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Application of CYP102A1M11H as tool for the generation of protein adducts of reactive drug metabolites

Adapted from: Application of CYP102A1M11H as tool for the generation of protein adducts of reactive drug metabolites
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ABSTRACT

Covalent binding of reactive metabolites (RMs) to proteins is considered to be one of the important mechanisms by which drugs can cause tissue damage. To facilitate the study of drug-protein adducts, we developed a potentially generic method for producing covalently modified proteins. A highly active drug metabolizing P450 BM3 mutant (CYP102A1M11H) is used for drug bioactivation. Because of its His-tag, CYP102A1M11H is easily removed by nickel affinity chromatography, facilitating subsequent characterization of the modified target protein. The applicability of our procedure is demonstrated by trapping of RMs of acetaminophen (APAP), clozapine (CLZ) and troglitazone (TGZ) with human glutathione-S-transferase P1-1 (hGST P1-1) as model target protein. Tryptic digests of hGST P1-1 were subjected to analysis by LC-MS/MS and modified peptides identified by comparative analysis of tryptic peptides of adducted and non-adducted hGST P1-1. Characteristic MS/MS ions of drug-modified peptides were identified by first searching for expected adduct-masses. Unanticipated drug-peptide adducts were subsequently identified in an unbiased manner by screening for diagnostic MS/MS ions of modified peptides. Reactive intermediates of APAP and CLZ adducted to cysteine-47 and mass shifts corresponded to alkylation of N-acetyl-p-benzoquinone imine (NAPQI) and the CLZ nitrenium ion, respectively. Adduction of TGZ appeared more complex, yielding three different types of adducts to cysteine-47, two adducts to cysteine-14 and a single adduct to cysteine-101. Together, these findings show that P450 BM3 mutants with high capacity to activate drugs into relevant RMs can be employed to produce protein adducts to study nucleophilic selectivity of highly reactive electrophiles.

INTRODUCTION

Adverse drug reactions pose a risk to patient health and are still a main problem in the development of drugs. These reactions are poorly understood and are often only identified after marketing of the drug. Reactive metabolites (RMs) are presumably involved in the etiology of many adverse drug reactions by covalent modification of proteins (1, 2). Currently, the standard method to detect and identify RMs is by trapping them with low molecular weight trapping agents, such as GSH or GSH-analogues, and subsequent LC-MS-analysis (3, 4). However, even non-toxic drugs can sometimes produce significant amounts of GSH-conjugates in in vitro studies (3), indicating that formation of GSH conjugates per se is not
predictive for toxicity. One hypothesis might be that toxic RMs might have higher selectivity to critical target proteins than non-toxic RMs (2). Therefore, it is important to develop analytical methods to identify critical target proteins and to characterize the protein damage caused by RMs. At the protein level, RMs can have selectivity for specific amino acids. Most toxic RMs have shown to react with cysteine which is the most intrinsically nucleophilic amino acid. Cysteine residues in proteins can have strongly different reactivities at physiological pH due to differences in microenvironment. Recently, it is shown that the most reactive cysteine residues are often important for protein functioning (5). Consistent with this, three of the 27 cysteines of human keap1, cys-151, cys-273 and cys-288, appeared to be particularly reactive to electrophiles and have critical regulatory roles (2). Therefore, characterization of the selectivity of RMs at the amino acid level might be important to distinguish critical from non-critical protein damage.

To characterize covalent protein modification, the production of the relevant RMs is a key step. In some cases, the chemical synthesis of RMs is feasible, examples of which are the formation of \( N \)-acetyl-\( p \)-benzoquinone imine (NAPQI) from acetaminophen (APAP) (6), the reactive nitrenium ion of clozapine (CLZ) (7) and the quinone imine metabolites of diclofenac (8). However, in case these reactive species are short-lived, they must be generated in situ. Recently, production of adducts of several reactive drug metabolites has been achieved by electrochemistry (9). Drug activation by electrochemistry has the ability to generate large amounts of RMs in the absence of biological matrices. However, it cannot mimic all reactions performed by P450 enzymes because it lacks the steric control that is determined by the active site of the P450 (9, 10). Therefore, for many drugs bioactivation to reactive intermediates is dependent on human liver microsomes or recombinant human P450s. Drawbacks of these systems are their relatively low bioactivation activity and the fact that these enzyme systems consist of complex mixtures of membrane-bound proteins.

Cytochrome P450 BM3 (CYP102A1), from \textit{Bacillus Megaterium}, is of particular interest as a biocatalyst for the formation of RMs since this enzyme has the highest catalytic activity ever recorded for a P450 mono-oxygenase (11). CYP102A1 is soluble, easily expressed in \textit{E. coli}, and catalytically self-sufficient due to a fusion between its reductase and catalytic domain (12). Despite its natural preference for long-chain fatty acids, by site-directed and random
mutagenesis, our lab (13-15) and others (16-18) have developed mutants of CYP102A1 with the ability to convert a variety of drugs into human relevant drug metabolites. The applicability of a number of these CYP102A1 mutants for the production of human relevant RMs of APAP, CLZ and diclofenac was subsequently described (19). Levels of GSH conjugates produced by CYP102A1M11H were up to 70-fold higher than with human liver microsomes. Hence, in a study in which human glutathione-S-transferase P1-1 (hGST P1-1) was used as a model target protein for RMs, covalent protein adducts of APAP were formed using CYP102A1M11H (20). Because hGST P1-1 is relatively small (23.5 kDa), which facilitates analysis by MS, and well characterized in terms of its structure (21), it is a useful model target protein. In addition, hGST P1-1 contains four cysteines (cys-14, cys-47, cys-101 and cys-169) with different accessibilities and reactivities (20, 22-24). Cys-47 is of particular interest since this residue is located near the GSH-binding site of the protein (21, 25) and has an unusually low pK_{a} value (4.2) (26). Hence, covalent modification of cys-47 has been observed for numerous reactive chemicals (22, 23, 27-29). The effect of covalent modification of cys-47 on hGST P1-1 activity can easily be assessed since both colorimetric (30) and fluorescence (31) activity assays have been described. From previous work with direct alkylating agents (20, 22, 32) and cysteine mutants of hGST P1-1 (24, 32), it appears that the presence of bulky adducts on cys-47 mainly contributes to the loss of activity of the protein following covalent modification.

