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# Cysteine amide adduct formation from carboxylic acid drugs *via* UGT-mediated bioactivation in human liver microsomes

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Although chemical trapping has been widely used to evaluate cytochrome P450-mediated drug bioactivation, thus far, only a few *in vitro*-trapping studies have been performed on UDP-glucuronosyltransferase (UGT)-mediated drug bioactivation. In this study, we used cysteine (Cys) as trapping agent to gain new insights into the UGT-mediated bioactivation involving acyl glucuronides of carboxylic acid drugs. Diclofenac, ketoprofen and ibuprofen were incubated in human liver microsomes with UDPGA and Cys, followed by analysis using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS). The *N*-acyl-Cys amide adduct of diclofenac was characterized by mass analysis and was detectable even in photodiode array analysis. Our data indicated that the formation of such adducts reflects the reactivity of the corresponding acyl glucuronides. In addition, it was suggested that the adduct formation requires an N-terminal Cys moiety with both a free amine and a free thiol group, from the results using various cysteine derivatives. We propose that the *S*-acyl-Cys thioester adduct can form via transacylation of an acyl glucuronide and can then form to an *N*-acyl-Cys amide adduct through intramolecular S- to N-acyl rearrangement. This series of the reactions has important implications as a possible bioactivation mechanism for covalent binding of carboxylic acid drugs to macromolecules.

## 1. Introduction

Glucuronidation is a major metabolic pathway for the elimination of endogenous substrates such as bilirubin, steroids and bile acids, as well as xenobiotic compounds including drugs, toxicants and other chemicals. This conjugative reaction with glucuronic acid is catalyzed by uridine 5'-diphosphoglucuronosyltransferase (UDP-glucuronosyltransferase, UGT), which requires UDP-glucuronic acid (UDPGA) as a cofactor. Common functional groups of glucuronidation substrates include hydroxyl, carboxyl, amino and thiol groups. Compounds with carboxyl groups can also be metabolized to ester-type glucuronides, known as acyl glucuronides. It has been proposed that acyl glucuronides of carboxylic drugs such as nonsteroidal anti-inflammatory drugs (e.g., tolmetin, zomepirac and diclofenac) cause idiosyncratic toxicities such as hepatotoxicity and anaphylactoid reactions (Spahn-Langguth and Benet 1992; Boelsterli et al. 1995; Boelsterli 2003; Regan et al. 2010). Acyl glucuronides are intrinsically reactive and can undergo intramolecular rearrangement including reversible acyl migration and anomerization of isomers (Faed 1984; Sallustio et al. 1997; Bailey and Dickinson 2003; Stachulski et al. 2006). Acyl glucuronides can also modify proteins by transacylation (with a loss of a glucuronic acid moiety) or by glycation with the open-chain isomer form (with retention of the glucuronic acid moiety) (Smith and Wang 1992; Liu et al. 1998; Chiou et al. 1999). Given the importance of acyl glucuronides as potentially toxic reactive species, the risk assessment of them has become an increasingly important field of study, recognized as a critical component of drug safety evaluation by the US Food and Drug Administration (US FDA Guidance on Safety Testing of Drug Metabolites, February 2008).

Chemical trapping is widely used for the detection and characterization of potentially reactive metabolites, especially those involved in cytochrome P450-mediated metabolism (Joshi et al. 2004; Castro-Perez et al. 2005; Ma and Subramanian 2006; Sleno et al. 2007). These experiments are often conducted in liver microsomes with reduced nicotinamide adenine dinucleotide phosphate (NADPH) and a nucleophilic trapping agent, such as glutathione (GSH). Many reactive metabolites can form GSH adducts that are detectable with liquid chromatography/tandem mass spectrometry (LC-MS/MS) and show characteristic ion fragmentation patterns. Comparatively few studies have been performed for detecting UGT-mediated bioactivation by using GSH-trapping methods, because acyl glucuronides are reported to be poorly reactive to GSH and to undergo mainly hydrolysis or isomerization (Grillo et al. 2003a). Other methods for evaluating the UGT-mediated bioactivation of carboxylic acid drugs include covalent binding studies with either a [$^{14}$C]-radiolabeled drug or UDPGA (Smith et al. 1990; Kretz-Rommel and Boelsterli 1994; Ebner et al. 1999; Georges et al. 1999). Some reports suggest that the reactivity of acyl glucuronides can be predicted from their degradation rate, including intra-molecular rearrangement (Ebner et al. 1999; Bolze et al. 2002a,b). Although the reactivity of acyl glucuronides has been extensively studied *in vitro* and *in vivo*, it is still difficult to

