Role of Residue 87 in the Activity and Regioselectivity of Clozapine Metabolism by Drug-Metabolizing CYP102A1 M11H: Application for Structural Characterization of Clozapine GSH Conjugates

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ABSTRACT:

In the present study, a site-saturation mutagenesis library of drug-metabolizing CYP102A1 M11H with all 20 amino acids at position 87 was applied as a biocatalyst for the production of stable and reactive metabolites of clozapine. Clozapine is an atypical antipsychotic drug in which formation of reactive metabolites is considered to be responsible for several adverse drug reactions. Reactive intermediates of clozapine can be inactivated by GSH to multiple GSH conjugates by nonenzymatic and glutathione transferase (GST)-mediated conjugation reactions. The structures of several GST-dependent metabolites have not yet been elucidated unequivocally. The present study shows that the nature of the amino acid at position 87 of CYP102A1 M11H strongly determines the activity and regioselectivity of clozapine metabolism. Some mutants showed preference for N-demethylation and N-oxidation, whereas others showed high selectivity for bioactivation to reactive intermediates. The mutant containing Phe87 showed high activity and high selectivity for the bioactivation pathway and was used for the large-scale production of GST-dependent GSH conjugates by incubation in the presence of recombinant human GST P1-1. Five human-relevant GSH adducts were produced at high levels, enabling structural characterization by H NMR. This work shows that drug-metabolizing CYP102A1 mutants, in combination with GSTs, are very useful tools for the generation of GSH conjugates of reactive metabolites of drugs to enable their isolation and structural elucidation.

Introduction

Cytochromes P450 (P450s) are involved in the metabolism of approximately 80% of the drugs currently on the market (Shimada et al., 1994; Evans and Relling, 1999). In some cases, drugs can be oxidized by P450s to electrophilic reactive intermediates, which subsequently can react with nucleophilic functional groups in biomolecules such as proteins and DNA. In addition, stable metabolites might possess pharmacological activities that might be responsible for undesired adverse drug reactions. It is for these reasons that the characterization of the biological properties of major metabolites is considered to be important for drug safety assessment (Smith and Obach, 2009). Therefore, methods are required to obtain the relevant drug metabolites in sufficient yield to allow structural elucidation and to study their pharmacological and toxicological properties. Metabolite production can be achieved by organic synthesis, electrochemical oxidation of parent drug, and by biosynthesis using specific P450s. In particular, mutants of the bacterial cytochrome P450 CYP102A1 (P450 BM3) are considered to have good perspective for the large-scale production of human-relevant drug metabolites because this very stable enzyme possesses the highest activity ever recorded for a P450 (Ost et al., 2000). By combinations of site-directed and random mutagenesis, many CYP102A1 mutants have been obtained that are able to convert drugs and drug-like molecules to human-relevant metabolites (van Vugt-Lussenburg et al., 2007; Yun et al., 2007; Sawayama et al., 2009; Kim et al., 2011). In our previous work, four mutants of CYP102A1 were evaluated as biocatalysts for the bioactivation of several drugs to reactive intermediates (Damsten et al., 2008). Drugs tested were acetaminophen, diclofenac, and clozapine. N-oxidation, whereas others showed high selectivity for bioactivation to reactive intermediates. The mutant containing Phe87 showed high activity and high selectivity for the bioactivation pathway and was used for the large-scale production of GST-dependent GSH conjugates by incubation in the presence of recombinant human GST P1-1. Five human-relevant GSH adducts were produced at high levels, enabling structural characterization by H NMR. This work shows that drug-metabolizing CYP102A1 mutants, in combination with GSTs, are very useful tools for the generation of GSH conjugates of reactive metabolites of drugs to enable their isolation and structural elucidation.

ABBREVIATIONS: P450, cytochrome P450; CYP102A1 M11H, cytochrome P450 102A1 mutant M11 His-tagged; hGST, human glutathione transferase; CLZ, clozapine; GST, glutathione transferase; PCR, polymerase chain reaction; CO, carbon monoxide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; VIS, visible; HPLC, high-performance liquid chromatography; COSY, correlation spectroscopy.
Recently, the highly active drug-metabolizing mutant CYP102A1 M11H (cytochrome P450 102A1 mutant M11 His-tagged) was used to investigate the role of human glutathione transferase (GST) in the inactivation of CLZ (Dragovic et al., 2010). CLZ is an atypical antipsychotic drug showing a low incidence of extrapyramidal side effects combined with excellent antipsychotic efficacy in schizophrenic and manic treatment-resistant patients (Safferman et al., 1991; Buchanan, 1995; Wagstaff and Perry, 2003). Approximately 1 to 2% of patients develop agranulocytosis. Enhanced serum transaminases were monitored with 37% of the patients, whereas 0.06% of the patients had liver failure (Hummer et al., 1997). It is still unknown which factors predispose part of the patient population to these forms of CLZ toxicity. On the basis of the identification of several GSH conjugates, formation of a reactive nitrenium ion by peroxidases, hypochlorite, and P450s 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). In vitro and in vivo studies of CLZ have shown the formation of four GSH conjugates with identical mass with a MH$^+$ ion at $m/z$ 632.2 and one dechlorinated GSH conjugate with a MH$^+$ ion at $m/z$ 598.3. All conjugates can be explained by direct conjugation of GSH at different positions of a reactive nitrenium ion and by chlorosubstitution of the nitrenium ion followed by reduction (see Fig. 1) (Maggs et al., 1995; Dragovic et al., 2010). Unequivocal structure determination by $^1$H NMR has been published for only two of the GSH conjugates of CLZ (Fischer et al., 1991; Liu and Uetrecht, 1995; Madsen et al., 2007). The major GSH conjugate (CG-1; Fig. 1) was found to be conjugated at position 6 of the chlorinated aromatic ring, whereas a minor GSH conjugate (CG-3; Fig. 1) was found to be conjugated at position 9 (Fischer et al., 1991; Liu and Uetrecht, 1995). A third GSH conjugate with a MH$^+$ ion at $m/z$ 632.2 was only identified in vitro incubations with human and rat liver microsomes and was tentatively assigned to position 7 (CG-4; Fig. 1). Two GSH conjugates, with a MH$^+$ ion at $m/z$ 632.2 and 598.3, were first discovered in the bile of treated mice, and rats and were originally proposed to originate from unidentified reactive intermediates formed in vivo (Maggs et al., 1995). However, we recently demonstrated that these GSH conjugates, CG-5 and CG-6 in Fig. 1, are formed at high levels when CLZ incubations with purified CYP102A1 M11H and human liver microsomes were supplemented with human glutathione transferases (hGSTs) (Dragovic et al., 2010). Three of the four tested hGSTs showed strongly increased total GSH conjugation and also resulted in formation of different regioisomeric GSH conjugates of CLZ (Fig. 1) (Dragovic et al., 2010).

