Characterization of Human Cytochrome P450s Involved in the Bioactivation of Clozapine

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ABSTRACT

Clozapine is known to cause hepatotoxicity in a small percentage of patients. Oxidative bioactivation to reactive intermediates by hepatic cytochrome P450s (P450s) has been proposed as a possible mechanism. However, in contrast to their role in formation of N-desmethyclozapine and clozapine N-oxide, the involvement of individual P450s in the bioactivation of reactive intermediates is much less well characterized. The results of the present study show that 7 of 14 recombinant human P450s were able to bioactivate clozapine to a glutathione-reactive nitrenium ion. CYP3A4 and CYP2D6 showed the highest specific activity. Enzyme kinetic characterization of these P450s showed comparable intrinsic clearance of bioactivation, implicating that CYP3A4 would be more important because of its higher hepatic expression, compared with CYP2D6. Inhibition experiments using pooled human liver microsomes confirmed the major role of CYP3A4 in hepatic bioactivation of clozapine. By studying bioactivation of clozapine in human liver microsomes from 100 different individuals, an 8-fold variability in bioactivation activity was observed. In two individuals bioactivation activity exceeded N-demethylation and N-oxidation activity. Quinidine did not show significant inhibition of bioactivation in any of these liver fractions, suggesting that CYP2D6 polymorphism is not an important factor in determining susceptibility to hepatotoxicity of clozapine. Therefore, interindividual differences and drug-drug interactions at the level of CYP3A4 might be factors determining exposure of hepatic tissue to reactive clozapine metabolites.

Introduction

Clozapine (CLZ) is an atypical antipsychotic drug that causes fewer extrapyramidal adverse effects than other neuroleptics (Safferman et al., 1991; Buchanan, 1995; Wagstaff and Perry, 2003). However, because of its associated risk of severe agranulocytosis, it is recommended only as a second-line drug for patients with schizophrenia who do not respond to typical neuroleptic drugs (Buchanan, 1995; Wagstaff and Perry, 2003). After agranulocytosis, mild hepatotoxicity has been reported as an adverse effect of CLZ in 37% of the patients. However, in 0.06% of the patients, it may progress to liver failure (Hummer et al., 1997). Although the exact mechanism is not yet known, local formation of reactive metabolites by myeloperoxidase in neutrophils and cytochrome P450s (P450s) in the liver has been proposed as a possible explanation for these adverse drug reactions (Fischer et al., 1991; Liu and Uetrecht, 1995; Maggs et al., 1995; Pirmohamed et al., 1995).

Analysis of urinary and serum metabolites from CLZ-treated patients and many in vitro studies using human liver microsomes (HLM) have shown that CLZ is extensively metabolized by P450s to multiple products (Fig. 1). The major metabolites in serum and urine appeared to be derived from N-demethylation, N-oxidation, and aromatic ring hydroxylation (Centorrino et al., 1994; Dain et al., 1997; Schaber et al., 2001). Several studies have been performed to identify the role of individual P450s in the oxidative metabolism of CLZ to its major metabolites, N-desmethyclozapine (DMCLZ; C-2) and clozapine N-oxide (CLZ-NO; C-1) (Fig. 1) (Pirmohamed et al., 1995; Eiermann et al., 1997; Linnet and Olesen, 1997; Fang et al., 1998; Tugnait et al., 1999; Olesen and Linnet, 2001; Zhang et al., 2008). The combined results of these in vitro studies show that both CYP1A2 and CYP3A4 are playing major roles in the biotransformation of CLZ to these two metabolites. In vivo studies indicate a major role of CYP1A2 in the pharmacokinetics of CLZ (Bertilsson et al., 1994). Furthermore, the involvement of polymorphic CYP2D6 and CYP2C19 in formation of DMCLZ and CLZ-NO has been reported (Fischer et al., 1992; Zhang et al., 2008). However, no association has been found between CLZ pharmacokinetics and debrisoquine (CYP2D6) or (S)-mephenytoin (CYP2C19) metabolizer status (Dahl et al., 1994; Arranz et al., 1995).

