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Correlation between glucuronidation and covalent adducts formation with proteins of nonsteroidal anti-inflammatory drugs

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) can cause idiosyncratic liver injury. Mechanisms involved in NSAID-induced liver injury are complex. Previous studies have suggested that acyl glucuronide of NSAIDs (NSAIDs-Glu) plays an important role in the development of liver injury via covalently binds to proteins and the resultant adduct induces immunological toxicity. As only some NSAIDs-Glu are commercially available, the evaluation of covalent protein adduct formation using ready-made NSAIDs-Glu is difficult and inconvenient. Moreover, glucuronidation potency varies with the NSAID, including stereoisomers. Therefore, in this study, we simultaneously examined the glucuronidation and covalent adduct formation using enantiomers of parent NSAIDs (ibuprofen, naproxen, pranoprofen, ketoprofen, and flurbiprofen) in rat liver microsomes. Glucuronides and covalent adducts were quantified by HPLC. The amount of covalent adduct increased with NSAIDs-Glu formation in the rat liver microsomes in a time-dependent manner. A significant positive correlation was observed between the *AUC* of NSAIDs-Glu and that of covalent adduct, except ketoprofen. Although ketoprofen exhibited the highest glucuronidation rate among the NSAIDs investigated, the amount of covalent adduct was similar to that for pranoprofen, which had the lowest glucuronidation rate. Thus, it may be difficult for ketoprofen glucuronide to covalently bind with proteins in the rat liver microsomes. Our results suggested that the amount of glucuronide formed is a key factor in predicting covalent bond formation with protein in NSAIDs, in addition to degradability and bindability with proteins of NSAIDs-Glu. Further studies are required to confirm the relationship between the tendency of glucuronidation and the formation of covalent adducts of NSAIDs.

1. Introduction

Drug-induced liver injury (DILI) is the primary cause for discontinuation of clinical studies and withdrawal of new drug from market. Among the various types of drugs, NSAIDs are the most common drugs that cause liver injury (Björnsson, 2010). In 1992, the incidence of NSAID-induced liver injury was reported to be 9 cases/100,000 people annually (García et al., 1992). Although the detailed mechanisms underlying NSAID-induced liver injury remains unclear, acyl glucuronide conjugates (AGs) of NSAIDs (NSAIDs-Glu) may play an important role in the development of liver injury.

Glucuronidation is a major metabolic pathway for carboxylic acid-containing drugs, including NSAIDs, in the liver. Almost all the AGs formed (i.e. AGs that generated from parent drugs) are excreted into bile and/or urine. However, owing to their chemical instability at physiological pH, AGs can undergo hydrolysis and isomerization at the anomeric center. Furthermore, AGs are reactive metabolites and form covalent bonds with endogenous proteins that defined as covalent adduct in this study. AG-bound proteins are suppressed their functions and induce immune reaction as an antigen. Interestingly, the degradation rate constant of AGs in the buffer containing albumin is significantly related to the extent of covalent binding with human serum albumin in several drugs (Benet et al., 1993). Consistently, the risk of idiosyncratic drug toxicity (e.g. DILI, Stevens-Johnson syndrome, and anaphylaxis) exhibited good correlation with instability of AGs in potassium phosphate buffer (Sawamura et al., 2010). Moreover, the exposure of AGs directly promotes the production of inflammatory cytokines in peripheral blood monocytes (Miyashita et al., 2014). Thus, both direct cytotoxicity and immune toxicity triggered by AGs could be the possible mechanisms underlying DILI.

