Chapter 5

Role of human glutathione-S-transferases in the inactivation of reactive metabolites of clozapine

Adapted from: Role of human glutathione-S-transferases in the inactivation of reactive metabolites of clozapine
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Chem Res Toxicol 2010, 23, p. 1467-1476
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ABSTRACT

Conjugation of reactive drug metabolites to GSH is considered an important detoxification mechanism that can be spontaneous and/or mediated by glutathione-S-transferases (GSTs). In case GSTs play an important role in GSH conjugation, genetically determined deficiencies in GSTs may be a risk factor for adverse drug reactions (ADRs) resulting from reactive drug metabolites. So far, the role of GSTs in the detoxification of reactive intermediates of clozapine (CLZ), a drug causing idiosyncratic drug reactions, has not been studied. In the present study, we investigated the ability of four recombinant human GSTs (hGST A1-1, hGST M1-1, hGST P1-1, and hGST T1-1) to catalyze the GSH conjugation of reactive metabolites of CLZ, formed in vitro by human and rat liver microsomes and drug metabolizing P450 BM3 mutant CYP102A1M11H. Consistent with previous studies, in absence of GSTs three GSH conjugates were identified derived from the nitrenium ion of CLZ. In presence of three of the GSTs, hGST P1-1, hGST M1-1, and hGST A1-1, total GSH conjugation was strongly increased in all bioactivation systems tested. Highest activity was observed with hGST P1-1, whereas hGST M1-1 and hGST A1-1 showed slightly lower activity. Polymorphic hGST T1-1 did not show any activity in catalyzing GSH conjugation of reactive CLZ metabolites. Interestingly, addition of hGSTs resulted in major changes in the regioselectivity of GSH conjugation of the reactive CLZ metabolite, possibly due to the different active site geometries of hGSTs. Two GSH conjugates found were completely dependent on the presence of hGSTs. Chlorine substitution of the CLZ nitrenium ion, which so far was only observed in in vivo studies, appeared to be the major pathway of hGST P1-1-catalyzed GSH conjugation, whereas hGST A1-1 and hGST M1-1 also showed significant activity. The second GSH conjugate, previously also only found in in vivo studies, was also formed by hGST P1-1 and to a small extent by hGST A1-1. These results demonstrate that human GSTs may play a significant role in the inactivation of reactive intermediates of CLZ. Therefore, further studies are required to investigate whether genetic polymorphisms of hGST P1-1 and hGST M1-1 contribute to the interindividual differences in susceptibility to CLZ-induced ADRs.
INTRODUCTION

Clozapine (CLZ) is an atypical antipsychotic drug, lacking extrapyramidal adverse effects and used in the treatment of refractory schizophrenia and with treatment-resistant patients (1-3). In spite of its advantages, an important side effect of CLZ is agranulocytosis occurring in 1-2% of the patients (2, 3). In addition, hepatotoxicity has been reported as a side effect of CLZ with 37% of the patients showing enhanced serum transaminases and 0.06% of the patients getting liver failure (4). So far, several in vitro studies have been performed to identify the mechanism of toxicity. Although the exact mechanism is not known yet, formation of reactive metabolites such as nitrenium ions has been proposed as a possible explanation for these Adverse Drug Reactions (ADRs) (5-8).

The large number of in vivo and in vitro studies, performed in humans and animal models, collectively show that CLZ undergoes extensive oxidative metabolism, followed by phase II reactions (7-12), as summarized in Figure 1. In human, N-demethylation and N-oxidation represent the major metabolic pathways. In addition, hydroxylation reactions have been demonstrated at positions 6, 7, 8, and 9 of the chlorinated ring of CLZ (9-11). These hydroxy-metabolites are subject to subsequent glucuronidation and sulfation reactions (Figure 1). In vitro studies have shown that several human cytochrome P450s (CYPs) are involved in oxidative metabolism of CLZ: CYP3A4 and CYP1A2 are involved in N-oxidation, whereas CYP2D6, CYP1A2 and CYP3A4 are involved in N-demethylation of CLZ (13).

Next to these stable metabolites, CLZ is known to be bioactivated to reactive intermediates by myeloperoxidases and CYPs (6-8). In vitro incubations of CLZ with hepatic microsomes (human and rat) in the presence of GSH or with human neutrophils and myeloid cells have shown formation of several GSH conjugates, with GSH conjugated at the 6-, 7-, and 9-position (6, 8) (Figure 1). These conjugates might result from a reactive nitrenium intermediate which can be conjugated to GSH at different positions of the quinoid ring (6).
**Figure 1:** Scheme of the oxidative metabolism of clozapine (CLZ) to stable and reactive metabolites, and subsequent phase II metabolism, as found in *in vitro* and *in vivo* studies (adapted from refs. 8-11). Major stable metabolites and GSH conjugates are shown with structures, others are indicated by abbreviations. The proposed reactive intermediate of CLZ is depicted in brackets and the structures of GSH adducts are presented as described in reference (8). Conjugates CG-5 and CG-6 were proposed to result from as yet unidentified reactive intermediates (8). DMCLZ, N-desmethylclozapine; CLZ-NO, clozapine N-oxide; 8-SCH$_3$-desCLZ, 8-thiomethyldeschloroclozapine; 7-SCH$_3$-CLZ, 7-thiomethylclozapine; 6-OH-CLZ, 6-hydroxyclozapine; 7-OH-CLZ, 7-hydroxyclozapine; 8-OH-desCLZ, 8-hydroxydeschloroclozapine; 9-OH-CLZ, 9-hydroxyclozapine.

In case of peroxidase- and P450-mediated bioactivation of CLZ, GSH conjugation mainly occurs at the 6-position of the chlorinated aromatic ring and to a lower extent at the 9-position (8). An additional minor GSH conjugate formed *in vitro* by human and rat liver microsomes was tentatively assigned to the 7-position (8). Two other GSH adducts were found only *in vivo* in bile of rats and mice, and were considered to be GSH conjugates resulting from still unidentified reactive intermediates (8). One GSH conjugate results from substitution of the chlorine by GSH. Identification of 8-methylthio-deschloroclozapine (8-SCH$_3$-desCLZ, Figure 1) in urine of patients (9), most likely resulting from initial substitution of the 8-chlorine by GSH, suggests that this bioactivation pathway is also represented in human. Another GSH conjugate found only *in vivo* was proposed to result from conjugation to the non-chlorinated ring of CLZ (8).