Many studies have shown that CLZ is bioactivated by HLM to a reactive nitrenium ion (Maggs et al., 1995; Pirmohamed et al., 1995; Damsten et al., 2008; Wen et al., 2008; Dragovic et al., 2010; Barbara and Castro-Perez, 2011). In addition, formation of iminium ions resulting from dehydrogenation of the piperazine-ring of CLZ, DMCLZ, and CLZ-NO were demonstrated using cyanide, which is able to trap hard electrophiles (Rousu et al., 2009; Argoti et al., 2005; Li et al., 2008). This article has supplemental material available at dmd.aspetjournals.org.
Theoretically, two different iminium ions can be formed by dehydrogenation of the two different pairs of nitrogen-methylene bonds of the piperazine-ring (Fig. 1). However, the structures of the corresponding cyanide adducts remain to be established. Thus far, no bioactivation studies have been performed using recombinant human P450 isoenzymes, because in none of the previous studies, GSH (glutathione) or cyanide was included to trap reactive intermediates. Therefore, the aim of the present study was to identify the isoenzymes of human P450s involved in the hepatic bioactivation of CLZ. The formation of reactive intermediates of CLZ was determined by quantifying adducts to GSH and cyanide in incubations with individual recombinant human P450s and by performing inhibition studies in incubations with pooled HLM using P450-isoform selective inhibitors. Finally, to study the interindividual variability in hepatic biotransformation and bioactivation of CLZ, incubations were performed with HLM from 100 individuals.

**Materials and Methods**

**Materials.** Supersomes containing cDNA-expressed human P450 enzymes were purchased from BD Biosciences (Breda, The Netherlands). The enzymes used were CYP1A1 (Lot No. 35400), CYP1A2 (Lot No. 21667), CYP2A6 (Lot No. 33769), CYP3A4 (Lot No. 38275), CYP3A5 (Lot No. 44743), CYP1B1 (Lot No. 26314), CYP2B6 (Lot No. 62543), CYP2C8 (Lot No. 62556), CYP2C9*1(Arg144) (Lot No. 41274), CYP2C18 (Lot No. 11301), CYP2C19 (Lot No. 62542), CYP2D6*1 (Lot No. 38273), CYP2E1 (Lot No. 44748), and CYP2J2 (Lot No. 456264). Human liver microsomes (HLM; Lot No. 0710619), pooled from 50 donors, were obtained from Xenotech (Lenexa, KS) and contained 20 mg protein/ml. DMCLZ and CLZ-NO were purchased from Sigma-Aldrich (St. Louis, MO); 7-Hydroxyclozapine (C-6) and 9-hydroxyclozapine (C-7) were prepared by the Udenfriend reaction as described previously (Slavik et al., 2011). All other chemicals and reagents were of analytical grade and obtained from standard suppliers. Expression and purification of human glutathione S-transferase (hGST)P1-1 was done as described previously (Dragovic et al., 2010). Protein concentrations were determined according to the method of Bradford (1976), with reagent obtained from Bio-Rad (Munich, Germany). The specific activity of the purified recombinant hGST P1-1, which was assayed according to Habig et al. (Habig et al., 1974), was 27.9 μmol/min/mg protein, using CDNB as a substrate.

**Incubations of CLZ with Recombinant Human P450s.** Incubations with recombinant human P450 were performed at CLZ concentrations of 10 and 100 μM, as was done previously (Pirmohamed et al., 1995; Eiermann et al., 1997; Linnet and Olesen, 1997; Fang et al., 1998; Tugnait et al., 1999; Olesen and Linnet, 2001; Zhang et al., 2008; Dragovic et al., 2010). Duplicate incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) at a final volume of 200 μl. The individual cDNA-expressed CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP1B1, CYP2B6, CYP2C8, CYP2C9*1, CYP2C18, CYP2C19, CYP2D6*1, and CYP2E1 were incubated for 30 minutes at 37°C with CLZ at a final P450 concentration of 50 nM. Formation of reactive nitrenium ion was determined by including 100 μM GSH and 8 μM hGSTP1-1 in the incubations. hGSTP1-1 was previously shown to be highly active in conjugation of the CLZ nitrenium ion (Dragovic et al., 2010). In addition to GSH, potassium cyanide (1 mM) was used as a trapping reagent to...
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detect formation of the reactive iminium ion resulting from oxidative bioactivation of the piperazine ring (Argoti et al., 2005). All incubations were initiated by the addition of 500 μM NADPH (final concentration). After 30 minutes, the reactions were terminated by the addition of 20 μl 10% ice-cold HClO4. To precipitate denatured proteins, the samples were centrifuged for 15 minutes at 14,000g. The supernatants were analyzed by high-performance liquid chromatography (HPLC), as described below.

