Toxicology in Vitro 30 (2015) 241–249

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/tiv

Toxicological evaluation of acyl glucuronides utilizing half-lives, peptide adducts, and immunostimulation assays

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ARTICLE INFO

Article history:
Received 26 June 2015
Received in revised form 6 October 2015
Accepted 30 October 2015
Available online 31 October 2015

Keywords:
Drug-induced liver injury
Acyl glucuronides
Half-lives assay
Peptide adducts assay
Immunostimulation assay

ABSTRACT

Chemical reactivity of acyl glucuronides (AGs) is believed to be involved in the toxicity of carboxylic acid-containing drugs. Both direct and immune-mediated toxicity have been suggested as possible mechanisms of toxicity; however, it remains unclear. In the present study, we performed assays of half-lives, peptide adducts, and immunostimulation to evaluate the potential risk of AGs of 21 drugs and analyzed the relationship to the toxic category. AGs of all withdrawn drugs tested in this study showed short half-lives and peptide adducts formation, but so did those of several safe drugs. In contrast, only AGs of withdrawn and warning drugs induced interleukin-8 (IL-8) in human peripheral blood mononuclear cells (hPBMCs). Using a DNA microarray assay, we found that zomepirac AG induced the mRNAs of 5 genes, including IL-8 in hPBMCs. In addition, withdrawn and warning drugs were distinguished from safe drugs by an integrated score of relative mRNA expression levels of 5 genes. The immunostimulation assay showed higher sensitivity, specificity, and accuracy compared with other methods. In preclinical drug development, the evaluation of the reactivity of AGs using half-lives and peptide adducts assays followed by the evaluation of immunostimulation by highly reactive AGs using hPBMCs can contribute to improved drug safety.

1. Introduction

Acyl glucuronidation is one of the major metabolic routes of carboxylic acid-containing drugs. Glucuronidation is an important phase II metabolic pathway for endogenous and exogenous substrates and is generally considered as a detoxification pathway. However, acyl glucuronides (AGs) are unstable under physiological conditions and consequently undergo hydrolysis or intramolecular rearrangement through the migration of the drug moiety from the 1-O-position to the 2-, 3-, or 4-positions on the glucuronic acid ring (Bailey and Dickinson, 2003; Benet et al., 1993; Smith et al., 1990). Because of their electrophilic nature and capacity to cause substitution reactions with nucleophilic groups in proteins or other macromolecules, AGs can covalently modify endogenous proteins leading to the adverse toxicity associated with carboxylic acid-containing drugs (Boelsterli, 2002; Faed, 1984). Till date, both direct and immune- and inflammation-mediated toxic pathways have been suggested as possible toxic mechanisms of AGs. Previous studies (Nakayama et al., 2009; Usui et al., 2009) have reported that zomepirac and bromfenac, which are carboxylic acid-containing drugs that have been withdrawn from the market, showed low covalent binding to proteins in human hepatocytes. In addition, the AGs of the widely used drugs naproxen, diclofenac, ketoprofen, and ibuprofen did not lead to cytotoxicity or genotoxicity in uridine 5′-diphosphate (UDP)-glucuronosyltransferase (UGT)–transfected human embryonic kidney 293 (HEK/UGT) cells and human hepatocytes (Koga et al., 2011). In contrast, it has been reported that mycophenolic acid AG induced tumor necrosis factor alpha (TNFα) and interleukin 6 (IL-6), and diclofenac AG induced IL-8 and monocyte chemoattractant protein 1 (MCP-1) in leukocytes (Miyashita et al., 2014; Wieland et al., 2000), suggesting that the induction of immune modulators could lead to immune- and/or inflammation-related adverse drug reactions.

Abbreviations: AG, acyl glucuronide; dKF, dansylated Lys-Phe; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK, human embryonic kidney; hPBMC, human peripheral blood mononuclear cell; HPLC, high-performance liquid chromatography; IL-1α, interleukin 1 alpha; IL-6, interleukin 6; IL-8, interleukin 8; KPB, potassium phosphate buffer; LC, liquid chromatography; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein 1; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MT, metallothionein; NAMPT, nicotinamide phosphoribosyltransferase; TNFα, tumor necrosis factor alpha; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; UPLC, ultra-performance liquid chromatography.

