Chapter 7

SUMMARY

Adverse Drug Reactions (ADRs) are a main cause of drug attrition and of withdrawal of marketed drugs (1, 2). Idiosyncratic adverse drug reactions (IADRs) are rare but very serious ADRs, which currently are unpredictable based on pre-clinical and clinical research (3, 4). Therefore, there is much interest in better understanding IADRs mechanistically and in the development of new approaches and methodologies to help predicting these reactions. Both the immune system and covalent protein binding by reactive metabolites (RMs) appear to play an important role in the onset of many IADRs (5, 6). Chapter 1 describes the formation of RMs by Cytochrome P450s (CYPs) and discusses the detoxification of RMs by glutathione-S-transferases (GSTs). Furthermore, this chapter provides an overview of the strategies to detect and characterize RMs of candidate drugs. While protein modification by RMs appears essential in the etiology of IADRs, gross covalent binding cannot distinguish toxic from non-toxic drugs (7, 8). Hence, the modification of individual critical proteins may determine whether alkylation results in toxicity (9, 10). The second part of chapter 1 focuses on methods to measure adduction of individual proteins and on approaches to identify adducted proteins in proteomes. Since target proteins critical for specific ADRs and IADRs still remain to be discovered, the use of glutathione-S-transferase P1-1 (GST P1-1) as a model target protein for RMs is discussed.

The main aim of this thesis was the development of new methods for the preparation and identification of drug-protein adducts resulting from CYP-dependent bioactivation of drugs to RMs. When this research started, several drug-metabolizing mutants of cytochrome P450 BM3 (CYP102A1) had been engineered in our laboratory (11-13). Some of these mutants were found to form similar RMs of drugs as human liver microsomes, but with much higher catalytic activities (13). Because the highest activity was generally observed for the his-tagged mutant CYP102A1M11H, this enzyme was applied for RM production in various studies (14-16). We employed CYP102A1M11H as biocatalyst for RM formation in chapter 2-4 of the current thesis, whereas GST P1-1 was used as the model target protein. GST P1-1 and other GSTs are also known to catalyze GSH conjugation of RMs (17, 18). A second aim of this thesis was therefore to investigate the role of all major cytosolic GSTs in the detoxification of RMs of drugs associated with IADRs. GST-catalyzed GSH conjugation of RMs is covered in chapter 5 and chapter 6.
In chapter 2 of this thesis, we have described a method to produce and analyze GST P1-1 adducts resulting from the bioactivation of several drugs by CYP102A1M11H. Because of its his-tag, this P450 BM3 mutant could easily be removed from incubations by nickel affinity chromatography, which facilitated LC-MS analysis of tryptic peptides of drug-modified GST P1-1. By applying this procedure to acetaminophen (APAP), clozapine (CLZ) and troglitazone (TGZ) we detected their anticipated adducts to cys-47, which is most reactive of the four cysteine residues in GST P1-1 (19). Analysis of tryptic peptides containing the anticipated cysteine adducts also resulted in identification of unmodified diagnostic fragments of these peptides. These diagnostic MS/MS fragments were subsequently used to identify unexpected drug modifications to the cysteine residues of GST P1-1. While screening for diagnostic fragment ions of GST P1-1 modified by APAP and CLZ only resulted in the detection of their anticipated drug adducts, modifications of unanticipated mass increments were observed to tryptic peptides of GST P1-1 modified by TGZ. It is concluded in chapter 2 that application of his-tagged drug-metabolizing CYP102A1 mutants in combination with screening for diagnostic MS/MS fragments of modified peptides might be a relatively easy and potentially generic procedure for the generation and characterization of cys-adducted proteins.