We have been conducting research on the hepatic disposition of NSAIDs using in situ liver perfusion (Ikuta et al., 2017; Uraki et al., 2016a). In our recent study, using sandwich-cultured hepatocytes, we observed a quantitative relationship among the amount of accumulated

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AG of diclofenac (DIC-Glu), extent of covalent binding, and cytotoxicity (Kawase et al., 2017). However, these methods are too complicated to evaluate formation of AGs and covalent adduct in the liver. Therefore, numerous studies have been conducted to evaluate the extent of covalent bond formation between NSAIDs-Glu and protein by incubating serum albumin with ready-made NSAIDs-Glu (Bischer et al., 1995a; Smith et al., 1990; Volland et al., 1991). Although protein binding assay using ready-made NSAIDs-Glu appears to be easy, it is difficult in practice due to their chemical instability in vitro. The half-lives of most NSAIDs-Glu are < 1 h in phosphate buffer at pH 7.4 and 37 °C (Sawamura et al., 2010). Furthermore, as the potency of glucuronidation varies with the NSAID (Magdalou et al., 1990), glucuronidation properties can affect subsequent covalent bond formation.

Although most NSAIDs, particularly 2-arylpropanoic acids, are used as a racemate in clinical practice, stereoselective pharmacokinetics (Foster and Jamali, 1988; Iwaki et al., 1995; Jamali et al., 1988), glucuronidation (Ikuta et al., 2017; Chakir et al., 1994; Mano et al., 2007), protein binding (Bischer et al., 1995b), and mitochondrial toxicity (Browne et al., 1999) have been reported in enantiomers of NSAIDs. These results indicate that development of NSAIDs-induced liver injury can depend on stereoselective character of NSAIDs. Therefore, selective use of enantiomer that exhibits lower glucuronidation and protein binding can bring to decrease incidence of liver injury.

In this study, we hypothesized that the extent of covalent bond formation of NSAIDs-Glu can be predicted from the kinetic profiles of glucuronidation of parent NSAIDs. The elucidation of correlation between glucuronidation and covalent binding may provide useful information for developing a simple screening assay to estimate the risk of DILI. To evaluate the stereoselective relationship between AGs formed and the extent of covalent binding of NSAIDs [enantiomers of ibuprofen (IB), naproxen (NA), pranoprofen (PR), ketoprofen (KE), and flurbiprofen (FL)], an in vitro metabolism assay was performed using liver microsomes.

2. Materials and methods

2.1. Chemicals

Enantiomers of IB and FL were obtained from Maluho Co. Ltd. (Kyoto, Japan) and Sumitomo Dainippon Pharma Co., Ltd. (Osaka, Japan). *S*- and *R*-NA were purchased from Sigma Aldrich (St. Louis, MO) and Syntex (Mississauga, Ontario, Canada). Enantiomers of PR and KE were obtained from SSP Co. Ltd. (Tokyo, Japan) and Towa pharmaceutical Co., Ltd. (Kyoto, Japan). All other compounds and reagents were commercial products of reagent grade.

2.2. Preparation of liver microsomes from rats

Male Wistar/ST rats (weighing 240–290 g, aged 8–9 weeks) were used in this study. All animals were allowed free access to food and water under a standard 12-h light/12-h dark cycle in a temperature-controlled room (24 ± 2 °C). Animals were acclimatized for a week before use. All animal experiments were performed in accordance with the requirements of Committee for the Care and Use of Laboratory Animals of the School of Pharmacy of Kindai University. Liver microsomes were prepared as previously described with minor modifications (Komura and Iwaki, 2005). The protein concentration of the microsomes was determined with a BCA protein assay kit (Thermo Scientific, Rockford, IL).

2.3. Glucuronidation assay in rat liver microsomes

The liver microsomes were diluted at 10 mg protein/mL in 100 mM Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 0.2% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM D-saccharic acid 1,4-lactone, and 1 mM of *S*- or *R*-NSAIDs. Glucuronidation assay was initiated by adding 6 mM of uridine 5′-diphosphoglucuronic acid in the reaction mixture after 5 min of preincubation at 37 °C. The reaction mixture was collected at designated time points to quantify the glucuronide of substrates and covalent adduct formed with microsomal protein by high performance liquid chromatography (HPLC).

