The involvement of CYP1A2 and CYP3A4 in the metabolism of clozapine

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Aims Clozapine (CLZ), an atypical neuroleptic with a high risk of causing agranulocytosis, is metabolized in the liver to desmethylclozapine (DCLZ) and clozapine *N*-oxide (CLZ-NO). This study investigated the involvement of different CYP isoforms in the formation of these two metabolites.

Methods Human liver microsomal incubations, chemical inhibitors, specific antibodies, and different cytochrome P450 expression systems were used.

Results K_m and V_{max} values determined in human liver microsomes were lower for the demethylation (61 ± 21 μM, 159 ± 42 pmol min^{-1} mg protein^{-1} mean ± s.d.; $n = 4$), than for the *N*-oxidation of CLZ (308 ± 1.5 μM, 456 ± 167pmol min^{-1} mg protein^{-1}; $n = 3$). Formation of DCLZ was inhibited by fluvoxamine (53 ± 28% at 10 μM), triacetyloleandomycin (33 ± 15% at 10 μM), and ketoconazole (51 ± 28% at 2 μM) and by antibodies against CYP1A2 and CYP3A4. CLZ-NO formation was inhibited by triacetyloleandomycin (34 ± 16% at 10 μM) and ketoconazole (51 ± 13% at 2 μM), and by antibodies against CYP3A4. There was a significant correlation between CYP3A content and DCLZ formation in microsomes from 15 human livers ($r = 0.67$; $P = 0.04$). A high but not significant correlation coefficient was found for CYP3A content and CLZ-NO formation ($r = 0.59$; $P = 0.09$). Using expression systems it was shown that CYP1A2 and CYP3A4 formed DCLZ and CLZ-NO. K_m and V_{max} values were lower in the CYP1A2 expression system compared to CYP3A4 for both metabolic reactions.

Conclusions It is concluded that CYP1A2 and CYP3A4 are involved in the demethylation of CLZ and CYP3A4 in the *N*-oxidation of CLZ. Close monitoring of CLZ plasma levels is recommended in patients treated at the same time with other drugs affecting these two enzymes.

Keywords: clozapine, human liver microsomes, CYP1A2, CYP3A4

Introduction

Clozapine is an atypical neuroleptic with some major advantages compared with classical neuroleptics used in the treatment of schizophrenia. It has much less extrapyramidal side effects [1, 2], and it is also effective in therapy resistant schizophrenic patients [2]. A major disadvantage of clozapine is the high risk of agranulocytosis occurring in 1–2% of the patients [1, 3], which makes frequent haematologic monitoring necessary.

The metabolism of clozapine is thought to occur mainly in the liver, with the two major metabolites being desmethylclozapine (DCLZ) and clozapine *N*-oxide (CLZ-NO) [1, 4, 5]. Considerable variation in plasma levels among patients given the same dose [6] may be caused by polymorphic cytochrome P450 enzymes (CYP) in the liver [7]. Fischer *et al.* [8] linked clozapine metabolism to CYP2D6, which is responsible for the metabolism of many classical neuroleptics such as perphenazine, zuclopenthixol, thioridazine and haloperidol [7]. In a recent study by Pirmohamed *et al.* [9] the *N*-demethylation of clozapine was linked to CYP1A2, while the *N*-oxidation was suggested to be catalysed by either CYP2C9 or CYP2E1.

In panels of healthy subjects Dahl *et al.* [10] found no association between the debrisoquine or S-mephenytoin hydroxylation phenotypes and clozapine disposition. Jerling *et al.* [11] and Hiemke *et al.* [12] found that concomitant treatment with clozapine and fluvoxamine, a potent inhibitor of CYP1A2 [13], caused a marked increase in clozapine plasma concentrations. Haring *et al.* [14] detected lower clozapine plasma levels in smokers than in non-smokers. The oral clearance of clozapine correlated with the CYP1A2 activity determined by a caffeine test in healthy subjects [15]. These studies taken together indicate that CYP1A2 is a major enzyme catalyzing the metabolism of clozapine. In addition, Jerling *et al.* [11] found an induction of clozapine elimination by carbamazepine, an inducer of CYP3A4 [16], indicating that CYP3A4 might also be involved in the metabolism of clozapine.

Our aim was to determine the role of different CYP450 enzymes in the biotransformation of clozapine. Therefore

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we have investigated the metabolism of clozapine *in vitro*, using human liver microsomes, cell line microsomes, chemical inhibitors and specific antibodies.