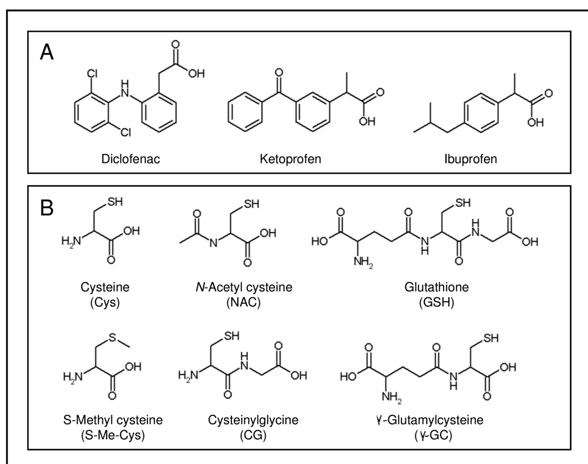


Fig. 1: Structures of (A) test drugs (diclofenac, ketoprofen and ibuprofen) and (B) cysteine derivatives (Cys, GSH, NAC, CG, γ-GC and S-Me-Cys).

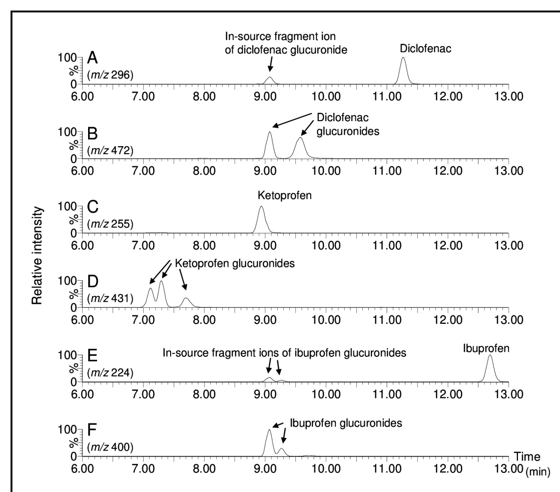


Fig. 2: Extracted ion mass chromatograms (in positive ion mode) obtained from human microsomal incubations with UDPGA, Cys, and test drugs. (A) diclofenac at *m/z* 296; (B) diclofenac glucuronides at *m/z* 472; (C) ketoprofen at *m/z* 255; (D) ketoprofen glucuronides at *m/z* 431; (E) ibuprofen at *m/z* 224; (F) ibuprofen glucuronides at *m/z* 400.

predict idiosyncratic toxicity in humans. Recently, it was proposed that acyl GSH thioesters or acyl CoA thioesters of carboxylic acids are more reactive intermediates than acyl glucuronides, and that acyl thioesters can be chemically generated from acyl glucuronides (Grillo and Benet 2001, 2002; Grillo et al. 2003a; Grillo and Hua 2003b; Grillo et al. 2003c). Therefore, it is suggested that *in vitro* studies of acyl glucuronide reactivity include consideration of acyl thioesters.

Previously, we found that UGT-mediated bioactivation of carboxylic acid drugs can be evaluated using N-acetyl cysteine (NAC) as a trapping agent by LC-MS/MS to detect NAC adducts (Harada et al. 2009). In the present study, we evaluated L-cysteine (Cys) and Cys derivatives as trapping agents for determining UDPGA-mediated bioactivation in human liver microsomal incubations of carboxylic acid drugs (diclofenac, ketoprofen and ibuprofen) (Fig. 1). Reaction products were detected using ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS).

