Characterisation of human cytochrome P450s involved in
the bioactivation of clozapine

Sanja Dragovic, Patrina Gunness, Magnus Ingelman-Sundberg, Nico P.E. Vermeulen and Jan
N.M. Commandeur

Affiliations of the authors:

Division of Molecular Toxicology, Leiden/Amsterdam Center for Drug Research, Faculty of
Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.
(SD, NPEV, JNMC)

Section of Pharmacogenetics, Department of Physiology and Pharmacology, Karolinska
Institutet, SE-17177 Stockholm, Sweden. (PG, MI-S)
Bioactivation of clozapine by human P450s

Corresponding author:
Jan N.M. Commandeur, Division of Molecular Toxicology, Leiden/Amsterdam Center for Drug Research, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. E-mail: j.n.m.commandeur@vu.nl

Number of text pages: 19
Number of Tables: 2
Number of Figures: 5
Number of References: 44
Number of words Abstract: 219
Number of words Introduction: 605
Number of words Discussion: 787

Abbreviations:
CLZ, clozapine; CLZ-NO, clozapine N-oxide; DMCLZ, N-desmethyloclozapine; hGST, human glutathione S-transferase; HLM, human liver microsomes; LC, liquid chromatography; MS/MS, tandem mass spectrometry; P450, cytochrome P450.
Abstract

Clozapine is known to cause hepatotoxicity in a small percentage of patients. Oxidative bioactivation to reactive intermediates by hepatic cytochrome P450s has been proposed as possible mechanism. However, in contrast to their role in formation of N-desmethylclozapine and clozapine N-oxide, the involvement of individual P450s in the bioactivation to reactive intermediates is much less well characterized. The results of the present study show that seven out of fourteen recombinant human P450s were able to bioactivate clozapine to a GSH-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 to 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.
1. Introduction

Clozapine (CLZ) is an atypical antipsychotic drug, which causes fewer extrapyramidal side effects than other neuroleptics (Saffereman et al., 1991; Buchanan, 1995; Wagstaff and Perry, 2003). However, because of its risk for 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). Next to agranulocytosis, mild hepatotoxicity has been reported as a side 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 known yet, 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 of CLZ-treated patients and many in vitro studies using human liver microsomes (HLM) have shown that CLZ is extensively metabolised by P450s to multiple products, as summarized in Figure 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 in order to identify the role of individual P450s in the oxidative metabolism of CLZ to its major metabolites, N-desmethylclozapine (DMCLZ; C-2) and clozapine N-oxide (CLZ-NO; C-1), see Figure 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 point to 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 the 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., 2011, Barbara and Castro-Perez, 2011). Theoretically, two different iminium ions can be formed by dehydrogenation of the two different pairs of nitrogen-methylene bonds of the piperazine-ring, Figure 1. However, the structure of the corresponding cyanide adducts remain to be established. So far no bioactivation studies have been performed using recombinant human P450 isoenzymes because in none of the previous studies GSH 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 CYPs 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 of 100 individuals was quantified.
2. Material and methods

2.1. Materials

Supersomes containing cDNA-expressed human cytochrome P450 (CYP) enzymes were purchased from BD Biosciences (Breda, 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, USA) and contained 20 mg protein/mL. DMCLZ and CLZ-NO were purchased from Sigma Aldrich (Netherlands). 7-Hydroxyclozapine (7-OH-CLZ; C-6) and 9-hydroxyclozapine (9-OH-CLZ; 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 hGSTP1-1 was done as described previously (Dragovic et al., 2010). Protein concentrations were determined according to the method of Bradford (Bradford, 1976) with reagent obtained from Bio-Rad (München, 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.

2.2. 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). Besides GSH, potassium cyanide (1 mM) was also used as trapping reagent to 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 min at 14000 rpm. The supernatants were analysed by HPLC, as described below.