Methods

Chemicals

Clozapine, clozapine *N*-oxide, and desmethylclozapine were kindly provided by Sandoz Pharmaceuticals, Basel, Switzerland. Fluvoxamine maleate was obtained from Solvay Duphar, Weesp, Netherlands, triacetyloleandomycin (TAO) from Pfizer Corporation, Brussels, Belgium, diethyldithiocarbamate (DDC), sulphaphenazole and NADPH from Sigma Chemicals Co., St Louis, MO, USA, and quinidine from Apoteksbolaget, Stockholm, Sweden. All other chemicals were purchased from commercial sources and were of analytical grade quality.

Human liver samples and preparation of human liver microsomes

Samples of human livers (HL 17, 18, 19, 22, 38, 39, 41, 42, 43, 45, 46, 48, 49, 50, and 51) were obtained from kidney transplant donors and microsomes were prepared as described earlier [17]. The protein content was estimated according to Lowry *et al.* [18]. All samples were stored at -80° C until used for incubations.

Investigation of clozapine kinetics in human liver microsomes

Time dependency was determined with incubations of 100 μM clozapine for 15, 30, 45, and 60 min. The formation of the metabolites desmethylclozapine and clozapine *N*-oxide was found to be linear within that time range.

Protein dependency was determined using 10 μM clozapine at microsomal protein concentrations of 0.25, 0.5, 0.75, and 1 mg. The formation of the two metabolites was found to be linear within that range.

Clozapine was incubated at eight different substrate concentrations (1, 5, 10, 25, 50, 100, 250, 500 μM) in microsomes from four livers (HL 38, 39, 49, 51). Incubations were performed at 37° C for 30 min in a shaker bath with a microsomal protein content of 0.75 mg in the presence of $MgCl_2$ (2.5 mM), tris-HCl (50 mM, pH 7.4) and NADPH (2 mM). The final incubation volume was 1 ml. Incubations were started by addition of NADPH and stopped by freezing in a mixture of ethanol and dry ice. Incubations for all studies were performed in triplicate.

Experiments with chemical inhibitors

Fluvoxamine (1, 10 μM) [13] was used as an inhibitor for CYP1A2, TAO (1, 10 μM) and ketoconazole (0.2, 2 μM) for CYP3A4, sulphaphenazole (2, 20 μM) for CYP2C9, quinidine (0.5, 5 μM) for CYP2D6, and DDC (10, 100 μM) for CYP2E1 [19, 20]. TAO and DDC were preincubated for 10 min in the presence of NADPH and the reaction started by addition of clozapine. Fluvoxamine, quinidine, and DDC were dissolved in water, TAO, sulphaphenazole, and ketoconazole in methanol. Methanol concentrations in incubations never exceeded 1% and were compared with appropriate blanks. All incubations were run in duplicate in three livers at clozapine concentrations of 50 μM.

Experiments with specific antibodies

Antiserum for human CYP1A1, 1A2 and monoclonal antibodies directed against CYP3A4, 3A5 (MAB 3A4/5) were purchased from Gentest, Woburn, MA, USA.

Antiserum for CYP1A1, 1A2 was preincubated for 30 min at room temperature. Incubations were performed with 0.05, 0.1, and 0.2 mg IgG for 30 min at 37° C and activities were compared with incubations containing normal rabbit serum.

MAB 3A4/5 were preincubated for 15 min on ice at concentrations of 0.05, 0.1, 0.2, and 0.5 mg IgG and then incubated for 20 min at 37° C. All incubations were run in duplicate in two livers at clozapine concentrations of 50 μM.

Correlation of clozapine metabolism with CYP1A2, CYP2C8, CYP2D6, CYP2E1, and CYP3A4 contents

For the correlation study microsomes from 15 human livers were incubated for 30 min with 50 μM clozapine. The formation of desmethylclozapine and clozapine *N*-oxide was measured and then correlated with the content of CYP 1A2, 2C8, 2D6, 2E1, and 3A4.

Western blotting was performed with the same microsomal preparations using standard procedures for SDS-polyacrylamide gel electrophoresis and semidry transfer of proteins to nitrocellulose sheets [21]. Human liver microsomes (15 μg of protein per lane) were separated on 10% polyacrylamide gels and one nitrocellulose sheet containing all microsome samples was prepared for each antibody to be tested. The mouse monoclonal antibody 114/2 was used to detect CYP2D6 [22]. Polyclonal rabbit antibodies against CYP1A2, CYP2C8 (recognizing essentially CYP2C8), CYP3A4 (recognizing both CYP3A4 and CYP3A5; since CYP3A5 is expressed only in about 20% of human livers [23], we will use CYP3A4 in the following), and CYP2E1 were kindly provided by Dr Philippe Beaune, Paris. They had been raised against purified expressed CYPs, and they recognize their specific antigens with a sensitivity of approximately 0.2 pmol but did not recognize 5 pmol of other P450 forms [24]. Bound antibodies were visualized with alkaline phosphatase conjugated anti-mouse or anti-rabbit IgG (Caltag Laboratories, San Francisco, CA, USA) and using 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) and *p*-nitro blue tetrazolium chloride (NBT) as substrates. The blots were densitometrically analyzed with an Elscript 400 scanner (Hirschmann, Neuried, Germany) and GelBase/GelBlot Pro V 3.0 software (UVP Ltd, Cambridge, U.K.).

