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# Inter-Individual Variation in Relative CYP1A2/3A4 Phenotype Influences Susceptibility of Clozapine Oxidation to CYP-Specific Inhibition in Human Hepatic Microsomes

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Abbreviations: CLZ, clozapine; CLZ *N*-oxide, clozapine *N*-oxide; CYP, cytochrome P450; norCLZ, norclozapine; EROD, 7-ethoxyresorufin O-deethylation; FMO, flavin-containing monooxygenase; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; SSRI, selective serotonin-reuptake inhibitor.

**ABSTRACT:**

The atypical antipsychotic drug clozapine (CLZ) is effective in a substantial number of patients who exhibit treatment-resistance to conventional agents. CYP1A2 is generally considered to be the major enzyme involved in the biotransformation of CLZ to its *N*-demethylated (norCLZ) and *N*-oxygenated (CLZ *N*-oxide) metabolites in liver, but several studies have also implicated CYP3A4. The present study assessed the interplay between these CYPs in CLZ biotransformation in a panel of hepatic microsomal fractions from fourteen individuals. The relative activity of CYPs 1A2 and 3A4 in microsomes was found to be a major determinant of the relative susceptibility of norCLZ formation to inhibition by the CYP-selective inhibitors fluvoxamine and ketoconazole. In contrast, the activity of CYP3A4 alone was correlated with the susceptibility of CLZ *N*-oxide formation to inhibition by these agents. These findings suggest that both CYPs may be dominant CLZ oxidases in patients and that the relative activities of these enzymes may determine clearance pathways. *In vivo* assessment of CYP1A2 and CYP3A4 activities, perhaps by phenotyping approaches, could assist the optimization of CLZ dosage and minimise pharmacokinetic interactions with coadministered drugs.

The atypical antipsychotic agent clozapine (CLZ) is effective in many patients who are resistant to conventional antipsychotic drugs, such as the phenothiazines and butyrophenones. However, its wider use is limited by inter-individual variation in efficacy and toxicity. *In vitro* evidence suggests that the biotransformation of CLZ to its major metabolites *N*-desmethyl-CLZ (norCLZ) and CLZ *N*-oxide is catalyzed by hepatic cytochromes P450 (CYPs) and flavin-containing monooxygenases (FMOs) (Pirmohamed et al., 1995). NorCLZ formation has been attributed to CYP1A2 and CYP3A4 (Linnet and Olesen, 1997; Pirmohamed et al., 1995) and CYPs 1A2 and 3A4, as well as the FMOs, catalyze CLZ *N*-oxygenation *in vitro* (Tugnait et al., 1997; Pirmohamed et al., 1995).

There is wide inter-individual variation in serum concentrations of CLZ and its active metabolite norCLZ and the potential for pharmacokinetic drug-drug interactions in psychotic patients is high because of the likelihood of concurrent drug treatment. There have been numerous clinical reports that coadministration of alternate substrates and inhibitors of CYP1A2, such as the selective serotinin reuptake inhibitor (SSRI) fluvoxamine, fluoroquinolone antibacterials and caffeine, inhibit CLZ clearance and mediate toxic interactions (Hiemke et al., 1994; Jerling et al., 1994). However, it has also been reported that drugs such as fluoxetine, paroxetine and erythromycin, that inhibit CYP3A4 rather than CYP1A2, may also precipitate toxicity (Wetzel et al., 1998; Centorrino et al., 1996). The factors that determine individual susceptibility to CYP1A2 and CYP3A4 inhibition that leads to clinical toxicity are presently unclear.

In a proportion of patients who receive CLZ therapeutic failure may occur because of rapid elimination of the drug and the inability to maintain effective plasma concentrations. Efficacy in some patients may be established by coadministration of the CYP1A2 inhibitor fluvoxamine, which decreases CLZ elimination and restores therapeutic concentrations of the

drug in serum (Ozdemir et al., 2001; Kontaxakis et al., 2005). Fluvoxamine is unlikely to be effective in patients who eliminate CLZ rapidly via CYP3A4.

In this study we tested the extent of individual variation in the oxidation of CLZ by CYPs 1A2 and 3A4 in a panel of human liver microsomal fractions and how this influenced susceptibility to CYP-specific inhibitors. NorCLZ formation was differentially inhibited in individual livers by the CYP3A4- and CYP1A2-specific inhibitors ketoconazole and fluvoxamine, whereas CLZ *N*-oxide formation was selectively inhibited by ketoconazole in the majority of livers. Inter-individual variation in the relative activities of CYPs 1A2 and 3A4 has emerged from these studies as a determinant of the susceptibility of CLZ oxidation to CYP-specific inhibition in human liver microsomes.

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## Materials and Methods

**Drugs and chemicals.** CLZ, 7-ethoxyresorufin, resorufin, fluvoxamine, ketoconazole, quinidine, tranylcypromine, benzydamine, norCLZ, CLZ *N*-oxide and biochemicals were from Sigma Aldrich (Castle Hill, NSW, Australia) or Roche Pty Ltd (Castle Hill, NSW, Australia). Microsomal fractions containing cDNA-directed CYPs or FMOs expressed in human B-lymphoblastoid or insect cells (Supersomes) were obtained from BD Biosciences (North Ryde, NSW, Australia). Reagents for electrophoresis were from Bio-Rad (Richmond, CA). HPLC-grade solvents were from Rhone-Poulenc (Baulkham Hills, NSW, Australia) and analytical reagents were from Ajax (Sydney, NSW, Australia). Hyperfilm-MP, Hybond-$N^+$ filters, and reagents for enhanced chemiluminescence were from Amersham GE Healthcare (Rydalmere, NSW, Australia). The preparation and characteristics of the anti-CYP peptide antibodies have been reported elsewhere (Edwards et al., 1998).

**Liver donors and preparation of microsomal fractions.** Experiments in human microsomal fractions were approved by the ethics committees of the Western Sydney Area Health Service and the Universities of New South Wales and Sydney, in accordance with the Declaration of Helsinki. Tissue from adult donors surplus to that used in the transplantation of pediatric recipients or biopsies from the normal margin of the liver during resection were obtained through the Queensland and Australian Liver Transplant Programs (Princess Alexandria Hospital, Brisbane, Queensland, and Royal Prince Alfred Hospital, Sydney, NSW, Australia, respectively). Tissue was perfused immediately with cold Viaspan solution (DuPont, Wilmington, DE, USA), transported on ice to the laboratory and frozen in liquid nitrogen. Samples from fourteen individuals were used in the present study; drug histories and activities of CYP-dependent biotransformation pathways are shown in Table 1. Washed microsomes were prepared by differential ultracentrifugation and then stored at -70°C as frozen suspensions in potassium phosphate buffer (50 mM, pH 7.4), that contained 20%

glycerol and 1 mM EDTA (Murray et al., 1986). Microsomal protein contents were determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

**HPLC analysis of CLZ biotransformation.** CLZ biotransformation in human hepatic microsomes was conducted at 37°C in 0.1 M potassium phosphate buffer (pH 7.4; final volume 250 µl). Incubations contained 0.2 mg microsomal protein and 100 µM CLZ and were initiated with NADPH (1 mM). After 15 min reactions were terminated with 1 mL of cold 0.1% formic acid. NorCLZ and CLZ *N*-oxide formation was linear under these conditions of protein and time. In kinetic experiments, incubations contained CLZ (25-500 µM) and data were analyzed by non-linear regression using Prism 4 (GraphPad Software, Inc., San Diego, CA). Reactions involving cDNA-expressed enzymes contained 200 µg protein, 100 µM CLZ and were incubated for 60 min.

Inhibitory effects of fluvoxamine, ketoconazole, quinidine, tranylcypromine and benzydamine on CLZ oxidation (100 µM) were determined in duplicate in microsomal fractions at two different inhibitor concentrations. $IC_{50}$ values for the CYP-selective inhibitors fluvoxamine and ketoconazole (5-7 concentrations, in duplicate) were determined in each of the microsomal fractions.

CLZ metabolites were extracted from microsomal incubations using Oasis HLB solid-phase cartridges (Waters Corp, Milford, MA) and separated on a Synergy Fusion-RP polar embedded $C_{18}$ column (250 x 4.6 mm, particle size 4 µm; Phenomenex Australia Pty Ltd, Pennant Hills, NSW) operating at 38$^{o}$C (Zhang et al., 2007). The mobile phase consisted of 3:2:5 acetonitrile:methanol:ammonium acetate buffer (20 mM, pH 5.0), containing *N*,*N*-dimethyloctylamine (0.4 mL/L), and the flow rate was 1.0 mL/min (260 nm detection). Retention times of authentic norCLZ, doxepin (internal standard) and CLZ *N*-oxide were 11.4, 13.1 and 16.9 min, respectively.

**Other assays of CYP function in human hepatic microsomes.** 7-Ethoxyresorufin *O*-deethylation (EROD) activity (0.05 mg protein/0.2 mL incubation; 7-ethoxyresorufin 2.5 μM) was measured in Tris-HCl buffer (0.1 M, pH 7.8) by the time-dependent formation of the fluorescent product resorufin using the excitation/emission wavelength pair of 560/580 nm (Prough et al., 1978).

