Zomepirac Acyl Glucuronide Is Responsible for Zomepirac-Induced Acute Kidney Injury in Mice

Atsushi Iwamura, Katsuhito Watanabe, Sho Akai, Tsubasa Nishinosono, Koichi Tsuneyama, Shingo Oda, Toshiyuki Kume, and Tsuyoshi Yokoi

Drug Metabolism and Pharmacokinetics Research Laboratory, Mitsubishi Tanabe Pharma Corporation, Saitama, Japan (A.I., T.K.); Department of Drug Safety Sciences, Nagoya University Graduate School of Medicine, Aichi, Japan (K.W., S.A., T.N., S.O., T.Y.); Department of Molecular and Environmental Pathology, Institute of Health Biosciences, Tokushima University Graduate School, Tokushima, Japan (K.T.); and Drug Metabolism and Toxicology, Faculty of Pharmaceutical Sciences, Kanazawa University, Ishikawa, Japan (A.I.)

Received January 19, 2016; accepted April 21, 2016

ABSTRACT

Glucuronidation, an important phase II metabolic route, is generally considered to be a detoxification pathway. However, acyl glucuronides (AGs) have been implicated in the toxicity of carboxylic acid drugs due to their electrophilic reactivity. Zomepirac (ZP) was withdrawn from the market because of adverse effects such as renal toxicity. Although ZP is mainly metabolized to acyl glucuronide (ZP-AG) by UDP-glucuronosyltransferase, the role of ZP-AG in renal toxicity is unknown. In this study, we established a ZP-induced kidney injury mouse model by pretreatment with tri-o-tolyl phosphate (TOTP), a nonselective esterase inhibitor, and L-buthionine-(S,R)-sulfoximine (BSO), a glutathione synthesis inhibitor. The role of ZP-AG in renal toxicity was investigated using this model. The model showed significant increases in blood urea nitrogen (BUN) and creatinine (CRE), but not alanine aminotransferase. The ZP-AG concentrations were elevated by cotreatment with TOTP in the plasma and liver and especially in the kidney. The ZP-AG concentrations in the kidney correlated with values for BUN and CRE. Upon histopathological examination, vacuoles and infiltration of mononuclear cells were observed in the model mouse. In addition to immune-related responses, oxidative stress markers, such as the glutathione/disulfide glutathione ratio and malondialdehyde levels, were different in the mouse model. The suppression of ZP-induced kidney injury by tempol, an antioxidant agent, suggested the involvement of oxidative stress in ZP-induced kidney injury. This is the first study to demonstrate that AG accumulation in the kidney by TOTP and BSO treatment could explain renal toxicity and to show the in vivo toxicological potential of AGs.

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 physiologic 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 position on the glucuronic acid ring (Smith et al., 1990a; Benet et al., 1993; Bailey and Dickinson, 2003). Because of their electrophilic nature and ability to cause substitution reactions with nucleophilic groups in proteins or other macromolecules, AGs can covalently modify endogenous proteins and have been postulated to cause the adverse toxicity associated with carboxylic acid–containing drugs (Faed, 1984; Boelsterli, 2002). To assess the toxicity of AGs, several in vitro assay systems, such as stability assay by measuring half-lives in potassium phosphate buffer, peptide adducts assay, and immunostimulation assay, have been proposed (Wang et al., 2004; Sawamura et al., 2010; Jinno et al., 2013; Miyashita et al., 2014; Iwamura et al., 2015). However, the toxicity of AGs has remained controversial because direct evidence of in vivo AG toxicity has not been provided.

Zomepirac (ZP), a nonsteroidal anti-inflammatory drug, was withdrawn from the market because of adverse effects such as anaphylaxis and renal toxicity (Smith, 1982; Miller et al., 1983; Heintz, 1995). ZP is mainly metabolized to acyl glucuronide (ZP-AG) in humans (Grindel et al., 1980; O’Neill et al., 1982). ZP-AG is more physicochemically unstable in phosphate buffer than the AGs of safe drugs, such as gemfibrozil, repaglinide, and telmisartan (Sawamura et al., 2010). ZP-AG also covalently modifies dipeptidyl peptidase IV in rat liver homogenates and microtubular protein in the bovine brain in vitro (Bailey et al., 1998; Wang et al., 2001). We previously demonstrated that ZP-AG showed the highest induction of the mRNA expression of immune- and inflammation-related genes in human peripheral blood mononuclear cells (PBMCs) in the AGs of 13 drugs (Iwamura et al., 2015). Although the toxicity of ZP-AG has been suggested, there is no evidence that ZP-AG is involved in ZP-induced toxicity in vivo in humans or laboratory animals because of the difficulty of toxicological