For two of the GSH conjugates that have been found previously, the structure has not been elucidated by NMR. Conjugate CG-4, which was found in incubations of CLZ with human liver microsomes, was tentatively assigned as the conjugate at position 7 (Maggs et al., 1995). GSH conjugate CG-5, which was identified in the bile of rats and mice, was proposed to result from GSH conjugation to the nonchlorinated ring (Maggs et al., 1995). The aim of the present study was to identify the structures of these GSH conjugates by $^1$H NMR by performing large-scale incubations of CLZ with selective CYP102A1 M11H mutants in the presence of glutathione transferase. Because GST P1-1 appeared to be the most active hGST in the formation of enzyme-dependent GSH conjugates (Dragovic et al., 2010), this enzyme was selected for large-scale production of GSH conjugates.

**Figs. 1.** Oxidative pathways of metabolism of CLZ by cytochrome P450 and nonenzymatic and enzymatic conjugation reactions of reactive CLZ nitrenium ion by GSH and GST: a, N-demethylation; b, N-oxidation; c, oxidative opening of piperazine ring; d, dehydrogenation to nitrenium ion.
Although CYP102A1 M11H was previously shown to produce high levels of CLZ metabolites, the most abundant metabolites appeared to be N-demethylclozapine and CLZ N-oxide (Damsten et al., 2008; Dragovic et al., 2010). As a consequence, GSH conjugates derived from these metabolites were also produced, which strongly complicates isolation of CG-4 and CG-5. We recently showed that by changing the active site residue at position 87 of CYP102A1 M11H, the regioselectivity of testosterone hydroxylation was strongly modified (Vottero et al., 2011). Therefore, in the present study, we first evaluated the effect of mutation at position 87 on the regioselectivity of CLZ metabolism to identify the most suitable biocatalyst for the bioactivation of CLZ and subsequent structural characterization of the formed GSH conjugates. The results show that the nature of the residue at position 87 strongly influences regioselectivity of CLZ metabolism and that by using a more selective P450 CYP102A1 M11H mutant, all five human-relevant GSH conjugates of CLZ could be produced in high levels, enabling structural elucidation by ³¹H NMR.

Materials and Methods

Materials. All chemicals were of analytical grade and obtained from standard suppliers.

Library Construction. Site-directed mutants of CYP102A1 M11H at position 87 were constructed by a mutagenic polymerase chain reaction (PCR) using the Stratagene QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) using 20 complementary pairs of mutagenesis primers (Vottero et al., 2011). The mutagenic PCR was applied to a pBluescript vector containing the gene of the drug-metabolizing CYP102A1 M11H flanked by EcoR1 and BamH1 restriction sites. CYP102A1 M11H contains mutations R47L, E64G, F81I, F87V, E143G, L188Q, Y198C, E267V, H285Y, and G415S compared with wild-type CYP102A1 (Damsten et al., 2008). The sequence of the forward primers was as follows: 5'-GCA GAC GGG TTA XXX ACT AGT TGG AGC CAT-3' and 5'-CAT GCC TCC AAC TAG TYY YTA ACC CGT CTC CTG C-3' in which the YYY is the reverse complement of codon XXX. The underlined bases indicate a new Spel digestion site. The following codons (XXX) were used: Ala, GCC; Arg, CGG; Asn,AAC; Asp, GAC; Cys, UGC; Gln, CAG; Glu, GAG; Gly, GGG; His, CAC; Ile, AUU; Leu, CGU, Lys, AAA; Met, AUG; Phe, UUC; Pro, CCC, Ser, UCC; Thr, ACC; Trp, UGG; Tyr, UAC; and Val, GUG. After mutagenic PCR, the plasmids were digested with EcoR1 and BamH1 restriction enzymes and the genes of mutated CYP102A1 M11H were cloned into a pET28a+ vector, which encodes for an N-terminal His-tag. The desired mutations in the P450 domain were confirmed by DNA sequencing (Basesclear, Leiden, The Netherlands).

Expression, Isolation, and Purification of Enzymes. Expression of the P450 CYP102A1 M11H mutants was performed by transforming competent Escherichia coli BL21 cells with the pET28+ vectors as described previously (Vottero et al., 2011). Proteins were purified using nickel-nitritotriacetic acid agarose, after which P450 concentrations were determined using a carbon monoxide (CO) difference spectrum assay. Purity of the enzymes was checked by SDS-polyacrylamide gel electrophoresis on a 12% gel and Coomassie staining. Protein purity was greater than 98% in all samples obtained.