The aim of this work is to develop a potentially generic method for the production and identification of protein adducts using his-tagged CYP102A1 mutants with high catalytic activity. Purified CYP102A1M11H was used as bioactivation system for APAP, CLZ and troglitazone (TGZ), whereas hGST P1-1 was used as model target protein. After separation of the His-tagged P450 from the modified target protein by affinity chromatography, protein adducts were characterized by LC-MS/MS analysis of peptides resulting from tryptic digestion of the target protein hGST P1-1. Because APAP and CLZ are known to be bioactivated by CYP102A1M11H to human relevant reactive intermediates with known structures, Figure 1, initially samples were analyzed by searching for peptides with expected mass increments of 149.1 and 324.1 Da, respectively. Fragmentation of tryptic peptides typically show cleavage of peptide bonds, enabling partial sequencing of the peptide and identification of modified amino acids. Because some fragments consist of amino acids not susceptible to alkylation by electrophiles, these fragments might be useful for screening of adducts of, as yet, unknown
mass. Therefore, after identification of unmodified diagnostic fragments, this potentially
generic procedure was applied to hGST P1-1 adducts of TGZ. Since bioactivation of TGZ is
highly complex, Figure 1, evaluation of its protein adducts provides a useful means of
validating our adduct preparation and identification strategy.

Figure 1: Structures of reactive intermediates produced by P450-mediated bioactivation of
acetaminophen (A), clozapine (B), troglitazone and troglitazone quinone (C). Sites of
nucleophilic attack are indicated by arrows. Structures of reactive intermediates derived from
troglitazone and troglitazone quinone are proposed by Kassahun et al. (37) and Tettey et al.
(40); R₁, chromane moiety; R₂, thiazolidinedione moiety. NAPQI, N-acetyl-p-benzoquinone
imine.
MATERIALS AND METHODS

Materials
Sequencing grade modified trypsin was from Promega (Madison, USA). HIS-select Nickel Affinity Gel was from Sigma-Aldrich (Saint Louis, USA). Benzyloxyresorufin was synthesized as previously described (33). All other chemicals were from standard suppliers and from the highest quality available.

Enzyme expression and activity measurements
_E. coli_ XL-1 Blue cells containing an expression plasmid for hGST P1-1 were a kind gift from Prof. B. Mannervik (Department of Biochemistry and Organic chemistry, Uppsala University, Sweden). GST P1-1 was expressed and purified by GSH affinity chromatography as previously described (34). Protein concentrations were determined with the Bradford method (35) using reagent from Bio-Rad. The activity of hGST P1-1 was determined by measuring conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to GSH, as previously described (30). The specific activity of purified hGST P1-1 was 27.9 µmol/min/mg protein.

Purified CYP102A1M11H was expressed and purified by nickel affinity chromatography according to Damsten et al. (19). Specific activity of CYP102A1M11H was determined with the benzyloxyresorufin O-dealkylation (BROD) assay using benzyloxyresorufin as substrate. Measurements were conducted in cuvettes in a final volume of 1.15 mL, containing 26 nM CYP102A1M11H, 22 µM benzyloxyresorufin and 175 µM NADPH in 0.1 M potassium phosphate (KPi) buffer pH 7.4. The BROD activity was measured for 1 min at an excitation wavelength of 530 nm and an emission wavelength of 586 nm. The specific activity of purified CYP102A1M11H was 9.42 nmol benzyloxyresorufin/min/nmol CYP102A1M11H.

Time-dependent inactivation of human Glutathione-S-Transferase P1-1 by reactive drug metabolites
Incubations were performed in a total volume of 250 µL 0.1 M KPi buffer pH 7.4, containing 250 nM CYP102A1M11H, 2 µM hGST P1-1 and 1 mM of APAP or CLZ. For TGZ a substrate concentration of 125 µM was used due to strong inhibition of hGST P1-1 by TGZ at higher concentrations (data not shown). Following a pre-incubation period of 5 min at 24 °C, reactions were started by addition of 0.4 mM NADPH, 0.3 mM glucose-6-phosphate and 0.4
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U/mL glucose-6-phosphate dehydrogenase (final concentrations). Immediately after starting, and after 60 and 120 min, a 75 µL aliquot of this incubation was used to determine time-dependent inactivation of hGST P1-1.

Because absorbance of NADPH strongly interferes with the spectroscopical CDNB-conjugation assay, activity of hGST P1-1 was measured fluorimetrically using monochlorobimane (MCB) as substrate. Measurements were conducted in cuvettes in a final volume of 500 µL of 0.125 M KPi pH 6.5, supplemented with 100 µM MCB and 1 mM GSH. GST catalysed conjugation of MCB with GSH was followed for 1 min in a fluorimeter (Perkin-Elmer LS-3 fluorescence spectrometer) using an excitation wavelength of 390 nm and an emission wavelength of 465 nm. The spontaneous conjugation of GSH with MCB was determined in control reactions in which hGST P1-1 was omitted.

Control incubations were performed in absence of drug, CYP102A1M11H or NADPH to establish whether GST inactivation was due to P450-dependent drug metabolism. To assess whether scavengers of soft or hard electrophiles protected against loss of hGST P1-1 activity, incubations were also supplemented with 5 mM GSH or 5 mM potassium cyanide. The hard nucleophile cyanide was previously shown to trap the nitrenium-ion formed by bioactivation of the piperazine-ring of CLZ, Figure 1B (36). In this study, the reactive intermediate of acetaminophen, NAPQI, was not trapped by cyanide.

Separation of modified target protein (hGSTP1-1) from CYP102A1M11H by affinity chromatography

For LC-MS/MS analysis, incubations containing CYP102A1M11H and alkylated hGST P1-1 were initially digested with trypsin without further cleanup. However, as previously noticed (20), the presence of the large number of tryptic peptides of CYP102A1 M11H (119 kDa) interfered with the analysis of peptides from the target protein (data not shown). Hence, nickel affinity chromatography was used to remove CYP102A1M11H from incubations prior to trypsin digestion.

Incubations (final volume 3 mL) containing 2 µM hGST P1-1 and 250 nM CYP102A1M11H in 0.1 M KPi buffer at pH 7.4 were concentrated to approximately 250 µL using centrifugal
filter tubes (10,000 MWCO, Millipore). A small aliquot (10 µL) was taken in order to measure the initial hGST P1-1 and CYP102A1M11H activity (as described above) and the concentrated incubation was mixed with 50 µL slurry of HIS-select Nickel Affinity Gel, resulting in 25 µL of settled gel. Following an incubation period of 30 min in an incubation block at room temperature, the beads were removed by centrifugation. Control incubations were not treated with the affinity gel but incubated at room temperature for the same period. Aliquots (10 µL) of treated and non-treated incubations were taken and the activity of hGST P1-1 and CYP102A1M11H determined.

The complete removal of CYP102A1M11H from incubations containing hGST P1-1 was confirmed by SDS-PAGE analysis of samples subjected to the cleanup procedure. Aliquots (10 µL) were mixed with 6X loading buffer (0.35 M Tris-HCl at pH 6.8, 10% SDS, 30% (v/v) glycerol, 0.6 M DTT, 0.175 mM Bromophenol Blue) and reduced by heating for 5 min at 95°C. Proteins were run on a 10% Tris-HCl Ready Gel precast gel (Bio-Rad) in running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) at 90V for 2 hours. Gels were washed with 3 loads of nanopure water and proteins subsequently stained by incubation with Bio-Safe Coomassie Stain (Bio-Rad) for one hour. Destaining was conducted with nanopure water.