2.3. 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. Based on these experiments, enzyme concentrations used were 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 $K_m$ and $V_{max}$ were determined by nonlinear regression according to the Michaelis-Menten equation, using GraphPad Prism software (San Diego, CA).
2.4. 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 DMSO 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 (SPZ, 10 μM), tranylcypromine (TCP, 25 μM), quinidine (2 μM), and diethylthiocarbamate (DDC, 20 μM), were used to investigate the involvement of CYP1A2, CYP3A (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 was dissolved in water. Reactions were initiated by the addition of 500 μM NADPH (final concentration) and incubated for 30 min at 37 °C. Incubations containing the mechanism-based inhibitors FURA, TCP and DDC were preincubated for 15 min in the presence of NADPH before addition of CLZ. The reactions were terminated by the addition of 25 μL of 10 % HClO₄ and centrifuged for 15 min at 14000 rpm. The supernatants were analyzed by HPLC and LC-MS/MS, as described below. Control incubations without CLZ were performed under the same conditions to ensure that the presence of inhibitors did not interfere with the quantification of formed metabolites. Incubations without inhibitor were performed as a control as well. All incubations were performed in duplo.
2.5. Incubations of CLZ with individual human liver microsomes

Liver microsomes were prepared of liver pieces from 100 individuals from a liver bank (approved by the Ethical Review Board) established at the Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden as described previously (Westlind et al., 1999). Protein contents were determined according to the method of Bradford (Bradford, 1976) with reagent obtained from Bio-Rad using bovine serum albumin as standard. The microsomes were stored in potassium phosphate buffer (100 mM, pH 7.4) at -80°C until use.

Incubations with HLM obtained from 100 individuals were performed for 30 min at 37°C with a final concentration of 0.1 mg microsomal protein/mL, in the presence of 5 mM GSH. The concentration of CLZ was 100 μM. The reaction was initiated with 0.5 mM NADPH and terminated by perchloric acid, as described above. Incubations were also performed in presence of 2 μM quinidine to investigate the involvement of CYP2D6 in the bioactivation of CLZ.

2.6. Analytical methods

All samples were analysed by reversed-phase HPLC as described previously, using LC-MS/MS for identification and UV/Vis detection at 254 nm for quantification of formed metabolites (Dragovic et al., 2010). Standard curves of commercially available DMCLZ and CLZ-NO references were used for quantification of these two metabolites. A standard curve of CLZ was used to estimate the concentrations of the formed GSH and cyanide conjugates, assuming that their extinction coefficients are equal to that of CLZ.
3. Results

3.1. Oxidative metabolism of CLZ by pooled human liver microsomes

Previously, CLZ was shown to be metabolised by pooled HLM in presence of hGST P1-1 to both 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, Figure 2. In total six GSH conjugates of CLZ were found in incubations of HLM in presence of hGST P1-1. The structures of conjugates CG-1, CG-3, CG-4, CG-5 and CG-6 are shown in Figure 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 resulting from partial degradation of the piperazine ring, CLZ-C\textsubscript{2}H\textsubscript{2} (C-3) and CLZ-C\textsubscript{3}H\textsubscript{4} (C-4) (Dragovic et al., 2010). Analysis by the highly sensitive LCMS-Q-TOF instrument revealed the formation of two minor peaks eluting between 15.5 and 18.5 minutes with \textit{m/z} value of 343.14 (C-6, and C-7), which corresponds to [M+H]\textsuperscript{+} of hydroxylated CLZ metabolites (see Supplemental Figure S1). Only C-6 could also be detected by UV-detection, Figure 2. Using the references obtained by the Udenfriend reaction (Slavik et al., 2011), the metabolites correspond to 7-hydroxyclozapine (C-6) and 9-hydroxyclozapine (C-7).

3.2 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 Figure 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 CYPs, KTZ inhibited GSH conjugation by 67.0 %. QND and DDC inhibited formation of GSH conjugates only to a low extent, 18.0 and 19.3 %, respectively. Results for inhibition of individual conjugates formation were matching those for total GSH conjugates. This can be seen in additional material, Figure S3. These results indicate that CYP3A-enzymes are the major isoenzyme involved in bioactivation of CLZ by pooled HLM.