Determination of Enzyme Kinetic Parameters of Oxidative Metabolism of CLZ. For the most active recombinant P450s and HLM, the enzyme kinetic parameters of product formation were determined. First, the ranges were determined where the enzyme activity is still linear with enzyme concentration and incubation time. On the basis of these experiments, enzyme concentrations used were 50 nM recombinant P450 and 1 mg/ml microsomal protein of HLM. Incubation time was 15 minutes. The incubations were performed using CLZ concentrations ranging from 1 to 1000 μM. Specific activities were calculated and plotted against substrate concentrations. Enzyme kinetic parameters Km and Vmax were determined by nonlinear regression according to the Michaelis-Menten equation, using GraphPad Prism software (version 4.0; San Diego, CA).

Inhibition of Metabolite Formation in Incubations of CLZ with Pooled Human Liver Microsomes by Isoenzyme-Specific Inhibitors of P450s. The contribution of individual P450s in metabolite formation were also studied by incubating CLZ with pooled HLM in presence or absence of specific inhibitors of individual P450 enzymes. The final concentration of HLM was 1 mg protein/ml. Incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) and at a final volume of 250 μl. The concentration of CLZ was 100 μM, and the final concentration of dimethylsulfoxide in incubations (used for stock solution of CLZ) was less than 1%. GSH conjugation was performed in addition of 100 μM GSH and 8 μM hGSTP1-1. P450 selective inhibitors furafylline (FURA; 10 μM), ketoconazole (KTZ; 2 μM), sulfaphenazole (10 μM), tranylcypromine (25 μM), quinidine (2 μM), and diethyldithiocarbamate (DDC; 20 μM) were used to investigate the involvement of CYP1A2, CYP1A1 (CYP3A4 and CYP3A5), CYP2C9, CYP2C19, CYP2D6, and CYP2E1, respectively (Baldwin et al., 1995; Newton et al., 1995; Ono et al., 1996; Lin et al., 2007; Khojasteh et al., 2011). All inhibitors except DDC were dissolved in methanol, and the final concentration of the solvent in the incubations was not exceeding 1%. DDC conjugation was performed in addition of 100 μM GSH and 8 μM hGSTP1-1. Identification and quantification of formed GSH conjugates (Damsten et al., 2008; Dragovic et al., 2010) were used to estimate the concentrations of the formed GSH and cyanide conjugates, assuming that their extinction coefficients are equal to that of CLZ.

Results

Oxidative Metabolism of CLZ by Pooled Human Liver Microsomes. Previously, CLZ was shown to be metabolized by pooled HLM in presence of hGST P1-1 to CLZ-NO, DMCLZ, and several GSH conjugates (Damsten et al., 2008; Dragovic et al., 2010). Consistent with these studies, CLZ-NO (C-1) and DMCLZ (C-2) were the major stable metabolites formed by pooled HLM (Fig. 2). In total, six GSH conjugates of CLZ were formed in incubations of HLM in presence of hGST P1-1. The structures of conjugates C-1, C-2, C-3, C-4, C-5, and C-6 are shown in Fig. 1. The minor conjugate CG-7 represents a secondary metabolite of CG-6, resulting from the presence of an additional glutathionyl group (Dragovic et al., 2010). In addition, two minor stable metabolites were formed as the result of