Investigation of clozapine kinetics with cytochromes expressed in yeast and lymphoblasts

Saccharomyces cerevisae strain W303-1B modified by the insertion of the *GAI10-CYC1* galactose-inducible promoter immediately upstream of the 5′ end of the YRED open reading frame [25] was used. The CYP1A2 cDNA was cloned from human liver total RNA by PCR-amplification

(forward primer 5′-GTC GGA TCC ATG GCA TTG TCC CAG TCT GTT CC-3′, reverse primer 5′-CTG GAA TTC TCA ATT GAT GGA GAA GCG CC-3′) and after digestion with *Bam*HI and *Eco*RI inserted into the pYeDP60 (pV60) expression vector [26]. The yeast was transformed with the pV60-CYP1A2 plasmid according to Cullin & Pompon [27] and cultured as described by Bellamine *et al.* [28]. After mechanical disruption of the yeast cells, microsomes were isolated by precipitation of the 20 000 *g* supernatant with polyethylene glycol 4000. Protein content was measured by the method of Lowry *et al.* [18]. The content of cytochrome P450 was 36 pmol mg^{-1} protein as determined by the reduced carbon monoxide difference spectra [29]. Expression of CYP1A2 was verified by Western-blot analysis using CYP1A2 specific polyclonal antiserum. The EROD (ethoxyresorufin *O*-deethylation), measured according to the method of Burke *et al.* [30] was determined to be 36.8 pmol min^{-1} mg^{-1} protein (turnover of 1.0 min^{-1}). The modified *Saccharomyces cerevisae* Strain W303-1B and the pYeD60vector were generous gifts from Dr Denis Pompon (CNRS, Gif-sur-yvette, France).

Microsomes from human lymphoblasts expressing CYP3A4 were purchased from Gentest, Woburn, MA, USA. Testosterone 6β-hydroxylase activity was determined to be 2350 pmol min^{-1} mg^{-1} protein (turnover of 31.3 min^{-1}). Incubations were performed in duplicate according to the protocol of the kinetic studies in human liver microsomes at protein concentrations of 0.25 and 0.15 mg in CYP1A2- and CYP3A4-expressing systems, respectively.

Analysis of clozapine metabolites

Ammonium carbonate buffer (1 ml 20 mM, pH 9.0) was added to 1 ml microsomal incubate. 1.8 ml was then transferred to a Sep-Pak light C18 Waters cartridge, washed with 2 ml water, 0.5 ml sodium hydroxide solution (30 mM, pH 9.0), 0.5 ml 3% acetonitrile in sodium hydroxide (30 mM, pH 9.0), 0.5 ml 20% acetonitrile in sodium hydroxide (30 mM, pH 9.0), and 0.2 ml 50% acetonitrile in phosphoric acid (10 mM), and then eluted with 0.3 ml 50% acetonitrile in phosphoric acid (10 mM). After evaporation to dryness in a vacuum centrifuge (Savant® Speed Vac Plus SC 110A, Farmingdale, NY, USA) the residue was dissolved in the buffer of the mobile phase and 50 μl injected into the h.p.l.c. system.

The h.p.l.c. system for the determination of clozapine *N*-oxide and desmethylclozapine consisted of a Shandon C_{18} Hypersil 3 μ 100 × 4.6 mm column (Cheshire, UK) connected with a 5 μ Hypersil BDS C_{18} procolumn (Cheshire, UK), and a u.v. detector set at 260 nm. Chromatograms were recorded and peak areas were integrated using the laboratory data system ELDS 900 (Chromatography Data System, Kungshög, Sweden). As mobile phase a solution of ammonium acetate buffer (10 mM, pH 5.0) with 30% acetonitrile and *N,N* dimethyl-n-octylamine (200 μl per 500 ml mobile phase) was used at a flow rate of 1 ml min^{-1}. The retention times were 3.0 and 4.4 min for desmethylclozapine and clozapine *N*-oxide, respectively. Mean recovery for desmethylclozapine was 91 ± 8.8% ($n = 16$; concentration range 100 nM–2 μM) and for clozapine *N*-oxide 80 ± 3.8% ($n = 16$; concentration range 100 nM–2 μM). A standard curve with concentrations ranging from 500 nM–25 μM for desmethylclozapine and clozapine *N*-oxide resulted in calibration curves with $r = 0.9996$ for desmethylclozapine and $r = 0.9994$ for clozapine *N*-oxide running through the origin.