Human GST P1-1 was prepared and purified as described previously (Dragovic et al., 2010). Protein concentration was determined according to the method of Bradford (1976) with reagents obtained from Bio-Rad Laboratories (Hemel Hempstead, UK). The specific activity of the purified GST was assayed according to Habig et al. (1974). The specific activity of the purified recombinant human GST P1-1 using 1-chloro-2,4-dinitrobenzene as a substrate was 27.9 µmol · min⁻¹ · mg protein⁻¹.

Metabolism of CLZ by CYP102A1 M11H Mutants in the Presence of Human GST P1-1. Incubations using CYP102A1 M11H mutants as a bioactivation system were performed at a final enzyme concentration of 250 nM as described previously (Damsten et al., 2008). All incubations were performed in potassium phosphate buffer (100 mM, pH 7.4) and at a final volume of 250 µl. The substrate CLZ was incubated at a concentration of 500 µM. GST P1-1 (8 µM) and GSH (100 µM) were added to the incubations to trap reactive CLZ nitrenium ion. Reactions were initiated by the addition of a NADPH-regenerating system (0.2 mM NADPH, 1 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, final concentrations) and performed for 30 min at room temperature. In this time period, product formation was linear, as described previously (Damsten et al., 2008). Reactions were terminated by the addition of 25 µl of 10% HCO₃ and centrifuged for 15 min at 14,000 rpm. The supernatants were analyzed by reversed-phase liquid chromatography using a Luna 5-µm C18 column (150 × 4.6 mm i.d.; Phenomenex, Torrance, CA) as stationary phase, protected by a 4- by 3-mm i.d. security guard (5 µm) C18 guard column (Phenomenex). The gradient used was constructed by mixing the following mobile phases: eluent A (0.8% acetonitrile, 99% water, and 0.2% formic acid) and eluent B (99% acetonitrile, 0.8% water, and 0.2% formic acid). The first 5 min were isocratic at 0% eluent B; from 5 to 30 min the percentage of eluent B linearly increased to 100%; and from 30 to 35 min linearly decreased to 0% B and maintained at 0% for re-equilibration until 40 min. The flow rate was 0.5 ml/min.

Samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) for identification and using UV/visible (VIS) detection at 254 nm for quantification. The Shimadzu Class VP 4.3 software package (Shimadzu, Kyoto, Japan) was used for determination of peak areas in the UV chromatograms. A standard curve of CLZ was used to estimate the concentrations of the metabolites, assuming that the extinction coefficients of the metabolites at 254 nm are equal to that of CLZ. The UV/VIS spectra of clozapine and its metabolites, as determined online by diode array detection (180–400 nm), all showed similar spectra with maxima at 240, 260, and 295 nm (data not shown). The standard curve of CLZ was linear between 1 and 100 µM; the limit of quantitative detection by UV/VIS was estimated to be 0.1 µM (data not shown).

For identification of the metabolites, an Agilent 1200 series rapid resolution liquid chromatography system was connected to a hybrid quadrupole-time-of-flight Agilent 6520 mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with an electrospray ionization source and operating in the positive mode. The MS ion source parameters were set with a capillary voltage at 3500 V; nitrogen was used as the desolvation and nebulizing gas at a constant gas temperature of 350°C, drying gas 8 L/min and nebulizer 40 psig. Nitrogen was used as a collision gas with collision energy of 25 V. Mass spectrometry spectra were acquired in full scan analysis over an m/z range of 50 to 1000 using a scan rate of 1.003 spectra/s. The MassHunter Workstation Software (version B.02.00; Agilent Technologies) was used for system operation and data collection. Data analysis was performed using Agilent MassHunter Qualitative analysis software.

Large-Scale Incubation. The CLZ GSH conjugates were produced on a preparative scale by large-scale incubation with the most selective CYP102A1 M11H mutant as a biocatalyst. A 5-ml reaction volume containing purified enzyme (1 µM), CLZ (500 µM), GSH (100 µM), GST P1-1 (8 µM), and an NADPH-regenerating system...
(0.2 mM NADPH, 1 mM glucose 6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase) was prepared in potassium phosphate buffer (100 mM, pH 7.4). The reaction was allowed to continue for 6 h at 25°C. To achieve maximal conversion of CLZ, the incubation was supplemented every hour with 40 μl of 120 μM M11 Phe87, 20 μl of 25 mM GSH, 500 μl of the NADPH-regenerating system, and 100 μl of 200 μM GST P1-1. The final incubation volume was 8.4 ml. The reaction was terminated by adding 0.84 ml of 10% HClO4 and centrifuged for 15 min at 14,000 rpm. The supernatant was applied to 25 mM GSH, 500 μl of the NADPH-regenerating system, and 100 μl of 200 μM GST P1-1. The final incubation volume was 8.4 ml. The reaction was terminated by adding 0.84 ml of 10% HClO4 and centrifuged for 15 min at 14,000 rpm. The supernatant was applied to a Strata X C18 solid-phase extraction column (200 mg/3 ml; Phenomenex). The column was washed with 5 ml of H2O to remove salts and proteins. CLZ and its metabolites were subsequently eluted using 2 ml of methanol. The sample was evaporated to dryness and reconstituted in 2 ml of eluent A (0.8% acetonitrile, 99% water, and 0.2% formic acid). The sample was applied by manual injection on a preparative chromatography Luna 5-μm C18(2) column (250 × 100 mm i.d.) from Phenomenex, which was previously equilibrated with 100 μl of eluent A. A flow rate of 2 ml/min and a gradient using eluent A (0.8% acetonitrile, 99% water, and 0.2% formic acid) and B (99% acetonitrile, 1% water, and 0.2% formic acid) was applied for separation of formed CLZ metabolites. The first 10 min were isocratic at 0% eluent B; from 10 to 65 min, the percentage of eluent B increased linearly to 65%; from 65 to 70 min was a further increase of eluent B to 100%; and from 70 to 80 min there was a linear decrease to 0% B, and re-equilibration was maintained until 120 min. Metabolites were detected using UV detection (254 nm) and collected manually. Collected fractions were first analyzed for purity and identity by the analytical high-performance liquid chromatography (HPLC) and LC-MS/MS methods as described under Metabolism of CLZ by CYP102A1 M11H Mutants in the Presence of Human GST P1-1. The samples were evaporated to dryness under a nitrogen stream and dissolved in 1 ml of deuterium oxide to exchange acidic hydrogen atoms by deuterium atoms. Samples were evaporated to dryness in the vacuum concentrator, the residues were redissolved in 500 μl of methyl alcohol-d6, and 1H NMR spectra were recorded at room temperature. 1H NMR analysis was performed on a Bruker Avance 500 (Milan, Italy) equipped with a cryoprobe. 1H NMR measurements were performed at 500.23 MHz.