![Fig. 2. HPLC chromatogram showing clozapine metabolites formed by HLM in vitro; 100 μM CLZ was incubated for 30 minutes in the presence of 100 μM GSH and 8 μM hGST P1-1. Identification and codes of all metabolites correspond to those described previously (Damsten et al., 2008; Dragovic et al., 2010). Background peak X was also present in the control without the substrate.](image-url)
partial degradation of the piperazine ring, CLZ-C3H2 (C-3), and CLZ-C3H4 (C-4) (Dragovic et al., 2010). Analysis by the highly sensitive liquid chromatography mass spectrometry–quadrupole–time of flight instrument revealed the formation of two minor peaks eluting from 15.5 through 18.5 minutes with mass-to-charge ratio value of 343.14 (C-6 and C-7), which corresponds to [M+H]+ of hydroxylated CLZ metabolites (Supplemental Fig. S1). Only C-6 could also be detected by UV-detection (Fig. 2). With use of the references obtained by the Udenfriend reaction (Slavik et al., 2011), the metabolites correspond to 7-hydroxylozapine (C-6) and 9-hydroxylozapine (C-7).

**Effect of Isoenzyme-Selective Inhibitors on Metabolism of CLZ by Pooled Human Liver Microsomes.** Figure 3 shows the effect of the isoenzyme-selective inhibitors on the formation of DMCLZ, CLZ-NO, and total of GSH conjugates by pooled HLM. Effects of inhibitors on individual GSH conjugates are shown in Supplemental Fig. S4. The results obtained are expressed as percentage of activity of the control HLM incubation in which no inhibitor was added.

The only inhibitor showing very strong inhibition of CLZ bioactivation was KTZ, which reduced the formation of total GSH conjugates by 58.8% at 2 μM. At this concentration, both CYP3A4 and CYP3A5 will be strongly inhibited (Khojasteh et al., 2011). At a concentration of 20 μM, at which KTZ is expected to inhibit multiple P450s, KTZ inhibited GSH conjugation by 67.0%. Quinidine and DDC inhibited formation of GSH conjugates only to a low extent, 18.0 and 19.3%, respectively. Results for inhibition of individual conjugate formation were matching those for total GSH conjugates by pooled HLM. Figure 3 shows the relative activities of individual recombinant human P450s in bioactivation of CLZ using GSH and potassium cyanide as trapping agents. Seven of the recombinant P450s were able to produce GSH conjugates to a variable extent (Table 1). At both 10 and 100 μM CLZ, CYP3A4 appeared to be the most active enzyme, followed by CYP2D6 (Fig. 4A). CYP1A1, CYP1A2, CYP1B1, CYP2J2, and CYP3A5 showed less than 20% of the activity of CYP3A4 activity at these two substrate concentrations. The ratios of the three major GSH conjugates formed in the presence of hGST P1-1 did not significantly change between the different enzymes, suggesting that all GSH conjugates are formed from the same reactive intermediate, as proposed previously (Rea et al., 2011).

When incubations of CLZ with individual P450s were performed in the presence of potassium cyanide, with most recombinant P450s, a product with mass-to-charge ratio 352.13 corresponding to the [M+H]+ of the cyano adduct of the CLZ iminium ion was observed. Consistent with previous studies, several P450s showed relatively high specific activity in N-demethylation (CYP2D6 > CYP3A4 > CYP1A2) and N-oxidation (CYP3A4 > CYP1A2, CYP2D6) of CLZ, whereas the other P450s showed only low or no activity (Pirmohamed et al., 1995; Eiermann et al., 1997; Linnet and Olesen, 1997; Fang et al., 1998; Tugnait et al., 1999; Olesen and Linnet, 2001; Zhang et al., 2008).