Testosterone 6β-hydroxylation (0.15 mg protein/0.4 mL incubation; $^{14}$C-testosterone 50 μM, 0.18 μCi) was measured as described previously (Murray, 1992). Reactions were performed in potassium phosphate buffer (0.1 M, pH 7.4) at 37°C for 2.5 min. Products were extracted with chloroform, separated by thin-layer chromatography, subjected to autoradiography and quantified by scintillation spectrometry (Murray, 1992).

Dextromethorphan *O*-demethylation (0.15 mg protein/0.25 mL incubation; dextromethorphan 16 μM) was measured as described by Vielnascher et al. (1996). Reactions were conducted in potassium phosphate buffer (0.1 M, pH 7.4) at 37$^{o}$C for 30 min and terminated by addition of ice-cold buffer; phenacetin was used as the internal standard. Products were loaded onto Oasis HLB solid-phase cartridges that had been conditioned with distilled water (1 mL) and methanol (1 mL). Cartridges were washed with 10% aqueous methanol (2x1 mL), and then eluted with methanol (2x1 mL). Quantification was by LC-mass spectrometry using a mobile phase of 50% aqueous acetonitrile, containing 0.1% formic acid.

Tolbutamide 4-hydroxylation activity (0.3 mg protein/0.4 mL reaction, tolbutamide 300 μM) was measured by HPLC according to Knodell et al. (1987). Linearity of product formation in all assays was established in preliminary experiments; substrate utilization was ≤15% in all cases.

Human liver microsomes (15 μg of protein per lane) were subjected to electrophoresis on 7.5% polyacrylamide gels in the presence of 5% 2-mercaptoethanol and 2% sodium

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dodecylsulfate (Laemmli, 1970) with minor modifications (Murray et al., 1986). Proteins were transferred to nitrocellulose sheets (Towbin et al., 1979) that were incubated with anti-CYP peptide antibodies (Edwards et al., 1998). Immunoreactive proteins were detected by enhanced chemiluminescence and autoradiography on Hyperfilm-MP (Amersham), and quantified by densitometry (Bio-Rad, Richmond, CA).

**DNA Extraction from human liver tissue and genotyping of CYP alleles.**

Genomic DNA was obtained from human liver by a standard phenol/chloroform/isoamyl alcohol extraction, quantified spectrophotometrically and diluted to 100 ng/μL; samples were stored at –20°C until used for CYP genotyping. Genotyping for allelic variants of CYPs 1A2 and 3A4 was done using the polymerase chain reaction (PCR) in a GeneAmp 2400 thermocycler (Perkin-Elmer Pty Ltd, Rowville, VIC, Australia). Primers were designed to amplify target sequences spanning the SNPs within each allele; Table 2 includes primer sequences and amplification conditions. All primers used for PCR analysis were custom synthesized by Geneworks Pty Ltd (Hindmarsh, SA, Australia).

Each PCR reaction incorporated genomic DNA (250 ng), forward and reverse primers (0.25 μM), 2.5X HotStarTaq MasterMix (20 μM; Quantum-Scientific, Milton, QLD, Australia) and sterile water to a final volume of 50 μL per reaction. Products were separated by electrophoresis on 2% agarose gels in Tris-Borate-EDTA buffer, stained with ethidium bromide and visualized under UV light (Gel-Doc 2000; Bio-Rad, Richmond, CA, USA).

The *CYP1A2*1D*, *CYP1A2*1F*, *CYP1A2*4*, *CYP1A2*6*, *CYP3A4*1B* and *CYP3A4*17* variant alleles were identified by direct sequencing of the PCR products (ABI prism Big Dye, DNA Analysis Facility, University of New South Wales, Sydney, Australia). The *CYP1A2*1C* allele was identified by restriction digestion of the amplified product using *Dde* I (Promega, Annandale, NSW, Australia). The variant is cleaved into 464 bp and 132 bp

fragments, whereas the wild-type allele is not digested; in preliminary studies the amplified products were confirmed by sequencing. To detect the *CYP3A4*2* and *CYP3A4*10* alleles amplicons were treated with the restriction endonucleases *Xcml* and *HpyCH4III*, respectively.

**Statistics**. Data are presented as means±SEM from measurements in hepatic fractions from individual subjects (n=14), unless otherwise indicated. Correlation and statistical analysis was performed with Statview (Abacus concepts, Berkeley, CA).

## Results

**Individual variation in the microsomal oxidation of CLZ and other substrates in human liver.** Fourteen individual human liver microsomal fractions were evaluated for CLZ oxidation capacity at a substrate concentration of 100 μM (Table 1). NorCLZ formation varied over an 8.8-fold range (43-378 pmol/mg protein/min; median 153) whereas CLZ *N*-oxide formation, which tended to be more extensive, varied over a 10.3-fold range (79-817 pmol/mg protein/min; median 306). By comparison EROD activity mediated by CYP1A2 varied over the range 7.3-49.1 pmol/mg protein/min; median 17.7 (n=14). The oxidative biotransformation of the CYP3A4 substrate testosterone varied over a 12-fold range (0.50-6.08 nmol/mg protein/min; Table 1). Significant variations in tolbutamide hydroxylation and dextromethorphan *O*-demethylation activities were also evident (Table 1).

Several cDNA-expressed CYPs mediated CLZ oxidation. CYP2D6 was highly active in norCLZ formation, followed by CYPs 3A4, 2C8 and 1A2 (12.59, 8.68, 5.48 and 5.40 pmol/pmol CYP/hr, respectively; Figure 1A). The remaining CYPs catalyzed norCLZ formation at lower rates (0.10-3.03 pmol/pmol CYP/hr). CLZ *N*-oxide formation by CYP3A4 and, to a lesser degree, CYP1A2 was extensive (48 and 6.1 pmol/pmol CYP/hr, respectively), whereas the remaining 12 CYPs were less efficient (0.03-3.65 pmol/pmol CYP/hr). As part of the present study, three FMO enzymes were also tested for CLZ oxidation capacity. While none supported norCLZ formation, FMO1 and FMO3 were active in CLZ *N*-oxygenation (43 and 19 pmol/ pmol/pmol enzyme/hr, respectively); FMO5 was inactive (Figure 1A).

**Relationship of CYP biotransformation pathways to other CYP-mediated reactions in human liver.** Regression analysis was used to relate the activities of CLZ oxidation pathways to those of well defined microsomal substrate oxidations mediated by CYPs 1A2, 3A4, 2D6 and 2C9. For norCLZ Spearman's correlations were significant with CYP1A2-catalyzed EROD activity ($\rho$=0.613, $p<0.02$; Figure 1B) and CYP3A4-mediated

testosterone 6β-hydroxylation (ρ=0.688, p<0.01; Figure 1B). CYPs 2C9 and 2D6, have previously been suggested to be alternate CLZ oxidases, but correlation of CLZ oxidation with rates of oxidation of tolbutamide and dextromethorphan were non-significant (ρ=0.212 and 0.198, respectively). The findings with CLZ *N*-oxide formation were similar, with significant Spearman's correlations observed with both testosterone 6β-hydroxylation (ρ=0.767, p<0.01; Figure 1B) and EROD (ρ=0.719, p<0.01; Figure 1B). In this set of microsomal fractions the expression and activity of CYP3A4 and 1A2 were also well correlated (ρ=0.70, p<0.01 and ρ=0.83, p<0.01, respectively).

**Expression of CYP proteins and allelic variants of CYPs 1A2 and 3A4 in human liver.** The relative microsomal contents of potential alternate CLZ oxidases CYPs 1A2, 3A4, 2C and 2D6 were quantified in human liver microsomal fractions using monospecific anti-CYP peptide antibodies (data not shown). The kinetics of CLZ metabolite formation were determined in seven of the microsomal fractions (Table 3). In the case of norCLZ the $K_m$ range varied 3.9-fold (35-135 μM; median 56 μM) whereas the $V_{max}$ varied over a 10.3-fold range (96-964 pmol/mg protein/min; median 445). These estimates are similar to those in previous literature reports (Eiermann et al., 1997). The $K_m$ for CLZ *N*-oxide formation varied over a 4.3-fold range (28-120 μM; median 76 μM) and the $V_{max}$ varied over a 3.3-fold range (311-1039 pmol/mg protein/min; median 405). Representative kinetic plots in two livers (HL16 and HL27) are shown in Figure 2A (norCLZ) and Figure 2B (CLZ *N*-oxide).