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http://dx.doi.org/10.1016/j.tiv.2015.10.013
0887-2333/

Several in vitro assay methods to assess the toxicity of AGs have been proposed. The first is an evaluation of the half-lives of AGs in potassium phosphate buffer (KPB). The half-lives of AGs in withdrawn drugs were shorter than those in safe drugs. Therefore, this assay is useful to predict the risk of toxicity (Jinno et al., 2013; Sawamura et al., 2010). The second method is a Lys-Phe adducts assay, wherein Lys-Phe is used as a novel trapping agent that forms glycation adducts via a Schiff base. In this assay, a correlation was observed between the formation of a peptide adduct and the rearrangement rate of the primary AG of 7 drugs (Wang et al., 2004). The third method is an immunostimulation assay using human peripheral blood mononuclear cells (hPBMCs), wherein cytokines and chemokines, such as IL-6 and IL-8, were induced in hPBMCs by treatment with AGs (Miyashita et al., 2014; Wieland et al., 2000). Although the predictability of the assay of half-lives for drug toxicity has been evaluated (Jinno et al., 2013; Sawamura et al., 2010), the relationship between the results of the other two assays and drug toxicity remains to be investigated. The purpose of the present study was to evaluate the relationship of the results of assays of half-lives, peptide adducts, and immunostimulation to the toxic category of carboxylic acid-containing drugs defined by description in drug package inserts, and then to compare the usability of the three assays to assess the risk of toxicity of AGs in preclinical drug discovery. We modified the assay of peptide adducts by using dansylated Lys-Phe (dKF), peptide-AG adducts of which were easily detectable by fluorescence.

2. Materials and methods

2.1. Chemicals and reagents

Oxaprozin, pranoprofen, etodolac, and dKF were prepared in-house by chemosynthesis. Probenecid was obtained from Wako Pure Chemical Industries (Osaka, Japan). Diclofenac sodium salt, tolmetin sodium salt dihydrate, zomepirac sodium salt, mefenamic acid, bumetanide, furosemide, flufenamic acid, meclofenamic acid sodium salt, ibuprofen, and repaglinide were obtained from Sigma-Aldrich (St. Louis, MO). Montelukast sodium and telmisartan were obtained from Kemprotec (Middlesborough, UK). Gemfibrozil was obtained from LKT Laboratories (St. Paul, MN). Naproxen sodium was obtained from Tocris Bioscience (Ellisville, MO). Ibufenac, benoxaprofen, piretanide, and AGs of zomepirac, benoxaprofen, tolmetin, ibufenac, diclofenac, mefenamic acid, probenecid, naproxen, gemfibrozil, furosemide, repaglinide, telmisartan, and ibuprofen were purchased from Toronto Research Chemicals (Ontario, Canada). Pooled human liver microsomes were obtained from XenoTech, LLC (Lenexa, KS). The UGT reaction mix solution (250 mM Tris–HCl (pH 7.4), 40 mM $MgCl_2$, and 0.125 mg/ml alamethicin) was purchased from Corning Gentest (Woburn, MA). Characterized hPBMCs (lot no. LP80), uncharacterized hPBMCs (10 individuals), and CTL-Test medium were obtained from Cellular Technology (Shaker Heights, OH). TRIzol and the High-Capacity cDNA Reverse Transcription Kit were obtained from Life Technologies (Carlsbad, CA). TaqMan Universal Master Mix, TaqMan Gene Expression Assays, and Human GAPDH Endogenous Control (FAM/MGB probe, non-primer limited) were obtained from Applied Biosystems (Darmstadt, Germany). The Human IL-8 ELISA Ready-SET-GO! Kit (2nd Generation) was obtained from eBioscience (San Diego, CA). Other chemicals were analytical grade or the highest grade commercially available.