The effect of the enzyme inhibitors on formation of stable metabolites of CLZ are consistent with previous studies, showing strong inhibition of CLZ-NO formation by CYP3A-inhibitor KTZ and significant inhibition of DMCLZ by CYP1A2-inhibitor FURA and, to a lesser extent, CYP3A-inhibitor KTZ (Figure 3).

3.3. Oxidative metabolism of CLZ by recombinant human P450 enzymes

Oxidative metabolism of CLZ by recombinant human P450s was determined at substrate concentrations of 10 and 100 µM CLZ. The specific activities by which each metabolite was formed by the individual recombinant P450's are shown in Table 1. All metabolites which are formed by HLM were also represented in incubations with recombinant P450 enzymes as shown in Supplemental Figure S2. Consistent with previous studies, several CYPs showed relatively high specific activity in N-demethylation (CYP2D6 > CYP3A4 > CYP1A2) and N-oxidation (CYP3A4 > CYP1A2, CYP2D6) of CLZ, whereas the other CYPs 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, Figure 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 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 m/z 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 only at this concentration peak areas at 254 nm could quantified. As shown in Figure 4B, the highest activity was found CYP2D6 and CYP2C18. CYP1A2, CYP2A6, and CYP3A4 showed approximately 25% of CYP2D6 activity, respectively.

3.4. 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 metabolism by HLM and recombinant P450s. For both recombinant CYPs 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 $V_{max}$ values and the intrinsic clearance, $V_{max}/K_M$, 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 $V_{max}$ values, respectively. For CLZ N-demethylation a $K_M$ of 268 μM and $V_{max}$ 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 ($V_{max}/K_M$) for total GSH conjugates than for DMCLZ and CLZ-NO, respectively. The $K_M$ and $V_{max}$ values for DMCLZ and CLZ-NO are somewhat higher than previously determined in 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). Also, 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. When comparing enzyme kinetic parameters of recombinant CYP3A4 and CYP2D6, both $K_M$ and $V_{max}$ 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.

3.5. Interindividual variability in CLZ bioactivation by human liver microsomes

To investigate the interindividual variability in microsomal biotransformation of CLZ, hundred individual 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 are displayed in Figure 5, with the samples ranked from highest to lowest GSH-conjugate formation. The total of GSH conjugates formation 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 fourteen individuals by Zhang et al. (Zhang et al., 2008). Interestingly, two HLM-fractions, indicated by stars in Figure 5, showed very high GSH conjugate formation when compared to formation of DMCLZ and CLZ-NO. When correlating formation of GSH-conjugates to DMCLZ and CLZ-NO relatively low correlations were found: DMCLZ vs. total CLZ-SG, $r^2 = 0.384$; CLZ-NO vs. total CLZ-SG, $r^2 = 0.624$. The low correlations might be explained by the fact that multiple P450's are involved in these metabolites, as shown in Table 1. Because inhibition of CYP2D6 had only minor effect in pooled HLM, also inhibition studies with quinidine were performed to investigate if in any of the liver fractions CYP2D6 plays a significant role in bioactivation of CLZ. The treatment of the panel of human liver microsomal samples (n = 100 subjects) with quinidine did not show significant inhibition of the formation of total GSH conjugates, see Supplemental Figure S5.

**Discussion**

Bioactivation of CLZ to reactive metabolites in the different target tissues is generally accepted as a cause for its ADRs. Occasional cases of liver injury in patients treated with CLZ might be the result from local bioactivation to a reactive nitrenium ion by hepatic P450s (Maggs et al., 1995). In addition, bioactivation of CLZ to a reactive iminium ion, which can be trapped by cyanide, has been demonstrated in microsomal incubations (Argoti et al., 2005; Rousu et al., 2009; Li et al., 2011; Barbara and Castro-Perez JM, 2011), might contribute to hepatotoxicity. Although these reactive CLZ metabolites have been demonstrated in many in
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 P450's and HLM in presence and absence of enzyme-specific inhibitors.