The possibility that allelic variants may contribute to the observed variations in expression and activity of major CYP CLZ oxidases was assessed. Genotyping for several CYP1A2 and CYP3A4 variant alleles was undertaken in DNA extracted from the available livers. The CYP1A2*1C allele was detected in one liver (HL21), which was also heterozygous for the CYP1A2*1D allele; the *1D allele was also detected in HL37 (Table 4). The CYP1A2*1F allele was detected in two of the livers but the non-synonymous variants –

CYPs 1A2*4 and 1A2*6 – which have been shown to encode variant enzymes with diminished catalytic activity (Zhou et al., 2004) - were not detected in any livers. Similarly, none of the CYP3A4 variant alleles (*1B, or the non-synonymous variants *2, *10 or *17 – that have been associated with altered function toward several substrates; Dai et al., 2001; Eiselt et al., 2001; Sata et al., 2000) were detected in any livers.

**Determinants of the differential inhibition of CLZ oxidation pathways in individual human livers.** The major focus of the present study was to test the hypothesis that CYPs 1A2 and 3A4 may contribute to CLZ oxidation to varying extents in individuals. Preliminary studies using cDNA-expressed CLZ oxidizing CYPs 1A2 and 3A4 confirmed the selectivity of their inhibition by fluvoxamine and ketoconazole, respectively (Fig 3A). The CYP2D6 and 2C inhibitors quinidine and tranylcypromine were inactive toward both CYPs 1A2 and 3A4. Similarly, benzydamine selectively inhibited CLZ *N*-oxygenation mediated by FMO3, and to a lesser extent FMO1, when tested at a concentration of 20 μM. However, at the higher concentration (250 μM), benzydamine also inhibited CLZ oxidation by cDNA-expressed CYPs 1A2 and 3A4 (Fig 3A).

Fluvoxamine and ketoconazole were tested against CLZ oxidation in 14 individual livers. There was considerable variation in the extent of inhibition of norCLZ formation by the CYP3A4-selective inhibitor ketoconazole (to 16-88% of control; median 66%, and to 34-120% of control; median 76%, at 2 μM and 0.2 μM, respectively; Figure 3B) and by the CYP1A2-selective inhibitor fluvoxamine (to 15-85% of control; median 51%, and to 29-117% of control; median 66%, at concentrations of 10 μM and 1 μM, respectively). In contrast, quinidine and tranylcypromine, were essentially inactive, although norCLZ formation in two livers (HL24 and HL28) was decreased to around 50% of control by tranylcypromine (50 μM).

Similar findings were made in the case of CLZ *N*-oxide formation. Thus, ketoconazole decreased *N*-oxide formation to 13-90% of control; median 55%, and to 30-102% of control; median 61%, at concentrations of 2 μM and 0.2 μM, respectively (Figure 3B). The CYP1A2-specific inhibitor fluvoxamine decreased *N*-oxide formation to 50-117% of control; median 77% and to 61-108% of control; median 85%, at 10 μM and 1 μM, respectively. Again, the microsomal formation of this metabolite was refractory to quinidine and tranylcypromine. The FMO-specific substrate benzydamine did not influence CLZ *N*-oxide formation in human liver microsomes when tested at the lower concentration of 20 μM that selectively inhibited cDNA-expressed FMO activity. However, at the higher concentration (250 μM), benzydamine inhibited microsomal formation of both CLZ metabolites. These findings implicated CYPs 1A2 and 3A4, but not CYPs 2D6, 2C or FMOs in the formation of CLZ metabolites by human liver microsomes.

The relative susceptibilities of CLZ oxidation pathways to the CYP1A2- and CYP3A4-selective inhibitors were tested in each of the human liver microsomal preparations. $IC_{50}$ values for fluvoxamine as an inhibitor of microsomal norCLZ formation were in the range 16-100 μM in eight of the livers but were lower in the remaining six ($IC_{50}$s 1.3-9.6 μM; Table 4). Similarly, ketoconazole inhibited microsomal norCLZ formation with $IC_{50}$ values between 11-130 μM in eight of the individual hepatic fractions, but was more potent against the activity in the remaining six livers ($IC_{50}$s 0.35-3.7 μM). Ketoconazole was also active against microsomal CLZ *N*-oxygenation in all livers (0.16-6.8 μM). By contrast, *N*-oxygenation of CLZ was inhibited weakly by fluvoxamine with relatively high $IC_{50}$ values (range 55 to >250 μM) in all livers except HL36 ($IC_{50}$ 14 μM).

As an indicator of relative CYP1A2:CYP3A4 inhibition potency $IC_{50}$ ratios for fluvoxamine and ketoconazole were determined in individual microsomal fractions and were in the ranges 0.012-174 (for norCLZ formation) and 7-590 (for CLZ *N*-oxide). In the case of

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three livers a ratio could not be determined for the inhibition of CLZ *N*-oxide formation because the $IC_{50}$s for fluvoxamine were large. The logarithm of the $IC_{50}$ ratio (fluvoxamine:ketoconazole) for the inhibition of norCLZ formation was inversely correlated with the logarithm of the EROD:testosterone 6β-hydroxylation activity ratio ($r=0.77$, $p<0.01$; Figure 4A). Thus, the relative potencies of CYP1A2/3A4 inhibitors against microsomal norCLZ formation were dependent on relative activities of the enzymes. In the case of CLZ *N*-oxygenation, the corresponding relationship was not significant. However, the logarithm of the $IC_{50}$ ratio was linearly related to the logarithm of the testosterone 6β-hydroxylation activity in the available microsomal fractions ($r=0.68$, $p<0.05$; Figure 4B). In contrast, the relationship between log $IC_{50}$ ratio and microsomal EROD activity was not significant. Thus, CYP3A4 activity appeared to be an important determinant of the susceptibility of microsomal CLZ *N*-oxygenation to inhibition.

The possibility that the CYP-selective inhibitors ketoconazole and fluvoxamine may interact to modulate CLZ biotransformation in microsomes was tested. Fixed concentrations of ketoconazole (2 μM) and fluvoxamine (10 μM) were added simultaneously to five separate microsomal fractions and the inhibition of CLZ oxidation was assessed relative to the separate effects of the individual inhibitors. The observed effect of the combined inhibitors on CLZ oxidation was also compared with the sum of the effects of the individual inhibitors. In the case of norCLZ formation the calculated values slightly underestimated the observed inhibition (by 9.6±4.1%) and for CLZ *N*-oxide formation a small overestimate was apparent (by 7.0±3.1%; Fig 5A). However, these discrepancies were within normal experimental variation. To corroborate these findings we also assessed whether the presence of additional recombinant proteins in incubations influenced the action of the CYP-selective inhibitors. As shown in Fig 5B, recombinant CYP2B6 and FMO3 minimally affected the extent to which CYP1A2-mediated norCLZ formation was inhibited by 10 μM fluvoxamine.

Similarly, CYP2C9 and FMO1 did not markedly affect the extent of inhibition by 2 μM ketoconazole of CYP3A4-dependent norCLZ formation. Inhibition of CYP1A2/3A4-mediated CLZ *N*-oxide formation by both chemicals was not significantly affected by the presence of either CYP2B6 or CYP2C9, but the recombinant FMOs greatly influenced the overall inhibition (Fig 5B). Thus, it emerges that FMOs are refractory to the action of fluvoxamine and ketoconazole. Considered together, these studies with microsomal fractions and recombinant enzymes suggest that the effects of the selective inhibitors on CYP-mediated CLZ biotransformation are not significantly affected by other enzymes.

## Discussion

Although several studies have implicated CYPs 1A2 and 3A4 in the oxidation of CLZ to its two major metabolites norCLZ and CLZ *N*-oxide, the interplay between these enzymes in CLZ oxidation has not been explored. The present findings suggest that the relative activity of the two enzymes is a major determinant of the susceptibility of microsomal CLZ oxidation to specific inhibitors and that this may have potential consequences for the profile of pharmacokinetic drug interactions observed in individual patients.

Thus, CLZ oxidation pathways in hepatic microsomal fractions from fourteen individuals were differentially inhibited by the CYP3A4- and CYP1A2-selective inhibitors ketoconazole and fluvoxamine, respectively. Low $IC_{50}$ values for ketoconazole, indicating significant potency, were noted in some of the microsomal fractions but others were much less susceptible to the inhibitor; the reactions in some of these fractions were inhibited by fluvoxamine. In addition, the reactions in certain livers were inhibited potently by both compounds. Kinetic studies in microsomal fractions in which CYP1A2, CYP3A4 or both emerged as the dominant CLZ oxidase suggested that CYP1A2 or CYP3A4 both catalyze CLZ biotransformation over an approximate four-fold range of $K_m$. This is consistent with the findings of Eiermann et al., (1997) who estimated $K_m$s of 42-89 μM for norCLZ formation in four livers and Tugnait et al. (1999) who estimated a $K_m$ of 121 μM in one liver. Although higher $K_m$s (>300 μM) have been reported in some studies for *N*-oxide formation (Tugnait et al., 1997; Eiermann et al., 1997), the present study found that $K_m$s were somewhat similar to those for norCLZ formation. This is consistent with the proposed role of CYP3A4 in both reactions, as suggested by Pirmohamed et al. (1995). Generally findings in previous studies were based on relatively few microsomal samples so that the differential contributions of CYPs were difficult to assess.