Results

Expression of CYP102A1 M11H Mutants. A saturation mutagenesis library with a different residue at position 87 of CYP102A1 M11H was recently created in our laboratory (Vottero et al., 2011). All 20 mutants were expressed in E. coli BL21 with pET28+ vectors; the P450 quantification was done by a CO difference spectrum. For the mutants Pro87, Asp87, and Ser87, the reduced CO difference spectra only showed a peak at 420 nm, suggesting that these amino acids negatively affect the folding and/or stability of CYP102A1 M11H. The mutant containing Asn87 showed a significant peak at 420 nm with an intensity almost equal to that at 450 nm. Mutants containing Met87, His87, and Gly87 showed a small shoulder at 420 nm next to the peak at 450 nm. All other mutants only produced peaks with maxima ranging from 448 to 450 nm (Vottero et al., 2011).

Activity and Regioselectivity of Metabolism of CLZ by CYP102A1 M11H Mutants. When CLZ was incubated at an analytical scale with the 20 different CYP102A1 M11H mutants in the presence of GSH and hGST P1-1, 13 different metabolites were found in total (Table 1), which is consistent with our previous studies (Damsten et al., 2008; Dragovic et al., 2010). Five of the metabolites result from N-oxidation (C-1), N-demethylation (C-2), piperazine ring cleavage (C-3), and combinations of these (C-4 and C-5) (Fig. 1). In total, eight different GSH conjugates were found resulting from bioactivation of CLZ to reactive intermediates. Five of these GSH conjugates result from addition reactions of the CLZ nitrenium ion with GSH (C-1, C-3, C-4, and C-5) and chlorine substitution (C-6). The LC-MS/MS spectra of these five GSH conjugates are tabulated in Table 2; the assignment of the fragments have been described elsewhere (Maggs et al., 1995; Damsten et al., 2008; Dragovic et al.,

### Table 1

| C-1   | C-2   | C-3   | C-4   | C-5   | CG-1 | CG-2 | CG-3 | CG-4 | CG-5 | CG-6 | CG-7 | CG-8 | Specific Activitya | Percentage Conversion |
|-------|-------|-------|-------|-------|------|------|------|------|------|------|------|------|------|--------------------|----------------------|
| Gly87 | 0.4   | 0.7   | 0.1   | N.D.  | N.D. | 1.1  | N.D. | 0.1  | N.D. | 1.2  | 2.7  | 0.5  | 0.4  | 1.2               | 1.4                  |
| Ala87 | 35.1  | 82.5  | 1.1   | 2.0   | 7.8  | 13.7 | 0.7  | 1.0  | 0.3  | 14.4 | 26.1 | 4.0  | 2.5  | 31.9             | 38.2                 |
| Val87 | 15.1  | 90.1  | 0.9   | 1.0   | 7.1  | 9.3  | 0.7  | 0.8  | 0.3  | 11.7 | 15.8 | 5.5  | 2.9  | 26.9             | 32.3                 |
| Leu87 | 1.0   | 9.0   | 0.3   | 0.1   | N.D. | 3.2  | 0.1  | 0.5  | 0.3  | 4.7  | 6.3  | 1.2  | 0.6  | 4.6              | 5.5                  |
| Ile87 | 6.8   | 71.4  | 0.5   | 0.3   | 0.6   | 9.6  | 0.5  | 0.8  | 0.4  | N.D. | 20.7 | 2.9  | 1.6  | 21.6             | 25.9                 |
| Phe87 | 8.7   | 10.5  | 0.2   | 0.2   | N.D. | 18.2 | 0.2  | 0.9  | 0.3  | 16.7 | 37.2 | 5.3  | 2.2  | 16.8             | 20.1                 |
| Trp87 | 11.6  | 46.9  | N.D.  | N.D.  | N.D. | 5.0  | N.D. | N.D. | 5.4  | 7.6  | 2.7  | 0.8  | 13.3            | 16.0                 |
| Met87 | 0.7   | 5.5   | 0.1   | 0.1   | N.D. | 2.8  | N.D. | 0.3  | N.D. | 3.5  | 5.1  | N.D. | 0.7  | 3.2              | 3.8                  |
| Pro87 | 1.4   | 4.6   | N.D.  | N.D.  | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.Q.             | 1.2                  |

**A**Polar uncharged side chain

| Thr87 | N.D.  | N.D.  | N.D.  | N.D.  | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D.             | N.D.                 |

**B**Polar charged side chain

| Asp87 | 8.5   | 13.6  | N.D.  | N.D.  | N.D. | 3.7  | N.D. | N.D. | 3.7  | 7.1  | 0.3  | 0.3  | N.Q.            | 7.4                  |
| Glu87 | 0.6   | 4.3   | 0.1   | 0.1   | N.D. | 1.7  | N.D. | N.D. | 1.7  | 3.1  | 0.5  | N.D. | 2.0  | 2.4              | 2.4                  |
| His87 | 0.9   | 1.3   | 0.3   | 0.1   | N.D. | 0.9  | N.D. | N.D. | 1.1  | 1.6  | N.D. | 1.0  | 1.2  | 1.0              | 1.2                  |
| Arg87 | 1.7   | 4.5   | N.D.  | N.D.  | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | 1.0              | 1.2                  |

N.D., not detected.