Bioactivation of drugs by CYP102A1M11H in presence of target protein or GSH
For LC-MS/MS analysis of drug-protein adducts, incubations (total volume 3 mL) were performed in 0.1 M KPi at pH 7.4, and contained 250 nM CYP102A1M11H, 2 µM hGST P1-1 and 1 mM APAP or CLZ. To characterize unmodified hGST P1-1, reactions were also performed in absence of drugs. Furthermore, incubations were performed in presence of 5 mM GSH to confirm formation of reactive intermediates by formation of GSH-conjugates, and to demonstrate protection of target protein from modification. After preincubation for 5 min at 24 °C, NADPH was added to a final concentration of 2 mM. Reactions were stopped after 2 h of incubation by cooling on ice and filtered through a Millipore Ultra-4 centrifugal 10,000 MWCO filter to remove unbound drug and metabolites.

In case of TGZ as drug, first it was investigated whether CYP102A1M11H produced the human relevant reactive intermediates by comparing formation of GSH conjugates with those formed by human liver microsomes. Incubations of TGZ (50 µM) were conducted in a total
volume of 1 mL 0.1 M KPi buffer at pH 7.4, containing 250 nM CYP102A1M11H or 1 mg/mL human liver microsomes and 5 mM GSH. Additional reactions were supplemented with 5 mM ascorbic acid, 500 U/mL superoxide dismutase, 500 U/mL catalase or a combination of catalase and superoxide dismutase. Following pre-incubation at 24 °C for 5 min, incubations were started by addition of 1 mM NADPH. Reactions were stopped after 1 h by addition of 1 mL ice cold acetonitrile and then centrifuged for 15 min at 4000g. Supernatant was dried under a steam of nitrogen, reconstituted in 0.2 mL 30% acetonitrile and then centrifuged for 15 min at 4000g. Supernatant was analyzed on a Luna C18(2) column (5 µm C18; 150 x 4.6 mm i.d.; Phenomenex) using a 20-100% acetonitrile/water gradient and a flow of 0.5 mL/min. GSH conjugates of TGZ were detected using UV detection at 254 nm and by LC-MS. Following confirmation that human relevant GSH conjugates of TGZ were formed by CYP102A1M11H, incubations of 125 µM TGZ with CYP102A1M11H and hGST P1-1 were prepared as described above.

Preparation of tryptic digests of alkylated human glutathione-S-transferase P1-1
Terminated incubations were concentrated to 250 µL by centrifugation (20 min, 2300g). To remove CYP102A1M11H, 50 µL HIS-select Nickel Affinity Gel was added to the concentrated incubations and allowed to incubate for 30 min at room temperature. Affinity beads containing the His-tagged CYP102A1 mutant were then pelleted by centrifugation for 1 min at 5000g. Supernatants were dried in a speed vacuum centrifuge operating at 35 °C. The dried samples were reconstituted in 200 µL of denaturation buffer (6 M guanidine HCL, 3 mM DTT, and 50 mM Tris-HCl pH 8) and subsequently incubated for 60 min at 60 °C. To alkylate free cysteines, iodoacetamide (IA) was added to the samples to a final concentration of 10 mM and reactions were incubated on a rollerbank for 30 min at room temperature. Salts and unreacted IA were then removed by gelfiltration using PD MiniTrap G-25 columns (GE healthcare, Buckinghamshire, UK) and desalted protein fractions were dried by speed vacuum centrifugation at 35 °C. For tryptic digestion, the desalted proteins were redissolved in 200 µL 50 mM ammonium bicarbonate and digested overnight at 37 °C with trypsin at a ratio of one equivalent of trypsin to 50 equivalents of hGST P1-1. Subsequently, samples were filtered using 0.2 µm regenerated cellulose filters (Phenomenex) and filtrate was stored at -80 °C until analysis by LC-MS/MS.
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**LC-MS/MS analysis of tryptic digests of hGST P1-1**

Analysis of protein digests was performed using an Agilent 1200 Series Rapid resolution LC equipped with a hybrid Quadrupole-Time-Of-Flight (Q-TOF) Agilent 6520 mass spectrometer (Agilent technologies, Waldbronn, Germany) and UV detection at 214 nm. Analytes were ionized by electrospray ionization. The Q-TOF was operated at a capillary voltage of 3500 V using nitrogen as drying gas (10 L/min) and nebulizer gas (pressure 50 psig). The gas temperature was held at 350°C during operation. Peptides selected for MS/MS analysis were fragmented at a collision energy voltage of 20 Volt using nitrogen as the collision gas.

After injection of 50 µL sample, tryptic peptides of hGST P1-1 were separated by reverse phase chromatography on a Vydac 218TP54 column (C18, 5 µm, 4.6 mm i.d. x 250 mm). Separations were conducted by a gradient composed of 98.9% water/1% acetonitrile/0.1% formic acid (solvent A) and 98.9% acetonitrile/1% water/0.1% formic acid (solvent B) using a flow rate of 400 µL/min. The following gradient was used: from 0 to 5 min 3% B, followed by a linear increase to 80% B at 60 min and subsequently to 100% at 65 min. A plateau at 100% B till 70 min was followed by a linear decrease to 3% B at 75 min and a re-equilibration period from 75 to 85 min at 3%B.

LC-MS/MS data acquisition was performed with the Mass Hunter Workstation software (version B.02.00). Data was acquired by operating the Q-TOF in positive mode using automated MS/MS analysis. Survey scans were recorded between m/z 200 and 3000, followed by fragmentation of the three most abundant ions with a charge of at least +2. Active exclusion of masses for 0.5 min after three MS/MS spectra was applied in order to acquire fragmentation spectra of low abundant peptides coeluting with abundant peptides. The LC-MS/MS data was analyzed using the Mass Hunter Qualitative Analysis software package (version B.01.03). Peak lists were generated using Mascot Distiller (version 2.3.2.0; Matrix Science) and peptide sequences were subsequently identified by database searching using the Mascot search program.
RESULTS

Cleanup of CYP102A1M11H from incubations containing target protein
To facilitate the mass spectrometric analysis of covalent modifications to hGST P1-1, first CYP102A1M11H was removed from the target protein using nickel affinity beads. Figure 2A shows the activity of CYP102A1M11H and hGST P1-1 after 30 min of incubation with affinity beads as described in the experimental procedures. In absence of nickel affinity beads, the activity of both proteins was unaffected by the incubation period. However, activity of CYP102A1M11H was completely abolished following treatment with nickel affinity beads, indicating complete removal of the bioactivation system. The activity of hGST P1-1 was only decreased to a relatively small extent (20%) by the cleanup procedure due to aspecific binding. To confirm that CYP102A1M11H was completely extracted by treatment with the affinity beads, incubations of hGST P1-1 and CYP102A1M11H subjected to the cleanup procedure were analyzed by SDS-PAGE. Following treatment with nickel affinity beads no protein band is detectable at 119 kDa, confirming the successful extraction of CYP102A1M11H from the incubation (Figure 2B).