The possibility that CYP1A2/3A4 genotype may influence phenotype was considered. Previous studies have demonstrated that non-synonymous alleles for these enzymes encode enzymes with altered catalytic properties (Dai et al., 2001; Chevalier et al., 2001; Eiselt et al., 2001; Sata et al., 2000; Zhou et al., 2004). However, none of the livers contained these variants and only variant alleles containing SNPs in non-coding regions were detected in the study population. The report of Jiang et al., (2006) found no relationship between CYP1A2 genotype and metabolic activity. The present study is consistent with the suggestion that epigenetic factors strongly influence the apparent phenotype of CYPs 1A2 and 3A4 in human liver microsomes.

*In vivo* studies in patients have largely concluded that CYP1A2 has the major role in CLZ elimination. Thus, drug interactions in which serum levels of CLZ are increased in patients by the CYP1A2 inhibitors and substrates fluvoxamine, ciprofloxacin and caffeine have been reported widely (Hiemke et al., 1994; Jerling et al., 1994; Raaska and Neuvonen, 2000). Indeed, fluvoxamine decreased CLZ clearance several-fold (Hiemke et al., 1994; Wang et al., 2004). Despite the established role of CYP1A2 there have also been a number of clinical reports that have implicated CYP3A4 in CLZ elimination. Thus, erythromycin increased CLZ plasma levels and precipitated seizures or other adverse drug interactions in some patients (Edge et al., 1997; Glassner Cohen et al., 1996; Funderburg et al., 1994). Similarly, coadministration of the SSRI drugs fluoxetine and paroxetine increased serum concentrations of CLZ up to 60% over control in some patients, possibly due to inhibition of CYP3A4 (Centorrino et al., 1994, 1996; Wetzel et al., 1998; Diaz et al., 2008). On the other hand there have also been reports that erythromycin, itraconazole and nefazodone do not elicit pharmacokinetic interactions with CLZ (Hagg et al., 1999; Raaska and Neuvonen, 1998; Taylor et al., 1999). However, on closer examination, some of the patients in these studies exhibited significantly impaired CLZ clearance when they received the CYP3A4

inhibitors concurrently. Thus, Raaska and Neuvonen (1998) studied 7 patients and, although there was no change overall, significant increases in CLZ serum concentrations occurred in two patients who received itraconazole (by about 40% over control). Again, although Taylor et al. (1999) found no overall increase in serum CLZ concentrations in six patients who also received nefazodone, closer inspection revealed increases in circulating CLZ and norCLZ concentrations in two subjects. Considered together these findings suggest that there may be a number of patients in whom CYP3A4 contributes importantly to clearance of CLZ.

The clinical efficacy of CLZ has been improved in certain patients by coadministration of fluvoxamine (Ozdemir et al., 2001; Kontaxakis et al., 2005). Thus, Ozdemir et al. reported a patient who, despite receiving a dose of CLZ that was close to the upper recommended limit, had only subtherapeutic serum concentrations of the drug. In vivo phenotyping with the CYP1A2 probe caffeine indicated very high clearance capacity in that patient. Thus, efficacy was compromised by rapid clearance of CLZ. After 28 days of concurrent treatment with fluvoxamine (25 mg/day increasing to 50 mg/day) serum CLZ concentrations had entered the therapeutic range and the patient's psychosis had improved. This approach is now valuable in a number of patients who otherwise would be unresponsive to the drug because of their high CYP1A2 clearance capacity. In view of the present in vitro findings that CYP3A4 is also an important CLZ oxidase in some microsomal fractions, it is now appropriate to assess individual variation in CYP-dependent CLZ clearance in vivo. Indeed, it is feasible that some cases of therapeutic failure with CLZ may be a result of high CYP3A4 activity in certain patients; an analogous strategy to that involving fluvoxamine, but instead using CYP3A4-specific inhibitors, may be developed. Further, because norCLZ appears to exert more significant toxicity toward human bone marrow cells than either CLZ or other stable metabolites (Gerson et al., 1994), it is conceivable that there may be additional therapeutic benefits from coadministered CYP inhibitors.

The finding that either CYP1A2 or CYP3A4 may dominate CLZ clearance now offers an explanation to account for apparently atypical interactions as reported for erythromycin and paroxetine, which do not inhibit CYP1A2. The prediction of the pharmacokinetic drug interaction profile would be advantageous in patients stabilized on CLZ. The present study provides support for the functional importance of both CYPs 1A2 and 3A4 in CLZ clearance and suggests that in vivo phenotyping with the CYP3A4 probe substrate midazolam as well as caffeine may help to avoid potential interactions with coadministered drugs. Such approaches may also assist clinicians in directing CLZ dosage to optimize efficacy and minimize toxicity.

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## References

Centorrino F, Baldessarini RJ, Frankenburg FR, Kando J, Volpicelli SA, and Flood JG (1996) Serum levels of clozapine and norclozapine in patients treated with selective serotonin reuptake inhibitors. *Am J Psychiatry* **153:**820-822.

Centorrino F, Baldessarini RJ, Kando J, Frankenburg FR, Volpicelli SA, Puopolo PR, and Flood JG (1994) Serum concentrations of clozapine and its major metabolites: effects of cotreatment with fluoxetine or valproate. *Am J Psychiatry* **151:**123-125.

Chevalier D, Cauffiez C, Allorge D, Lo-Guidice JM, Lhermitte M, Lafitte JJ, and Broly F (2001) Five novel natural allelic variants -951A>C, 1042G>A (D348N), 1156A>T (I386F), 1217G>A (C406Y) and 1291C>T (C431Y)- of the human CYP1A2 gene in a French Caucasian population. *Hum Mutat* **17:**355-356.

Dai D, Tang J, Rose R, Hodgson E, Bienstock RJ, Mohrenweiser HW, and Goldstein JA (2001) Identification of variants of CYP3A4 and characterization of their abilities to metabolize testosterone and chlorpyrifos. *J Pharmacol Exp Ther* **299:**825-831.

Diaz FJ, Santoro V, Spina E, Cogollo M, Rivera TE, Botts S, and de Leon J (2008) Estimating the size of the effects of co-medications on plasma clozapine concentrations using a model that controls for clozapine doses and confounding variables. *Pharmacopsychiatry* **41:**81-91.

Edge SC, Markowitz JS, and Devane CL (1997) Clozapine drug-drug interactions: a review of the literature. *Human Psychopharmacol* **12:**5-20.

Edwards RJ, Adams DA, Watts PS, Davies DS, and Boobis AR (1998) Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. *Biochem Pharmacol* **56:**377-387

Eiermann B, Engel G, Johansson I, Zanger UM, and Bertilsson L (1997) The involvement of CYP1A2 and CYP3A4 in the metabolism of clozapine. *Brit J Clin Pharmacol* **44:**439-446.

Eiselt R, Domanski TL, Zibat A, Mueller R, Presecan-Siedel E, Hustert E, Zanger UM, Brockmoller J, Klenk HP, Meyer UA, Khan KK, He YA, Halpert JR, and Wojnowski L (2001) Identification and functional characterization of eight CYP3A4 protein variants. *Pharmacogenetics* **11:**447-458.

Funderburg L, Vertrees J. True J, and Miller A (1994) Seizure following addition of erythromycin to clozapine therapy. *Am J Psychiatry* **151:**1840-1841.

Gerson SL, Arce C, and Meltzer HY (1994) N-desmethylclozapine: a clozapine metabolite that suppresses haemopoiesis. *Br J Haematol* **86:**555-561.

Glassner Cohen L, Chesley S, Eugenio L, Flood JG, Fisch J, and Goff DC (1996) Erythromycin-induced clozapine toxic reaction. *Arch Intern Med* **156:**675-677.

Jiang Z, Dragin N, Jorge-Nebert LF, Martin MV, Guengerich FP, Aklillu E, Ingelman-Sundberg M, Hammons GJ, Lyn-Cook BD, Kadlubar FF, Saldana SN, Sorter M, Vinks AA, Nassr N, von Richter O, Jin L, and Nebert DW (2006) Search for an association between the human CYP1A2 genotype and CYP1A2 metabolic phenotype. *Pharmacogenet Genom* **16:**359-367.

Hagg S, Spigset O, Mjorndal T, Granberg K, Persbo-Lundqvist G, and Dahlqvist R (1999) Absence of interaction between erythromycin and a single dose of clozapine. *Eur J Clin Pharmacol* **55:**221-226.

Hiemke C, Weigmann H, Hartter S, Dahmen N, Wetzel H, and Muller H (1994) Elevated levels of clozapine in serum after addition of fluvoxamine. *J Clin Psychopharmacol* **14:**279-281.

Jerling M, Lindstrom L, Bondesson U, and Bertilsson L (1994) Fluvoxamine inhibition and carbamazepine induction of the metabolism of clozapine: evidence from a therapeutic drug monitoring service. *Ther Drug Monit* **16:**368-374.