Calculations

For statistical analysis comparing groups we used the unpaired Student's *t*-test. Multiple regression analysis was applied to the correlation study. *P* values of 0.05 or less were regarded as significant.

Enzyme kinetics were investigated using the non-linear regression program (Enzfitter, Biosoft, Cambridge, England). Calculated parameters were maximum rate of formation (V_{max}) and Michaelis-Menten constant (K_m). Eadie-Hofstee plots were used to examine the involvement of more than one enzyme. The computer program GraphPad prism (GraphPad Software, San Diego, CA, USA) applied a one enzyme model and a two enzyme model to the data and compared fit using a '*F*' ratio test. Intrinsic clearance (CL_{int}) was calculated from $CL_{int} = V_{max}/K_m$.

Results

Clozapine kinetics

The formation of the metabolites desmethylclozapine and clozapine *N*-oxide followed Michaelis-Menten kinetics (Figures 1 and 2). The K_m for the formation of desmethylclozapine varied from 42 to 89 μM in the four livers investigated and the V_{max} ranged from 107 to 204 pmol min^{-1} mg^{-1} protein, respectively (Table 1). A one enzyme and a two enzyme model were applied to the data. The one enzyme model resulted in a better fit in all livers except HL 51. However, the standard errors of K_m and V_{max} using the two enzyme model in this liver were greater than 50% of the value or even higher than the value estimated.

Both K_m and V_{max} for the formation of clozapine *N*-oxide were higher than those determined for the formation

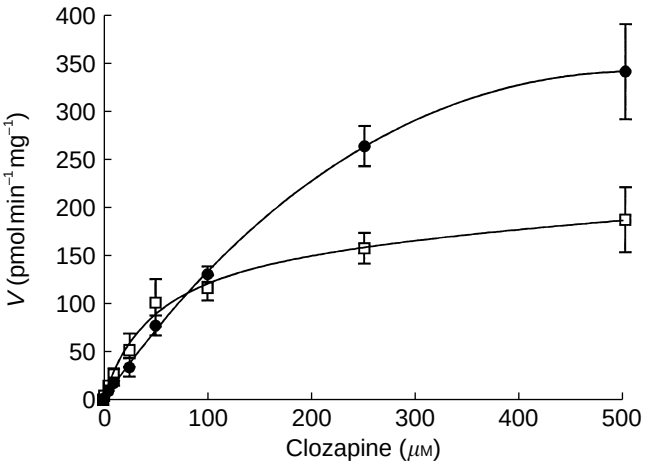

Figure 1 Michaelis-Menten-kinetics of the formation of desmethylclozapine (□) and clozapine *N*-oxide (●) in HL 39. Each point represents mean ± s.d. ($n = 3$).

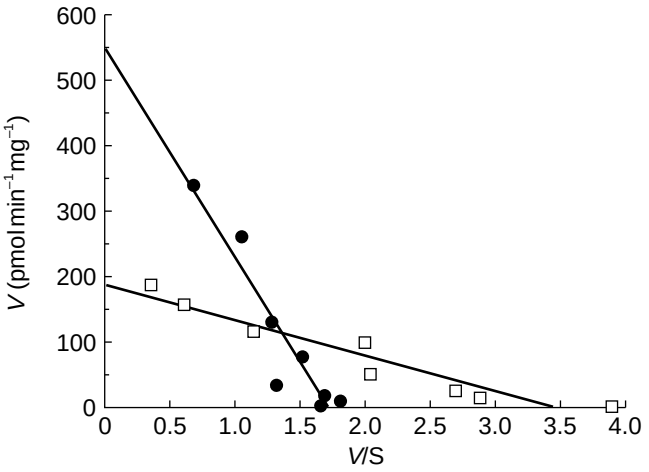

Figure 2 Eadie-Hofstee plot of clozapine metabolism (● clozapine-*N*-oxide, □ desmethylclozapine) in HL 39. Each point represents mean ($n=3$).

of desmethylclozapine (Table 1). One has to keep in mind that the K_m-values of 308, 310, and 307 μM were all close to the highest substrate concentration used (500 μM clozapine). In HL 51 enzyme saturation was not achieved, resulting in an uncertain K_m-value of 1298 μM. Eadie-Hofstee plots (not shown) gave weak hints of the involvement of two enzymes in the formation of clozapine *N*-oxide in HL 51.