## 2. Investigations, results and discussion

### 2.1. Detection and characterization of reaction products

When the test compounds (diclofenac, ketoprofen and ibuprofen) were incubated with human liver microsomes in the presence of UDPGA and Cys, their glucuronides were observed in MS analysis (Fig. 2). These glucuronides were detected as protonated molecular ions for diclofenac and ketoprofen and ammonium adduct ions for ibuprofen, showing a mass shift of 176 amu in comparison with their parent compounds. It was reported that the glucuronide group contains 1-*O*-β-acyl glucuronide or its acyl migration isomers (Bolze et al. 2002b; Harada et al. 2009).

The extracted ion chromatograms for products of the diclofenac reaction, their product ion spectra and their postulated fragmentation patterns are shown in Fig. 3. An unknown product, D1 correspond to a Cys adduct of diclofenac at *m/z* 399, increasing by 103 amu compared with diclofenac (Fig. 3C). D1 was not detected in the incubations that did not contain either UDPGA or Cys. This indicates that the formation of D1 is a UGT-mediated reaction, depending on the presence of UDPGA. In addition, another unknown product, D2 was observed in the extract stored at 4 °C for 24 h after quenching the incubation mixture with acetonitrile (Fig. 3D). D2 was detected at *m/z* 518, increasing by 222 amu compared with diclofenac. Fig. 3F shows the product ions obtained from D1 at *m/z* 399. The fragment ion at *m/z* 278 corresponds to a loss of 121 amu (the Cys moiety). D1 also produced

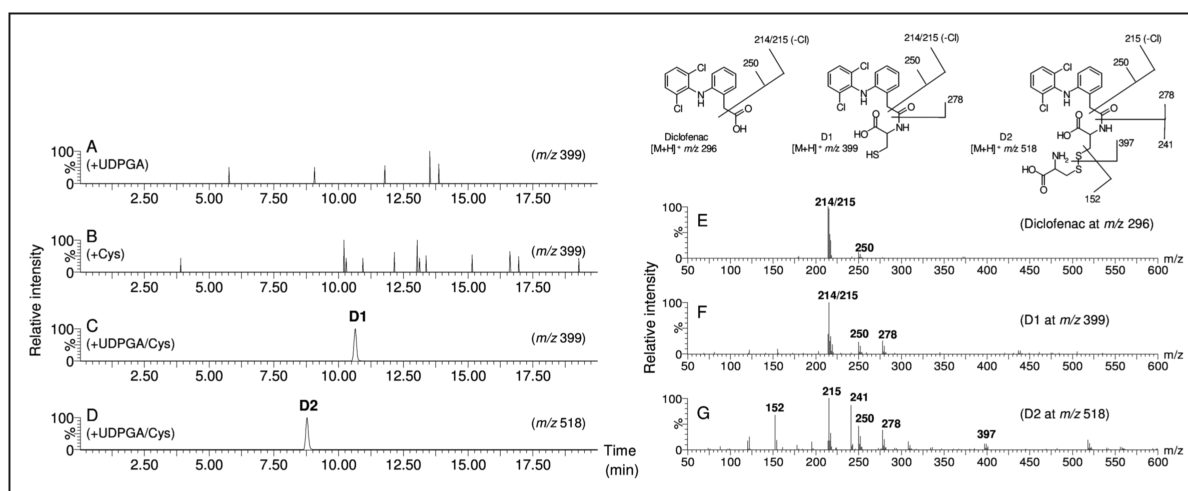


Fig. 3: Extracted ion mass chromatograms (A)-(D) and product ion spectra (E)-(G) of diclofenac derivatives in human microsomal incubations. Incubations were performed in the presence of (A) UDPGA, (B) Cys, or (C) and (D) UDPGA/Cys. Samples were kept at 4 °C and analyzed (C) just after or (D) 24 h after quenching incubation reaction with acetonitrile.