*Presented as nmol product/nmol CYP102A1/30 min.

*N.Q., not quantified because P450 concentration could not be quantified (CO difference spectrum only showed a peak at 420 nm).*
2010). GSH conjugates designated CG-2 and CG-8 were found to be secondary GSH conjugates resulting from bioactivation of N-demethylclozapine to its corresponding nitrenium ion and subsequent addition (CG-2) and chlorine substitution (CG-8). CG-7 corresponds to a di-GSH conjugate that most likely results from GSH conjugation to the GSH containing nitrenium ion formed after chlorine substitution of the CLZ nitrenium ion (Fig. 1).

As shown in Table 1, the nature of the amino acid residue at position 87 has a strong influence on the activity and regioselectivity of formation of CLZ metabolites. As indicated in the last column, the highest activity was generally observed with mutants containing polar amino acids at position 87. The mutants containing Ala87, Val87, and Ile87 showed the highest activity, followed by Phe87 and Trp87. Mutants containing Leu87, Met87, and Glu87, and Ile87 showed the highest activity, followed by Phe87 and Trp87. Among the mutants containing polar uncharged residues, Tyr87 and Gln87 were the most active, showing 25.7 and 9.4% conversion, respectively. The mutants containing Ala87, Val87, and Thr87 at position 87 did not show any activity.

Figure 2 shows the effect of amino acid residue 87 on the relative amounts of the stable metabolites formed via pathways a, b, and c (Fig. 1) and the relative amounts of GSH conjugates resulting from bioactivation of CLZ (pathway d, Fig. 1). For all of the mutants the major stable metabolite was N-demethylclozapine (C-2), followed by CLZ N-oxide (C-1) (Fig. 2A). Significant differences were observed in the ratios of C-2 to C-1. In the case of mutants containing Ile87, Leu87, Met87, and Glu87, N-demethylation was up to 10-fold more abundant than N-oxidation. In contrast, in mutants containing Phe87, His87, Asp87, Gly87, and Gln87 the ratio of N-demethylation to N-oxidation ranged from 1.2 to 2. The other stable metabolites (C-3, C-4, and C-5) represented only minor metabolites for all of the mutants.

As illustrated in Fig. 2B, the metabolic profile of the formed GSH adducts appears to be relatively constant for all of the active mutants, supporting the hypothesis that all GSH conjugates originate from the CLZ nitrenium ion (Dragovic et al., 2010). In all cases, GSH conjugate CG-6 is the major metabolite and accounts for on average 41 ± 5% of the total GSH conjugates. Considering the fact that conjugate CG-7 most likely also originates from the same intermediate nitrenium ion (Fig. 1), the chlorine substitution pathway represents 47 ± 4% of the total GSH conjugation in the presence of hGST P1-1. GSH conjugate CG-5, which was tentatively assigned to the conjugate in which the GSH moiety is attached to the nonchlorinated aromatic ring, represents 3.5 ± 2.3% of the total GSH conjugates.

The secondary GSH conjugates CG-2 (MH+ ion at m/z 618.23) and CG-8 (MH+ ion at m/z 584.25), derived from N-demethylclozapine,
and CG-7 (MH$^+$ ion at $m/z$ 903.35), derived from CG-6 (MH$^+$ ion at $m/z$ 598.27), have not been found in human studies and therefore were not further characterized.

To select the most appropriate CYP102A1 M11H mutant for large-scale production of GSH conjugates, which mutant showed a combination of high overall activity and high selectivity toward the biotransformation pathway was investigated (pathway d in Fig. 1). Figure 3 shows the ratio of total of GSH conjugates to stable metabolites for each active mutant, ranked from low to high ratio. As shown in Fig. 3, for the four most active mutants having active-site amino acids Ala$_{87}$, Val$_{87}$, Tyr$_{87}$, and Ile$_{87}$, less than 35% of the total of metabolites represented GSH conjugates. However, for mutants containing Gln$_{87}$, Phe$_{87}$, and Gly$_{87}$, approximately 80% of the metabolites found were GSH conjugates, indicating that for these mutants biotransformation to CLZ nitrenium ion (pathway d, Fig. 1) is the major pathway of metabolism. Because the mutant containing Phe$_{87}$ has the highest activity, this mutant was selected for large-scale production of GSH conjugates for structural elucidation by $^1$H NMR.

Large-Scale Incubation and NMR Identification of Isolated GSH Conjugates. Figure 4 shows the preparative HPLC chromatogram, with UV detection at 254 nm, obtained after large-scale incubation of CLZ with mutant Phe$_{87}$. After isolation of the individual metabolites by preparative HPLC, their purity and identity were first analyzed by analytical HPLC and LC/MS/MS methods, resulting in the assignment of metabolites and parent compound as presented in Fig. 4. By hourly additions of enzymes and cofactors, over 90% of CLZ was converted, according to the strong decrease in parent compound. On the basis of the peak areas, approximately 98% of the metabolites found were GSH conjugates. This higher percentage of GSH conjugation, compared with the analytical-scale incubations, can be explained by further biotransformation of the stable metabolite C-2, producing CG-8 and CG-2. The low yield of CLZ N-oxide (C-1) might be explained by nonenzymatic reduction of the N-oxide by NADPH and GSH that was added hourly to the incubation (Pirmohamed et al., 1995).