Figure 4 shows the relative activities of individual recombinant human P450s in bioactivation of CLZ using GSH and potassium cyanide as trapping agents. Seven of the recombinant P450s were able to produce GSH conjugates to a variable extent (Table 1). At both 10 and 100 μM CLZ, CYP3A4 appeared to be the most active enzyme, followed by CYP2D6 (Fig. 4A). CYP1A1, CYP1A2, CYP1B1, CYP2J2, and CYP3A5 showed less than 20% of the activity of CYP3A4 activity at these two substrate concentrations. The ratios of the three major GSH conjugates formed in the presence of hGST P1-1 did not significantly change between the different enzymes, suggesting that all GSH conjugates are formed from the same reactive intermediate, as proposed previously (Rea et al., 2011).

When incubations of CLZ with individual P450s were performed in the presence of potassium cyanide, with most recombinant P450s, a product with mass-to-charge ratio 352.13 corresponding to the [M+H]+ of the cyano adduct of the CLZ iminium ion was observed. Table 1 shows the specific activities at a CLZ concentration of 100 μM, because peak areas at 254 nm could be quantified only at this concentration. As shown in Fig. 4B, the highest activity was found in CYP2D6 and CYP2C18. CYP1A2, CYP2A6, and CYP3A4 showed approximately 25% of CYP2D6 activity.

**Enzyme Kinetic Characterization of P450-Dependent Metabolism of CLZ by Pooled Human Liver Microsomes and Recombinant Human CYP2D6 and CYP3A4.** The enzyme kinetic parameters of pooled HLM and two most active CYP enzymes for the bioactivation of CLZ (CYP3A4 and CYP2D6) were determined by varying CLZ substrate concentrations from 1 to 1000 μM. Supplemental Figure S3 shows the concentration dependence of CLZ

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**Figure 3.** Effect of P450 inhibitors on the metabolism of clozapine by HLM to clozapine N-oxide (CLZ-NO), N-desmethylclozapine (DMCLZ), and total of GSH conjugates (CG-1, CG-4, CG-5, and CG-6). Data are expressed as percentage of control activity and represent mean of duplicate determinations. QND, quinidine; SPZ, sulfaphenazole; TCP, tranicyclopramide.
metabolism by HLM and recombinant P450s. For both recombinant P450s, substrate inhibition was observed at concentrations above 250 μM. The enzyme kinetic parameters for these isoenzymes were therefore estimated from the initial part of the substrate-velocity plots; the last two points at 750 and 1000 μM were excluded. The \( K_M \) and \( V_{\text{max}} \) values and the intrinsic clearance, \( V_{\text{max}}/K_M \), of the P450 enzymes are shown in Table 2.

In the pooled HLM incubations, the enzyme kinetic parameters for total GSH conjugate formation were 126 μM and 1266 nmol/min/mg protein for \( K_M \) and \( V_{\text{max}} \) values, respectively. For CLZ N-demethylation, a \( K_M \) of 268 μM and \( V_{\text{max}} \) of 3215 nmol/min/mg protein was found. For CLZ N-oxidation, these values were 250 μM and 2130 nmol/min/mg protein, respectively (Table 2). These results led to the 1.1- and 1.2-fold higher intrinsic clearance (\( V_{\text{max}}/K_M \)) for total GSH conjugates than for DMCLZ and CLZ-NO, respectively. The \( K_M \) and \( V_{\text{max}} \) values for DMCLZ and CLZ-NO are somewhat higher than previously determined in the literature (Eiermann et al., 1997; Tugnait et al., 1999; Zhang et al., 2008), although higher values (≥300 μM) have also been reported for N-oxide formation (Eiermann et al., 1997; Tugnait et al., 1999). In addition, our results are in agreement with Zhang et al. (Zhang et al., 2008), who showed that \( K_M \) values for DMCLZ and CLZ-NO are similar.

**Fig. 4.** Formation of GSH- and cyanide-reactive metabolites of clozapine by recombinant human P450s. Rates of formation were measured as total of GSH conjugates (CG-1, CG-5, and CG-6) (A) and cyanide adduct (B) when incubated with 10 μM and 100 μM CLZ. Values represent the mean of duplicate measurements and are given as percentage, where 100% correspond to the activity of the most active enzyme. CN, cyanide.