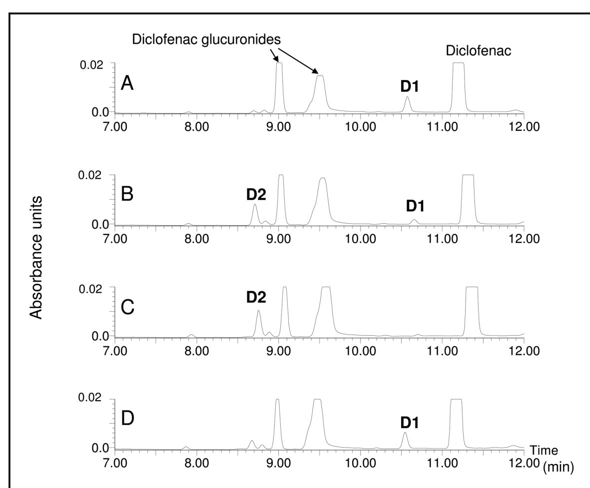


Fig. 4: PDA chromatograms (extracted at 225 nm) of human microsomal incubations of diclofenac with UDPGA/Cys. Samples were kept at 4 °C and analyzed (A) just after, (B) 24 h after or (C) 48 h after quenching incubation reaction with acetonitrile. Finally, the sample 48 h after quenching was analyzed (D) with addition of DTT.

the fragment ions at *m/z* 250 and 215, showing a fragmentation pattern similar to that of diclofenac. Product ion spectrum of D2 at *m/z* 518 is shown in Fig. 3G. The fragment ion at *m/z* 278 corresponds to a loss of 240 amu. Additionally, D2 produced fragment ion at *m/z* 241 (the cystine moiety), which is likely to be the *N*-acyl-Cys-Cys disulfide amide of diclofenac with an amide linkage.

D1 and D2 were also observed in photodiode array (PDA) analysis of the microsomal incubations for diclofenac (Fig. 4). D1 was detected in the extract just after quenching the incubation mixture with acetonitrile. D1 gradually degraded to D2 in the extract at 4 °C, to almost disappear after 48 h. When dithiothreitol (DTT) was added to the extract after 48 h, D2 disappeared, while D1 appeared in its place. These results indicated that D1 is the *N*-acyl-Cys amide adduct with a free thiol group and an amide linkage, because the thiol group can form a disulfide bond. In PDA analysis of the microsomal incubations for the test compounds with UDPGA and Cys, no such corresponding products were detected for ketoprofen and ibuprofen unlike diclofenac (Fig. 5). However, the Cys adduct of ketoprofen was detectable by MS analysis, but not for ibuprofen. This difference may reflect the relative reactivity of the corresponding acyl glucuronides. It has been reported that the *in vitro* hydolysis rate order of various acyl glucuronides was associated with the inherent reactivity and specific structural features of drug carboxylic acid groups; generally, a rate order of phenyl acetic acid-type (diclofenac) > phenyl propionic acid-type (ketoprofen and ibuprofen) > benzoic acid-type was observed (Stachulski et al. 2006; Ebner et al. 1999).

### 2.2. Comparison of various Cys derivatives as the trapping agent

In a previous study, we used NAC as a trapping agent for drug acyl glucuronides and found that UGT-mediated bioactivation can be detected as NAC adducts by using LC-MS/MS, despite having relatively unstable thioester bonds (Harada et al. 2009). No NAC adducts were detectable in that study by UV chromatograms, presumably because of their low production. In the current study, the incubations were conducted in the presence of the various cysteine derivatives as the trapping agent (Fig. 6). As described above, the *N*-acyl-Cys amide adduct is stable and detectable, perhaps because of its amide linkage. Interestingly, an unknown product, D3 was observed when *N*-L-cysteinylglycine (CG) was used in place of Cys (Fig. 6B). In MS analysis, D3, *N*-acyl-CG amide adduct of diclofenac was detected at *m/z* 456, increasing by 160 amu compared with diclofenac, and showed a characteristic fragmentation pattern (Fig. 7). Meanwhile, no such corresponding adducts of diclofenac were observed, in the case of GSH, NAC, *N*-L-γ-glutamyl-L-cysteine (γ-GC) or *S*-methyl-L-cysteine (*S*-Me-Cys) as the trapping agent. It was suggested that the formation of the amide adduct requires an N-terminal Cys moiety with not only a free amine group but also a free thiol group. Additionally, comparable reactions were conducted with the addition of 3% formic acid to the quenching solution in an attempt to stabilize the thioesters, but no marked changes were observed in PDA chromatograms (data not shown).