Figure 2: Removal of CYP102A1M11H from incubations containing hGSTP1-1 and CYP102A1M11H. Samples containing hGST P1-1 and CYP102A1M11H were incubated in presence (cleanup) or absence (no cleanup) of Ni-NTA for 30 min. The cleanup was evaluated by measuring the activity of hGSTP1-1 and CYP102A1M11H (A) and by SDS-PAGE (B). Results in panel A are averages (±S.D.) from three independent experiments.
LC-MS/MS analysis of hGST P1-1 peptides and potential diagnostic MS/MS ions

First, tryptic digests of hGST P1-1 from incubations in absence of drug were analyzed in order to characterize tryptic peptides derived from hGST P1-1 after reduction and modification of cysteine residues by iodoacetamide (IA). A total of 13 tryptic peptides of hGST P1-1 were identified by Mascot analysis. Measured m/z values corresponded to singly, doubly or triply charged ions of the predicted tryptic peptides (Supporting information, Table S1). Collectively, these peptides cover 74% of the protein sequence. Although hGST P1-1 contains four cysteine residues, at position 14, 47, 101 and 169, only peptides containing cys-47 (45 ASCLYGQLPK54) and cys-14 (14 CAALR18) were identified. Peptide 45-54 modified by IA appeared as doubly charged ion with m/z 568.79. Collision-induced fragmentation of this ion showed fragments with masses corresponding to fragmentation y2, y3, y5, y6, y7 (Figure 3A). The fragments 978.52 and 301.10 corresponded to the y8 and b3 fragments containing the alkylated cysteine residue (Figure 3A). Peptide 14-18 modified by IA also appeared as doubly charged ion with m/z 295.66. Collision-induced fragmentation of ion 295.66 showed fragments y1, y2, y3 and y4 (Figure 3B). Peptides containing cys-101 and cys-169 were not found in tryptic digests when subjected to Mascot analysis or by manual inspection. Cys-101 is expected to appear as dipeptide 101 CK102 after tryptic digestion, which may have insufficient retention in reverse phase chromatography. The absence of cys-169 is probably due to the large size of its expected tryptic fragment (1+, m/z 4649.44), consisting of 42 amino acids between residue 141 and 182. Although other proteases might be more suitable for producing peptides of lower size, these were not evaluated in the present study because it has been shown previously that cys-169 is resistant to alkylation by electrophiles (27).
Figure 3: LC-MS/MS spectra of peptides obtained after tryptic digestion of iodoacetamide (IA)-alkylated glutathione S-transferase P1-1. Panel A: IA-modified peptide 45-54. Panel B: IA-modified peptide 14-18. Peaks indicated by diamonds correspond to m/z of the parent ion.

When comparing UV-Vis and LC-MS/MS chromatograms of the tryptic digest of hGST P1-1 obtained from incubations with drugs and CYP102A1M11H with the tryptic digest of unmodified hGST P1-1, several modified peptides were identified. In case of APAP, a modified peptide was found with retention time 31.6 min. The m/z value of this peptide was 614.81, which corresponds to a doubly charged peptide 45-54 with a mass increase of 149.1 Da. This mass increase is expected from covalent binding of NAPQI, the reactive intermediate of APAP (Figure 1). Figure 4A shows the mass spectrum obtained after collision-induced fragmentation of the doubly charged ion m/z 614.81. MS/MS analysis shows that the site of adduction is localized on cys-47 as demonstrated by the +149.1 Da shifts of the y8 (m/z 1070.54) and b3 (m/z 411.14) fragments. In presence of 5 mM GSH, the intensity of the APAP-modified peptide was strongly reduced, whereas a GSH conjugate was found with mass 456.1 (data not shown).
Figure 4: LC-MS/MS spectra of peptide 45-54 obtained after tryptic digestion of glutathione-S-transferase P1-1 modified by reactive intermediates produced in incubations of CYP102A1M1H with (A) acetaminophen (APAP); (B) clozapine (CLZ); (C) troglitazone (TGZ). Peaks indicated by diamonds correspond to m/z of the parent ion.
When analyzing the tryptic digest of hGST P1-1 obtained from incubations in presence of CLZ and CYP102A1M11H, a modified peptide was found with a retention time of 36.9 min. In LC-MS this modified peptide appeared as m/z 702.34 and m/z 468.57, which can be rationalized as the doubly and triply charged peptide 45-54, respectively, with a mass increase of 324.1 Da. This mass increase is expected from covalent binding of the CLZ nitrenium ion formed by CYP102A1M11H (Figure 1). Collision-induced fragmentation of m/z 468.57 showed that the y-ion series was unmodified up to y6 (Figure 4B). Although y7 was not detected, masses of y8$^{+2}$ and y9$^{+2}$ were found to be increased by 324.1 Da, restricting the site of the modification to $^{47}$CL$^{48}$. Unambiguous evidence for alkylation of cys-47 was obtained by the mass shift of +324.1 Da of fragment b3, whereas b2 was not modified.

By comparison with human liver microsomes, we have previously shown that relevant GSH conjugates are formed in reactions of APAP and CLZ with CYP102A1M11H and GSH (19, 34). Hence, prior to evaluation of TGZ adducts to the target protein, incubations of CYP102A1M11H and human liver microsomes were performed in presence of 5 mM GSH. The most abundant GSH conjugate in these reactions has a m/z value of 747. A GSH conjugate with this mass was previously found in reactions with human and rat liver microsomes (37, 38), human hepatocytes (39), as well as in the bile of rats dosed with TGZ (37, 40). Minor human or rat relevant GSH conjugates formed by TGZ bioactivation with CYP102A1M11H consisted of m/z 733, 737, 751, 763, 765, 781 and 797 (data not shown), all of which were previously identified (37, 40-42).

When analyzing tryptic digests of hGST P1-1 isolated from incubations in presence of TGZ, an adduct was observed eluting at 49.1 min by visual inspection of UV-trace and total ion chromatograms. This peptide showed m/z values of 759.87 and 1518.70, which corresponds to doubly and singly charged ions, respectively, of peptide 45-54 with a mass increment of 439.1 Da. This mass shift is consistent with that observed for the major GSH conjugate of TGZ (m/z 747). Collision-induced fragmentation of m/z 759.87 resulted in the mass spectrum shown in Figure 4C. Major fragments were m/z 1079.56 and 540.29 corresponding to singly and doubly charged peptide ASCLYGQLPK, respectively, and m/z 440.16 which represents the TGZ-moiety which is cleaved off from the cysteine-residue. The lower abundant ions in the MS/MS spectrum correspond to the unmodified fragments y2, y3, y5, y6 and y7 of the peptide 45-54.
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backbone. Based on the presence of the intact y-ions series up to y7, and of b2, the site of adduction is also located at cys-47.