Knodell RG, Hall SD, Wilkinson GR, and Guengerich FP (1987) Hepatic metabolism of tolbutamide: Characterization of the form of cytochrome P450 involved in methyl hydroxylation and relationship to *in vivo* disposition. *J Pharmacol Exp Ther* **241:**1112-1119.

Kontaxakis VP, Ferentinos PP, Havaki-Kontaxaki-BJ, Paplos KG, Roukas DK, and Christodoulou GN (2005) Case studies of adjunctive agents in clozapine-resistant schizophrenic patients. *Clin Neuropharmacol* **28:**50-53.

Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (Lond)* **227:**680-685

Linnet K, and Olesen OV (1997) Metabolism of clozapine by cDNA-expressed human cytochrome P450 enzymes. *Drug Metab Dispos* **25:**1379-1382.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* **93:**265–275.

Murray M, Zaluzny L, and Farrell GC (1986) Drug Metabolism in cirrhosis: Selective changes in cytochrome P-450 isozymes in the choline-deficient rat model. *Biochem Pharmacol* **35**:1817-1824.

Murray M (1992) Metabolite intermediate complexation of microsomal cytochrome P450 2C11 in male rat liver by nortriptyline. *Mol Pharmacol* **42:**931-938.

Ozdemir V, Kalow W, Okey AB, Lam MS, Albers LJ, Reist C, Fourie J, Posner P, Collins EJ, and Roy R (2001) Treatment-resistance to clozapine in association with ultrarapid CYP1A2 activity and the C>A polymorphism in intron 1 of the CYP1A2 gene: effect of grapefruit juice and low-dose fluvoxamine. *J Clin Psychopharmacol* **21:**603-607.

Pirmohamed M, Williams D, Madden S, Templeton E, and Park BK (1995) Metabolism and bioactivation of clozapine by human liver *in vitro. J Pharmacol Exp Ther* **272:**984-990.

Prough RA, Burke MD, and Mayer RT (1978) Direct fluorometric methods for measuring mixed function oxidase activity. *Methods Enzymol* **52:**372-377.

Raaska K, and Neuvonen PJ (1998) Serum concentrations of clozapine and *N*-desmethylclozapine are unaffected by the potent CYP3A4 inhibitor itraconazole. *Eur J Clin Pharmacol* **54:**167-170.

Raaska K, and Neuvonen PJ (2000) Ciprofloxacin increases serum clozapine and *N*-desmethylclozapine: a study in patients with schizophrenia. *Eur J Clin Pharmacol* **56:**585-589.

Sata F, Sapone A, Elizondo G, Stocker P, Miller VP, Zheng W, Raunio H, Crespi CL, and Gonzalez FJ (2000) CYP3A4 allelic variants with amino acid substitutions in exons 7 and 12: evidence for an allelic variant with altered catalytic activity. *Clin Pharmacol Ther* **67:**48-56.

Taylor D, Bodani M, Hubbeling A, and Murray R (1999) The effect of nefazodone on clozapine plasma concentrations. *Int Clin Psychopharmacol* **14:**185-187.

Towbin H, Staehlin T, and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:**4350-4354.

Tugnait M, Hawes EM, McKay G, Rettie AE, Haining RL, and Midha KK (1997) *N*-oxygenation of clozapine by flavin-containing monooxygenase. *Drug Metab Dispos* **25:**524-527.

Tugnait M, Hawes EM, McKay G, Eichelbaum M, and Midha KK (1999) Characterization of the human hepatic cytochromes P450 involved in the *in vitro* oxidation of clozapine. *Chem-Biol Interact* **118:**171-189.

Vielnascher E, Spatzenegger M, Mayrhofer A, Klinger P, and Jäger W (1996) Metabolism of dextromethorphan in human liver microsomes: a rapid HPLC assay to monitor cytochrome P450 2D6 activity. *Pharmazie* **51:**586-588.

Wang CY, Zhang ZJ, Li WB, Zhai YM, Cai ZJ, Weng YZ, Zhu RH, Zhao JP, and Zhou HH (2004) The differential effects of steady-state fluvoxamine on the pharmacokinetics of olanzapine and clozapine in healthy volunteers. *J Clin Pharmacol* **44:**785-792.

Wetzel H, Anghelescu I, Szegedi A, Wiesner J, Weigmann H, Harter S, and Hiemke C (1998) Pharmacokinetic interactions of clozapine with selective serotonin reuptake inhibitors: differential effects of fluvoxamine and paroxetine in a prospective study. *J Clin Psychopharmacol* **18:**2-9.

Zhang WV, Ramzan I, and Murray M (2007) Impaired microsomal oxidation of the atypical antipsychotic agent clozapine in hepatic steatosis. *J Pharmacol Exp Ther* **322:**770-777.

Zhou H, Josephy PD, Kim D, and Guengerich FP (2004) Functional characterization of four allelic variants of human cytochrome P450 1A2. *Arch Biochem Biophys* **422:**23-30.

**Footnotes:**

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**Figure legends**

Figure 1 (A) Oxidation of CLZ to norCLZ and CLZ *N*-oxide by cDNA-expressed CYPs and FMOs. As described in Materials and Methods incubations contained 200 µg of cDNA-expressed enzyme, 100 µM CLZ and were run for 60 min. Metabolites were isolated by solid-phase extraction on Oasis HLB cartridges and resolved by HPLC. (B) Correlations between CYP1A2 activity (EROD), CYP3A4 activity (Test 6β; testosterone 6β-hydroxylation) and formation of norCLZ and CLZ *N*-oxide in human hepatic microsomal fractions (n=14). Values of Spearman's (rank) correlation coefficients are indicated.

Figure 2 Kinetic plots for the formation of (A) norCLZ and (B) CLZ *N*-oxide in human liver microsomal fractions are shown: (●) HL16, (■) HL27.

Figure 3 (A) Selective inhibition of cDNA-expressed CYP1A2- and CYP3A4-mediated norCLZ and CLZ *N*-oxide formation by fluvoxamine (Fluvox) and ketoconazole (Keto), respectively, but not by inhibitors of CYP2D6 (quinidine; Quin), CYP2C (tranylcypromine; Tranyl) or FMO (benzydamine; Benz). (B) Box plots showing individual variation in the inhibition of norCLZ and CLZ *N*-oxide formation in human hepatic microsomes (n=14) by the CYP-selective inhibitors. The points indicate the extremes of the observed extent of inhibition in individual fractions. Inhibitor data are means of duplicate determinations and varied by less than 8% from the stated values.

Figure 4 (A) Relationship between $\log_{10}$ ($IC_{50}$) ratio (Fluvoxamine:ketoconazole) for the inhibition of norCLZ formation and the $\log_{10}$ (EROD/Test 6β activity ratio) in human hepatic microsomes. (B) Relationship between $\log_{10}IC_{50}$ ratio (Fluvoxamine:ketoconazole) for the inhibition of CLZ *N*-oxide formation and the $\log_{10}$ (Test 6β activity). Simple linear correlation coefficients are shown.

Figure 5 (A) Effects of ketoconazole (2 μM; solid bars) and fluvoxamine (10 μM; open bars) on CLZ biotransformation in microsomal fractions from five individual human livers. The observed effects of the combined inhibitors (hatched bars) vary from the sum of the separate effects of the inhibitors (stacked) by the percentage values shown at the right of each bar. The mean±SEM variations (observed-calculated) were +9.6±4.1% and -7.0±3.1% for norCLZ and CLZ *N*-oxide, respectively. Data are means from duplicate incubations and varied by less than 10% from the stated mean values. (B) Effect of additional recombinant enzymes on the extent of inhibition of CYP1A2-mediated CLZ biotransformation by fluvoxamine (10 μM) and CYP3A4-mediated CLZ biotransformation by ketoconazole (2 μM). The additional enzymes included in incubations are indicated below the bars.

