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Mini-dialysis tubes as tools to prepare drug-protein adducts of P450-dependent reactive drug metabolites

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ABSTRACT

The modification of critical cellular proteins by reactive metabolites (RMs) resulting from P450-dependent drug bioactivation is considered essential to the onset of many idiosyncratic drug reactions. In this study, we report a novel method that can be used to prepare and study drug-protein adducts. Drug bioactivation by P450s was performed in a small container containing a mini-dialysis tube with the model target protein human glutathione-S-transferase P1-1 (hGST P1-1), allowing RMs to translocate from P450 to hGST P1-1 via a semi-permeable membrane (6–8 kDa). GST P1-1 modification was evaluated by LC–MS analysis of intact protein adducts and following digestion of protein with trypsin. As proof of principle, the described methodology was first applied to the direct electrophile monochlorobimane. A highly active P450 BM3 mutant (CYP102A1M11H) was subsequently used for bioactivation of acetaminophen, clozapine, diclofenac (DF) and mefenamic acid (MFA), but hGST P1-1 adducts were only observed for the latter two drugs. CYP2C9 and CYP3A4, which metabolize DF to *p*-benzoquinone imines, were tested to investigate the applicability of human P450s. Finally, it was evaluated whether bioactivation of MFA by human and rat liver microsomes resulted in modification of hGST P1-1. The results show that our adduct preparation method can also be used in combination with membrane-bound P450 bioactivation systems, as long as formed RMs have sufficient life-time to reach hGST P1-1 inside the dialysis tube.

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1. Introduction

Idiosyncratic adverse drug reactions (IADRs) are rare but very serious adverse reactions that have resulted in withdrawal of several marketed drugs. The mechanisms leading to IADRs are largely unknown, but bioactivation of drugs to reactive metabolites (RMs) and subsequent covalent modification of proteins appear important events in the onset of many IADRs [1]. To identify compounds with IADR potential, pharmaceutical companies typically evaluate total covalent binding to biological material by radiolabeled drug candidates [2]. However, total covalent binding alone cannot properly distinguish toxic from non-toxic drugs, because high levels of covalent binding are also observed for safe drugs [3–5]. Therefore, alkylation of individual critical proteins by reactive intermediates may be important for toxicity [6]. In this respect, it is essential to develop methodologies to evaluate the selectivity of modification of individual proteins and to prepare drug-protein adducts for further mechanistic studies.

Different methods have been applied to prepare drug-protein conjugates. The RMs of some drugs can be obtained by chemical synthesis, which allows incubation of the pure RM with the protein of interest. Electrochemistry has also been employed to mimic oxidative drug metabolism and prepare drug-modified proteins in absence of biological matrices [7,8]. Although high amounts of drug-protein conjugates were generated of drugs activated by dehydrogenation [8], electrochemical oxidation cannot mimic all P450 reactions because it lacks steric control.

Therefore, there is a need for approaches that use P450 enzymes to prepare drug-protein adducts. Because human P450s have relatively low activity, cytochrome P450 BM3 (CYP102A1) from *Bacillus Megaterium* has gained interest as an alternative biocatalyst. CYP102A1 is soluble, self-sufficient due to a fusion between its reductase and catalytic domain, and has the highest activity ever recorded for a P450 [9]. By rational and random

Abbreviations: 4′-OH-DF, 4′-hydroxydiclofenac; 5-OH-DF, 5-hydroxydiclofenac; APAP, acetaminophen; CLZ, clozapine; CYP102A1, Cytochrome P450 BM3; DF, diclofenac; hGST P1-1, human glutathione-S-transferase P1-1; HLMs, human liver microsomes; IADRs, idiosyncratic adverse drug reactions; MCB, monochlorobimane; MFA, mefenamic acid; RMs, reactive metabolites.

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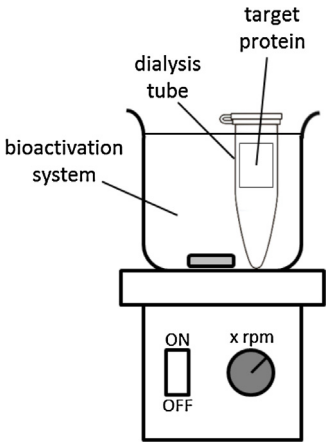

Fig. 1. Schematic representation of the protein alkylation procedure applied in the present study. A dialysis tube with the target protein is incubated in a solution of drug and the P450 bioactivation system. Reactive metabolites can migrate freely through the semi-permeable membrane (6–8 kDa MWCO) to form adducts to the target protein.

mutagenesis of CYP102A1, drug-metabolizing mutants have been engineered that form human relevant RMs in higher amounts than human liver microsomes (HLMs) [10]. Purified mutant CYP102A1M11H was previously used to prepare protein-adducts of acetaminophen (APAP), clozapine (CLZ) and diclofenac (DF) [11,12]. Because this CYP102A1 mutant is his-tagged, the P450 can be easily separated from the drug-modified protein by affinity chromatography. However, this procedure is not applicable to human P450s, which consist of a complex mixture of membrane-bound enzymes.