Accurate MS data for the test drugs and their derivatives are shown in the Table. The *m/z* values of all the compounds as protonated molecular ions for diclofenac and ketoprofen and ammonium adduct ions for ibuprofen were measured with good accuracy. Additionally, the monoisotopic molecular weights of diclofenac and the derivatives were detected with their distinguishable isotopes which contain $^{37}$Cl, increasing their molecular weight by 2 amu.

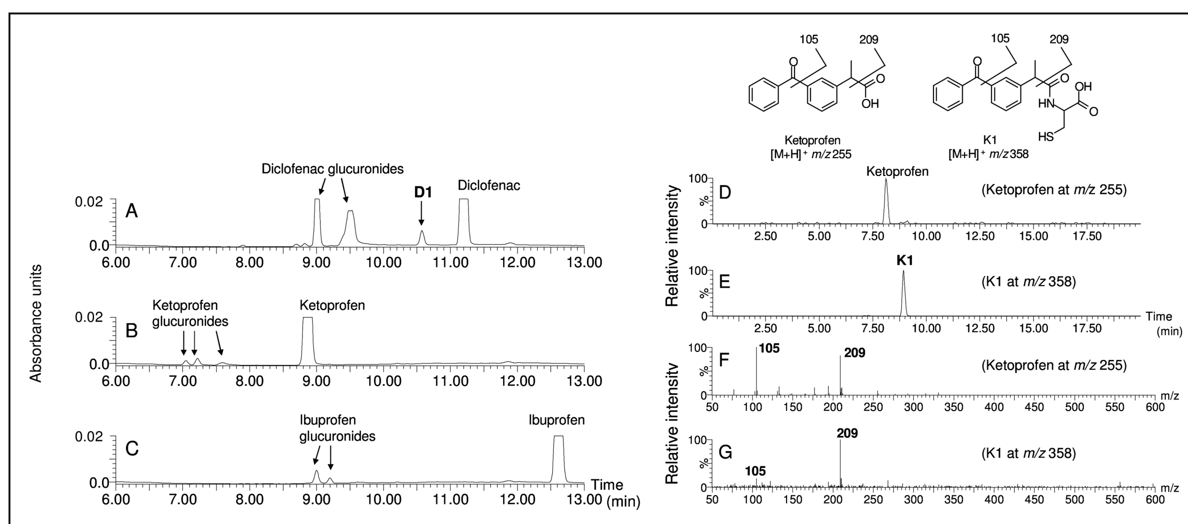


Fig. 5: PDA chromatograms (extracted at 225 nm) of human microsomal incubations in the presence of UDPGA, Cys, and test drug (A, diclofenac; B, ketoprofen; C, ibuprofen) and MS data of ketoprofen and K2 (D and E, extracted ion mass chromatograms; F and G, product ion mass spectra).

**Table: Accurate mass measurements of diclofenac, ketoprofen, ibuprofen and their derivatives by using UPLC-QTOF/MS**

| Compound | Ionized form | Measured mass | Calculated mass | Elemental composition |
|---|---|---|---|---|
| Diclofenac | $[M+H]^+$ | 296.0248 | 296.0245 | $C_{14}H_{12}NO_2Cl_2$ |
| Diclofenac glucuronide | $[M+H]^+$ | 472.0568 | 472.0566 | $C_{20}H_{20}NO_8Cl_2$ |
| D1 | $[M+H]^+$ | 399.0336 | 399.0337 | $C_{17}H_{17}N_2O_3SCl_2$ |
| D2 | $[M+H]^+$ | 518.0385 | 518.0378 | $C_{20}H_{22}N_3O_5S_2Cl_2$ |
| D3 | $[M+H]^+$ | 456.0545 | 456.0552 | $C_{19}H_{19}N_3O_4SCl_2$ |
| Ketoprofen | $[M+H]^+$ | 255.1026 | 255.1021 | $C_{16}H_{15}O_3$ |
| Ketoprofen glucuronide | $[M+H]^+$ | 431.1349 | 431.1342 | $C_{22}H_{23}O_9$ |
| K1 | $[M+H]^+$ | 358.1121 | 358.1113 | $C_{19}H_{20}NO_4S$ |
| Ibuprofen | $[M+NH_4]^+$ | 224.1653 | 224.1651 | $C_{13}H_{22}NO_2$ |
| Ibuprofen glucuronide | $[M+NH_4]^+$ | 400.1973 | 400.1971 | $C_{19}H_{30}NO_8$ |