ABBREVIATIONS: AG, acyl glucuronide; ALT, alanine aminotransferase; BSO, L-buthionine-(S,R)-sulfoximine; BUN, blood urea nitrogen; CRE, creatinine; GSH, glutathione; GSSG, disulfide glutathione; HO-1, heme oxygenase 1; HPLC, high-performance liquid chromatography; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MDA, malondialdehyde; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; PBMC, peripheral blood mononuclear cell; RT, reverse-transcription; S100A9, S100 calcium-binding protein A9; TBARS, thiobarbituric acid reactive substances; TOTP, tri-o-tolyl phosphate; UGT, UDP-glucuronosyltransferase; ZP, zomepirac.
assessment under the conditions required for sufficient exposure to ZP-AG in vivo.

The level of AG production is determined by glucuronidation catalyzed by UDP-glucuronosyltransferase (UGT) and enzymatic hydrolysis. The enzymatic hydrolysis of AG is catalyzed by esterases such as acyleptide hydrolyase and α/β hydrolyase domain-containing 10 (Suzuki et al., 2010; Iwamura et al., 2012). It was reported that the plasma clearance of ZP-AG in the guinea pig was decreased by phenylmethylsulfonyl fluoride, a general esterase inhibitor, suggesting that ZP-AG is hydrolyzed by esterases (Smith et al., 1990b). In other reports, esterases were potently inhibited by tri-o-tolyl phosphate (TOTP), a nonselective esterase inhibitor, in mice and rats in vivo (Silver and Murphy, 1981; Kobayashi et al., 2012). ZP-AG conjugates with glutathione (GSH) in rat hepatocytes and bile (Grillo and Hua, 2003). Therefore, the ZP-AG level is regulated via hydrolysis by esterases and GSH conjugation against the generation by UGT. It is assumed that increased exposure to ZP-AG in vivo by TOTP and t-buthionine-(S,R)-sulfoximine (BSO), a glutathione synthesis inhibitor, after ZP administration may show that ZP-AG, rather than ZP, is involved in ZP-induced toxicity in vivo. The purpose of the present study was to establish the ZP-induced kidney injury mouse model and to investigate the role of ZP-AG in kidney injury.

Materials and Methods

Chemicals and Reagents. Reduced GSH, oxidized GSH, and BSO were purchased from Wako Pure Chemical Industries (Osaka, Japan). Zomepirac sodium and 4-hydroxy-2,6,6-tetramethylpiperidine-1-oxyl (tempol) were obtained from Sigma-Aldrich (St. Louis, MO). ZP-AG and TOTP were purchased from Toronto Research Chemicals (Ontario, Canada) and Acros Organics (Morris Plains, NJ, respectively). β-NAPDH and GSH reductase were obtained from Oriental Yeast (Tokyo, Japan). A ReverTra Ace qPCR RT kit was obtained from Toyobo (Osaka, Japan). RNAsin Plus and SYBR Premix Ex Taq (Tli RNaseH Plus) were obtained from Takara (Osaka, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Fuji DRI-CHROM slides of GPT/ALT-PIII, BUN-PIII, and CRE-PIII, which were used to measure alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine (CRE), respectively, were from Fujifilm (Tokyo, Japan). Rabbit polyclonal antibody against myeloperoxidase (MPO) was purchased from DAKO (Carpinteria, CA). A thiothiobarbituric acid reactive substances (TBARS) assay kit was obtained from Oxoid Biomedical Research (Oxford, MI). Other chemicals used in this study were of analytical grade or the highest grade commercially available.

Animals. Nine- to 11-week-old female BALB/cCrSlc mice were obtained from Japan SLC (Hamamatsu, Japan). The animals were housed under a 12-hour light/dark cycle (lights on 0900–2100 hours) in a controlled environment (temperature 23 ± 2°C and humidity 55 ± 10%) in the institutional animal facility. All animals were allowed free access to food and water, except when fasting was being conducted. The animals were acclimatized before use in the experiments. All procedures were carried out in accordance with the guidelines established by the Institute for Laboratory Animal Research of the Medical School of Nagoya University.