Although formation of multiple GSH conjugates of CLZ has been demonstrated in many *in vitro* studies, the role of glutathione-S-transferases (GSTs) in catalyzing GSH conjugation of reactive CLZ metabolites has remained unexplored. GSTs are a family of enzymes whose main role is detoxifying electrophilic xenobiotics forming stable and more hydrophilic GSH conjugates for easier excretion from the body (16-19). An increasing number of GST genes are being recognized as polymorphic. In humans, marked interindividual differences exist in the expression of class Alpha, Mu, Pi and Theta GSTs (20). Genetic polymorphism of GSTs has been associated with increased susceptibility of several forms of cancers, alcoholic liver
disease, and toxic hepatitis caused by chemicals and drugs (21-25). Several clinical studies have demonstrated an increased susceptibility to idiosyncratic drug-induced liver injury by the combined GST M1-T1 double-null genotype (22, 23).

So far, only few in vitro studies have been performed to investigate the role of GSTs in the inactivation of electrophilic drug metabolites (26-29). Several rat and human GSTs catalyze the GSH conjugation reaction of synthetical N-acetyl-p-benzoquinone imine, the reactive metabolite of acetaminophen (26). In case of valproic acid, it was found that non-enzymatic GSH conjugation to its reactive diene metabolite results in a single GSH conjugate. Addition of GSTs, however, resulted in a second GSH conjugate, which was also found in vivo in rats treated with valproic acid (27). For felbamate (28) and zileuton (29), the rate of GSH conjugation to their electrophilic metabolites, 2-phenylpropenal and 2-acetylbenzothiophene, respectively, was increased in presence of all three studied human GST isoforms (hGST A1-1, hGST M1-1 and hGST P1-1). In all of these studies, the GST incubations were performed in presence of a synthetical electrophilic drug metabolite. However, this approach will not be applicable to highly reactive, short-lived reactive drug metabolites, or metabolites which are not or poorly accessible by organic synthesis. Therefore, in the present study, the role of human GSTs in the GSH conjugation of reactive CLZ metabolites was studied in incubations of CLZ with human and rat liver microsomes and a drug-metabolizing bacterial P450 BM3 mutant (CYP102A1M11H) as bioactivation systems. Purified CYP102A1M11H was selected because it is able to bioactivate CLZ to all relevant metabolites at much higher activity than human and rat liver microsomes (12). The results demonstrate that hGSTs play an important role in inactivation of reactive metabolites of CLZ and explain the formation of several GSH conjugates previously only found in in vivo studies.

MATERIALS AND METHODS

Enzymes and plasmids
The bacterial P450 BM3 mutant, CYP102A1M11H, was prepared and purified as described previously (12). Rat liver microsomes were prepared according to the protocol already used in our laboratory (30). Human liver microsomes (Lot No. 0710619), pooled from 50 donors, were obtained from Xenotech (Lenexa, USA) and contained 20 mg/mL protein. E. Coli XL-1 Blue
cells containing the expression plasmids for human GST A1-1, M1-1 (B allele) and P1-1 (A allele) were a kind gift from Prof. Mannervik (Department of Biochemistry and Organic chemistry, Uppsala University, Sweden). The plasmid pet20b-hGSTT1 (33), coding for human GST T1-1 with a C-terminal his-tag, was a kind gift from Prof. Hayes (Biomedical Research Centre, University of Dundee, Scotland, UK). All other chemicals and reagents were of analytical grade and obtained from standard suppliers.

Expression and purification of human GST A1-1, GST M1-1 and GST P1-1

Overnight precultures of E. Coli XL-1 Blue cells containing the expression plasmids for human GSTs were prepared in 5 mL LB medium supplemented with 50 µg/mL ampicillin and grown at 37 °C and 175 rpm. For large scale enzyme expression, 500 mL 2YT medium fortified with 50 µg/mL ampicillin was inoculated with 5 mL of an overnight culture and cells were grown at 37 °C until OD600 reached 0.3. Enzyme expression was then induced by addition of IPTG to a final concentration of 0.2 mM and cells were incubated overnight at 37 °C and 150 rpm. The following procedures were all carried out at 4 °C. Cells were harvested by centrifugation at 4000g for 15 min. Pellets were resuspended in 10 mM Tris-HCl pH 7.8 containing 1 mM DTT and 0.2 mg/mL lysozyme and allowed to incubate for 1 h. Cells were subsequently disrupted by sonication (Branson sonifier 250, 4x 30 sec, at 60% full power) and cell debris removed by centrifugation in an eppendorf centrifuge (50 min at 20,000g). Lysate was incubated with a 50% slurry of GSH sepharose 4B (GE healthcare) on a rollerbank for 90 min. Nonspecifically bound proteins were removed by washing of the beads with three changes of 10 mM Tris-HCl pH 7.8 containing 1 mM DTT. GSTs were eluted with 50 mM Tris-HCl pH 8 supplemented with 10 mM GSH and 1 mM DTT. The GSH was subsequently removed by repeated washing with 10 mM Tris-HCl pH 7.8 containing 1 mM TCEP in a Vivaspin 20 filtration tube (10,000 MWCO PES, Sartorius) at 4000g. The washing procedure was continued until the GSH concentration was below 500 nM. Enzymes were aliquoted and stored at -20°C until use.