**TABLE 1**

Specific activities of oxidative metabolism of CLZ by recombinant human P450s

<table>
<thead>
<tr>
<th>Variable</th>
<th>DMCLZ</th>
<th>CLZ-NO</th>
<th>Total of GSH Conjugates</th>
<th>Cyanide Adduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLZ conc.</td>
<td>10 μM</td>
<td>100 μM</td>
<td>10 μM</td>
<td>100 μM</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>0.64 ± 0.02</td>
<td>0.56 ± 0.03</td>
<td>0.24 ± 0.01</td>
<td>0.20 ± 0.02</td>
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<td>CYP1A2</td>
<td>1.12 ± 0.01</td>
<td>8.71 ± 0.1</td>
<td>0.55 ± 0.01</td>
<td>3.70 ± 0.1</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>1.16 ± 0.01</td>
<td>0.94 ± 0.05</td>
<td>0.84 ± 0.01</td>
<td>0.64 ± 0.07</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.92 ± 0.01</td>
<td>0.89 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.075 ± 0.006</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.33 ± 0.02</td>
<td>0.22 ± 0.01</td>
<td>0.065 ± 0.005</td>
<td>0.057 ± 0.01</td>
</tr>
<tr>
<td>CYP2C18</td>
<td>4.08 ± 0.05</td>
<td>5.71 ± 0.02</td>
<td>0.025 ± 0.005</td>
<td>0.065 ± 0.01</td>
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<tr>
<td>CYP2C19</td>
<td>6.62 ± 0.03</td>
<td>5.11 ± 0.02</td>
<td>0.71 ± 0.03</td>
<td>0.58 ± 0.005</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>8.43 ± 0.2</td>
<td>23.7 ± 1.1</td>
<td>0.51 ± 0.01</td>
<td>1.62 ± 0.1</td>
</tr>
<tr>
<td>CYP2F2</td>
<td>0.70 ± 0.07</td>
<td>1.40 ± 0.1</td>
<td>0.05 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>2.98 ± 0.01</td>
<td>15.3 ± 0.1</td>
<td>2.37 ± 0.02</td>
<td>5.62 ± 0.01</td>
</tr>
<tr>
<td>CYP3A5</td>
<td>0.24 ± 0.04</td>
<td>1.29 ± 0.02</td>
<td>0.32 ± 0.04</td>
<td>0.88 ± 0.03</td>
</tr>
</tbody>
</table>

CLZ, clozapine; CLZ-NO, clozapine N-oxide; DMCLZ, N-desmethylclozapine; GSH, glutathione; n.d., not detectable; P450, cytochrome P450.

* Involvement of individual P450s in CLZ bioactivation is expressed as formation of total of GSH conjugates and cyanide adduct after 30 minutes incubations of 10 μM CLZ, 50 nM recombinant human P450s, in the presence of 100 μM GSH and 8 μM KCN (human glutathione S-transferase) P1-1 or 1 mM KCN, respectively. The values represent means of two measurements; standard deviations were less than 10%.
enzyme kinetic parameters of recombinant CYP3A4 and CYP2D6, both $K_M$ and $V_{\text{max}}$ values for the formation of total GSH conjugates were somewhat higher for CYP3A4 (30.3 μM and 3.1 nmol/min/nmol CYP) than those determined for CYP2D6 (21.9 μM and 2.8 nmol/min/nmol CYP). Because of its lower $K_M$ value, a 26% higher intrinsic clearance was found for CYP2D6.

**Interindividual Variability in CLZ Bioactivation by Human Liver Microsomes.** To investigate the interindividual variability in microsomal biotransformation of CLZ, 100 HLM fractions were incubated at a CLZ concentration of 100 μM. The concentrations of the formed metabolites (DMCLZ, CLZ-NO, and total GSH conjugates) after 30 minutes of incubation were shown in Fig. 5, with the samples ranked from highest to lowest GSH-conjugate formation. The total of GSH-conjugate formations varied over an 8.3-fold range (0.26–2.16 μM; median, 0.62 μM), whereas DMCLZ formation varied over a 5.1-fold range (0.43–2.19 μM; median, 0.95 μM) and CLZ-NO over a 9.4-fold range (0.17–1.59 μM; median, 0.50 μM).