The mass increase of 439.1 Da might result from modification of the cys-47 by TGZ quinone methide or from attachment of cys-47 to the thiazolidinedione moiety of TGZ, as was previously observed with GSH as nucleophile (37, 38, 40). The present LC-MS/MS analysis is not able to distinguish between these two possibilities, however.

To study whether cysteine-containing peptide 14-18 (CAALR) and peptide 101-102 (CK) was also alkylated by the reactive TGZ-metabolite, it was investigated whether corresponding adducts, with mass increment of 439.1, were formed in TGZ-incubations. A TGZ-dependent peak eluting at 51 min showed a m/z of 486.73 which corresponds to a doubly charged adduct to peptide 14-18 (predicted mass (CAALR + 439.1)/2 = 486.72). Collision-induced fragmentation showed fragments with m/z 533.29 and 440.16, which correspond to the cleavage of CAALR and the bound TGZ-moiety, respectively (data not shown).

Although peptide 101-102 was not found after modification by IA, a modified peptide 101-102 was found in tryptic digest of TGZ-modified hGST P1-1. By direct search of peptide 101-102 with mass increment of 439.1 Da, a peptide with the predicted mass 689.29 (singly charged) was found. Collision-induced fragmentation resulted in ions representing the peptide and bound TGZ-moiety, as was observed in case of peptide 14-18 and peptide 45-54.
Application of diagnostic ions of peptide 45-54 for the identification of drug adducts to hGST P1-1

When comparing the mass spectra obtained by collision-induced fragmentation, we found that all adducts to peptide 45-54 have three relatively abundant fragments in common: m/z 244.17 (y2), 542.33 (y5), and 705.39 (y6). Because these fragments do not contain the cysteine residue, screening for peptides containing all three diagnostic fragments therefore might facilitate identification of cys-47-adducts of unknown mass.

In case of both APAP and CLZ, analysis of the tryptic digests of alkylated hGST P1-1 by inspection of reconstructed ion chromatograms of the diagnostic ions m/z 244.17, 542.33 and 705.39 only a single peptide contained all three fragments. These peptides corresponded to peptide 45-54 adducted by NAPQI and the CLZ nitrenium ion, respectively. The intensity of these modified peptides was strongly decreased when bioactivation of the drug was performed in presence of 5 mM GSH (data not shown).

Interestingly, when analyzing tryptic digests of hGST P1-1 modified by reactive TGZ-metabolites by the diagnostic ions 244.17, 542.33 and 705.39, several additional adducts to peptide 45-54 were identified, next to the adduct eluting at 49.1 min (Figure 5). Incubation in presence of excess GSH prevented the formation of these peptide adducts.

![Figure 5: Ion traces of diagnostic MS/MS ions of peptide 45-54 of hGST P1-1 after exposure to RMs of TGZ. Drug adducts already identified by a directed search for anticipated modifications are labeled with an arrow, while adducts identified by screening diagnostic ions are labeled with an asterisk (*).](image-url)
Adducts of TGZ to hGST P1-1 with a mass shift (+455.1 Da) that corresponds to opening of the thiazolidinedione ring of TGZ (37) were observed at 44.6 and 45.8 min in the chromatogram of diagnostic MS/MS ions of peptide 45-54 (Figure 5). Both peaks contain ions of the doubly charged (2+, m/z 767.86) and singly charged modified peptide (1+, m/z 1534.70). Inspection of the Extracted Ion Chromatograms (EICs) of these ions (Figure 6A) revealed a further adduct with the same mass shift at 47.4 min. Fragmentation spectra of the adducts at 44.6 and 45.8 min were identical, hence Figure 6B depicts the spectrum (precursor 2+, m/z 767.86) of the most intense peak at 44.6 min. The y-ion sequence is unmodified up to y7. The absence of y8 (m/z 921.49) in combination with presence of y8 + 455.1 and of adducted y8-H2O+2 is consistent with alkylation of cys-47. Fragmentation of the doubly charged ion m/z 767.86 at 47.4 min resulted in protonated adduct (1+, m/z 440.16) and oxidized peptide 45-54 (1+, m/z 1095.57), suggesting adduction of the RM of TGZ with mass shift +439.1 Da to the sulfenic acid of peptide 45-54 (data not shown).

The peaks at 42.9, 44.3, 44.9 and 45.5 min in the chromatogram of the diagnostic MS/MS ions of peptide 45-54 correspond to TGZ adducts with a mass shift of + 471.2 Da (Figure 5). Selected ion chromatograms of the doubly (2+, m/z 775.86; Figure 6C) and triply charged ions (3+, m/z 517.59) of these TGZ adducts also demonstrate the presence of two additional peaks (43.2 and 43.6 min) of the same mass in samples exposed to RMs of TGZ but not in similar GSH-fortified controls. Interestingly, two different type of fragmentation spectra were observed for these TGZ adducts. The peaks at 42.9, 44.3, 44.9 and 45.5 min exhibited identical MS/MS spectra; hence, fragmentation of the most abundant peak at 44.9 min is shown in Figure 6D. Sequence analysis from the b-ion series was poor since only b2 was being detected. The y-ion series contained, among others, y6 and y7 as well as y8 + 471.2 Da. Together, these data locate the alkylation to cys-47. For peaks at 43.2 and 43.6 min (Figure 6C), little backbone fragmentation was observed. MS/MS analysis yielded intact peptide 45-54 (1+, m/z 1079.56) and adducted TGZ moiety (1+, m/z 472.2) (data not shown).
Figure 6: LC-MS/MS analysis of TGZ adducts (+455.1 Da and +471.2 Da) to peptide 45-54 of hGST P1-1. Panel A and C: ion traces of doubly charged peptide 45-54 modified by reactive TGZ metabolites leading to mass increment of 455.1 Da (2+, m/z 767.86; panel A) and 471.2 Da (2+, m/z 775.86; panel C). EICs of hGST P1-1 exposed to RM of TGZ in absence of GSH (i) and in reactions fortified with 5 mM GSH (ii) are shown. Drug adducts are labeled with an asterisk (*). Panel B and D: MS/MS spectra of peptide 45-54 with TGZ-dependent mass shift of +455.1 Da (2+, m/z 767.86; panel B) and +471.2 Da (2+, m/z 775.86; panel D). Peaks indicated by diamonds correspond to m/z of the parent ion.
The observed mass increments (439.1, 455.1 and 471.2 Da) to peptide 45-54 after TGZ adduction were different by the mass of one or two oxygens. For TGZ adducts with mass shifts of +455.1 and 471.2 Da, multiple peaks of adducted peptide were observed (Figure 6). This large number of TGZ adducts with identical mass has not been reported in the literature. Because CYP102A1M11H is not likely able to directly oxygenate TGZ protein adducts, we hypothesized that some of the formed adducts with mass increments of 455.1 and 471.2 Da might result from bioactivation of stable oxygenated TGZ-metabolites, such as troglitazone quinone (TGZQ, Figure 1C) or by the oxygenation of TGZ adducts by reactive oxygen species. Since products formed by uncoupling of CYP102A1M11H may be responsible for the formation of the unusually large number of adducts, the effect of antioxidants on GSH conjugation to TGZ was investigated. Figure 7 shows that in the presence of ascorbic acid, a lower number of GSH conjugates with mass 763 was detected, indicating that part of these TGZ-GSH conjugates result from oxidative modifications. The remaining GSH conjugates of this mass might result from consecutive oxidative modification of TGZ, as described by Kassahun et al. (37).