Protein concentrations were determined according to the method of Bradford (31) with reagent obtained from Bio-Rad. The specific activity of the purified GSTs was assayed according to Habig et al. (32). The specific activities of the purified recombinant human GSTs using CDNB as a substrate were as follows: 20.4 µmol/min/mg protein for GST A1-1, 55.6 µmol/min/mg protein for GST M1-1, 26.9 µmol/min/mg protein for GST P1-1.
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Expression and purification of human GST T1-1

*E. Coli* BL21 DE3 competent cells were transformed with pet20b-hGSTT1 by heatshock. For expression, 400 ml LB containing 50 µg/mL ampicillin was inoculated with 3 mL of an overnight culture and cells were grown at 37 °C and 150 rpm. IPTG was added to the culture in a final concentration of 0.5 mM when OD600 reached 0.7. After an induction period of approximately 6 h, cells were harvested (4000g, 15 min, 4 °C) and pellets frozen overnight at -20°C.

The following steps were conducted at 4 °C. Cell pellets were reconstituted in NaP buffer (50 mM sodium phosphate buffer, pH 8, 0.2 M NaCl) supplemented with 0.2 mg/mL lysozyme. Following an incubation period of 30 min, cells were disrupted using a French Press (1000 psi, 3 repeats). Cell debris was removed by ultracentrifugation (120,000g; 70 min) and the supernatant passed through a 0.45 µm filter (Whatman).

The GST was purified using Ni-NTA agarose (Sigma). A 50% slurry of Ni-NTA in NaP buffer was added to the lysate and incubated on the rollerbank for 2 h. The solution was then applied to a disposable column (Pierce, Rockford, USA). Nonspecifically bound proteins were removed by extensive washing with NaP buffer fortified with 2 mM imidazole. His-tagged hGST T1-1 was eluted with NaP buffer supplemented with 250 mM imidazole and then dialyzed overnight against NaP buffer. For storage, this buffer was exchanged for 25 mM Tris-HCl pH 7.7 containing 1 mM TCEP and 20% glycerol by repeated washing in a Vivaspin 20 filtration tube (10,000 MWCO PES, Sartorius) at 4000g and by overnight dialysis. Purified enzyme was stored at -80 °C until use. The protein concentration of purified hGST T1-1 was determined according to the method of Bradford (31) with reagent obtained from Bio-Rad. The activity of hGST T1-1, using 1,2-epoxy-3-(p-nitrophenoxy)propane as a substrate, was determined essentially as described (34). The specific activity of his-tagged hGST T1-1 using 1,2-epoxy-3-(p-nitrophenoxy)propane was 1.83 µmol/min/mg protein.

Bioactivation of clozapine by CYP102A1M11H in presence of GSH and human recombinant glutathione-S-transferases

Incubations using purified CYP102A1M11H as bioactivation system were performed at a final enzyme concentration of 250 nM, as described previously (12). All incubations were performed in 100 mM potassium phosphate buffer (pH 7.4) and at a final volume of 250 µL.
The substrate CLZ was incubated at a concentration of 500 µM. The final DMSO concentration from the CLZ stock solution was less than 1% in the incubations. Non-enzymatic GSH conjugation was investigated at different GSH concentrations (0.0125, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, and 5 mM). Reactions were initiated by the addition of 500 µM NADPH (final concentration) and performed for 30 min at room temperature. In this time period, product formation was linear as described previously (12). Reactions were terminated by addition of 25 µL 10% HClO₄, and centrifuged for 15 min at 4000 rpm. Supernatants were analyzed by HPLC and LC-MS, as described below.

Enzymatic GSH conjugation by recombinant human GSTs was investigated by adding 8 µM (final concentration) hGST A1-1, hGST M1-1, hGST P1-1, or hGST T1-1 to the incubations. Incubations with hGSTs were performed in presence of 100 µM of GSH. hGST T1-1 was also incubated in presence of 5 mM GSH because of its lower affinity to GSH (33). Finally, hGSTs, showing activity in catalyzing GSH conjugation of CLZ metabolites, were also tested at 0.25, 0.5, 1, 2, 4, and 8 µM to investigate linearity of product formation with protein concentration.

Bioactivation of clozapine by human and rat liver microsomes in presence of GSH and human recombinant glutathione-S-transferases
To confirm that the catalytic effects of hGSTs, as observed in P450 BM3 incubations, were also applicable for incubations with mammalian liver fractions, CLZ was also incubated with human liver microsomes and and rat liver microsomes, each at a final microsomal protein concentration of 1 mg/mL, 100 µM GSH, and in absence or presence of 8 µM hGST. Reactions were initiated by the addition of 500 µM NADPH (final concentration) and were incubated for 30 min at 37 °C. Reactions were terminated by addition of 25 µL 10% HClO₄, and centrifuged to remove precipitated proteins (4000 rpm, 15 min). Supernatants were analyzed by HPLC and LC-MS, as described below.

Analytical methods
The metabolites of CLZ were analyzed by reversed-phase liquid chromatography. A Luna 5 µm C18 column (150 mm x 4.6 mm i.d.) from Phenomenex was used as stationary phase, protected by a 4.0 mm × 3.0 mm i.d. security guard (5 µm) C18 guard column (Phenomenex, Torrance, CA, USA). The gradient used was constructed by mixing the following mobile phases: solvent A (1% acetonitrile/99% water/0.2% formic acid); solvent B (99%)
acetonitrile/1% water/0.2% formic acid). The first 5 min were isocratic at 0% solvent B; from 5 to 30 min the concentration of solvent B linearly increases to 100%; from 30 to 35 min linear decreased to 0% B and maintained at 0% for re-equilibration until 40 min. The flow rate was 0.5 mL/min. Samples were injected at an injection volume of 50 µL.

Samples were analyzed using LC-MS/MS for identification and by LC-UV/Vis (254 nm) for quantification. A standard curve of CLZ was used to estimate the concentrations of the formed GSH conjugates, assuming that the extinction coefficients of the GSH adducts are equal to that of CLZ. The standard curve of CLZ was linear between 1 and 100 µM; the limit of quantitative detection by UV/VIS was estimated to be 0.2 µM (data not shown). The Shimadzu Class VP 4.3 software package was used for determination of peak areas in the UV chromatograms.