**Table 1. Individual variation in oxidation of CLZ and CYP-specific substrates in human hepatic microsomes**

| liver | medication | CLZ metabolite | | EROD | testosterone 6β-hydroxylation | tolbutamide hydroxylation | dextromethorphan demethylation |
|---|---|---|---|---|---|---|---|
| | | norCLZ | CLZ *N*-oxide | | | | |
| | | *nmol/mg protein/min* | | *pmol/mg protein/min* | *nmol/mg protein/min* | | *pmol/mg protein/min* |
| HL12[c] | spironolactone | 353 | 817 | 40.5 | 2.75 | ND[a] | 224 |
| HL16[b,c] | dopamine, desmopressin | 378 | 332 | 42.5 | 2.34 | 3.22 | 64.6 |
| HL21[d] | unknown | 194 | 194 | 11.2 | 2.36 | 3.56 | 27.0 |
| HL22[c] | unknown | 193 | 417 | 18.5 | 6.08 | 3.24 | 252 |
| HL24[d] | flucloxacillin, ceftriaxone | 180 | 262 | 11.4 | 2.72 | 5.87 | 156 |
| HL27[b,e] | dopamine, desmopressin | 150 | 370 | 20.9 | 1.61 | 2.71 | 10.0 |
| HL28 | unknown | 79 | 127 | 11.7 | 0.50 | 0.86 | 50.9 |
| HL29[c] | simvastatin | 158 | 481 | 45.7 | 3.06 | 8.23 | 384 |
| HL30 | adrenaline, ranitidine, penicillin | 43 | 256 | 7.3 | 0.66 | 3.84 | 1.1 |
| HL35[b] | dopamine | 75 | 197 | 9.2 | 0.82 | 2.51 | 2.1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HL36 | unknown | 84 | 79 | 13.1 | 0.92 | 2.63 | 149 |
| HL37[b,c] | spironolactone, thyroxine | 334 | 473 | 49.1 | 2.37 | 3.46 | 163 |
| HL38 | prednisone | 61 | 171 | 17.0 | 1.30 | 3.46 | 258 |
| HL40[c] | unknown | 155 | 449 | 24.6 | 2.39 | 7.23 | 5.7 |
| Median | | 153 | 306 | 17.7 | 2.35 | 3.46 | 107 |
| Range | | (43-378) | (79-817) | (5.6-49.1) | (0.50-6.08) | (0.86-8.23) | (1.1-384) |

[a]ND, not determined

[b]cigarette smoker.

[c]norCLZ and CLZ N-oxide formation both exceeded the median.

[d]norCLZ formation exceeded the median.

[e]CLZ N-oxide formation exceeded the median.

**Table 2. Primer sequences and PCR amplification conditions used in genotyping of CYP alleles in human liver**

| *Allele* | *product size* | *primer sequences* | *PCR conditions* |
|---|---|---|---|
| *(genomic sequence)* | | | |
| CYP1A2*1C | 596 bp | Forward primer: 5'-GCTACAACATGATCGAGCTATAC-3' | 94ºC for 2 min, 35 cycles of |
| *(-3994 to -3164)* | | Reverse primer: 5'-CAGGTCTCTTCACTGTAAAGTTA-3' | 94ºC for 30 sec, 55ºC for 30 sec, 72ºC for 30 sec and 72ºC for 10 min |
| CYP1A2*1D | 250 bp | Forward primer: 5'-TGCACACACCTGTGATTGTGGT-3' | as for CYP1A2*1C |
| *(-2570 to -2157)* | | Reverse primer: 5'-AGGAGTCTTTAATATGGACCCAG-3' | |
| CYP1A2*1F | 242 bp | Forward primer: 5'-CCCAGAAGTGGAAACTGAGA -3' | as for CYP1A2*1C |
| *(-281 to -41)* | | Reverse primer: 5'-GGGTTGAGATGGAGACATTC -3' | |
| CYP1A2*4 | 255 bp | Forward primer: 5'-AGCTCTGCTTGTCCTCTGTG -3' | as for CYP1A2*1C |
| *(2359 to 2612)* | | Reverse primer: 5'-AGGTCGGGAAGGAGATGCT -3' | |
| CYP1A2*6 | 252 bp | Forward primer: 5'-CTCAACAGAAGTCTCCCTC-3' | 94ºC for 2 min, 35 cycles of |
| *(4974 to 5236)* | | Reverse primer: 5'-ATGGCCAGGAAGAGGAAGAT-3' | 94ºC for 30 sec, 56ºC for 30 |

| | | | |
|---|---|---|---|
| | | | sec, 65ºC for 30 sec and 65ºC for 10 min |
| CYP3A4*1B *(-673 to -82)* | 592 bp | Forward primer: 5'-AACAGGACGTGGAAACACAAT-3' Reverse primer: 5'-CTTTCCTGCCCTGCACAG-3' | 94ºC for 2 min, 35 cycles of 94ºC for 30 sec, 63ºC for 30 sec, 72ºC for 30 sec and 72ºC for 10 min |
| CYP3A4*2 *(15555 to 15977)* | 423 bp | Forward primer: 5'-ATCTTTCTCCACTCAGCGTCTTTG-3' Reverse primer: 5'-GGCAGAAAGTTGATTAGTGGTTGCATA-3' | 94ºC for 2 min, 35 cycles of 94ºC for 30 sec, 58ºC for 30 sec, 72ºC for 30 sec and 72ºC for 10 min |
| CYP3A4*10 *(14205 to 14505)* | 301 bp | Forward primer: 5'-ATGTCCTTCTGGGACTAGAG-3' Reverse primer: 5'-GGGAGAAGATCCTTTTCCTC-3' | 94ºC for 2 min, 35 cycles of 94ºC for 30 sec, 58ºC for 30 sec, 72ºC for 30 sec and 72ºC for 10 min |
| CYP3A4*17 | 286 bp | Forward primer: 5'-GCTGATTTTATTTTTCCACATCTTTCTC-3' | as for CYP3A4*10 |

*(15536 to 15821)* Reverse primer: 5’-CTGTATATTTTAAGTGGATGAATTACATGGTG-3’

**Table 3. Kinetic parameters of CLZ oxidation in individual human livers**

| | CLZ metabolite | | | | | |
|---|---|---|---|---|---|---|
| | norCLZ | | | CLZ N-oxide | | |
| Liver | $K_m$ *(μM)* | $V_{max}$ *(pmol/mg protein/min)* | $V_{max}/K_m$ *(pmol/mg protein/ min/μM)* | $K_m$ *(μM)* | $V_{max}$ *(pmol/mg protein/min)* | $V_{max}/K_m$ *(pmol/mg protein/ min/μM)* |
| HL12 | 54 | 597 | 11.1 | 120 | 531 | 4.43 |
| HL16 | 56 | 238 | 4.25 | 109 | 665 | 6.10 |
| HL22 | 56 | 396 | 7.07 | 109 | 1039 | 9.53 |
| HL27 | 107 | 964 | 9.01 | 76 | 891 | 11.7 |
| HL35 | 135 | 445 | 3.30 | 35 | 311 | 8.89 |
| HL36 | 47 | 96 | 2.04 | 61 | 339 | 5.56 |
| HL37 | 35 | 574 | 16.4 | 28 | 405 | 14.5 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Median | 56 | 445 | 7.07 | 76 | 405 | 8.89 |
| Range | (35-135) | (96-964) | (3.30-16.4) | (28-120) | (311-1039) | (4.43-14.5) |

**Table 4. $IC_{50}$ values for fluvoxamine and ketoconazole against CLZ oxidation pathways, CYP activity ratios and CYP1A2 genotypes in human livers**

| | $IC_{50}$ (μM) | | | | $IC_{50}$ ratio | | | |
|---|---|---|---|---|---|---|---|---|
| Liver | ketoconazole | | fluvoxamine | | (fluvoxamine/ ketoconazole) | | CYP activity | CYP1A2 |
| | norCLZ | CLZ *N*-oxide | NorCLZ | CLZ *N*-oxide | norCLZ | CLZ *N*-oxide | ratio[a] | alleles |
| HL12 | 11 | 0.33 | 9.6 | 195 | 0.87 | 590 | 14.7 | |
| HL16 | 96 | 4.2 | 1.3 | 110 | 0.012 | 26 | 18.2 | |
| HL21 | 0.55 | 4.2 | 54 | 72 | 98 | 17 | 4.7 | *1C, *1D |
| HL22 | 0.61 | 0.16 | 58 | 55 | 95 | 344 | 3.0 | |
| HL24 | 0.35 | 0.23 | 61 | 116 | 174 | 504 | 4.2 | |
| HL27 | 1.2 | 0.68 | 4 | >250 | 3.3 | ND | 13.0 | *1F |
| HL28 | 18 | 6.8 | 5.6 | >250 | 0.31 | ND | 23.5 | |
| HL29 | 45 | 0.34 | 7.9 | 66 | 0.18 | 194 | 14.9 | *1F |
| HL30 | 3.7 | 2.6 | 100 | 55 | 27 | 21 | 11.1 | |
| HL35 | 2.5 | 0.28 | 66 | >250 | 26 | ND | 11.2 | |
| HL36 | 65 | 2.0 | 21 | 14 | 0.32 | 7.0 | 14.2 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| HL37 | 33 | 3.3 | 16 | 73 | 0.49 | 22 | 20.7 | *1D |
| HL38 | 130 | 1.6 | 66 | 66 | 0.51 | 41 | 13.1 | |
| HL40 | 52 | 1.3 | 1.9 | 160 | 0.037 | 123 | 10.3 | |

[a]Ratio of EROD/testosterone 6β-hydroxylation activities in microsomal fractions (Table 1).