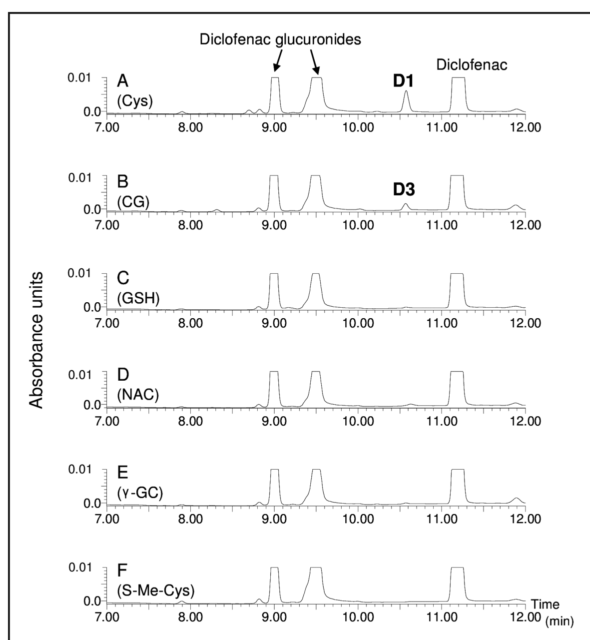


Fig. 6: PDA chromatograms (extracted at 225 nm) of human microsomal incubations of diclofenac with UDPGA and Cys derivative (A, Cys; B, CG; C, GSH; D, NAC; E, γ-GC; F, S-Me-Cys).

### 2.3. Postulated mechanism of amide adduct formation

The postulated reaction pathway for the formation of *N*-acyl-Cys amide adduct is shown in Fig. 8. The *N*-acyl-Cys amide adduct of a carboxylic compound is likely to be formed *via* a route beginning with an acyl glucuronide. Initially, the *S*-acyl-Cys thioester adduct could be formed *via* transacylation in which cysteine displaces a reactive 1-*O*-acyl glucuronide, with the resulting acyl thioester undergoing intramolecular S- to N-acyl rearrangement to generate the *N*-acyl-Cys amide adduct. Interestingly, the γ-glutamyltranspeptidase (γ-GT)-mediated degradation of the *S*-acyl-GSH conjugates (S-G) of various carboxylic acid drugs leads to the formation of *N*-acyl-cysteinylglycine conjugates (N-CG) (Tate. 1975; Grillo and Benet 2001; Grillo and Hua 2003b; Grillo et al. 2008). In addition, the N-CG disulfide of clofibric acid is an oxidative product of the N-CG. It is presumed that the *S*-acyl-cysteinylglycine conjugate (S-CG) is produced by the cleavage of S-G, followed by an intramolecular S- to N-rearrangement, forming the more stable amide product, N-CG. This reaction proceeds chemically at physiological conditions (pH 7.4). The postulated mechanism described above is based on the reactivity of the thioester bond. Recent findings suggest that thioester-linked compounds such as *S*-acyl GSH conjugates and *S*-acyl CoAs may be unstable, and therefore more reactive species than esters (Grillo and Benet 2002; Grillo et al. 2003c; Sidenius et al. 2004; Olsen et al. 2005; Mitamura et al. 2007; Skonberg et al. 2008).