For identification of metabolites an Agilent 1200 Series Rapid resolution LC system was connected to a hybrid quadrupole-time-of-flight (Q-TOF) Agilent 6520 mass spectrometer (Agilent Technologies, Waldbronn, Germany), equipped with electrospray ionization (ESI) 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 voltage of 25V. MS spectra were acquired in full scan analysis over an m/z range of 50–1000 using a scan rate of 1.003 spectra/s. The Mass Hunter Workstation Software (version B.02.00) was used for system operation and data collection. Data analysis was performed using Agilent Mass Hunter Qualitative analysis software.
RESULTS

Role of GSTs in inactivation of CLZ metabolites formed by CYP102A1M11H

It was previously shown that purified CYP102A1M11H metabolizes CLZ to all metabolites produced by human and rat liver microsomes but at significantly higher levels (12). As shown in Table 1, when incubated in presence of 5 mM GSH, but in absence of hGSTs, approximately 30% of CLZ concentration was converted to nine metabolites of which four were GSH dependent, CG-1, CG-2, CG-3, and CG-4, consistent with the previous study (12). The characteristics and identity of the different metabolites found are listed in Table 2.

Table 1: Effect of glutathione concentration on the concentration of metabolites formed in incubations of clozapine with CYP102A1M11H in absence of human GSTs

<table>
<thead>
<tr>
<th>Concentration of clozapine metabolites (µM)</th>
<th>100 µM GSH</th>
<th>5 mM GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSH conjugates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CG-1</td>
<td>5.9 ± 0.2</td>
<td>26.3 ± 0.5</td>
</tr>
<tr>
<td>CG-2</td>
<td>0.3 ± 0.2</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>CG-3</td>
<td>0.5 ± 0.2</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>CG-4</td>
<td>ND</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>CG-5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CG-6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CG-7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CG-8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Other metabolites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td>18.8 ± 0.3</td>
<td>19.5 ± 0.9</td>
</tr>
<tr>
<td>C-2</td>
<td>84.5 ± 0.5</td>
<td>88.2 ± 1.6</td>
</tr>
<tr>
<td>C-3</td>
<td>1.6 ± 0.2</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>C-4</td>
<td>2.7 ± 0.1</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>C-5</td>
<td>3.3 ± 0.2</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>118 ± 1.9</td>
<td>147 ± 3.8</td>
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</table>
### Table 2: Metabolites of clozapine formed by CYP102A1M11H in presence and absence of glutathione and recombinant human GSTs

Metabolites were formed by incubating clozapine (500 µM) for 30 min with 250 nM CYP102A1M11H and 500 µM NADPH. *tₚ*, retention time. *m/z* values correspond to the singly protonated molecular ion [(M+1H)/1]. Molecular composition and change of the parent drug are proposed. Identification was based on identity of mass spectra and order of elution as described previously by Maggs et al. (8).

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>tₑ <em>a</em> (min)</th>
<th><strong>Exact mass</strong></th>
<th><strong>Elemental composition</strong></th>
<th><strong>Change of parent drug</strong></th>
<th><strong>Identity</strong> <em>d</em></th>
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<tr>
<td><strong>GSH-conjugates</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CG-1</td>
<td>17.6</td>
<td>632.230</td>
<td>C₂₃H₃₈ClN₂O₆S</td>
<td>ClZ + GS</td>
<td>C-6 glutathionyl clozapine</td>
</tr>
<tr>
<td>CG-2</td>
<td>17.2</td>
<td>618.216</td>
<td>C₂₃H₃₇ClN₂O₆S</td>
<td>ClZ-CH₂ + GS</td>
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</tr>
<tr>
<td>CG-3</td>
<td>17.4</td>
<td>632.230</td>
<td>C₂₃H₃₇ClN₂O₆S</td>
<td>ClZ + GS</td>
<td>C-9 glutathionyl clozapine</td>
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<tr>
<td>CG-4</td>
<td>16.7</td>
<td>632.230</td>
<td>C₂₃H₃₇ClN₂O₆S</td>
<td>ClZ + GS</td>
<td>C-7 glutathionyl clozapine</td>
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<tr>
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<td>17.9</td>
<td>632.230</td>
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<td>ClZ + GS</td>
<td>C(1-4) glutathionyl clozapine</td>
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<tr>
<td>CG-6</td>
<td>15.0</td>
<td>598.269</td>
<td>C₂₉H₃₄N₂O₆S</td>
<td>ClZ-Cl + GS</td>
<td>C-8 glutathionyl deschloroclozapine</td>
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<tr>
<td>CG-7</td>
<td>15.4</td>
<td>903.348</td>
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<td>ClZ-Cl + 2GS</td>
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<tr>
<td>CG-8</td>
<td>14.7</td>
<td>584.253</td>
<td>C₂₇H₃₅N₇O₆S</td>
<td>ClZ-Cl-CH₂ + GS</td>
<td></td>
</tr>
<tr>
<td><strong>Other metabolites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1</td>
<td>19.9</td>
<td>343.147</td>
<td>C₁₈H₂₃ClN₄O</td>
<td>ClZ + O</td>
<td>clozapine N-oxide</td>
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<tr>
<td>C-2</td>
<td>18.2</td>
<td>313.135</td>
<td>C₁₇H₂₁ClN₄</td>
<td>ClZ-CH₂</td>
<td>N-desmethyloclozapine</td>
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<td>C-3</td>
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<td>301.134</td>
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<tr>
<td>C-4</td>
<td>16.2</td>
<td>287.118</td>
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<td>C-5</td>
<td>19.5</td>
<td>329.132</td>
<td>C₁₇H₁₉ClN₄O</td>
<td>ClZ-CH₂ + O</td>
<td></td>
</tr>
</tbody>
</table>

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*a* Metabolites were formed by incubating clozapine (500 µM) for 30 min with 250 nM CYP102A1M11H and 500 µM NADPH.

*b* tₑ, retention time.

*c* m/z values correspond to the singly protonated molecular ion [(M+1H)/1]. Molecular composition and change of the parent drug are proposed.