For the five primary GSH conjugates of CLZ, having an MH$^+$ ion at $m/z$ 632.23 (CG-1, CG-3, CG-4, and CG-5) and an MH$^+$ ion at $m/z$ 598.25 (CG-6), $^1$H NMR spectra were recorded to identify the position of GSH conjugation. Figure 5 shows the signals of the aromatic hydrogen atoms of the CLZ moiety of these GSH conjugates. The correlation spectroscopy (COSY) spectra of these signals, which were recorded to facilitate the assignment of the aromatic hydrogen atoms as shown in Table 2, can be found in the supplemental material. The signals of the aliphatic hydrogen atoms were consistent with the glutathionyl moiety (data not shown).

Figure 5A shows the $^1$H NMR spectrum of conjugate CG-1, which is the major GSH conjugate formed in the absence of glutathione transferases (Damsten et al., 2008; Dragovic et al., 2010). This conjugate, which eluted after 32.5 min in the preparative HPLC (Fig. 4), was previously identified as C-6 glutathionyl clozapine. The spectrum shown in Fig. 5A is in full agreement with the $^1$H NMR spectra of C-6 glutathionyl clozapine, which was identified previously as the major
GSH conjugate formed by peroxidases and electrochemical oxidation of CLZ (Fischer et al., 1991; Liu and Uetrecht, 1995; Madsen et al., 2007). Two doublets at 6.96 and 7.23 ppm correspond to the protons at positions 9 and 7, respectively, with a small coupling constant of 2.5 Hz due to the proton in the meta position. Fischer et al. (1991) previously assigned a doublet at 6.96 ppm to H₉ and the signal at 7.23 ppm to H₇. However, Madsen et al. (2007) assigned a doublet at 6.96 ppm to H₉ and the signal at 7.23 ppm to H₇ (Madsen et al., 2007). Which signal corresponds to which proton could not be determined unequivocally only based on chemical shift and coupling pattern (Liu and Uetrecht, 1995). However, this does not affect the identification of the position of GSH conjugation because each theoretically possible GSH conjugate is expected to have its own unique combination of multiplicity and coupling pattern. Therefore, for signals that could not be assigned unequivocally to specific aromatic protons, two possibilities are shown in Table 2. The assignments before the slashes correspond to the second possibility, whereas the assignments after the slashes correspond to the first possibility. Furthermore, the signals at 6.96, 7.37, and 7.45 ppm could be attributed to protons H₆, H₉, and H₇ of the chlorinated ring according to the COSY spectrum (Supplemental Fig. 4). This confirms that addition of GSH conjugates are shown in Fig. 5, C and E, respectively. These typical triplets could not be found in the spectrum of CLZ (Fischer et al., 1991). The signals are given in Table 2. The corresponding chemical shifts and coupling constants for the observed signals are given in Table 2.

Two more conjugates having a MH⁺ ion at m/z 632.2 eluted at retention times of 30.9 and 33.1 min (Fig. 4) and appeared to correspond to conjugates CG-4 and CG-5 (Dragovic et al., 2010). On the basis of the order of elution and small differences in fragmentation patterns in LC-MS/MS (Maggs et al., 1995), the structure of these GSH conjugates were previously tentatively assigned to C-7 glutathionyl clozapine (CG-4) and a conjugate with GSH bound to the nonchlorinated ring (CG-5). However, so far no ¹H NMR spectra have been reported that confirm the exact positions. Spectra for these two conjugates are shown in Fig. 5, C and E, respectively.

Figure 5E shows the ¹H NMR spectrum of the aromatic region of CG-5, which is found at high levels when GSH conjugation is catalyzed by GST P1-1 (Fig. 4). This spectrum can only be explained by conjugation of GSH at the C-9 position of clozapine. Two signals at 6.83 and 7.02 ppm only showed a coupling of 8 Hz and are therefore assigned to the neighboring H₆ and H₇ protons. The COSY spectrum of conjugate CG-3 (Supplemental Fig. 2) was identical to that of C-9 glutathionyl clozapine as published previously (Liu and Uetrecht, 1995). The corresponding chemical shifts and coupling constants for the observed signals are given in Table 2.

Figure 4 shows the preparative HPLC-UV chromatogram of metabolites obtained by largescale incubation of CLZ with CYP102A1 M11H Phe87 in the presence of hGST P1-1 and GSH.
coupling of 1.5 Hz at 6.95 ppm corresponds to H₄ by meta coupling by proton H₂. Conjugate CG-4 is the fourth GSH conjugate with an MH⁺ ion at m/z 632.2 and was previously tentatively assigned to C-7 glutathionyl clozapine (Dragovic et al., 2010). However, this theoretically can also represent a conjugate with GSH bound to one of the other positions of the nonchlorinated aromatic ring. This GSH conjugate eluted at 30.9 min with preparative HPLC (Fig. 4). Because this conjugate is produced at very low yield, several large-scale incubations were performed to obtain enough material to record a ¹H NMR spectrum with sufficient signal-to-noise ratio. Although this small amount of the conjugate appeared to be contaminated by an unknown compound, the COSY spectrum allowed us to solve the spectrum despite the strong contaminant signal at 7.1 ppm (Fig. 5C; Supplemental Fig. S3). On the basis of these spectra, this GSH conjugate is identified as C-7 glutathionyl clozapine, which is consistent with the previous proposals (Maggs et al., 1995; Dragovic et al., 2010). First, two triplets at 7.04 and 7.37 ppm correspond to the protons H₂ and H₃ at the nonchlorinated aromatic ring. The signals centered at 7 and 7.3 ppm correspond to protons H₁ and H₄, as demonstrated by the combination of ortho and meta coupling by protons H₂ and H₃. The signals at 6.82, 7.01, and 7.12 ppm correspond to protons H₆, H₇, and H₉, respectively, on the basis of the coupling patterns and COSY spectrum. On the basis of this spectrum and the mass spectrum, it was confirmed that this conjugate corresponds to C-8 glutathionyl deschloroclozapine.

