer, pH 7.4. The reaction was started by addition of 2.5 pmol of CYP enzyme (6 pmol for CYP1A2), which was stored on ice and thawing. After incubation periods of 15 min at 37°C under air, the reaction was stopped by the addition of 1.0 ml of ice-cold 0.5 M carbonated bicarbonate buffer, pH 10.5, and the compounds were extracted shortly thereafter. The enzymatic reactions were linear with regard to time and added amount of enzyme within the ranges used in the present study. Enzymatic activity was expressed as micromoles of product formed per hour per mole of CYP enzyme.

Assay of N-Demethylclozapine and Clozapine N-Oxide. The two metabolites were measured in separate assays. N-Demethylclozapine was extracted by addition of 5 ml of heptane/isomylalcohol, 98:2 (v/v), after addition of 50 μl of 6 μM (E)-10-hydroxyamitriptyline as internal standard. After shaking for 5 min and centrifugation at 1500g, the aqueous layer was frozen by placing tubes in a cooling bath of dry ice and ethanol. The organic layer was decanted into point tubes and evaporated to dryness at 60°C in a gentle stream of nitrogen. The residue was dissolved in 75 μl of mobile phase, of which 65 μl was injected into the HPLC apparatus.

Clozapine N-oxide was subsequently extracted from the aqueous phase, which was thawed using tepid water. If the clozapine substrate concentration in the experiment had been more than 200 μM, the extraction procedure described above was repeated but the organic layer was discarded. The water phase was thawed again and, after addition of 7.5 ml of ethyl acetate with 50 μl of 10 μM imipramine as internal standard for clozapine-N-oxide determination, clozapine-N-oxide was extracted by shaking for 5 min. After centrif-
1. CYP2C9 and 1A2 had involvement in the significant metabolic transformations of clozapine. Literature reports on CYP isoform distributions in the human liver served for a comparison of the activities of these enzymes. The kinetic parameter values for the N-demethylation and N-oxide formation of clozapine by CYP enzymes are listed in Table 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>CYP Enzymes</th>
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<tbody>
<tr>
<td></td>
<td>1A2</td>
</tr>
<tr>
<td>N-Demethylation</td>
<td></td>
</tr>
<tr>
<td>$K_M$ (µM)</td>
<td>25 ± 6.5</td>
</tr>
<tr>
<td>$V_{max}$ (mol/hr/mol CYP)</td>
<td>44 ± 1.5</td>
</tr>
<tr>
<td>$K_M/V_{max}$ (10$^6$ liters/hr/mol CYP)</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>N-Oxide reaction</td>
<td></td>
</tr>
<tr>
<td>$K_M$ (µM)</td>
<td>120 ± 6.9</td>
</tr>
<tr>
<td>$V_{max}$ (mol/hr/mol CYP)</td>
<td>120 ± 5.8</td>
</tr>
<tr>
<td>$K_M/V_{max}$ (10$^6$ liters/hr/mol CYP)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE ($N = 3$ independent series).

HPLC of N-demethylclozapine was performed with a Zorbax 300 SB C$_{18}$ column (150 × 4.6 mm, with a particle size of 3.5 µm). The mobile phase was a mixture (62:38, v/v) of buffer (0.025 M KH$_2$PO$_4$, 0.01 M triethylamine, pH 5.5) and acetonitrile. The flow rate was 0.9 ml/min. UV detection was at 260 nm. HPLC of clozapine-N-oxide was carried out with a Supelco C$_{18}$ (250 × 4.6 mm, 5 µm) column. The mobile phase was a mixture as described above, with the modification that the buffer/acetonitrile ratio was 60:40 (v/v), and the pH was 2.5. The flow rate and UV detection wavelength were as described above. The lower level of quantification was 7.5 pmol for N-demethylclozapine and 15 pmol for clozapine-N-oxide. The interday coefficient of variation was <10% for both compounds.

**Assay of CYP Enzyme Activities.** Before use in the clozapine experiments, the activities of the individual CYP enzymes were checked by established marker reactions. CYP1A2 activity was monitored by the 3-demethylation of caffeine to 1,7-dimethylxanthine (10). Amitriptyline N-demethylation to nortriptyline served to assess the activity of CYP3A4 (11). CYP2C9 activity was evaluated by formation of 4′-hydroxydiclofenac (12). CYP2C19 activity was assessed by the conversion of (S)-mephénytoin to 4′-hydroxy-(S)-mephénytoin (13). The activity of CYP2D6 was assessed by conversion of nortriptyline to (E)-10-hydroxynortriptyline (14). Finally, the conversion of chlorzoxazone to 6-hydroxycloazoxazone served as an index of 2D6 activity (15).