*d* Identification was based on identity of mass spectra and order of elution as described previously by Maggs et al. (8).
In incubations with 100 µM GSH, the concentration of metabolites C1 - C-5 did not change significantly, indicating that the activity of CYP102A1M11H is not influenced by GSH. As expected, at 100 µM GSH significantly lower amounts of the corresponding GSH conjugates were found; the concentration of GSH conjugate CG-4 was too low to quantify. However, the total concentration of GSH conjugates was only 4.8-fold lower than that found at 5 mM GSH, suggesting that even at 100 µM GSH already a significant fraction of the reactive intermediates is trapped by GSH. To test this hypothesis, the dependence of the non-enzymatic GSH conjugation on GSH concentration was investigated using concentrations ranging from 0 - 5 mM. As shown in Figure 2, between 0 and 100 µM of GSH, the concentration of GSH conjugates increased almost linear with increasing concentration of GSH. At GSH concentrations higher than 1 mM, only relatively small increases in concentration of GSH conjugates was observed, suggesting that at the high GSH concentrations, the reactive intermediates are trapped almost quantitatively. Assuming that the observed saturation curve in Figure 2 is described by the equation $y = a \times (1 - e^{-bx})$, the maximal yield of GSH-conjugates would be 38 µM at infinitive GSH concentration. From this it is estimated that at 100 µM GSH approximately 18% of the reactive intermediates formed is trapped non-enzymatically by GSH, whereas at 5 mM GSH approximately 85% is trapped by GSH.

**Figure 2:** Dependence of non-enzymatic GSH conjugation of reactive clozapine metabolites formed by CYP102A1M11H on GSH concentration. Insert: detail from 0 to 100 µM GSH.
To investigate whether hGSTs are able to catalyze formation of GSH conjugates, incubations were performed in presence of 8 µM hGST and 100 µM GSH, as done previously (29). At this low GSH concentration, hGST activity will still be close to maximal, whereas the competing non-enzymatic reaction will be minimized.

**Effect of human GST P1-1**

As shown in Figure 3A, addition of 8 µM hGST P1-1 to the incubation of CLZ with P450 BM3 and 100 µM GSH resulted in major changes in the profile of GSH conjugates. Addition of hGST P1-1 resulted in the formation of significant amounts of four additional GSH conjugates, CG-5, CG-6, CG-7, and CG-8. Amounts of CG-1, CG-2, and CG-3 did not change significantly. By assuming that extinction coefficients of all GSH conjugates at 254 nm are equivalent, the total amount of GSH conjugates was increased 3.7-fold by hGST P1-1 when compared to corresponding incubations in absence of hGST (Table 3). CG-6 and CG-5 were the major GSH conjugates in presence of hGST P1-1, representing 49.6 and 31.6% of the total amount of GSH conjugates, respectively (Table 3).

LC-MS analysis of CG-6 showed a protonated molecular ion \([M + H]^+\) of m/z 598.3 ((CLZ-SG – Cl + H\(^+\)). The MS/MS of this ion displayed product ions of m/z 580.3 ((CLZ-SG – Cl – H\(_2\)O + H\(^+\)); m/z 541.2 ((CLZ-SG – Cl – C\(_3\)H\(_7\)N (piperazine ring) + H\(^+\)); m/z 469.2 ((CLZ-SG – Cl – glutamic acid + H\(^+\)); m/z 412.2 ((CLZ-SG – Cl – glutamic acid – C\(_3\)H\(_7\)N(piperazine ring) + H\(^+\)); m/z 325.2 ((CLZ-SG – glutamic acid – glycine – C\(_3\)H\(_7\)NO(GSH-moiety) + H\(^+\)) and 268.1 ((CLZ-SG – glutamic acid – glycine – C\(_3\)H\(_7\)NO(GSH-moiety) – C\(_3\)H\(_7\)(piperazine ring) + H\(^+\)). Loss of the γ-glutamyl moiety ([M+1-129]\(^+\)) giving m/z 469.2 and scission of the S-CH\(_2\) linkage with hydrogen transfer to the CLZ thiyl moiety [M+1-273]\(^+\) giving m/z 325.2 are typical characteristics for this GSH adduct. Based on the identical m/z value and fragmentation pattern, this product is identified as C-8 glutathionyl deschloroclozapine, which previously was only found in bile of rat and mice administered CLZ (12). This product was not found in incubations in absence of P450 BM3 or NADPH, and therefore should represent a GSH conjugate of an oxidative CLZ metabolite. Formation of C-8 glutathionyl deschloroclozapine can be rationalized by enzymatic substitution of the chlorine atom of the CLZ nitrenium ion by GSH, followed by reduction by NADPH and/or GSH to restore aromaticity.
Figure 3: HPLC-UV chromatograms of incubations of clozapine using bacterial CYP102A1M11H mutant as bioactivation system, in absence and presence of GSH and/or hGSTs. (A) Addition of 8 µM hGST P1-1. (B) Addition of 8 µM hGST A1-1. (C) Addition of 8 µM hGST M1-1. Traces: (i) incubations in absence of GSH and GST, (ii) incubations with 100 µM GSH, and (iii) incubations in the presence of 100 µM GSH and 8 µM hGSTs.
LC-MS analysis of the second major GSH conjugate, CG-5, showed a protonated molecular ion [M + H]⁺ of m/z 632.2 [CLZ-SG + H]⁺ and therefore apparently corresponds to a regioisomer of CG-1 and CG-3, which both result from addition of GSH to the CLZ nitrenium ion. CG-5 showed the following fragmentation pattern: m/z 614.2 ([CLZ-SG – H₂O + H]⁺); m/z 575.2 ([CLZ-SG – C₃H₇N(piperazine ring) + H]⁺); m/z 503.2 ([CLZ-SG – glutamic acid + H]⁺); m/z 446.1 ([CLZ-SG – glutamic acid – C₃H₇N(piperazine ring) + H]⁺); m/z 359.1 ([CLZ-SG – glutamic acid – glycine – C₃H₇NO(GSH-moiety) + H]⁺) and 302.1 ([CLZ-SG – glutamic acid – glycine – C₃H₇NO(GSH-moiety) – C₃H₇N (piperazine ring) + H]⁺). Because CG-1 and CG-3 were previously identified by ¹H-NMR as GSH conjugates at the C-6 and C-9 positions of the nitrenium ion, CG-5 might correspond to an adduct to the C-7 position, the only remaining position on the chlorinated ring, or a GSH conjugate to the non-chlorinated ring, as suggested previously (8).