The fifth GSH conjugate for which a ¹H NMR spectrum is recorded is CG-6, which was the major GSH conjugate found in the incubations in the presence of hGST P1-1 and which showed a MH⁺ ion at m/z 598.25 by LC-MS/MS analysis. In the preparative HPLC system used, this GSH conjugate eluted at 27.6 min (Fig. 4). Figure 5D and Supplemental Fig. S5 show the ¹H NMR and COSY spectra obtained. Two triplets centered at 7.04 and 7.35 ppm with small meta couplings correspond to the protons H₃ and H₂ at the nonchlorinated aromatic ring. The signals centered at 7 and 7.3 ppm correspond to protons H₁ and H₄, as demonstrated by the combination of ortho and meta coupling by protons H₂ and H₃. The signals at 6.82, 7.01, and 7.12 ppm correspond to protons H₆, H₇, and H₉, respectively, on the basis of the coupling patterns and COSY spectrum. On the basis of this spectrum and the mass spectrum, it was confirmed that this conjugate corresponds to C-8 glutathionyl deschloroclozapine.

**Discussion**

Currently, there is an increasing interest in developing novel methodologies to produce human-relevant drug metabolites on a large scale to enable structural characterization of metabolites and test their pharmacological and toxicological properties. One of the approaches is to make use of genetically engineered cytochromes P450 that are developed for the catalysis of regio- and stereoselective hydroxylation of chemicals at high activity. In particular, the bacterial cytochrome P450 CYP102A1 from *Bacillus megaterium* has high potential as a biocatalyst for these purposes because this enzyme is the most active P450 discovered so far and because the substrate selectivity and
metabolic profile can be manipulated by site-directed and/or random mutagenesis (Yun et al., 2007; Sawayaama et al., 2009). One of the CYP102A1 mutants that show high activity in drug metabolism is CYP102A1 M11H, which contains 10 different amino acid substitutions compared with wild-type CYP102A1. This CYP102A1 mutant was shown to be highly active in metabolizing various drugs to human-relevant metabolites, including reactive intermediates (van Vugt-Lussenburg et al., 2007; Damsten et al., 2008; Dragovic et al., 2010). We have recently performed a saturation mutagenesis study in which the active-site residue at position 87 was mutated to all 20 possible amino acids (Vottero et al., 2011). In CYP102A1 M11H the residue at this position is Val87, which was introduced at an early stage of the mutagenesis process to expand the substrate selectivity to drug metabolism (Lussenburg et al., 2005). In the saturation mutagenesis study in which all amino acids were evaluated at position 87, we recently demonstrated that the type of amino acid at position 87 has strong effect on substrate selectivity when comparing a series of alkoxyresorufins (Vottero et al., 2011). In this study, it was also demonstrated that the nature of the amino acid at position 87 strongly influences the regioselectivity of testosterone hydroxylation of CYP102A1 M11H.

In the present study, the library of CYP102A1 M11H mutants with different amino acids at position 87 was evaluated with CLZ as a substrate. CLZ is a drug that can be metabolized by peroxidases and P450s to multiple metabolites, including reactive nitrenium ions that might be involved in adverse drug reactions associated with CLZ therapy. CYP102A1 M11H has been shown to produce high levels of most human-relevant metabolites of CLZ, which are represented in Fig. 1. All metabolites can be explained by four different initial oxidative pathways: N-demethylation (pathway a), N-oxidation (pathway b), piperazine ring opening (pathway c), and dehydrogenation to a reactive nitrenium ion (pathway d). Although CYP102A1 M11H with residue Val87 produced significant amounts of reactive nitrenium ion (as identified as GSH conjugates), the major pathways of metabolism are N-demethylation and N-oxidation, which explain approximately 70% of the total of metabolites. The aim of this study was to investigate whether residue 87 also controls the regioselectivity of CLZ metabolism and to investigate whether a mutant could be identified with higher selectivity toward the bioactivation to the toxicologically relevant CLZ nitrenium ion. A more selective P450 CYP102A1 mutant would be more useful for the generation of high levels of CLZ GSH conjugates that still require structural confirmation by 1H NMR. So far, the structures of only two of the GSH conjugates shown in Fig. 1 have been elucidated by 1H NMR and mass spectrometry. However, the structures of the GSH conjugates found in the bile of rats and mice, and that also have MH ions at m/z 632.2 (Maggs et al., 1995), have not yet been characterized by 1H NMR.

As shown in Table 1, changing the amino acid residue at position 87 of CYP102A1 M11H has strong effects on the total activity and regioselectivity of CLZ oxidation. The mutants Ala87, Val87, and Ile87 were found to be the most active, as was found previously with alkoxyresorufins and testosterone (Vottero et al., 2011). These mutants have a small and apolar residue in position 87. This seems consistent with the previous hypothesis that replacement of the bulky Phe87 in wild-type CYP102A1 by smaller amino acids creates space for the bulky substrates that allows for better positioning with respect to the activated oxygen species, resulting in higher activities and coupling efficiencies (Carmichael and Wong, 2001; Li et al., 2001; Landwehr et al., 2006). However, in the present study relatively high activities were also found in the CYP102A1 M11H mutants containing the relatively bulky amino acids Phe87, Tyr87, and Trp87. Previously, replacing Phe87 by Tyr87 in wild-type CYP102A1 was found to be detrimental for activity toward long-chain fatty acids, probably by disruption of the hydrophobic interaction by the phenol group. In the case of CYP102A1 M11H, the presence of these bulky amino acids apparently is less restrictive for bulky substrates because in presence of Phe87 and Tyr87 testosterone (Vottero et al., 2011) and CLZ are metabolized at high activity. Apparently, by the combination of 10 mutations present in CYP102A1 M11H, the topology of the active site and/or substrate access channel has changed significantly, explaining the much wider substrate selectivity compared with wild-type CYP102A1.