Administration of ZP, TOTP, and BSO. ZP was dissolved in potassium phosphate buffer (pH 7.4, 5–15 mg/ml) and intraperitoneally administered to the mice at a dose of 50–150 mg/kg. TOTP was dissolved in corn oil (10 mg/ml) and orally administered at a dose of 50 mg/kg to mice 12 hours before ZP administration.

BSO was dissolved in saline (70 mg/ml) and intraperitoneally administered at a dose of 700 mg/kg to mice 1 hour before ZP administration after overnight fasting. The BSO dosage was confirmed at 700 mg/kg because a decreased hepatic GSH level was previously reported at this dose (Shimizu et al., 2009).

Administration of Antioxidant Agent. Mice were intraperitoneally administered tempol, an antioxidant agent (200 mg/kg in sterilized phosphate-buffered saline) at 6 and 18 hours after ZP administration. The plasma was collected at 12 and 24 hours after ZP administration.

Concentrations of ZP and ZP-AG in Plasma, Liver, and Kidney. Plasma was obtained from heparinized blood by centrifugation at 13,000g for 5 minutes at 4°C, and tissues were frozen by liquid nitrogen immediately after collection. Plasma and tissues were stored at −80°C prior to analysis. Mice livers and kidneys were quickly homogenized in homogenate buffer (10 mM Tris–HCl (pH 7.4), 20% glycerol, 1 mM EDTA (pH 8.0)) on ice. Plasma and tissue concentrations of ZP and ZP-AG were determined by high-performance liquid chromatography (HPLC) according to a previous study (Smith et al., 1985a), with slight modifications. In brief, 5 μl of plasma or 1.25 mg of tissue homogenates was mixed with 20 μl of acetonitrile and 35 μl of 8% HClO4 to precipitate the protein. Smith et al. (1985a) previously demonstrated that adding acids and organic solvents stabilized ZP-AG. The mixture was centrifuged at 13,000g for 5 minutes, and a 40-μl sample of the supernatant was subjected to HPLC. The HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), and an L-2400 UV detector (Hitachi) equipped with a Mightysil RP-18 C18 GP column (5-μm particle size, 4.6 × 150-mm i.d.: Kanto Chemical, Tokyo, Japan). The eluent was monitored at 313 nm. The mobile phases were 47% methanol/10 mM acetate buffer (pH 5.0). The quantification of ZP and ZP-AG was performed by comparing the HPLC peak areas with those of authentic standards. Standard samples were prepared in blank plasma and tissue homogenates for ZP and ZP-AG. A preliminary study confirmed that blank plasma and tissue homogenates did not affect standard curves of ZP and ZP-AG when standards were taken through the same steps as measured samples.

Histopathological Examination. Kidney specimens were fixed in 10% neutral-buffered formalin. The fixed samples were dehydrated with alcohols and embedded in paraffin. Serial sections were stained with H&E for histopathological examination. Neutrophil infiltration was assessed by MPO immunostaining. A rabbit polyclonal antibody against MPO was used for kidney immunohistochemical staining, as previously described (Kumada et al., 2004). Two visual fields at a 200× magnification (0.2 mm² each) were randomly selected from each MPO-immunostained specimen. The average number of MPO-positive cells from two randomly selected visual fields was compared between the specimens.

Real-Time Reverse-Transcription Polymerase Chain Reaction. The RNA from mice livers and kidneys was isolated using RNAiso Plus according to the manufacturer’s instructions. The mRNA expression of interleukin 1α (IL-1α), IL-6, macrophage inflammatory protein-2 (MIP-2/CXCL2), intercellular adhesion molecule-1 (ICAM-1/CD54), S100 calcium-binding protein A9 (S100A9), and heme oxygenase 1 (HO-1) was quantified using real-time reverse-transcription (RT) polymerase chain reaction (PCR). RT was performed using a ReverTra Ace RT-qPCR kit, according to the manufacturer’s instructions. In brief, 1 μg of total RNA was mixed with an appropriate volume of 5-fold RT buffer, enzyme mix, primer mix, and nuclelease-free water to adjust the total volume to 20 μl, and the RT reaction was carried out at 37°C for 15 minutes and 98°C for 5 minutes. Real-time RT-PCR was performed using an Mx3000P (Agilent Technologies, Santa Clara, CA), and the