The two minor GST P1-1 dependent adducts CG-7 (t_{ret} 15.4 min) and CG-8 (t_{ret} 14.7 min) (Figure 3A) showed protonated molecular ions of m/z ratio 903.3 and 584.2, respectively, and did not show a chlorine isotope pattern. CG-7 most likely represents a secondary metabolite of CG-6, resulting from addition of an additional glutathionyl group. CG-8 most likely results from chlorine substitution of the corresponding nitrenium ion of desmethylclozapine (C-2), the major metabolite of CLZ (Table 1).

**Effect of human GST A1-1**

Figure 3B shows the effect of 8 µM hGST A1-1 on GSH conjugation of reactive metabolites of CLZ. Similar to GST P1-1, a significant amount of CG-6 was produced although to a 5-fold lower extent. Only minor amounts of CG-5 were produced by hGST A1-1. In contrast to hGST P1-1, addition of 8 µM hGST A1-1 resulted in a 25% increase of CG-1, the major GSH conjugate in absence of hGST. By assuming equivalent extinction coefficients for each GSH conjugate, the total amount of GSH conjugates was increased by 52% by the addition of hGST A1-1.
Effect of human GST M1-1
As shown in Figure 3C, addition of 8 µM hGST M1-1 to CYP102A1M11H incubations resulted in a more than 2-fold increase in the amount of CG-1. Furthermore, hGST M1-1 catalyzed the formation of CG-6 at amounts comparable to that formed by GST A1-1. Interestingly, GST M1-1 also produced significant amounts of GSH adduct CG-4, which was also found as a minor metabolite in incubations in presence of 5 mM GSH (Table 1), and in trace amounts in incubations with hGST P1-1 (Table 3). LC-MS-analysis of this GSH conjugate also showed a protonated molecular ion [M + H]⁺ of m/z 632.2 [CLZ-SG + H]⁺, and a fragmentation pattern highly similar to that of the other GSH conjugates with the same molecular weight: m/z 614.2 ([CLZ-SG – H₂O + H]⁺); m/z 575.2 ([CLZ-SG – C₃H₇N(piperazine ring) + H]⁺); m/z 503.2 ([CLZ-SG – glutamic acid + H]⁺); m/z 446.1 ([CLZ-SG – glutamic acid – glycine – C₃H₇NO(GSH-moiety) + H]⁺) and 302.1 ([CLZ-SG – glutamic acid – glycine – C₃H₇NO(GSH-moiety) – C₃H₇N (piperazine ring) + H]⁺). CG-4 might also correspond to an adduct to the C-7 position, or a GSH conjugate to the non-chlorinated ring, as suggested previously (8). Because of its ability to catalyze formation of CG-1, CG-4 and CG-6, the total amount of GSH conjugate was increased 2.7-fold, assuming extinction coefficients of GSH conjugates are comparable.

Effect of human GST T1-1
Addition of 8 µM GST T1-1 did not show any effect on the formation of CLZ-GSH conjugates, both in the presence of 100 µM or 5 mM GSH (data not shown). The role of this enzyme was therefore not further evaluated.
Table 3: Effect of recombinant human glutathione-S-transferases (hGST) on the formation of GSH conjugates of reactive metabolites formed by oxidative bioactivation of clozapine

Clozapine (0.5 mM) was incubated for 30 min with different bioactivation systems in presence of 100 µM of glutathione, and in absence or presence of different recombinant hGSTs (at 8 µM). Relative quantification was performed by integrating peaks of LC-UV-chromatograms and assuming that the extinction coefficients of the different GSH conjugates at 254 nm are identical.  

\[ \text{Relative amount of GSH conjugate} = \left( \frac{\text{Peak area of individual GSH conjugate}}{\text{Sum of peak areas of all GSH conjugates}} \right) \times 100\% \]

\[ \text{Relative amount of total GSH conjugates} = \left( \frac{\text{Sum of peak areas of all GSH conjugates}}{\text{Sum of peak areas of GSH conjugates in absence of recombinant human GST}} \right) \times 100\% \]

Assigned structures, based on identity of mass spectra and order of elution (8): CG-1, C-6 glutathionyl clozapine; CG-3, C-9 glutathionyl clozapine; CG-4, C-7 glutathionyl clozapine; CG-5, C(1-4)-glutathionyl clozapine; CG-6, C-8 glutathionyl deschloroclozapine.  

\[^d\text{ND, not detectable because it is below the level of detection (0.2 µM).}\]
**Effects of hGST concentration on GSH conjugation of CLZ metabolites**

Three of the GSH conjugates, CG-4, CG-5, and CG-6, were only found in incubations of CLZ with CYP102A1M11H when hGSTs were added to the incubation (Table 3). To study the effect of protein concentration of hGST on the formation of these GSH conjugates, incubations were performed at hGST concentrations ranging from 0.25 to 8 µM. Figure 4A shows the effects of different concentrations of hGSTs on the formation of GSH conjugate CG-6. For hGST P1-1, hGST A1-1, and hGST M1-1, the formation of CG-6 was linear with the concentration of hGST. GST P1-1 showed the highest activity, being approximately five times more active than the other two isoenzymes. GST M1-1 and GST A1-1 showed similar activity.

The formation of GSH adduct CG-5 was only catalyzed by GST P1-1 and, at 40-fold lower activity, by GST A1-1 (Table 3). When varying the concentration of hGST P1-1, the amount of CG-5 was proportional to the hGST P1-1 concentration (Figure 4B). In case of hGST A1-1, CG-5 formation was proportional to GST concentration at concentrations higher than 2 µM. Because of its lower activity compared to GST P1-1, at concentrations lower than 2 µM GST A1-1, this GSH conjugate was below the level of detection.