As shown in Fig. 2A, the nature of amino acid 87 has a strong effect on the regioselectivity of CLZ metabolism. When considering the stable metabolites that are formed via pathways a, b, and c (Fig. 1), the major metabolite with all mutants was N-demethylclozapine (C-2), although the N-oxide (C-1) was also produced at significant levels. However, the ratio of N-demethylation to N-oxidation appeared to be quite dependent on the nature of the amino acid residue at position 87. For example, for the mutants containing Leu87 and Ile87, N-demethylation was almost 10-fold higher than N-oxidation. In the mutant containing Phe87, N-demethylclozapine and CLZ N-oxide were formed in almost the same amount. However, the relative contribution of N-oxidation in all incubations might be somewhat underestimated because all incubations were performed in the presence of GSH, which is known to reduce CLZ N-oxide back to CLZ (Tugnait et al., 1999). Therefore, in the case of Phe87, N-oxidation of CLZ might even be higher in the absence of reductive agents. In case of the human P450s, it has been shown that CYP1A2 preferentially metabolizes CLZ by N-demethylation, whereas CYP3A4 is mainly responsible for production of CLZ N-oxide (Tugnait et al., 1999). However, the factors that determine the ratio of N-demethylation and N-oxidation are still unclear. Different presentation of the piperazine N-methyl group to the oxidative species at the active site might explain why some human P450s preferentially catalyze N-demethylation whereas others predominantly catalyze N-oxidation.

One of the aims of the present study was to identify mutants with high activity and selectivity for bioactivation of CLZ to the reactive nitrenium ion. As shown in Fig. 2A, several mutants produced high levels of GSH conjugates (CG-total), indicative of relatively high selectivity in the formation of the reactive nitrenium ion. Other mutants showed strong preference in catalyzing the formation of N-demethylclozapine and CLZ N-oxide. However, from the results it is unclear which features of the amino acid side chain determine selectivity for bioactivation. For example, the CYP102A1 M11H mutant containing the bulky Phe87 showed high selectivity and activity in the formation of GSH conjugates, whereas the mutants containing the bulky Tyr87 and Trp87 preferentially catalyze formation of stable metabolites. Future detailed protein modeling studies including those evaluating protein dynamics and substrate mobility might help to rationalize the different regioselectivities observed. Figure 2B shows the relative amounts of the different GSH conjugates that are formed in incubations of CLZ with CYP102A1 mutants in the presence of recombinant hGST P1-1. Consistent with our previous study, the major pathway of GST P1-1-catalyzed GSH conjugation is substitution of the chlorine atom of the CLZ nitrenium ion (Dragovic et al., 2010). The resulting GSH-bound nitrenium ion is subsequently reduced by NADPH or GSH to form CG-6 or further conjugated to GSH to form CG-7 (Fig. 1). The fact that with all mutants the same ratio of GSH conjugates is formed strongly suggests that all form from the same reactive intermediate.

Mutant Phe87 was selected for the large-scale biosynthesis of GSH conjugates because this mutant combined high activity with high...
preference for the bioactivation pathway (Fig. 3). Previous studies aiming at the characterization of GSH conjugates of CLZ showed that nonenzymatic GSH conjugation to the CLZ nitrenium ion, formed by peroxidases or electrochemically, mainly produced a GSH conjugate bound at the C-6 position of CLZ and minor amounts of conjugate bound at the C-9 position. The structures of these two GSH conjugates have been elucidated by $^1$H NMR. However, in vitro studies with rats and mice have shown that in bile two major GSH conjugates are excreted that do not correspond to these two conjugates (Maggs et al., 1995). Also, incubations with rat liver microsomes showed small amounts of a fifth GSH conjugate with an MH$_1$ ion at $m/z$ 632.2 (Maggs et al., 1995). It was initially concluded that these GSH conjugates might originate from an as yet unidentified reactive intermediate produced in vivo. However, we recently demonstrated that these alternative GSH conjugates probably are resulting from GST-catalyzed inactivation of the CLZ nitrenium ion (Dragovic et al., 2010). By using mutant Phe87, we were able to produce significant amounts of all GSH conjugates for which the structures were not yet elucidated unequivocally by $^1$H NMR. Because four GSH conjugates were found with an MH$_1$ ion at $m/z$ 632.2, it was previously concluded that for at least one of the conjugates, GSH is bound to the nonchlorinated aromatic ring of CLZ. The present study shows that conjugate designed CG-5, which is a major product in the presence of hGST P1-1, has the GSH moiety bound to the nonchlorinated ring at position 2 or 3 (Fig. 5). For the minor conjugate CG-4, we were able to confirm binding at the 7 position, as it was tentatively assigned based on the fragmentation pattern in LC-MS/MS (Maggs et al., 1995).

In conclusion, the present study shows that mutation of residue 87 in drug-metabolizing mutant CYP102A1 M11H has a strong influence on the activity and regioselectivity of CLZ metabolism. Using a mutant that combined high activity and high selectivity for CLZ bioactivation, we were able to produce sufficient amounts of as yet tentatively assigned GSH conjugates to characterize their structures by $^1$H NMR. This study confirms the high potential of CYP102A1 mutants as tools to characterize human-relevant metabolites.

**Authorship Contributions**

**Participated in research design:** Rea, Dragovic, Vermeulen, and Commandeur.

**Conducted experiments:** Rea, Dragovic, Boerma, and de Kanter.

**Contributed new reagents or analytic tools:** Boerma.

**Performed data analysis:** Rea, Dragovic, de Kanter, and Commandeur.

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

**References**


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