The methodologies developed in chapter 2 were applied in chapter 3 to investigate protein modification by the nonsteroidal anti-inflammatory drug diclofenac (DF). Therapy of patients with DF is associated with a low/rare incidence of hepatotoxicity, which may be due to DF bioactivation to p-benzoquinone imines, o-imine methides or arene oxides (20-22). In previous work, bioactivation of 4’-hydroxydiclofenac (4’-OH-DF) and 5-hydroxydiclofenac (5-OH-DF) by CYP102A1M11H resulted in formation of GSH conjugates from their p-benzoquinone imines (14). Remarkably, when we used GST P1-1 as a nucleophile, only adducts of the p-benzoquinone imine of 5-OH-DF were detected after bioactivation by CYP102A1M11H. Incubation with DF confirmed these findings and also showed protein modification by DF o-imine methide. By unbiased screening using diagnostic ions of DF-modified tryptic peptides, three unanticipated DF adducts were observed for the first time. Two of these adducts resulted from further reaction of the p-benzoquinone imine of 5-OH-DF, whereas the third adduct was from protein modification by a previously uncharacterized metabolite of DF. Importantly, since none of these adducts was formed in DF incubations of CYP102A1M11H in presence of GSH, our results show that adduction of cysteine residues in proteins is not necessarily reflected by GSH conjugation.
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The drug-protein adduct preparation and identification strategy used in the previous chapters requires the removal of his-tagged P450 by affinity chromatography. Drug bioactivation can therefore not simply be performed with human liver microsomes, which consist of a complex mixture of membrane-bound proteins. Chapter 4 describes a procedure for the preparation of drug-modified proteins which allows membrane-bound P450s to be used for bioactivation. Drug bioactivation was performed in a small container which also contained a mini-dialysis tube with the model target protein hGST P1-1. Following drug bioactivation, RMs can translocate from the P450 compartment to the GST P1-1 compartment via a semi-permeable membrane (6-8 kDa). This novel procedure was evaluated by performing metabolic activation with P450 bioactivation systems of different complexity. Bioactivation of drugs by CYP102A1M11H showed that some RMs were unable to modify GST P1-1, possibly because these intermediates are too short-lived. However, GST P1-1 adducts of DF and mafenamic acid were observed in incubations of CYP102A1M11H, and after metabolic activation by recombinant human P450s and liver microsomes, respectively. This new approach may prove useful for the characterization of drug-protein adducts resulting from bioactivation by human liver microsomes and it allows intact drug-modified protein to be analyzed by LC-MS without laborious cleanup.

Cells have developed sophisticated defense mechanisms to prevent the covalent modification of proteins. The tripeptide glutathione (GSH) is the major cellular nucleophile available for reaction with electrophiles (23). While spontaneous conjugation is presumably dominant at high GSH concentration, enzymatic conjugation by cytosolic GSTs may become more important when GSH levels are low. Previously, it was found that GSTs played a role in GSH conjugation of cytostatic drugs and in the detoxification of the RMs of zileuton and felbamate (18, 24, 25). In chapter 5 we investigated whether cytosolic GSTs were involved in GSH conjugation of the reactive nitrenium ion of CLZ, which may be responsible for CLZ-induced agranulocytosis. Bioactivation of CLZ was performed with CYP102A1M11H and with human and rat liver microsomes, while GST A1-1, M1-1, P1-1 and T1-1 were evaluated for their ability to catalyze GSH conjugation of the nitrenium ion of CLZ. We generally observed that GSTs increased GSH adduct levels and changed the regioselectivity of the GSH conjugation. In addition, the formation of two GSH adducts appeared completely GST-dependent. These data suggest that GSTs play an important role in detoxification of the CLZ nitrenium ion.
Patients with polymorphisms in these enzymes might therefore be at increased risk for developing agranulocytosis during CLZ therapy.

By mass spectrometric analysis of DF-modified proteins formed by CYP-mediated DF bioactivation (chapter 2), we identified an unanticipated adduct resulting from substitution of the chlorine of DF by a protein thiol. Interestingly, the corresponding GSH conjugate [2’-(glutathione-S-yl)-deschloro-diclofenac (DDF-SG)] was only formed when GST P1-1 was added to DF incubations, suggesting formation of DDF-SG is completely GST-dependent. Since none of the previously described RMs of DF (p-benzoquinone imine, arene oxide, o-imine methide) could explain formation of the chlorine-substituted GSH conjugate of DF (20-22), the aim of chapter 6 was to investigate which RM of DF was responsible for DDF-SG formation. Remarkably, when DF metabolism was evaluated using recombinant CYPs, all tested enzymes appeared to form DDF-SG, while the mono-oxidized metabolites of DF were only catalyzed by CYP2C9 and CYP3A4. Further mechanistic studies using CYP2C9, CYP3A4 and other heme-containing enzymes were subsequently performed under various reaction conditions. Our findings showed that formation of DDF-SG resulted from non-enzymatic heme-dependent bioactivation of DF, presumably to a nitrogen-centered radical cation, which then undergoes GST P1-1-catalyzed GSH conjugation. It remains to be established whether this bioactivation pathway is relevant to DF-induced hepatotoxicity.

**CONCLUSIONS AND PERSPECTIVES**

The occurrence of serious idiosyncratic drug toxicity poses a risk to patient health and it can result in withdrawal of a drug that is safe in the majority of the population. An increasing body of evidence suggests that drug-modified proteins play an important role in the onset of IADRs (26). Therefore, in industry, drug candidates are evaluated for their ability to form RMs by screening for GSH conjugates or by measurement of gross covalent protein binding (27, 28). Because covalent modification of proteins does not necessarily lead to a toxic response, total covalent binding cannot properly distinguish toxic from safe drugs (7, 8). Hence, the selectivity of RMs for specific proteins or for amino acids within proteins may be a more important determinant of the toxic outcome (10).