**Figure 7:** Effect of ascorbic acid presence on formation of GSH conjugates of TGZ. Ion traces of GSH conjugates of TGZ with m/z 763 (panel A) and m/z 747 (panel B). Shown are incubations conducted in absence (black) and presence (grey) of ascorbic acid.
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Application of diagnostic ions of peptide 14-18 for the identification of drug adducts to hGST P1-1

Fragmentation of the peptide 14-18 adduct of TGZ with a mass increase of 439.1 Da did not result in fragmentation of the peptide backbone (data not shown). However, the mass spectrum of IA-modified peptide 14-18 (Figure 3B) contained two relatively abundant peptide fragments of significant mass: m/z 359.24 (y3) and 430.28 (y4). Since none of the identified peptide 14-18 fragments contains cysteine, these fragments might be useful for the identification of unanticipated adducts to cys-14.

Screening for the diagnostic MS/MS ions of peptide 14-18 resulted in the identification of a TGZ adduct, with a mass shift of +455.1 Da, at 44.8 min (Figure 8A). A second adduct peak with lower abundance was observed in the ion traces of doubly charged (2+, m/z 494.73) and singly charged (1+, m/z 988.45) ions of the modified pentapeptide (Figure 8B). MS/MS analysis of the major adduct (2+, 494.73 at 44.8 min) demonstrated that y1 to y4 were unmodified, suggesting modification at cys-14 (Figure 8C).
Figure 8: Identification of TGZ-adducted peptide 14-18 with mass increment of 455.1 Da.
Panel A: merged ion traces of diagnostic MS/MS ions (m/z 359.24, m/z 430.28) of peptide 14-18. Panel B: EIC (2+, m/z 494.73) of peptide 14-18 with mass shift of +455.1 Da. For both panels, traces of hGSTP1-1 exposed to RMs of TGZ in absence (i) and presence (ii) of 5 mM GSH are shown. Drug adducts are labeled with an asterisk (*). Panel C: fragmentation of TGZ-modified peptide 14-18 (2+, m/z 494.73) at 44.8 min.

Inhibition of hGST P1-1 by reactive drug metabolites
Alkylation of cys-47 of hGST P1-1 by thiol-reactive chemicals is known to result in enzyme inactivation (20, 22, 32). Loss of enzyme activity might therefore reflect the degree of protein modification. The estimated extent of modification of peptide 14-18 and peptide 45-54 by RMs of APAP, CLZ and TGZ is shown in Table 1. In addition, the effect of RMs on hGST P1-1 activity was determined by measuring GSH conjugation of MCB into a fluorescent GSH conjugate. Complete inhibition of hGST P1-1 by RMs of APAP occurs within 120 min. No
significant inactivation was observed in controls lacking one of the reaction components (data not shown). Incubations fortified with 5 mM GSH (Table 1), but not with 5 mM KCN (data not shown), fully protected against enzyme inactivation, suggesting that thiol-reactive metabolites are responsible for the inactivation.

In contrast to incubations with APAP, hGST P1-1 inhibition was only 33% in incubations performed with CLZ in presence of CYP102A1M11H (Table 1). Addition of GSH completely protected against inactivation by reactive CLZ metabolites, whereas addition of KCN had no effect. This strongly suggests that inactivation is due to the thiol-reactive nitrenium ion of CLZ which is produced by CYP102A1M11H (19, 34). Bioactivation of the piperazine-ring of CLZ, which produce a cyanide-reactive RM (36) apparently does not contribute to hGST P1-1 inactivation.

In case of TGZ, activity of hGST P1-1 was abolished within 120 min of incubation. Interestingly, addition of 5 mM GSH did not fully protect against inactivation (Table 1) which might be explained by the formation of strongly inhibitory GSH conjugates.

**Table 1: Overview of the degree of modification and inactivation of hGST P1-1 by RMs of the drugs tested**

*Semiquantitative evaluation of the extent of modification was conducted by determination of AUCs of IA-modified tryptic peptides in samples containing adducted and non-adducted hGST P1-1. To correct for small differences between runs, AUCs determined in each run were divided by the AUC for peptide 75-81, which served as reference peptide. Percentage modification equals 100 – ( (AUC\text{mod.} / AUC\text{ref}) / (AUC\text{unmod.} / AUC\text{ref}) * 100). bNot determined.

<table>
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<tr>
<th>Drug</th>
<th>Degree of modification (%)a</th>
<th>Degree of inactivation (%)</th>
<th>-GSH</th>
<th>+GSH</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Pep 45-54</td>
<td>Pep 14-18</td>
<td></td>
<td></td>
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<tr>
<td>APAP</td>
<td>47.8 (±8.9)</td>
<td>NDb</td>
<td>98.7 (±1.4)</td>
<td>6.2 (±8.7)</td>
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<td>ND</td>
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<td>0.6 (±6.2)</td>
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<td>TGZ</td>
<td>71.6 (±7.8)</td>
<td>41.7 (±2.7)</td>
<td>99.1 (±1.7)</td>
<td>57.0 (±3.6)</td>
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DISCUSSION

The aim of the present investigation was the development of a potentially generic method for the production and identification of protein adducts. Using a highly active P450 BM3 mutant (CYP102A1M11H) for drug bioactivation, APAP, CLZ and TGZ were metabolized and the formed RMs were subsequently trapped with the model target protein hGST P1-1. After the covalent modification of hGST P1-1, CYP102A1M11H was removed completely using affinity beads (Figure 2), resulting in relatively clean samples of adducted target protein. Alkylated hGST P1-1 was digested with trypsin and analyzed by LC-MS/MS. Comparative analysis of adducted and non-adducted hGST P1-1 indicated that peptide 45-54 was targeted by RMs of all tested drugs, whereas peptide 14-18 was only adducted by RMs of TGZ. After a directed search for expected adducts of IA, APAP, CLZ and TGZ to these peptides (Figure 3 and 4), a set of diagnostic MS/MS ions was identified for each of the targeted peptides. By screening for the presence of these diagnostic MS/MS ions, TGZ modifications to peptide 45-54 with unanticipated mass shifts were identified.