The results summarized in Table 1 and Figure 6, show that bioactivation of CLZ, when quantified as the total of GSH conjugates, appears to be a relatively important pathway when compared to N-demethylation and N-oxidation pathways. DMCLZ formation which is the major pathway of CLZ metabolism was on average only 1.6-fold higher than bioactivation. In the set of 100 HLM fractions, two individuals even showed higher activity of bioactivation when compared to the N-demethylation and N-oxidation pathways, Figure 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. Also also relatively high activity was observed with recombinant CYP2D6, Figure 4A. When determining enzyme kinetic parameters for bioactivation, it appeared that CYP2D6 displayed a 1.3 higher intrinsic clearance for bioactivation when compared to 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, Figure 4B. However, considering the fact that CYP3A4 is on average almost 20-fold more abundant in HLM than CYP2D6 (Shimada et al., 1994), we conclude that CYP3A4 is most likely 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, Figure 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, but also the
interactions with other xenobiotics that influence its activity might cause interindividual differences that could lead to the susceptibility for CLZ adverse drug reactions. Although it appears that CYP3A4 is without common functional polymorphisms (Hiratsuka, 2012), it has been demonstrated that nonsynonymous alleles for CYP3A4 encode enzymes with altered catalytic properties (Dai et al., 2001; Eiselt 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 (Figure 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 which were genotyped as poor or extensive metabolisers of CYP2D6. Also, 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 cytoxic in human cell lines transfected with CYP3A4 (Hosomi et al., 2011; Thompson et al., 2012), down regulation of Nrf-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 cytochrome 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 part of the patients to hepatotoxic effects of CLZ.
Authorship Contributions

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

Conducted experiments: Dragovic, Gunness

Contributed new reagents or analytical tools: Dragovic, Gunness

Performed data analysis: Dragovic, Gunness, Commandeur

Wrote of contributed to the writing of the manuscript: Dragovic, Commandeur
References


Footnotes

This research was performed within the framework of project D3-201 of the Dutch Top Institute Pharma.
Legends to the Figure

**Figure 1.** Metabolic scheme of identified oxidative metabolites of clozapine formed by cytochrome P450s. Structures of proposed reactive nitrenium and iminium ions are shown between brackets, and are based on identified structures of adducts to GSH and cyanide.

**Figure 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.

**Figure 3.** Effect of CYP450 inhibitors on the metabolism of clozapine by HLM to clozapine N-oxide (CLZ-NO), N-desmethyloclozapine (DMCLZ) and total of GSH conjugates (CG-1, CG-4, CG-5 and CG-6). Data are expressed as % of control activity and represent mean of duplicate determinations.

**Figure 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 %, where 100 % correspond to the activity of the most active enzyme.