2.4. Quantitation of glucuronides

NSAIDs-Glu (AGs and its isomers) were determined by HPLC as previously described with minor modifications (Uraki et al., 2016b; Nagao et al., 2003; Iwaki et al., 1999). In brief, to quantify the glucuronide metabolites formed, 170 μL of 2% phosphate containing internal standards (indomethacin for IB, 3-indoleethanol for NA and PR, proxicam for KE, etodolac for FL) diluted in methanol were added to 25 μL of collected samples, and the samples were centrifuged at 12,000 × *g* for 10 min at 4 °C. The resultant supernatant was used as measurement sample for HPLC method. IB glucuronide was analyzed using Fluofix column (4.6 × 250 mm, 5 μm, Wako Pure Chemical Industries, Osaka, Japan) maintained at 40 °C, with a mobile phase comprising acetonitrile, water, and phosphate at a ratio of 40:60:0.1, *v*/v. The flow rate of mobile phase was 2.0 mL/min and IB glucuronide was detected by UV absorption at 237 nm (Shimadzu-SPD-6A, Shimadzu, Kyoto, Japan). The glucuronides of NA, KE, and FL were analyzed with Cosmosil 5C18-AR (4.6 × 250 mm, 5 μm, Nacalai Tesque, Kyoto, Japan) maintained at 35 °C. The mobile phases used for NA, KE, and FL glucuronides were acetonitrile/0.05 M ammonium acetate (30:70, *v*/v, pH 6.0), acetonitrile/2 mM tetrabutylammonium hydrogen sulfate containing 0.05 M phosphate buffer (30:70, *v*/v, pH 5.0), and acetonitrile/2 mM tetrabutylammonium hydrogen sulfate containing 0.05 M phosphate buffer (35:65, v/v, pH 5.5), respectively. The flow rate for all three mobile phases was 1.0 mL/min. NA glucuronide was detected using a fluorescence detector (Shimadzu-RF-535, Shimadzu); the excitation and emission wavelengths were set at 275 and 355 nm, respectively. KE and FL glucuronides were detected by UV absorption at 254 nm. PR glucuronide was analyzed with Inertsil ODS-2 column (4.6 × 250 mm, 5 μm, GL Science, Tokyo, Japan) maintained at 35 °C. The mobile phase for PR glucuronide was acetonitrile, water, and phosphate at a ratio of 25:75:0.1, *v*/v, and the flow rate was 1.3 mL/min. The excitation and emission wavelengths of the fluorescence detector were set at 300 and 355 nm, respectively.

2.5. Quantitation of covalent adducts

Covalent adducts with rat liver microsomal proteins were quantified according to a previously described method (Zia-Amirhosseini et al., 1994). In brief, ice-cold isopropanol (0.5 mL) and methanol/phosphate (3:0.01, *v*/v, 2 mL) were added to the collected samples (0.5 mL) and the samples were centrifuged at 300 × *g* for 10 min at 4 °C. The resultant pellet was washed seven times with methanol/diethyl ether (3:1, *v*/v, 3 mL). Then, the pellet was heated with 1 mL of 0.5 M KOH at 80 °C for overnight. The mixture was acidified by adding phosphate (50 μL of 17% solution in water) and after the addition of internal standards, the liberated parent drugs from microsome proteins were extracted with 4 mL of dichloromethane. The organic phase of each sample was dried under vacuum and reconstituted into the mobile phase and quantified by HPLC.

IB, PR, and FL were analyzed with Inertsil ODS-2 column (4.6 × 150 mm, 5 μm, GL Science). The mobile phase used was acetonitrile/water/phosphate (IB, 55:45:0.1, *v*/v; PR, 22:78:0.1, v/v; FL, 50:50:0.1, *v*/v). NA and KE were analyzed with Inertsil ODS-3 column (4.6 × 250 mm, 5 μm, GL Science) and the mobile phase used for KE was acetonitrile/0.05 M ammonium acetate (30:70, *v*/v, pH 6.0). Other conditions of HPLC analysis for liberated parent NSAIDs were performed same as NSAIDs-Glu.