The inactivation and covalent modification of hGST P1-1 by NAPQI, the RM of APAP, was previously investigated (20). Although protein modification was mainly performed with synthetic NAPQI, metabolic activation of APAP by CYP102A1M11H was also used to produce hGST P1-1 adducts. Both synthetic and in situ generated NAPQI preferentially adducted to cys-47, which is consistent with results obtained from this study (Figure 4A). However, in a previous work (20), CYP102A1M11H was not removed prior to digestion with trypsin and LC-MS/MS analysis. Because the molecular weight of His-tagged CYP102A1 is about 120 kDa, it results in more than 75 tryptic peptides of at least 500 Da. These peptides may interfere with the tryptic digest of the target protein. Therefore, the cleanup step provides significant advantages in terms of sample complexity and hence facilitates the identification of modifications to the protein of interest.

Activation of CLZ by CYP102A1M11H and trapping of formed RMs with hGST P1-1 produced a single adduct to peptide 45-54 with mass shifts equivalent to that of CLZ alkylation (+324.1 Da; Figure 4B). Reactions of CYP102A1M11H with CLZ in presence of GSH contained a GSH conjugate (2+, m/z 316.61) with identical mass increase as major adduct (19, 34). The mass increase of 324.1 Da corresponds to modification by the CLZ nitrenium...
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intermediate, which can be conjugated to cys-47 at different positions of the quinoid ring (Figure 1). Structural characterization of CLZ-GSH conjugates (GSH + 324.1 Da) generated by peroxidises (43, 44) and chemical synthesis (7) demonstrated the main GSH conjugate to be adducted via C-6 of CLZ. Whether CLZ adducts to cys-47 through the C-6 position, or whether the microenvironment of this residue only allows the covalent binding through another carbon, remains to be determined.

By visual inspection of peptide profiles and subsequent screening of diagnostic fragments, in total 14 different TGZ-modified peptides were identified in incubations of TGZ with CYP102A1M11H (see Table 2). Mass shift of +439.1 Da was observed with peptide 14-18, peptide 45-54 and peptide 101-102, whereas a mass shift of +455.1 Da was observed with peptides 14-18 and 45-54. GSH conjugates of TGZ with these mass increases were observed in incubations with human liver microsomes and CYP102A1M11H in this study, and previously in the bile of rats dosed with TGZ (37, 40). Interestingly, adducts to peptide 45-54 with a mass shift of +471.2 Da were also observed in hGST P1-1 samples exposed to RMs of TGZ (Figure 6). Adducts to GSH with this mass increase have not been reported in the literature (37-42, 45) and were not observed in incubations of CYP102A1M11H with TGZ in the presence of GSH (data not shown). Previously it was shown that the oxidation product of the skin sensitizer p-phenylenediamine did not react to GSH and N-acetylcysteine, whereas adducts to cys-47 of hGST P1-1 were detected presumably because of its higher reactivity due to the neighboring lysine-54 (28). The observed mass increase of 471.2 Da is exactly 16 Da higher than that observed for the TGZ adduct of +455.1 Da, which might result from bioactivation of a stable oxygenated TGZ metabolite, such as TGZ quinone (Figure 1), or by oxygenation of the TGZ adduct of +455.1. Possible explanations of this mass increase might be that part of cys-47 in GST P1-1 is present as sulfenic acid as was shown previously by Callan et al. (29). Alternatively, part of the initially formed adducts to cys-47 might be further oxygenated by reactive oxygen species produced by TGZ-induced uncoupling of CYP102A1M11H.
Table 2: Overview of the covalent modifications of RMs of TGZ to hGST P1-1

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<th>m/z; charge</th>
<th>Mass shift (Da)</th>
<th>Adduct type&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>494.73; 2+</td>
<td>+455.1</td>
<td>+TGZ+O</td>
</tr>
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<td>44.8</td>
<td>494.73; 2+</td>
<td>+455.1</td>
<td>+TGZ+O</td>
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<td>51.0</td>
<td>486.73; 2+</td>
<td>+439.1</td>
<td>+TGZ</td>
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<tr>
<td>Adducts to peptide 45-54</td>
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<tr>
<td>42.9</td>
<td>775.86; 2+</td>
<td>+471.2</td>
<td>+TGZ+2O</td>
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</tr>
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<td>45.5</td>
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<td>+471.2</td>
<td>+TGZ+2O</td>
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<tr>
<td>45.8</td>
<td>767.86; 2+</td>
<td>+455.1</td>
<td>+TGZ+O</td>
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<td>+TGZ+O</td>
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<td>49.1</td>
<td>759.87; 2+</td>
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<td>+TGZ</td>
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<td>Adducts to peptide 101-102</td>
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<tr>
<td>46.8</td>
<td>689.29; 1+</td>
<td>+439.1</td>
<td>+TGZ</td>
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The metabolism of APAP and TGZ by CYP102A1M11H resulted in complete inactivation of hGST P1-1, while CLZ caused 33% loss of hGST P1-1 activity (Table 1). The protection offered by GSH may suggest adduction to highly nucleophilic cys-47, which is most reactive and in close proximity to the GSH-binding site of the protein (21, 25). Hence, the degree of protein inactivation may be related to the extent of modification at cys-47 of hGST P1-1. Semiquantitative analysis of cys-47 modification involved comparative analysis of the area under curve of carbamidomethylated peptide 45-54 (1+, m/z 1136.58; 2+, m/z 568.79) in incubations containing modified hGST P1-1 and similar reactions supplemented with GSH (Table 1). The relation between hGST P1-1 activity and the percentage alkylation of cys-47 was previously investigated with iTRAQ labelling (20). Reactions of synthetic NAPQI with hGST P1-1 at equimolar ratio showed that 30% of cys-47 was covalently modified, resulting in
about 60% loss of hGST P1-1 activity. In this work, 48% modification of cys-47 of hGST P1-1 by APAP was accompanied by complete loss of hGST P1-1 activity. Jenkins et al. suggested that alkylation of cys-47 in one of the subunits was sufficient for disruption of the functional dimer (20), as has been shown for several other multimeric enzymes. Our results with APAP are compatible with this theory. In contrast, modification of cys-47 of hGST P1-1 by CLZ was approximately 27%, with a similar loss (33%) in enzyme activity. Complete inhibition of hGST P1-1 activity by TGZ was accompanied by 72% modification of cys-47 (Table 1). In case of TGZ, inactivation of hGST P1-1 may also be affected by the observed TGZ adducts to cys-14 (42% modification) and cys-101. However, adduction of cys-101 of hGST P1-1 by a quinone methide had only minor effects on enzyme activity (22). Incorporation of a second adduct, most-likely at cys-47, completely inactivated hGST P1-1. Several studies that investigated the interaction of thiol-reactive compounds with WT and cysteine mutants of hGST P1-1 confirm that alkylation of cys-47 has the most pronounced effect on enzyme activity (24, 32, 46). The importance of modification at cys-47 is further demonstrated by the observation that this residue is about two- or three-fold more reactive than cys-14 (23). Together, these data indicate that adduction of RMs of TGZ to cys-47 presumably is the major contributing factor to the hGST P1-1 inactivation observed in this study (Table 1).