Yukinaga and co-workers recently detected APAP and raloxifene protein adducts resulting from bioactivation by HLMs and CYP3A4-expressing microsomes [13]. Fractionation by HPLC was used to isolate the (modified) target protein from the mixture of microsomal proteins. Although this procedure significantly improved detection of drug-adducts, fractionation by HPLC is time-consuming and the collected target protein might be contaminated with co-eluting microsomal proteins.

In the present paper, we report an alternative procedure for the preparation of drug-protein adducts. The target protein was added to mini-dialysis tubes which were then incubated in buffer solution containing drug bioactivation system (Fig. 1). Because the dialysis membrane (6–8 kDa cut-off) provides a physical barrier between the bioactivation system and the target protein, microsomal preparations may be used for bioactivation and target protein does not require cleanup post-modification. Human glutathione-S-transferase P1-1 (hGST P1-1) was selected as model target protein to evaluate this alkylation procedure. Covalent modification of the cysteine residues in hGST P1-1 (cys-14, cys-47, cys-101 and cys-169) was investigated by analysis of intact and of trypsin digested hGST P1-1 by mass spectrometry. Initially, we demonstrated the concept of our method by incubating hGST P1-1 with the direct electrophile monochlorobimane (MCB) (Fig. 2). It was then determined whether bioactivation of APAP, CLZ and DF by CYP102A1M11H resulted in formation of adducts to hGST P1-1 in dialysis tubes. CYP2C9 and CYP3A4 catalyze formation of *p*-benzoquinone imines of DF resulting from 4′-hydroxydiclofenac (4′-OH-DF) and 5-hydroxydiclofenac (5-OH-DF), respectively [14]. These enzymes were therefore used to evaluate whether human P450s could be used to prepare hGST P1-1 adducts. Finally, it was investigated whether hGST P1-1 adducts could be detected in incubations of CYP102A1M11H and liver microsomes with mefenamic acid (MFA), a nonsteroidal anti-inflammatory drug which is associated with liver and renal injury [15,16]. Previously, GSH conjugates resulting from oxidative metabolism of MFA have been observed [17,18]. Corresponding protein adducts have not yet been studied.

2. Materials and methods

2.1. Materials

Mini dialysis tubes (GeBAflex; volume 250 μL) were obtained from Generon (Maidenhead, UK). 4′-hydroxydiclofenac (4′-OH-DF) and 5-hydroxydiclofenac (5-OH-DF) were from Toronto Research Chemicals (North York, Canada). The compounds monochlorobimane (MCB), acetaminophen (APAP), diclofenac (DF) sodium salt, and mefenamic acid (MFA) were bought from Sigma (Steinheim, Germany), whereas clozapine (CLZ) was from Duchefa Farma (Haarlem, The Netherlands). Sequencing grade modified trypsin was from Promega (Madison, USA). All other reagents were from standard suppliers and of analytical grade.

2.2. Enzyme expression

The expression of hGST P1-1 and its purification by GSH-affinity chromatography was performed as described previously [19]. Recombinant human CYP3A4 and CYP2C9 were co-expressed with human NADPH cytochrome P450 reductase in *Escherichia coli* DH5α cells. Isolation of membranes was conducted according a previously described procedure [20]. CYP102A1M11H was expressed and purified by nickel affinity chromatography as reported elsewhere [10].

2.3. Modification of human glutathione-S-transferase P1-1 by monochlorobimane

Incubations were conducted in glass scintillation vials containing 10 mL potassium phosphate (KPi) buffer (pH 7.4; 0.1 M). MCB was added to a final concentration of 250 μM. After washing mini-dialysis tubes according to the manufacturer's guidelines, tubes were filled with 180 μl 35 μM hGST P1-1 and placed in the incubation mixture. Reactions were performed in the dark on a magnetic stirring plate at room temperature and quenched after 18 h by addition of 4 mM GSH. Samples were analyzed directly by LC–MS (TOF) or digested with trypsin prior to LC–MS/MS (Q-TOF) analysis.

2.4. Bioactivation of drugs by cytochrome P450 enzymes in presence of human glutathione-S-transferase P1-1

Reactions (10-mL) were performed in glass scintillation vials in KPi buffer (pH 7.4; 0.1 M). APAP, CLZ and DF were incubated at 1 mM final concentration. MFA and the monohydroxylated DF metabolites were incubated at 500 μM and 50 μM, respectively. All incubations were conducted with 250 nM P450 or with 2 mg/mL microsomes. In case of incubations of DF or its monohydroxylated metabolites with CYP102A1M11H, superoxide dismutase (10 U/mL) was added to prevent further oxygenation of protein adducts by reactive oxygen species [12]. Reactions were started by the addition of NADPH regenerating system (final concentration: 50 μM NADPH, 2.5 mM glucose-6-phosphate, 0.5 U/mL glucose-6-phosphate dehydrogenase). Mini dialysis tubes, pre-rinsed according the manufacturer's instructions, were filled with 180 μL 35 μM hGST P1-1 and placed in the incubation mixtures (Fig. 1). Reactions of CYP102A1M11H were performed on magnetic stirring plates incubated at room temperature, whereas hCYPs were incubated in shaking water baths at 37 °C. Incubations were terminated after 18 h. In case samples were subsequently subjected to tryptic digestion, reactions were quenched with 4 mM GSH and

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