Experiments with chemical inhibitors

Inhibition studies using six different inhibitors for different CYP450 enzymes revealed the involvement of CYP1A2 and CYP3A4 in the demethylation and mainly CYP3A4 in the *N*-oxidation of clozapine (Figure 3).

Fluvoxamine inhibited the demethylation by 34 ± 27% at 1 μM and 53 ± 28% at 10 μM. TAO reduced the formation of DCLZ by 32 ± 11% and 33 ± 15% at 1 and 10 μM, respectively. Ketoconazole inhibited the demethylation by 28 ± 20% and 68 ± 15% at 0.2 and 2 μM, respectively. Sulfaphenazole, quinidine and DDC caused only minor changes in the formation of DCLZ at the lower concentrations used. However, DDC inhibited the demethylation by 38 ± 9.2% at 100 μM.

CLZ-NO formation was inhibited by ketoconazole by 41 ± 14% and 51 ± 13% at 0.2 and 2 μM, respectively. TAO reduced the *N*-oxidation of CLZ by 16 ± 22% and 34 ± 16% at 1 and 10 μM, respectively. Fluvoxamine caused an inhibition by 12 ± 21% and 22 ± 18% at 1 and 10 μM, respectively. Sulfaphenazole and quinidine had no inhibitory effect at either concentration used, while DDC inhibited the *N*-oxidation only at the higher concentration by 28 ± 15%.

Experiments with specific antibodies

The results of the inhibition study using antiserum for human 1A1/1A2 and MAB3A4 in two livers are shown in Figure 4a and b. Inhibition varied to a great extent between the two livers investigated. Antiserum for human 1A1/1A2 caused a maximum decrease of the demethylation in HL 46 by 37% and by 12% in HL 48. The formation of clozapine *N*-oxide was only slightly inhibited in one liver (19% in HL 46), while there was no effect seen in HL 48. MAB3A4 reduced mainly the formation of CLZ-NO by 39% and 56% in HL 46 and HL 48, respectively. DCLZ formation was decreased in HL 46 by 11% and in HL 48 by 39%.

Correlation of clozapine metabolism with CYP1A2, CYP2C8, CYP2D6, CYP2E1, and CYP3A4 contents

Since more than one enzyme seem to be involved in the formation of DCLZ and CLZ-NO, multiple regression analysis was applied to the data of the correlation study to investigate the influence of all enzymes (CYP1A2, CYP2C8, CYP2D6, CYP2E1 and CYP3A4) on the formation of each metabolite at the same time (Table 2). CYP3A4 correlated with the formation of DCLZ ($r=0.67$; $P=0.04$) and

Table 1 Michaelis-Menten kinetics of the formation of desmethylclozapine and clozapine N-oxide in microsomes of four human livers.

	HL 38	*HL 39*	*HL 49*	*HL 51*
			Desmethylclozapine	
V_{max} (pmol min^{-1} mg^{-1} protein)	107 ± 2.6	204 ± 12	145 ± 3.8	180 ± 12
K_m (μM)	48 ± 4.1	66 ± 12	42 ± 3.9	89 ± 17
CL_{int} (μl min^{-1} mg^{-1} protein)	2.3	3.1	3.4	2.0
			Clozapine N-oxide	
V_{max} (pmol min^{-1} mg^{-1} protein)	263 ± 10	558 ± 47	548 ± 29	(2190 ± 446)*
K_m (μM)	308 ± 25	310 ± 52	307 ± 32	(1298 ± 345)*
CL_{int} (μl min^{-1} mg^{-1} protein)	0.85	1.80	1.79	(1.69)*

*Uncertain as enzyme saturation was not achieved at the highest clozapine concentration (500 μM).
Data are mean ± s.d. (n = triplicate incubations with microsomes from each liver).