The larger variation for CLZ-NO formation than for DMCLZ is in agreement with previous results obtain with liver fractions obtained from 14 individuals by Zhang et al. (Zhang et al., 2008). Inducers that increase the activity of microsomal CYPs (Maggs et al., 1995) could be more important in the CLZ bioactivation. This is without common functional polymorphisms (Hiratsuka, 2012), it is possible that metabolism and protein adducts (Pirmohamed et al., 1995). Genetic polymorphisms of CYP3A4 and CYP2D6, and the interactions with other xenobiotics that influence its activity might contribute to hepatotoxicity. Although these reactive CLZ metabolites have been demonstrated in many in vitro studies using HLM, only very limited information is available on the role of individual P450s in bioactivation of CLZ (Pirmohamed et al., 1995). Therefore, in the present study, experiments were performed with recombinant P450s and HLM in the presence and absence of enzyme-specific inhibitors.

The results summarized in Table 1 and Fig. 5 show that bioactivation of CLZ, when quantified as the total of GSH conjugates, appears to be a relatively important pathway when compared with N-demethylation and N-oxidation pathways. DMCLZ formation, which is the major pathway of CLZ metabolism, was an average of only 1.6-fold higher than bioactivation. In the set of 100 HLM fractions, two individuals even showed higher activity of bioactivation when compared with the N-demethylation and N-oxidation pathways (Fig. 5).

When comparing 14 different recombinant human P450s, it appeared that CYP3A4 showed the highest specific activity in bioactivation of CLZ according to the formation of GSH conjugates. In addition, relatively high activity was observed with recombinant CYP2D6 (Fig. 4A). When determining enzyme kinetic parameters for bioactivation, it appeared that CYP2D6 had a 1.3 higher intrinsic clearance for bioactivation when compared with CYP3A4. When using cyanide as trapping agent, CYP2D6 also seems to be the most active enzyme involved in the bioactivation of CLZ to reactive iminium ion, with CYP2C18 and CYP3A4 having 30 and 70% lower activity, respectively (Fig. 4B). However, considering that CYP3A4 is a mean of almost 20-fold more abundant in HLM than in CYP2D6 (Shimada et al., 1994), we conclude that CYP3A4 is most likely to be the major enzyme involved in hepatic CLZ bioactivation. This is supported by the fact that only the CYP3A-selective inhibitor ketoconazole was able to cause significant inhibition of bioactivation of CLZ by pooled HLM (Fig. 3). These results are consistent with the observation of Pirmohamed et al. that ketoconazole significantly inhibited the formation of GSH conjugates and protein adducts (Pirmohamed et al., 1995). Genetic polymorphisms of CYP3A4 and the interactions with other xenobiotics that influence its activity might cause interindividual differences that could lead to the susceptibility for CLZ-related adverse reactions. Although it appears that CYP3A4 is without common functional polymorphisms (Hiratsuka, 2012), it has been shown that non-synonymous alleles for CYP3A4 encode enzymes with altered catalytic properties (Dai et al., 2001; Eissel et al., 2001; Zhang et al., 2008). Inducers that increase the activity of CYP3A4 (Cohen et al., 1996; Jerling et al., 1994) could be more
important than genetic polymorphism of this enzyme for the individual variability in CLZ bioactivation.

Although CYP2D6 showed relatively high specific activity in all three oxidative pathways of CLZ-metabolism (Table 1), the CYP2D6-specific inhibitor quinidine showed no or only minor inhibition of these pathways in incubations of CLZ with the 100 individual HLM fractions (Supplemental Fig. S5). These results support previous observations of Pirmohamed et al. (Pirmohamed et al., 1995), in which no significant difference was observed in covalent protein binding between incubations of microsomes of a limited number of individuals who were genotyped as poor or extensive metabolizers of CYP2D6. In addition, no significant differences in the pharmacokinetic parameters of CLZ were observed between poor and extensive metabolizers of debrisoquine (Dahl et al., 1994), suggesting that the genetic polymorphism of CYP2D6 has little clinical relevance for CLZ pharmacokinetics and CLZ bioactivation.