The formation of GSH conjugate CG-4, which was only produced to a significant extent by GST M1-1, also increased with GST M1-1 concentration, although not linearly (Figure 4C). At the lowest GST concentration, the amount of CG-4 was below the level of detection.

**Role of hGSTs in inactivation of CLZ metabolites formed by human and rat liver microsomes**

Table 3 shows the effect of hGSTs on the GSH conjugation of reactive CLZ metabolites formed by human and rat liver microsomes. In both human and rat liver microsomal incubations, hGST P1-1 was the most active enzyme in catalyzing GSH conjugation, increasing the total amount of GSH conjugates 3.7-fold and 2.4-fold, respectively, when compared to the corresponding incubations in absence of hGST. By adding hGST P1-1, CG-6 and CG-5 again appeared to be the major GSH conjugates, consistent with the observations with CYP102A1M11H incubations. The addition of hGST A1-1 or hGST M1-1 to the microsomal incubations increased the total GSH conjugation to approximately the same extent, from 34 to 53%, with slightly higher activity for GST M1-1. In case of human liver microsomes incubations, a significant amount of CG-4 was already found in absence of
recombinant hGSTs, consistent with previous studies (8). This GSH conjugate, which was originally assigned to C-7 of the chlorobenzoid ring, was previously proposed to be a product of microsomal GSTs (8).

Figure 4: Effect of different GST concentrations on the formation of GSH adduct forms in the incubation of clozapine with CYP102A1M11H. Panel A: Influence of the concentration of hGST P1-1, A1-1 and M1-1 on the formation of CG-6. Panel B: Influence of the concentration of hGST P1-1 (scale: left axis) and A1-1 (scale: right axis) on formation of CG-5. Panel C: Influence of hGST M1-1 concentration on the formation of CG-4.
DISCUSSION

It is generally accepted that the ADRs caused by CLZ are the result of the bioactivation to reactive metabolites in the different target tissues. The mild hepatotoxicity observed in 37% of patients treated with CLZ might be the result from local bioactivation by hepatic P450s, whereas the idiosyncratic agranulocytosis might result from myeloperoxidase-mediated bioactivation in neutrophils and their bone marrow precursors. Based on the observation that in presence of GSH the same GSH conjugates are produced in both P450 and peroxidase-mediated reactions, a common reactive intermediate is implicated in both types of ADRs (8). As reactive intermediate, a nitrenium ion was proposed, formed by a two-electron oxidation pathway. In microsomal incubations, the nitrenium ion can be trapped chemically by GSH leading to several GSH conjugates. The principle GSH conjugate results from GSH addition to the C-6-position while a minor conjugate was assigned to GSH addition to the C-9 position. Furthermore, a third conjugate with identical mass was tentatively assigned to a conjugate to the C-7 position (8). Furthermore, two GSH conjugates were only identified in vivo in bile of treated rats and mice, and were considered to involve an unknown reactive intermediate.

Although GSH conjugation of reactive CLZ metabolites has been observed in many in vitro studies, the role of the cytosolic GSTs has not yet been investigated. Because several cytosolic GSTs are known to be polymorphic, genetically determined deficiency in GSTs might be considered as a risk factor for drug-induced idiosyncratic drug reactions if these enzymes play an important role in GSH conjugation of reactive intermediates. The results of the present investigation, as summarized in Table 3, clearly show for the first time that hGSTs have a significant activity in catalyzing GSH conjugation of reactive CLZ metabolites formed by CYPs. The most active human GST in all experiments appeared to be hGST P1-1, which, at 8 µM, resulted in a 3.7-fold increase in the total amount of GSH conjugates formed in incubations with CYP102A1M11H and human liver microsomes. This increase is only slightly lower than the 4.8-fold increase observed by raising the GSH concentration 50-fold, from 100 µM to 5 mM (Table 1). Next to hGST P1-1, hGST M1-1, and hGST A1-1 were able to catalyze GSH conjugation (Table 3). Although their activity appeared to be lower than that of GST P1-1 when measured at equal concentration, the role of GST A1-1 and GST M1-1 in vivo might be more important due to their 13- and 8-fold higher protein concentration in the liver (Table 4).
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(36, 37) and the fact that the enzymatic GSH conjugation is proportional to the concentration of GST (Figure 4).

Table 4: Estimated concentration of individual GST isoenzymes in human liver (adapted from ref 36)

<table>
<thead>
<tr>
<th>Hepatic GST concentration (µg/mg cytosolic protein)</th>
<th>(µg/gr liver)^a</th>
<th>µM^b</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGST A1-1</td>
<td>20.3</td>
<td>1810</td>
<td>~ 72</td>
</tr>
<tr>
<td>hGST A2-2</td>
<td>10.7</td>
<td>952</td>
<td>~ 38</td>
</tr>
<tr>
<td>hGST M1-1</td>
<td>12.1^c</td>
<td>1080</td>
<td>~ 43</td>
</tr>
<tr>
<td>hGST P1-1</td>
<td>1.5</td>
<td>133</td>
<td>~ 5.3</td>
</tr>
<tr>
<td>hGST T1-1</td>
<td>0.3</td>
<td>26.7</td>
<td>~ 1.1</td>
</tr>
</tbody>
</table>

Two of the active GSTs, hGST P1-1 and hGST M1-1, are known to be genetically polymorphic. Individuals lacking the gene of hGST M1-1 therefore theoretically might have an increased risk for CLZ-induced hepatotoxicity. For hGST P1-1, four alleles have been described resulting from the polymorphic substitutions I105V and A113V (40, 41). However, it remains to be established whether these polymorphic substitutions affect the ability of hGST P1-1 to inactivate the reactive nitrenium ion of CLZ. Because hGST T1-1 did not show any activity in catalyzing GSH conjugation, deficiency of this GST is not likely to be a risk factor for CLZ. The clinical relevance of these GST polymorphisms to CLZ-induced ADRs, however, still remains to be confirmed in case control studies.