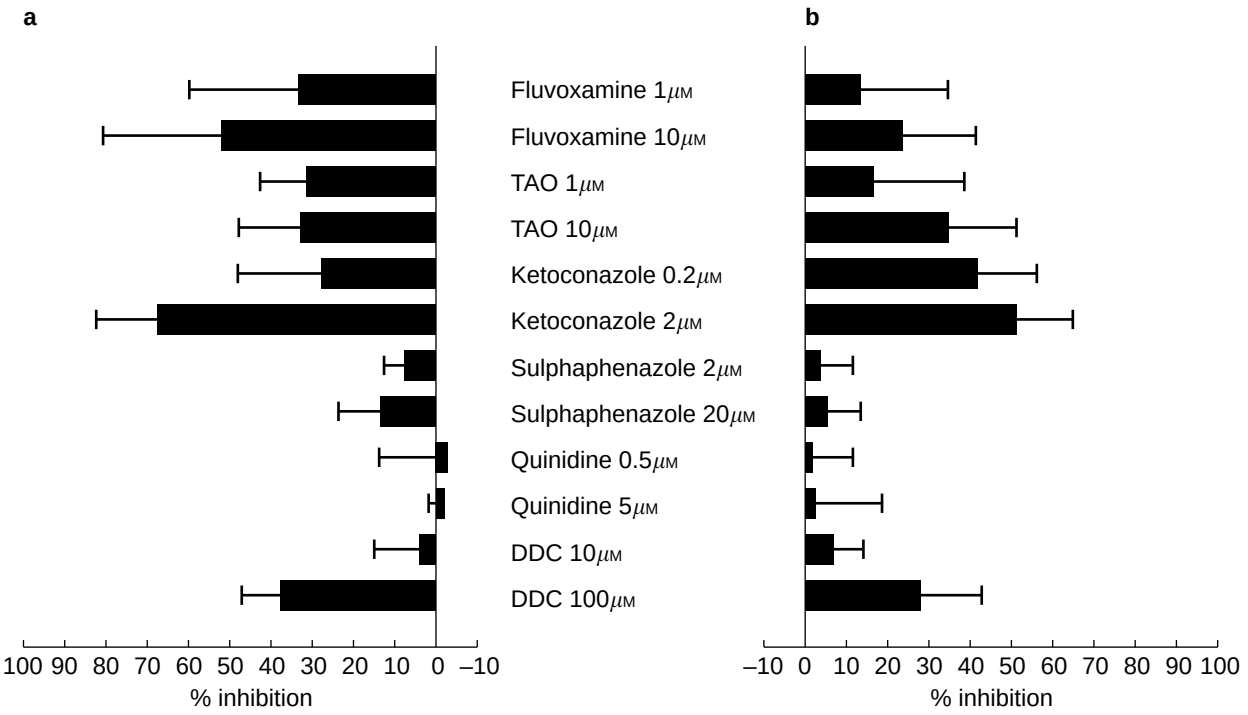

Figure 3 Effect of various CYP inhibitors on the formation of desmethylclozapine (a) and clozapine *N*-oxide (b). Each bar represents mean of duplicate determinations in three livers + s.d.

Table 2 Summary of multiple regression analysis of the effect of CYP1A2, CYP2C8, CYP2D6, CYP2E1, CYP3A4 on the formation of desmethylclozapine (DCLZ) and clozapine *N*-oxide (CLZ-NO).

	DCLZ		*CLZ-NO*	
	r *value*	P *value*	r *value*	P *value*
CYP1A2	0.39	0.12	0.21	0.43
CYP2C8	−0.36	0.24	0.11	0.75
CYP2D6	0.24	0.29	0.04	0.89
CYP2E1	0.38	0.14	0.14	0.61
CYP3A4	0.67	0.04*	0.59	0.09

*$P<0.05$.

CLZ-NO ($r=0.59$; $P=0.09$). CYP1A2 was the second strongest determinant, although not significant, in the formation of both metabolites. CYP2C8, CYP2D6, and CYP2E1 showed no correlation with the formation of either metabolite.

Cytochromes P450 expressing systems

Results of the incubations with microsomes from CYP1A2-expressing yeast and CYP3A4-expressing lymphoblasts are listed in Table 3. Both enzymes catalyzed demethylation and *N*-oxidation of clozapine. K_m and V_{max} values were lower in CYP1A2-expressing yeast for DCLZ and CLZ-NO compared with CYP3A4-expressing lymphoblasts. The intrinsic clearance (CL_{int}) measured in yeast expressing CYP1A2 was higher for demethylation than for *N*-oxidation. In contrast, for CYP3A4 expressed in lymphoblasts the CL_{int} was higher for the *N*-oxidation than for the demethylation (Table 3).

Discussion

The present investigation suggests that the formation of desmethylclozapine is mainly catalysed by CYP1A2 and CYP3A4 and the formation of clozapine-*N*-oxide is mainly mediated by CYP3A4.

Clozapine kinetics determined in microsomes from four human livers revealed monophasic Michaelis-Menten kinetics (Figures 1 and 2). An increase in substrate concentrations might result in biphasic kinetics, but would lead to unphysiological clozapine concentrations much higher than those occurring *in vivo*. Monophasic Michaelis-Menten kinetics with K_m and V_{max} for the *N*-oxidation similar to ours were also found by Tugnait *et al*. [31]. The fact that monophasic Michaelis-Menten kinetics are observed but more than one enzyme is involved in the formation of a metabolite, might suggest only minor differences in the K_m-values for the different enzymes. The K_m-values determined with stable expressed CYP1A2 and CYP3A4 show for the demethylation a difference by a factor of 16, while it was less than 10 for the *N*-oxidation (Table 3). In addition the V_{max} values are in the same order of magnitude and different expression systems have been used to investigate the enzyme kinetics of the distinct cytochromes. Thus, the difference may be too small to reveal biphasic kinetics in human liver microsomes. However, inhibition studies with drugs and specific antibodies, and studies using various CYP450 expression systems clearly show, that two enzymes are involved in the demethylation and *N*-oxidation of clozapine.