Adducts of APAP, amodiaquine and CLZ to β-lactoglobulin were previously generated by electrochemical oxidation of the drugs (47). In the case of APAP, both adducted and non-adducted β-lactoglobulin were observed after drug activation, but the degree of protein modification was not determined. Electrochemical oxidation of CLZ resulted in complete modification of β-lactoglobulin. In contrast, in our study, modification of the target protein by CLZ was lowest, with an estimated 33% modification (Table 1). Activation of APAP, CLZ and amodiaquine by dehydrogenation can be conducted by electrochemical oxidation and may therefore result in high levels of modified protein. Other phase I reactions such as aliphatic hydroxylations and epoxidations are not readily reproduced by electrochemistry (10).

Electrochemical oxidation of TGZ resulted in TGZ quinone methide, which could be trapped as GSH conjugate. However, no GSH conjugates resulting from activation of the thiazolidinedione ring of TGZ were detected after electrochemical oxidation (48), while these conjugates were observed previously in incubations with human liver microsomes (37). In this study, by using CYP102A1M11H for TGZ bioactivation, adducts resulting from opening of the
thiazolidinedione ring were found to GSH, peptide 45-54 (Figure 6AB) and peptide 14-18 (Figure 8) of hGST P1-1. These findings demonstrate the limitations of electrochemistry for the preparation of adducts of TGZ.

The concept of diagnostic fragment ions as tool to screen for adducts was previously recognized by Onkenhout et al. (49). By examination of a series of synthetically prepared mercapturic acid methyl esters, three diagnostic ions from the N-acetylcysteine methyl ester part of the mercapturic acids were identified. These ions may be useful for the analysis of mercapturic acids of unknown masses. Screening for GSH conjugates by LC-MS/MS is facilitated by the identification of characteristic neutral losses instead of characteristic fragments ions. Among others, neutral losses of 75 Da (glycine) and 129 Da (pyroglutamic acid) are most frequently used for screening GSH conjugates. In this way, adducts to GSH can be determined without prior knowledge of the RM involved (50). In the present work, screening for diagnostic MS/MS ions of targeted peptides allowed for the identification of adducts without prior knowledge of the RM responsible. For peptide 45-54 of hGST P1-1, y2 (1+, m/z 244.17), y5 (1+, m/z 542.33) and y6 (1+, m/z 705.39) were selected as characteristic ions on the basis of mass spectrometric analysis of expected adducts of IA, APAP, CLZ and TGZ (Figure 3A and Figure 4). Previously, MS/MS ions y5 and y6 of peptide 45-54 were observed as prominent fragments upon cys-47 adduction of this peptide by the quinone methide of a food preservative (22), and upon fragmentation of cys-47 adducts of two model electrophiles (23). In addition, LC-MS/MS analysis of cys-47 adducts of the oxidation products of sulfamethoxazole and p-phenylenediamine showed that y2 of peptide 45-54 was among the most abundant fragment ions (28, 29). Together, these studies confirm the importance of y2, y5 and y6 of peptide 45-54 as characteristic fragment ions, irrespective as to the nature of the attached electrophile.

In conclusion, the present study shows that application of a highly active purified His-tagged drug-metabolizing P450 BM3 mutant in combination with screening for diagnostic fragments of modified peptides might be a potentially generic procedure for the generation, and characterization, of protein adducts, especially for reactive intermediates which are not accessible by organic synthesis or electrochemistry, or which are produced at only low levels by human liver fractions. Although in the present study only considered cytosolic hGST P1-1
as model protein, this strategy might be applicable to other proteins (cytosolic or membrane-bound) that can be purified to homogeneity. However, in case of membrane-bound proteins amino acids might become alkylated which normally are shielded by the membrane.

The current P450 BM3 mutant, CYP102A1M11H, was shown to activate APAP, CLZ, diclofenac and TGZ to human-relevant reactive drug metabolites (19, 34, present study). However, in case of other toxicants this biocatalyst might be less useful in case no or non-relevant RMs are produced. By site-directed and random mutagenesis a panel of catalytically diverse P450 BM3 mutants have been developed recently by us and others (17, 51), which will enable the selection of a more appropriate bioactivating enzyme than CYP102A1M11H by first screening for their ability to generate human-relevant GSH conjugates.

Next to the application to characterize the nature of protein damage, the current methodology will allow biosynthesis of protein adducts to support biomonitoring studies. Protein adducts have long been used as biomarkers of internal exposure to reactive chemicals (52). For example, we previously described a strategy that relies on detection of drug adducts to cys-34 of human serum albumin for monitoring exposure to reactive intermediates (53). The methodology developed here can be employed for biosynthesis of the relevant reference adducts. Finally, an application of drug-protein adducts generated by the P450 BM3-system can be found in the production of antibodies against drug-modified proteins. For example, covalent modification of keyhole limpet hemocyanin by RMs may be used for raising antibodies in test animals. These antibodies might be used for immunohistochemical approaches to localize covalent binding in complex protein mixtures.

REFERENCES


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**SUPPORTING INFORMATION**

**Table S1: Overview of observed hGSTP1-1 peptides**

Theoretical masses of tryptic peptides were obtained by in silico digestion of hGSTP1-1 (Uniprot sequence P09211-1) using tools from the ExPASY Proteomics Server (www.expasy.ch). Peptide masses based on adduction of 1A to free cysteines. Singly charged ions are shown when only theoretical mass is available. "Not detected.

<table>
<thead>
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<th>Peptide</th>
<th>Sequence</th>
<th>Theoretical mass(^a)</th>
<th>Measured mass</th>
</tr>
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<tbody>
<tr>
<td>1-11</td>
<td>PPYTVVFYPVR</td>
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<td>669.376(^{+2})</td>
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<td>GR</td>
<td>232.140(^{+1})</td>
<td>ND(^b)</td>
</tr>
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<td>CAALR</td>
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<td>295.660(^{+2})</td>
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<td>TFFIVGDQISFADYNLLDLLLIHEVLAPGCLDAFPLLSSAYVGR</td>
<td>4649.436(^{+1})</td>
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<td>183-188</td>
<td>LSARPK</td>
<td>671.420(^{+1})</td>
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<td>189-190</td>
<td>LK</td>
<td>260.197(^{+1})</td>
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<td>191-208</td>
<td>AFLASPEYVNLPINGNGK</td>
<td>952.500(^{+2})</td>
<td>952.509(^{+2})</td>
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<td>209-209</td>
<td>Q</td>
<td>147.076(^{+1})</td>
<td>ND</td>
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