Next to an increase in the total amount of GSH conjugation of reactive CLZ metabolites, addition of the hGSTs also resulted in striking changes in regioselectivity of GSH conjugation (Table 3). Consistent with previous studies, in absence of GSTs in all bioactivation systems used, the major GSH conjugate formed was the adduct at the C-6-position of CLZ (CG-1), whereas the adduct at the C-9 position (CG-3) was a minor adduct. In the presence of hGST
P1-1, however, the major GSH conjugate observed appeared to be C-8 glutathionyl deschloroclozapine (CG-6), representing approximately 50-60% of the total of GSH conjugates. The formation of this GSH conjugate can be rationalized by a substitution reaction of the chlorine of the nitrenium ion of CLZ, followed by a reduction reaction, presumably by GSH and/or NADPH (Figure 5). In the absence of NADPH or P450 enzyme fraction this GSH conjugate was not found, excluding a direct chlorine substitution reaction on CLZ itself. Next to hGST P1-1, also hGST A1-1 and hGST M1-1 were able to catalyze this substitution reaction, at apparently 5-fold lower activity (Figure 3). From the fact that CG-6 was observed only in bile of CLZ-treated animals, but not in in vitro microsomal incubations, it was previously concluded that this GSH conjugate was derived from a distinct pathway of bioactivation in vivo (8). The present study, however, clearly demonstrates that CG-6 most likely originates from enzymatic GSH conjugation of the reactive CLZ nitrenium ion. Although the present in vitro incubations were performed at low GSH concentration to minimize non-enzymatic GSH conjugation, the fact that CG-6 and CG-4 were found in bile of rats and mice in amounts comparable to that of CG-1 (8), shows that GSTs contribute significantly to GSH conjugation in vivo. Furthermore, C-8 methylthio deschloroclozapine and C-7 methylthio clozapine are the only thioethers so far identified in urine of man treated with CLZ (9, 10), and are most likely resulting from catabolism of CG-6 and CG-4, respectively. The fact that formation of both CG-6 and CG-4 is completely dependent on presence of GSTs, suggests that GSTs play an important role in GSH conjugation of reactive CLZ metabolites in man (9). However, the excretion of products derived from GSH conjugates in urine of CLZ-treated patients still remains to be further characterized. The highly sensitive LC-MS/MS methodology recently developed for analysis of N-acetyl-L-cysteine conjugates of CLZ and other drugs might be useful in human studies when high analytical sensitivity and selectivity is required (39). In the latter study, human urine was spiked with N-acetyl-L-cysteine conjugates of CLZ generated by incubating CLZ with rat liver microsomes in presence of N-acetyl-L-cysteine. Unfortunately, no urine samples of CLZ-treated patients were analyzed, which would have shown the actual urinary profile of N-acetyl-L-cysteine conjugates of CLZ. Consistent with the present study, no N-acetyl-L-cysteine conjugate was found resulting from chloro substitution of the CLZ nitrenium ion (39), confirming the dependence of conjugating enzymes for this specific regioisomeric conjugate.
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**Figure 5**: Role of hGSTs in the regioselective GSH conjugation of the reactive nitrenium ion formed by cytochrome P450. Structures of CG-4 and CG-5 were tentatively assigned in reference (8).

The results summarized in Table 3 show that four GSH conjugates are formed with m/z 632, consistent with GSH conjugates 3a-d described previously (8). GSH conjugate CG-4 was found in incubations of CLZ with human liver microsomes in absence of cytosolic GSTs, and therefore most likely corresponds to GSH conjugate 3b which was found by Maggs et al. in incubations of CLZ with human and rat liver microsomes (8). This GSH conjugate was tentatively identified by these authors as the thioether substituted ortho to the chlorine, i.e. C-7 glutathionyl clozapine, and was suggested to be a product of enzymatic GSH conjugation by the microsomal GSTs (8). As shown in Table 3 and Figure 4C, hGST M1-1 is also contributing to the formation of CG-4. As mentioned above, a methylthio-adduct at the C-7 position of CLZ has been identified in urine of man (9, 10), supporting the relevance of this metabolic pathway for man.
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The fact that CG-1, CG-3 and CG-4 all represent GSH conjugates in which GSH is bound to the chlorinated ring, implicates that the fourth GSH conjugate with m/z 632, CG-5, results from GSH conjugation to the non-chlorinated ring. This GSH conjugate therefore most likely corresponds to conjugate 3d of Maggs et al., which was only found in vivo in the bile of CLZ-treated rats and mice (8). In the present study, CG-5 was found at significant amounts (18 to 32% of total of GSH conjugates) when CLZ incubations were performed in the presence of hGST P1-1 and at much lower amounts in presence of hGST A1-1 (Table 3). GSH conjugation to the non-chlorinated ring of CLZ might be explained by reaction to the CLZ nitrenium ion, involving delocalization of the positive charge to the non-chlorinated ring, or by a distinct P450-dependent reactive intermediate such as an arene oxide. The fact that aromatic hydroxylation to the non-chlorinated ring was not observed in the present in vitro incubations and has never been found in previous in vitro and in vivo studies (10, 11) seems to rule out involvement of arene oxides and suggests that all GSH conjugates originate from a common reactive nitrenium ion, as illustrated in Figure 5. Therefore, the different regioselectivity in GSH conjugation observed is most likely a reflection of the different binding orientation of the nitrenium ion in the active sites of these GSTs.

In summary, the results of the present study indicate that at least three hGSTs are able to catalyze the GSH conjugation of the reactive CLZ nitrenium ion resulting in different regioisomeric GSH conjugates (Figure 5). Genetically determined deficiency of hGST or drug-drug interaction at the level of hGST, therefore, might be risk factors for adverse side effects associated with CLZ treatment. Case control studies correlating GST genotypes with susceptibility to CLZ side effects, however, remain to be performed. The fact that several GSH conjugates are formed only in presence of hGST implicates that analysis of corresponding thioethers (N-acetyl-L-cysteine- and/or thiomethyl conjugates) in urine of CLZ-treated patients might support these case control studies.
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