For the identification of the enzymes involved in clozapine metabolism studies using chemical inhibitors were performed. The CYP1A2 inhibitor fluvoxamine and the CYP3A4 inhibitors TAO, and ketoconazole had a strong inhibitory effect on the demethylation. DDC inhibited the

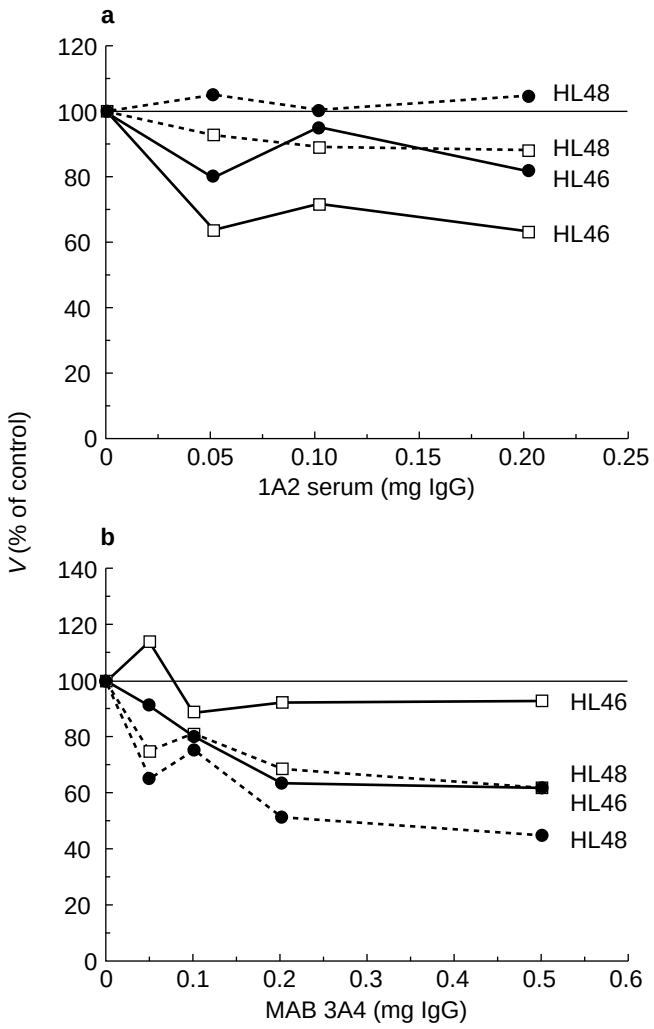

Figure 4 Inhibition of desmethylclozapine (□) and clozapine *N*-oxide (●) formation in HL 46 (—) and HL 48 (---) by 1A2 antiserum (a) and MAB3A4 (b). Each point represents mean of duplicate determinations.

demethylation of clozapine only at the higher concentration (100 μM) used, suggesting the possible involvement of CYP2E1, although at this concentration DDC inhibits other CYP isoforms [19]. No inhibition was seen at 10 μM, and there was no significant correlation between the CYP2E1 content and the rate of demethylation determined in 15 livers.

Clozapine *N*-oxide formation was mainly inhibited by ketoconazole and TAO. Inhibition by fluvoxamine was less pronounced. DDC caused a reduction of the formation of clozapine *N*-oxide by 28% at 100 μM. However, as with the demethylation CYP2E1 content did not correlate with CLZ-NO formation as determined in the correlation study, and there was no effect seen using 10 μM of DDC as an inhibitor.

These results partly confirm the study of Pirmohamed *et al.* [9], who detected a strong inhibition by the CYP1A2 inhibitor furafylline on the formation of desmethylclozapine, but not on the formation of clozapine *N*-oxide. Ketoconazole neither influenced the *N*-demethylation nor the *N*-oxidation in low (<5 μM) concentrations, which are specific for CYP3A4. In contrast we found a strong inhibition of the *N*-oxidation and demethylation of clozapine by TAO and ketoconazole. The differences in effects might be explained by the fact, that Pirmohamed *et al.* used only one liver for the inhibition study. Effects of inhibitors vary widely in different livers as we could detect using three livers.