Similar reactions have been reported, not only in drug metabolism, but also in macromolecular science. The chemoselective reaction of two unprotected peptides to give a single ligation product is widely used for protein synthesis, and is known as native chemical ligation (NCL) (Dawson et al. 1994; Johnson and Kent 2006; Baslé et al. 2010). It relies on an S- to N-rearrangement of a thioester into an amide. A peptide containing a C-terminal thioester group reacts with another peptide containing an N-terminal Cys residue to give a thioester-linked intermediate, which spontaneously rearranges through intramolecular nucleophilic attack by the Cys amino group to form a native amide bond. NCL proceeds non-enzymatically in aqueous solutions at a neutral pH. This shows that the

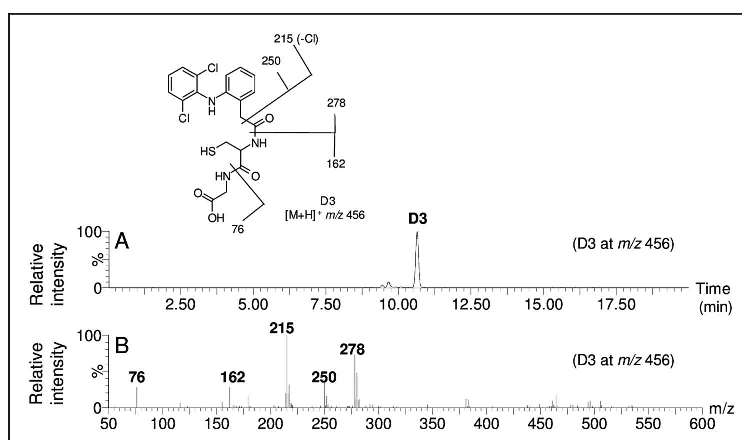


Fig. 7: MS data of D3. (A) extracted ion mass chromatogram and (B) product ion mass spectrum.

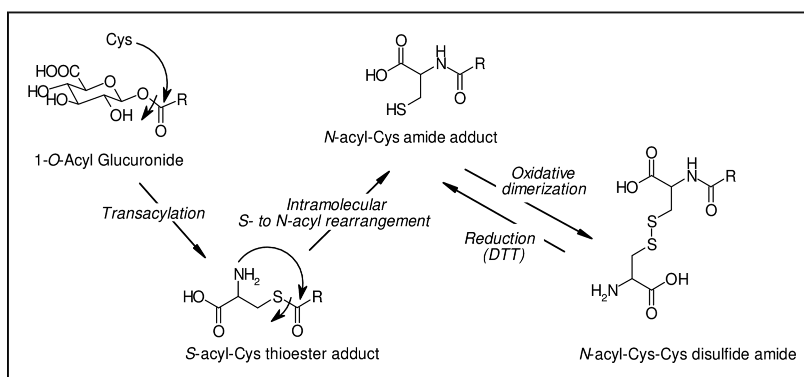


Fig. 8: Proposed reaction pathways for the formation of an *N*-acyl-Cys amide adduct (R = xenobiotic moiety).

thioesters are highly reactive and that S- to N-acyl rearrangement is a common and reasonable reaction in physiological conditions.

The mechanism of the NCL reaction also indicates that thioester linked-compounds could react with body macromolecules such as peptides, proteins and other N-containing components *in vivo*. 2-Phenylpropionyl-*S*-acyl-CoA was reported to cause covalent binding to bovine serum albumin in vitro, indicating the formation of *N*-amide-linked protein adducts (Li et al. 2002). The S- to N-rearrangement is also involved in the interaction between 2-mercaptobenzamide thioester compounds and the HIV-1 nucleocapsid protein (NCp7), which is widely regarded as a potential target for the development of antiviral drugs (Miller Jenkins et al. 2007). The proposed reaction mechanism for this interaction is an acyl transfer from the thioester of the compound to a Cys sulfur and then to a lysine nitrogen in the C-terminal zinc-binding domain of NCp7. In addition, several *in vivo* NCL reactions have been described. A cell-based native chemical ligation approach was developed for cyclization of the N-terminal Src homology 3 (SH3) domain from murine c-Crk adapter protein in *Escherichia coli* cells (Camarero et al. 2001). An *in vivo* biotinylation approach was developed in living cells for protein microarray applications (Yeo et al. 2004). It has also proposed that bile acid acyl adenylates with a phosphoester bond can form covalent bile acid adducts with peptides and proteins (Goto et al. 2001). Furthermore, bile acid acyl adenylates can cause the formation of the bile acid adduct on the lysine residues in the human histone H3 amino-terminal tail domain (Mano et al. 2004). Collectively, these examples confirm that various activated esters are sufficiently reactive to modify macromolecules *in vivo*. It is likely that a carboxylic acid group obtains higher reactivity by activation through acyl glucuronidation and subsequent thioesterification.