In conclusion, the results of the present study show that CYP3A4 is the main enzyme involved in the bioactivation of CLZ in human liver microsomes. Although two recent studies showed that clozapine was not cytotoxic in human cell lines transfected with CYP3A4 (Hosomi et al., 2011; Thompson et al., 2012), down regulation of Nuclear factor (erythroid-derived 2)-like 2 by siRNA resulted in cytotoxicity of clozapine in CYP3A4-transfected HepG2-cells (Hosomi et al., 2011). This may be rationalized by the reduced activity of protective phase II enzymes, such as hGSTs. We have shown previously that several hGSTs, including polymorphic GST M1-1 and GST P1-1, have a significant activity in catalyzing GSH conjugation of reactive CLZ metabolites formed by P450s (Dragovic et al., 2010). Therefore, a high activity of bioactivation by CYP3A4 in combination with reduced activity of protective hGSTs might explain high susceptibility of some of the patients to hepatotoxic effects of CLZ.

**Authorship Contributions**

**Participated in research design:** Dragovic, Ingelman-Sundberg, Vermeulen, Commandeur.

**Conducted experiments:** Dragovic, Gunness.

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**Fig. 5.** Metabolism of CLZ by different individual human liver microsomes at substrate concentration of 100 μM. Concentrations of total of GSH conjugates (A), DMCLZ (B), and CLZ-NO (C) were measured after 30 minutes of incubation of 0.1 mg/ml HLM with 100 μM CLZ in presence of 5 mM GSH. Two individuals with high bioactivation activity, compared with N-demethylation and N-oxidation, are marked by gray column and asterix.
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Contributed new reagents or analytic tools: Dragovic, Gunnoss.

Performed data analysis: Dragovic, Gunnoss, Commandeur.

Wrote or contributed to the writing of the manuscript: Dragovic, Commandeur.

References


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Figure S1.

Extracted ion chromatograms of $m/z$ 343.14 of incubations of CLZ with HLM, recombinant human CYP3A4 and CYP2D6. C-1, clozapine N-oxide; C-6, 7-hydroxyclozapine; C-7, 9-hydroxydiazepam.
**Figure S2:** HPLC-UV chromatograms of incubation of CLZ with cDNA-expressed CYP1A2 (A), CYP2D6 (B), and CYP3A4 (C) in presence of 100 µM GSH and 8 µM GSTP1-1.
Figure S3: Michaelis-Menten plots of incubations of CLZ with HLM (A), CYP2D6 (B) and CYP3A4 (C) Metabolites: CLZ-NO (■), DMCLZ (△), and total GSH conjugates (●). Mean values (solid points) and error bars (SD) are shown.
**Figure S4:** Effect of CYP450 inhibitors on the formation of individual GSH conjugates. Relative ratios between the formed conjugates are taken into account. Data are expressed as % inhibition of control activity and represent mean of duplicate determinations. KTZ, ketoconazole; QND, quinidine, SPZ, sulfaphenazole; FURA, furafylline; TCP, tranylcypromide; DDC, diethyldithiocarbamate.
Figure S5: Variability of formation of GSH-conjugates in incubations of individual HLM fractions (numbered) and pooled HLM (HLM) with 100 µM CLZ and effect of CYP2D6-inhibitor quinidine (2 µM) on formation of GSH-conjugates. Data are expressed in µM of formed GSH conjugates and represent mean of duplicate determinations.
Table S1 Involvement of individual CYPs in CLZ bioactivation and formation of individual GSH conjugates (nmol/min/nmol CYP) after 30 minutes incubations of 10 µM or 100 µM CLZ, 50 nM of CYPs, in the presence of 100 µM GSH and 8 µM GST P1-1.

<table>
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<tr>
<th>Total GSH conjugates</th>
<th>CLZ conc.</th>
<th>10µM</th>
<th>100µM</th>
<th>10µM</th>
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