MAB3A4 antibodies inhibited the *N*-oxidation of clozapine by a mean of 47% (maximum mean of HL 46 and HL 48; Figure 4b), while the demethylation was reduced by a mean of 24% indicating the involvement of CYP3A4 in both metabolic steps. The effect of antiserum for human CYP1A1/2 was weaker. The demethylation was reduced by 25% (maximum mean of HL 46 and 48), while there was almost no effect seen on the *N*-oxidation. In the correlation study CYP3A4 content, determined through Western blotting, correlated with the formation of DCLZ and CLZ-NO. CYP1A2 was the second strongest determi-

Table 3 Michaelis-Menten kinetics of the formation of desmethylclozapine and clozapine *N*-oxide using CYP1A2-expressing yeast and CYP3A4-expressing lymphoblasts.

	CYP1A2 expressed in yeast	*CYP3A4 expressed in human lymphoblasts*
	Desmethylclozapine	
V_{max} (pmol min^{-1} mg^{-1} protein)	160 ± 14	404 ± 30
V_{max} (pmol min^{-1} $pmol^{-1}$ CYP)	4.4 ± 0.39	5.4 ± 0.40
K_m (μM)	18 ± 6.8	304 ± 45
CL_{int} (μl min^{-1} mg^{-1} protein)	8.8	1.3
	Clozapine N-oxide	
V_{max} (pmol min^{-1} mg^{-1} protein)	44 ± 3.6	289 ± 17
V_{max} (pmol min^{-1} $pmol^{-1}$ CYP)	1.2 ± 0.1	3.9 ± 0.22
K_m (μM)	12 ± 3.9	100 ± 16
CL_{int} (μl min^{-1} mg^{-1} protein)	3.8	2.9

nant but failed to reach statistical significance. Neither CYP2C8, 2D6, nor CYP2E1 correlated with the rate of formation of DCLZ or CLZ-NO. Regarding correlation studies one has to keep in mind, that correlation analysis are of limited value, if more than one enzyme is involved in the formation of one metabolite. This is especially the case, when one of the major forms of CYP450 enzymes (like CYP3A4) is involved [32].

Results of the investigation with CYP1A2 and CYP3A4 expression systems reveal the capability of both enzymes to metabolize clozapine. The determined K_m and V_{max} values for the demethylation and *N*-oxidation in CYP1A2-expressing yeast were lower than those determined in CYP3A4-expressing lymphoblasts (Table 3). Intrinsic clearance was highest for demethylation in CYP1A2-expressing yeast, suggesting that demethylation of clozapine is the major metabolic reaction. However, to determine the exact contribution of the two enzymes to clozapine metabolism the relative levels of these CYP450 enzymes in human liver have to be taken into account. *In vivo* studies and case reports show, that comedication with drugs being metabolized by either CYP1A2 [33] or CYP3A4 [33] or drugs which induce CYP3A4 [11] as well as smoking [14] influence clozapine plasma levels in patients and healthy subjects.

The discrepancy between K_m values of CLZ-NO found in systems with expressed enzymes (CYP1A2: $K_m = 12$ μM; CYP3A4 $K_m = 100$ μM) and in human liver microsomes ($K_m \sim 300$ μM) might be explained by the involvement of other enzymes such as flavin-containing monooxygenases in CLZ-NO formation. Tugnait *et al.* [31] described a decrease in CLZ-NO formation (83% inhibition) after heating of human liver microsomes, which inactivates flavin-containing monooxygenases. However, the contribution of FMO_3 to the *N*-oxidation of clozapine *in vivo* is difficult to determine. The K_m measured in expressed FMO_3 determined by Tugnait *et al.* is two orders of magnitude higher than therapeutic plasma concentrations [5], and the V_{max} is relatively low. The overall problem is to extrapolate and compare K_m- and V_{max}-values from different artificial systems, such as cell lines or yeast, with the *in vivo* situation.

Taking the results together the increased plasma levels of clozapine during concomitant intake of fluvoxamine [33] or caffeine [34] can be explained. Both caffeine and fluvoxamine are substrates for CYP1A2 [35–37]. Lower clozapine plasma levels in smokers compared with non-smokers found by Haring *et al.* [14] may also be explained, since smoking induces CYP1A2 [35].

Erythromycin, a substrate and inhibitor of CYP3A4 [35], led to elevated plasma levels with severe side effects in two patients treated with clozapine [33, 38]. Jerling *et al.* [11] detected induced elimination of clozapine under carbamazepine treatment. Both findings corroborate the involvement of CYP3A4 in clozapine metabolism.

It is concluded that CYP1A2 and CYP3A4 are the major enzymes forming desmethylclozapine and CYP3A4 forming clozapine-*N*-oxide. Close monitoring of clozapine plasma levels is advisable in cases of co-treatment with other drugs being metabolized by those two enzymes.

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