These findings show that this reaction is not only useful for detecting unstable thioester intermediates as the amide adducts and for evaluating the reactivity of them, but also important in the potential bioactivation mechanism of the covalent binding of carboxylic acid drugs to macromolecules. It has been proposed that Cys residues of proteins are presumably involved in the mechanism of stable adduct formation through acyl transfer. This has significant implications for the consideration of potential toxicity mechanisms for investigational drugs.

In conclusion, the Cys amide adducts of carboxylic acid drugs via UGT-mediated bioactivation were detectable and able to be characterized using UPLC-QTOF/MS. This reaction using Cys as a trapping agent can potentially simplify the evaluation of bioactivation for investigational drugs. Additionally, our findings have important implications not only for evaluating the reactivity of acyl glucuronides, but also for a possible bioactivation mechanism of the covalent binding of carboxylic acid drugs to macromolecules via S- to N-acyl rearrangement.

## 3. Experimental

### 3.1. Materials

Diclofenac, ketoprofen, ibuprofen, UDP-glucuronic acid (UDPGA), L-cysteine (Cys), glutathione (GSH), *N*-acetyl-L-cysteine (NAC), *N*-L-cysteinylglycine (CG), *N*-L-γ-glutamyl-L-cysteine (γ-GC), *S*-methyl-L-cysteine (*S*-Me-Cys) and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). Pooled male human liver microsomes were purchased from XenoTech (Lenexa, KS, USA). Acetonitrile and distilled water were purchased from Kanto-Chemicals (Tokyo, Japan). Ammonium acetate was purchased from Nacalai Tesque (Kyoto, Japan). Other chemicals and reagents were of the highest available quality.

### 3.2. Incubation with human liver microsomes

Individual incubation mixtures (final volume of 0.1 mL) contained 0.1 mM test compound (diclofenac, ketoprofen or ibuprofen), 1.0 mg protein/mL human liver microsomes, 5 mM UDPGA and 5 mM cysteine derivative (Cys, GSH, NAC, CG, γ-GC or S-Me-Cys) in 50 mM potassium phosphate buffer (pH 7.4). The incubation buffers containing the respective cysteine derivatives were tested for pH. Reactions were conducted at 37 °C in a water bath and terminated after 60 min by the addition of 0.1 mL of acetonitrile. After quenching the reaction, the samples were centrifuged at 10,000 × g for 5 min, and the resulting supernatants were analyzed by UPLC-QTOF/MS system.

### 3.3. UPLC-QTOF/MS analysis

Liquid chromatography was performed on an Acquity UPLC system (Waters, Tokyo, Japan). The separations were performed on an Acquity UPLC BEH C18 (1.7 μm, 2.1 × 50 mm; Waters). The column was eluted by gradient elution with solvent A (10 mM ammonium acetate, pH 5.0) and solvent B (acetonitrile) at a flow rate of 0.2 mL/min and set at 40 °C. The eluting gradient conditions were as follows: 0–3 min, 15% B; 3–13 min, 15–60% B; 13–16 min, 60% B; 16–20 min, 15% B. The wavelength of the photodiode array (PDA) detector ranged from 200 to 400 nm.

Mass spectrometry was performed on a Xevo G2 QTof accurate mass spectrometer (Waters) with an electrospray ionization (ESI) interface. MS conditions in the positive ion mode were as follows: capillary voltage, 3.0 kV; sampling cone voltage, 20 V; extraction cone voltage, 3.0 V; source temperature, 120 °C; desolvation temperature, 350 °C; cone gas, 50 L/h; desolvation gas, 800 L/h. MS resolution was more than 20,000 (full width at half maximum, FWHM) in resolution mode. The $MS^E$ scan was conducted under the following conditions: scan range *m/z*, 200–800; collision energy, 6 eV (low) and 15–35 eV (high). Accurate mass calibration was performed using sodium formate. All mass spectra were corrected using Leu-enkephalin, introduced by means of a lockspray device. Data were acquired and analyzed using MassLynx software V.4.1 (Waters).

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