**Data Analysis.** Rates of metabolism ($V$, expressed as moles per hour per mole of CYP) as functions of the substrate concentration ($S$) were fitted by nonlinear regression according to the Michaelis-Menten equation, using GraphPad Prism software (San Diego CA). $V_{max}$ and $K_M$ were estimated, and the efficiency of the different CYP enzymes was calculated as $V_{max}/K_M$ (liters/hr/mol CYP). The experimentally obtained $K_M$ and $V_{max}$ values together with literature reports on CYP isoform distributions in the human liver served as a basis for simulation of metabolic patterns.

**Results**

Initial screening of the CYP enzymes 1A2, 3A4, 2C9, 2C19, 2D6, and 2E1 for activity towards clozapine at concentrations of 2, 10, and 100 µM showed that 1A2, 3A4, 2C9, 2C19, and 2D6 all displayed significant N-demethylation activity, but only 3A4 was able to catalyze N-oxide formation. CYP2E1 did not mediate any measurable conversion of clozapine.

Plots of velocity of metabolite formation as a function of substrate concentration for the CYP enzymes 2C19, 2C9, 1A2, 2D6, and 3A4, mentioned in decreasing order of $K_M$ values, showed the presence of saturation kinetics. The $K_M$ and $V_{max}$ values and the efficiency of the CYP enzymes, expressed as $V_{max}/K_M$ values, displayed in table 1 give an impression of the relative importance of the five CYP enzymes for the N-demethylation and N-oxidation of clozapine. Expressing per mole of CYP enzyme, 2C19 and 2D6 are the most important CYP enzymes mediating N-demethylation, with relatively low $K_M$ values (13–25 µM) and high $V_{max}$ values (149–366 mol/hr/mol CYP) (table 1). CYP2C9 and 1A2 had $K_M$ values in the same range as 2C19 and 2D6, but their $V_{max}$ values were smaller (27–44 mol/hr/mol CYP). CYP3A4 displayed the highest $K_M$ (230 µM) of the five CYP enzymes for demethylation, and the $V_{max}$ value was at an intermediate level (89 mol/hr/mol CYP). The $K_M$ for clozapine-N-oxide formation was somewhat lower (122 µM), and the $V_{max}$ value was 115 mol/hr/mol CYP.

To judge the relative importance of the CYP enzymes in vivo, one has to take into account the average relative amounts of CYP enzymes present in the liver and the possible occurrence of saturation (16). On this basis, the study by Shimada et al. (17), CYP1A2 is expected to constitute about 10% of the total CYP content and 3A4 about 30%. The amount of 2C19 has not been quantified, but the total amount of 2C has been estimated to account for about 20% of the CYP content (17). This pool is constituted by 2C8, 2C9, 2C18, and 2C19 (18). Somewhat arbitrarily, one may set each subtype equal to a quarter of the total amount, i.e. 5%. The average relative amount of 2D6 was estimated to be 1.5% (17). Taken together, these results indicate a relative distribution of 10:30:5:5:1.5 for the amounts of 1A2, 3A4, 2C8, 2C9, 2C18, and 2C19.

To simulate hepatic metabolism, estimates of drug concentrations in hepatic tissue are necessary. With ordinary therapeutic dosing of clozapine, serum concentrations extend up to about 3 µM (19, 20). Assuming hepatic tissue concentrations of 10 times the serum levels, the clinically relevant range for hepatic tissue concentrations amounts to about 30 µM (21).

Fig. 1A displays the hepatic reaction rate (in moles per hour) for clozapine conversion per mole of CYP isozone for the substrate concentration interval of 0.5–50 µM, estimated from the experimentally obtained $K_M$ and $V_{max}$ values, and fig. 1B shows the simulated relative contributions of the CYP enzymes given the average relative distribution in the liver (see above). Within 10 times the therapeutic range for serum concentrations, (fig. 1, dashed lines), the contribution of the 3A4 isofrom (N-demethylation + N-oxidation) was estimated to increase from 30 to 45%, whereas that of CYP2C19 decreased from 35 to 25% due to saturation. The isoforms CYP2C9, 1A2, and 2D6, with low $K_M$ values, showed a more steady contribution, ranging from 5 to 15% at therapeutic clozapine concentrations. The N-oxide formation was estimated to constitute 20–30% of the total main metabolite production.

**Discussion**

Our results point to the involvement of several CYP isofroms in the major metabolic transformations of clozapine. Five cDNA-expressed CYP enzymes, i.e., 2C19, 2C9, 2D6, 1A2, and 3A4, were able to mediate N-demethylation, and 3A4 mediated N-oxide formation. The involvement of several CYP isofroms makes it a difficult task to elucidate the role of individual CYP forms, both in the context of in vivo human studies and with regard to in vitro studies based on hepatic microsomal preparations. It should also be stressed that simulation
and extrapolation methods like the one used in our study may not reflect the true \textit{in vivo} quantitative contribution of the different CYP enzymes. For clozapine, the \textit{in vivo} disposition may be heavily influenced by intestinal metabolism.