**Figure 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 to N-demethylation and N-oxidation are marked by grey column and asterix.
Table 1  Specific activities of oxidative metabolism of CLZ by recombinant human CYPs.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>DMCLZ</th>
<th>CLZ-NO</th>
<th>Total of GSH conjugates(^b)</th>
<th>Cyanide adduct (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10µM 100µM</td>
<td>10µM 100µM</td>
<td>10µM 100µM</td>
<td>100µM</td>
</tr>
<tr>
<td>CYP 1A1</td>
<td>0.64±0.02 0.56±0.03</td>
<td>0.24±0.01 0.20±0.02</td>
<td>0.30±0.008 0.32±0.01</td>
<td>0.024±0.003</td>
</tr>
<tr>
<td>CYP 1A2</td>
<td>1.12±0.01 8.71±0.1</td>
<td>0.55±0.01 3.70±0.1</td>
<td>0.060±0.003 0.25±0.004</td>
<td>0.20±0.01</td>
</tr>
<tr>
<td>CYP 1B1</td>
<td>1.16±0.01 0.94±0.05</td>
<td>0.84±0.01 0.64±0.07</td>
<td>0.15±0.01 0.15±0.004</td>
<td>0.010±0.001</td>
</tr>
<tr>
<td>CYP 2A6</td>
<td>n.d. n.d.</td>
<td>n.d. n.d.</td>
<td>n.d. n.d.</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>CYP 2C8</td>
<td>0.92±0.01 0.89±0.01</td>
<td>0.10±0.01 0.075±0.006</td>
<td>n.d. n.d.</td>
<td>0.058±0.002</td>
</tr>
<tr>
<td>CYP 2C9</td>
<td>0.33±0.02 0.22±0.01</td>
<td>0.065±0.005 0.057±0.01</td>
<td>n.d. n.d.</td>
<td>0.010±0.001</td>
</tr>
<tr>
<td>CYP 2C18</td>
<td>4.08±0.05 5.71±0.02</td>
<td>0.025±0.005 0.065±0.01</td>
<td>n.d. n.d.</td>
<td>0.41±0.02</td>
</tr>
<tr>
<td>CYP 2C19</td>
<td>6.62±0.03 5.11±0.02</td>
<td>0.71±0.03 0.58±0.005</td>
<td>n.d. n.d.</td>
<td>0.030±0.003</td>
</tr>
<tr>
<td>CYP 2D6</td>
<td>8.43±0.2 23.7±1.1</td>
<td>0.51±0.01 1.62±0.1</td>
<td>0.47±0.01 1.60±0.05</td>
<td>0.64±0.07</td>
</tr>
<tr>
<td>CYP 2J2</td>
<td>0.70±0.07 1.40±0.1</td>
<td>0.05±0.01 0.10±0.01</td>
<td>0.009±0.001 0.03±0.03</td>
<td>0.05±0.01</td>
</tr>
<tr>
<td>CYP 3A4</td>
<td>2.98±0.01 15.3±0.1</td>
<td>2.37±0.02 5.62±0.01</td>
<td>0.87±0.01 1.92±0.01</td>
<td>0.18±0.01</td>
</tr>
<tr>
<td>CYP 3A5</td>
<td>0.24±0.04 1.29±0.02</td>
<td>0.32±0.04 0.88±0.03</td>
<td>0.15±0.01 0.30±0.01</td>
<td>0.08±0.01</td>
</tr>
</tbody>
</table>
a) Specific activities (nmol/min/nmol P450) were calculated by peak areas at 254 nm assuming that extinction coefficients of GSH-conjugates and cyanide adducts are the same as that of CLZ.

b) Involvement of individual CYPs in CLZ bioactivation is expressed as formation of total of GSH conjugates and cyanide adduct after 30 minutes incubations of 10 µM or 100 µM CLZ, 50 nM recombinant human CYPs, in the presence of 100 µM GSH and 8 µM hGST P1-1 or 1 mM KCN, respectively. The values represent averages of two measurements; standard deviations were less than 10%; n.d. not detectable.
Table 2

Enzyme kinetic parameters for formation of clozapine metabolites by HLM and recombinant human CYP3A4 and CYP2D6.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Kinetic Parameters</th>
<th>Enzyme fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CYP3A4</td>
</tr>
<tr>
<td>Total GSH conjugates</td>
<td>$K_M$ (µM)</td>
<td>30.3 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$ (nmol/min/nmol CYP)</td>
<td>3.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>$V_{max}/K_M$ (ml/min/nmol CYP; x10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>101.9</td>
</tr>
<tr>
<td>Clozapine N-Oxide</td>
<td>$K_M$ (µM)</td>
<td>92.7 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$ (nmol/min/nmol CYP)</td>
<td>9.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>$V_{max}/K_M$ (ml/min/nmol CYP; x10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>102.5</td>
</tr>
<tr>
<td>N-Demethyl Clozapine</td>
<td>$K_M$ (µM)</td>
<td>47.6 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$ (nmol/min/nmol CYP)</td>
<td>19.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>$V_{max}/K_M$ (ml/min/nmol CYP; x10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>419</td>
</tr>
</tbody>
</table>

a. $V_{max}$ values for HLM are expressed as nmol/min/mg protein.

b. $V_{max}/K_M$ values for HLM are expressed in ml/min/mg protein.
Figure 4

A. Clozapine GSH-Conjugates

- 10 μM CLZ
- 100 μM CLZ

B. Clozapine CN-adduct

- 100 μM CLZ