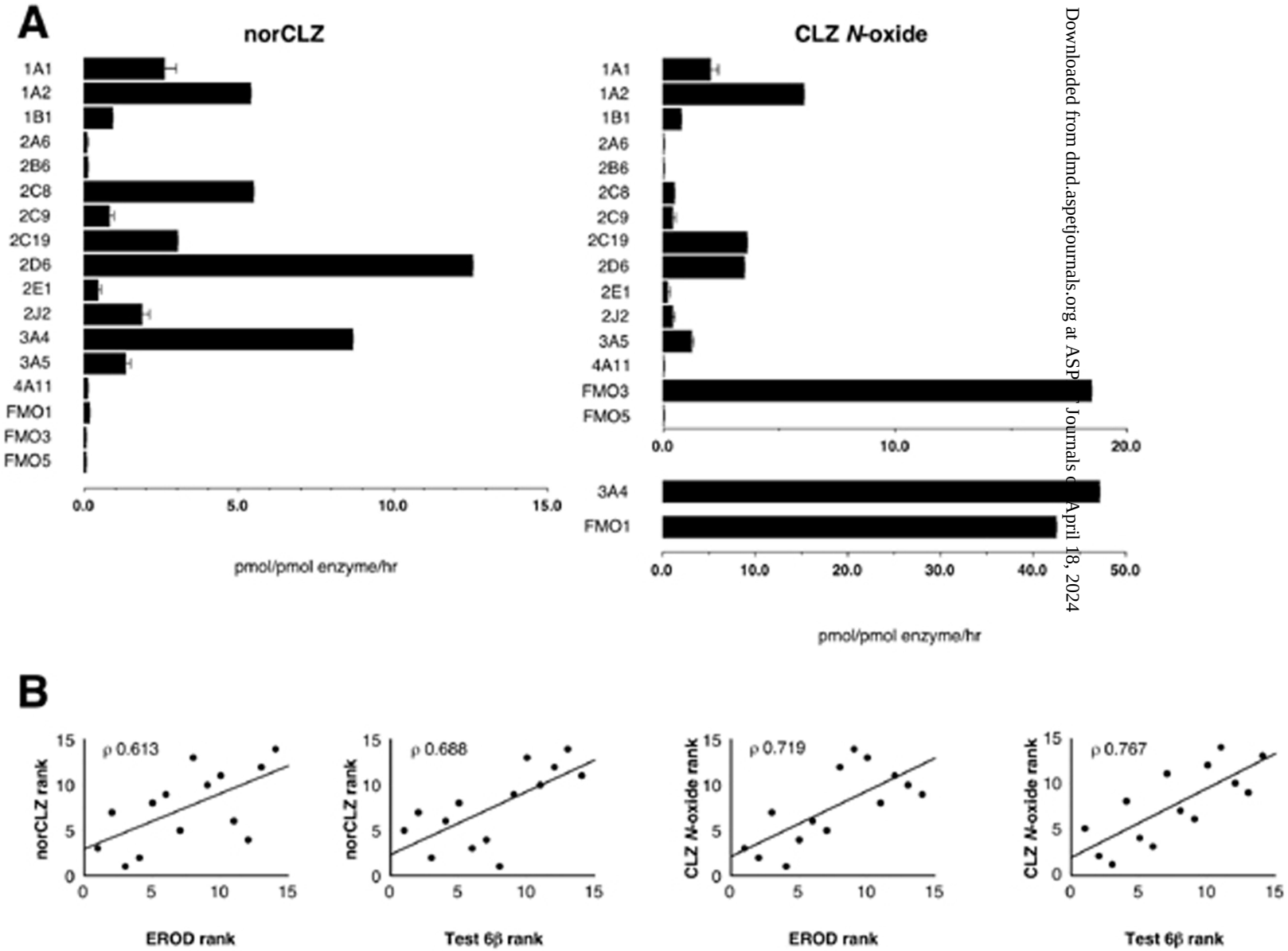



Figure 1

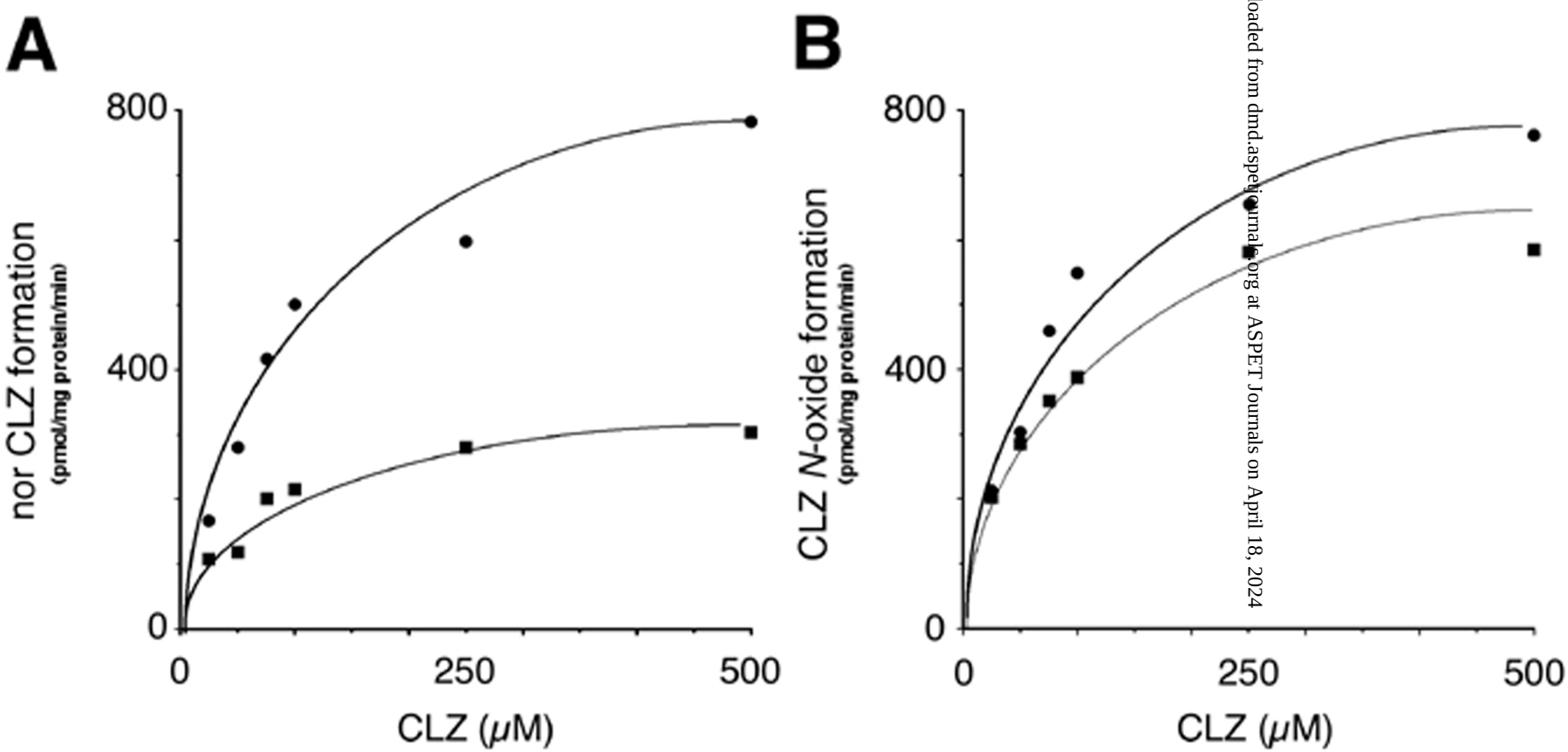
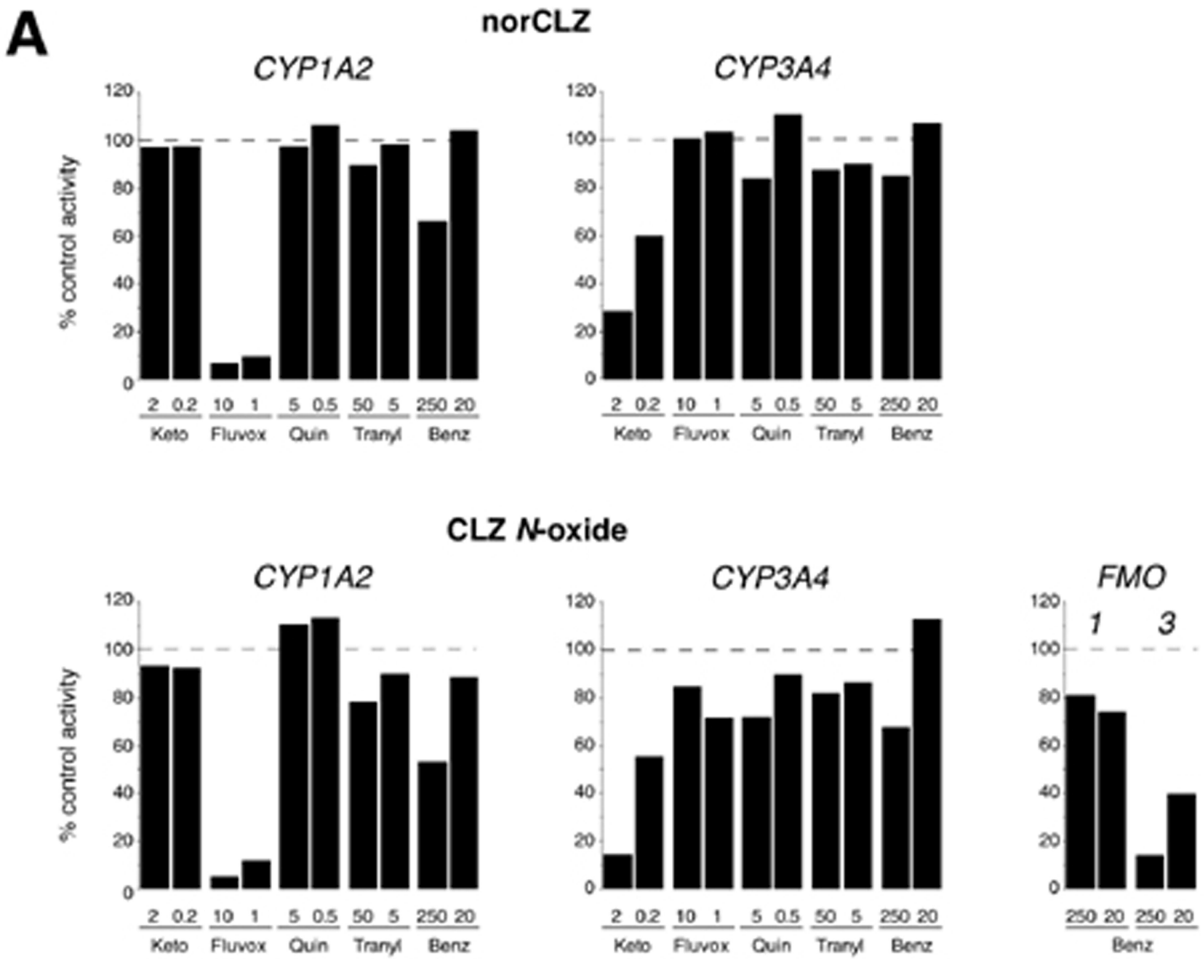