\textit{In vivo} human studies have pointed to a role for CYP1A2. Bertilsson \textit{et al.} (6) found a correlation between clozapine metabolic rate and a marker for CYP1A2 activity, \textit{i.e.} caffeine \textit{N}-demethylation. Furthermore, smokers tend to have increased metabolic rates, which may be ascribed to an induction of CYP1A2 by smoking (22). Finally, fluvoxamine, which is a strong inhibitor of CYP1A2, increases serum concentrations of clozapine severalfold (23). With regard to \textit{in vitro} studies, Pirmohamed \textit{et al.} (4) also found support for a role of CYP1A2. The specific CYP1A2 inhibitor furaphylline decreased the rate of \textit{N}-demethylation of clozapine observed in a human hepatic microsomal preparation (4). Our study points to a somewhat modest role for CYP1A2, corresponding to mediation of about 10\% of the metabolism at therapeutically relevant concentrations in an "average" subject (fig. 1B). One should, however, recognize that the influence of smoking was only observed in men in the study cited above, and in another study no association was detected between smoking and serum concentrations of clozapine (24). Furthermore, one should take into account the fact that fluvoxamine is not a specific CYP1A2 inhibitor, but CYP2C19 and 2D6 may also be subject to inhibition by this drug (25, 26). Thus, the results of the considered human studies do not necessarily imply that CYP1A2 should play a dominant role in clozapine metabolism.

Our study suggests that CYP3A4 is very important for clozapine metabolism, being responsible for 30 – 45\% of the hepatic metabolism under ordinary circumstances (fig. 1B). The involvement of CYP3A4 is supported by case reports on the pharmacokinetic interaction between erythromycin and clozapine, implying that co-treatment with this antibiotic raises serum concentrations of clozapine (27, 28). Furthermore, the induction of clozapine metabolism by carbamazepine observed during therapeutic drug monitoring supports a role for CYP3A4 (23). Finally, the \textit{in vitro} study by Pirmohamed \textit{et al.} (4) mentioned above also pointed to some role for CYP3A4.

Involvement of CYP3A4 may also explain the rather low bioavailability of clozapine. Cheng \textit{et al.} (29) performed hepatic clearance studies with clozapine that suggested that the bioavailability should be about 80\% and not the observed 20 – 30\%. The discrepancy points to extrahepatic presystemic metabolism in the intestines. Our observation that CYP3A4, which is located in the intestines as well as in the liver, may mediate both \textit{N}-demethylation and \textit{N}-oxide formation is in accordance with the hypothesis of extrahepatic metabolism put forward by Cheng \textit{et al.} (29). The fact that \textit{N}-oxide formation is a significant metabolic pathway is apparent from the study by Centorino \textit{et al.}, which showed that the \textit{N}-oxide metabolite constituted about 20\% of the total drug metabolite level in patients at steady state with regard to clozapine (19).

The involvement of CYP2C19, corresponding to the mediation of about 30\% of metabolism, is apparently not supported by previous studies. Dahl \textit{et al.} (7) found no association between clozapine metabolic rate and the (S)-mephenytoin genetic polymorphism in a panel study. However, in a situation with several involved CYP enzymes, such a relationship may be difficult to determine, because other CYP enzymes may take over when a particular isozyme, such as CYP2C19, is lacking. CYP2C19 has been found to be involved in \textit{N}-demethylation reactions for several psychotropic drugs, \textit{e.g.} the antidepressants imipramine and amitriptyline (30, 31). Thus, an involvement also in clozapine \textit{N}-demethylation would not be unexpected.

Concerning CYP2D6, the literature reports have been somewhat contradictory. Fischer \textit{et al.} (5) reported in an \textit{in vitro} study that CYP2D6 was involved in clozapine metabolism. However, CYP2D6 apparently did not mediate formation of the major metabolites, but only the formation of some minor ones (5). Furthermore, Dahl \textit{et al.} (7) found no association between clozapine metabolic rate and the

**Fig. 1. Contributions of CYP enzymes to clozapine metabolism.**

A. Reaction rate (moles per hour) per mole of CYP isoform. B. Percentage contributions simulated for an “average” liver with a relative distribution of 10:30:5:5:1.5 for CYP1A2, 3A4, 2C9, 2C19, and 2D6.
CYP2D6 genetic polymorphism. According to our simulation, CYP2D6 appears to play a minor role (fig. 1B). This may be regarded as analogous to the minor contribution of CYP2D6 to N-demethylation reactions of tricyclic antidepressants (8, 32). Finally, the participation of CYP2C9 in the N-demethylation reaction of clozapine parallels the recent finding that CYP2C9 contributes to the N-demethylation reaction of amitriptyline (32).

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References