2.2. Formation of AGs and degradation in KPB in assay of half-lives

A chemical stability study was performed according to the methods described by Chen et al. (2007), with slight modifications. The test compounds were incubated at concentrations of 500 μM at 37 °C for 60 min with pooled human liver microsomes (2.0 mg protein/ml) in 100 mM Tris–HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 50 μg/ml alamethicin, and 4 mM UDP-glucuronic acid. The volume of each reaction mixture was 300 μl, and the reaction was stopped by adding an equal volume of acetonitrile. The reaction-stopped mixture was transferred to new reaction tubes and mixed with 4 volumes of 100 mM KPB (pH 7.4). The samples were incubated at 37 °C and aliquots were removed at 0, 10, 20, 40, 90, 180, and 300 min. The reaction was stopped by adding an equal volume of acetonitrile/methanol (90/10) containing 1% formic acid and the internal standard (0.2 μM niflumic acid). The reaction mixture was centrifuged at 3000 rpm at 4 °C for 5 min, and then the supernatants were collected and stored at −20 °C until analysis.

2.3. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of AGs in assay of half-lives

To determine half-lives, the 1-O-β-AGs in samples prepared as described above were measured by LC–MS/MS. An Acquity system (Waters, Milford, MA) equipped with a Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 mm × 100 mm) and a triple quadrupole mass spectrometer (Quattro Premier and Xevo TQ MS; Waters) were used. The AGs were separated from other isomers and metabolites using an elution gradient. The mobile phases were 0.1% formic acid and acetonitrile. The column was eluted at a flow rate of 0.4 ml/min at 50 °C. The elution program and the MS/MS condition for detection of AGs are shown in Supplementary Tables 1 and 2, respectively.

2.4. Calculating the half-lives of AGs

The degradation rate constant (K) of each AG was determined from the LC–MS/MS peak area of 1-O-β-AG versus time curve by the linear regression of the semi-logarithmic plot. The half-lives ($T1/2$) were calculated from K by the following equation:

$$T1/2 = \ln\ 2/K.$$

2.5. Formation of AG–dKF adducts

The test compounds were incubated at concentrations of 500 μM with pooled human liver microsomes (2.0 mg protein/ml) in 88 mM KPB (pH 7.4) containing 8 mM $MgCl_2$, 25 μg/ml alamethicin, and 4 mM UDP-glucuronic acid at 37 °C for 60 min. The volume of the mixture was 500 μl, and the reaction was stopped by adding 1 ml of ice-cold acetonitrile, followed by centrifugation at 14,000 rpm at 4 °C for 5 min. The supernatant (1.4 ml) was transferred into another test tube, evaporated to dryness under a N_2 stream at 40 °C, and resuspended in 500 μl of acetonitrile/KPB (15/85) containing 10 mM dKF. After 3 h incubation at 37 °C, the mixture was evaporated to dryness under a N_2 stream at 40 °C, resuspended in 500 μl of acetonitrile/water (50/50), and filtrated (0.2-μm filter) by centrifugation at 4500 g at 4 °C for 10 min. The AG–dKF adducts were analyzed by LC–mass spectrometry (MS) and fluorescence as follows.

2.6. LC–MS analysis of AG–dKF adducts

Samples prepared as described above were subjected to LC–MS. An Acquity system (Waters) equipped with a Waters Acquity UPLC HSS T_3 column (1.8 μm, 2.1 mm × 50 mm) was used. Mobile phases were 0.1% formic acid (solvent A) and 90% acetonitrile containing 0.1% formic acid (solvent B). The column was eluted at a flow rate of 0.6 ml/min at 40 °C. Conditions for elution were as follows: 20% solvent B (0–1 min), 20%–50% B (1–7 min), 50%–90% B (7–13 min), 90% B (13–14 min), and 20% B (14.1–15.5 min). The dKF adducts were detected by fluorescence (excitation 340 nm, emission 525 nm) using a fluorescence detector (2475 Multi λ Fluorescence Detector; Waters) and mass spectrometry using a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP; AB Sciex, Framingham, MA). Parameters for mass spectrometers were as follows: ion mode, positive-