Figure 2



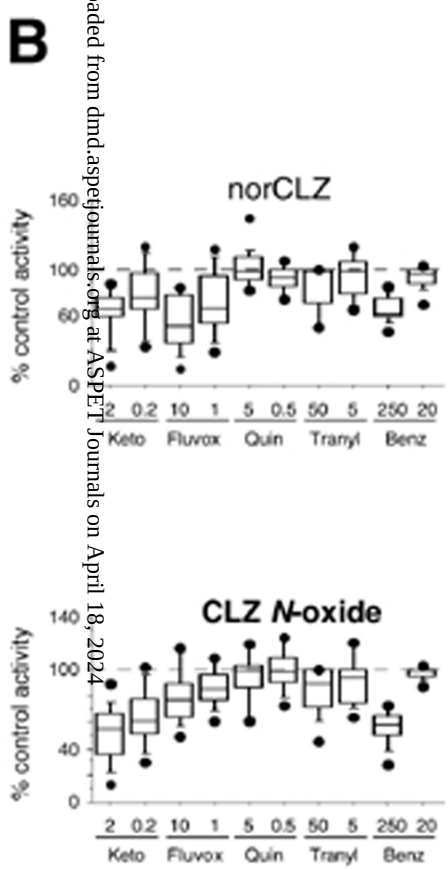




Figure 3

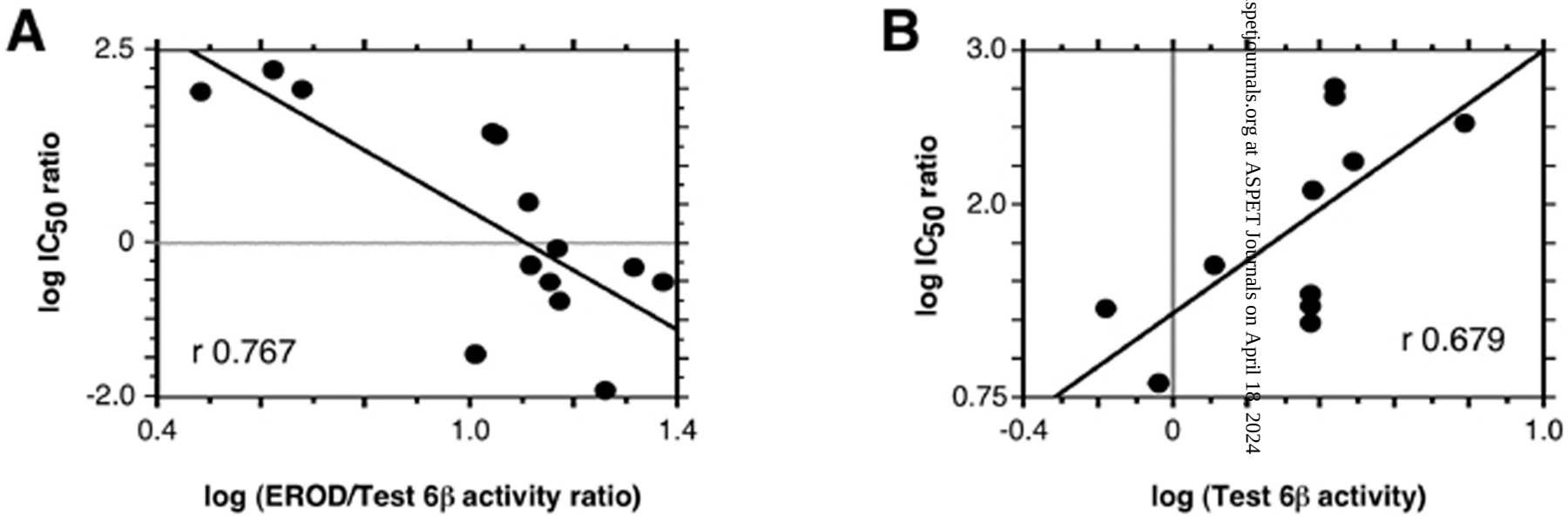



Figure 4

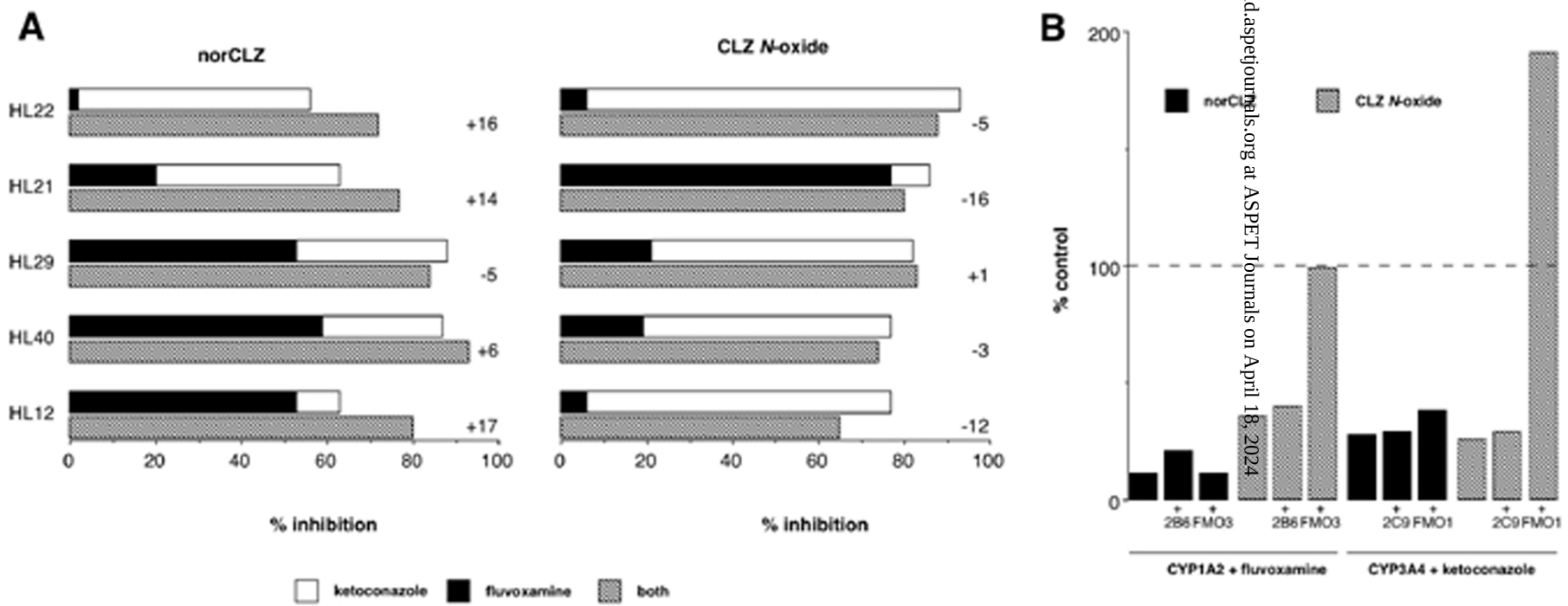



Figure 5