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequences</th>
<th>NCBI Accession No</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>5′- TTACAGTGAAACACGAAGAC -3'</td>
<td>NM_010554.4</td>
</tr>
<tr>
<td>AS IL-1α</td>
<td>5′- GATCTTGCAAGTCTCATGAA -3'</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>5′- CCTAGCTACCTGGAGTACA -3'</td>
<td>NM_031681.1</td>
</tr>
<tr>
<td>AS IL-6</td>
<td>5′- GGAATGGTGGTAGAAGAGA -3'</td>
<td></td>
</tr>
<tr>
<td>MIP-2</td>
<td>5′- AAAGTGGCTCTGGACCTGAA -3'</td>
<td>NM_009430.2</td>
</tr>
<tr>
<td>AS MIP-2</td>
<td>5′- ATCCAGTGATCCAGCCTTCC -3'</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5′- GCTACCATCACCCTGGATACC -3'</td>
<td>NM_001043.2</td>
</tr>
<tr>
<td>S100A9</td>
<td>5′- TGAATTTGCTGGCTTTCTCG -3'</td>
<td></td>
</tr>
<tr>
<td>AS S100A9</td>
<td>5′- AGTGGCCCAAAAGACAGCTT -3'</td>
<td></td>
</tr>
<tr>
<td>HO-1</td>
<td>5′- GCCACCTGAGGGTCAAGCACA -3'</td>
<td>NM_001442.2</td>
</tr>
<tr>
<td>AS HO-1</td>
<td>5′- ATCCATGCGAGTCCCTTACAA -3'</td>
<td></td>
</tr>
</tbody>
</table>

AS, antisense; S, sense; NCBI, National Center for Biotechnology Information.
PCR conditions included denaturation at 95°C for 30 seconds, followed by 40 amplification cycles of 95°C for 5 seconds and 60°C for all targets. The amplified products were monitored directly by measuring the increase in the intensity of the SYBR Green I dye binding to the double-stranded DNA amplified by PCR, and a dissociation curve analysis was conducted to confirm the amplification of the PCR product. The primer sequences used in this study are shown in Table 1.

Renal and Hepatic GSH Levels. Mice livers and kidneys were homogenized in ice-cold 5% sulfosalicylic acid and centrifuged at 8000 g for 10 minutes at 4°C. The supernatant was collected, and the total GSH and disulfide glutathione (GSSG) concentrations were measured as previously described (Tietze, 1969; Griffith, 1980). The reduced GSH contents were calculated from the total GSH and GSSG contents.

Renal and Hepatic Malondialdehyde Levels. Mice livers and kidneys were homogenized in homogenate buffer. A half volume of trichloroacetic acid (1 g/mL) was added to tissue homogenates to precipitate proteins and acidify the samples. The samples were then centrifuged at 12,000 g for 5 minutes at 4°C. The supernatant was collected, and the malondialdehyde (MDA) concentrations were measured using a TBARS assay kit (Oxford Biomedical Research) according to the manufacturer's protocol. The TBARS were detected by fluorescence (excitation 532 nm, emission 585 nm) using a PowerScan4 (DS Pharma Biomedical, Osaka, Japan).

Fig. 1. Changes in plasma CRE (A), BUN (B), and ALT (C) levels in female BALB/c mice after ZP administration. TOTP (50 mg/kg in corn oil, by mouth), BSO (700 mg/kg in saline, i.p.), and ZP (150 mg/kg in potassium phosphate buffer, i.p.) were administered as described in Materials and Methods. The plasma CRE, BUN, and ALT levels were measured 24 hours after ZP administration. The data are shown as the mean ± S.E.M. (n = 5–7). The statistical analyses were performed using one-way analysis of variance followed by Dunnett’s test. **P < 0.01 compared with the vehicle-treated (corn oil, saline, and potassium phosphate buffer) group. #P < 0.05 and ##P < 0.01 compared with ZP only–treated group.

Fig. 2. Dose-dependent changes in plasma CRE (A), BUN (B), and ALT (C) levels after ZP administration in mice. TOTP and BSO were administered as described in Materials and Methods. ZP was administered at a dose of 50, 100, and 150 mg/kg. The plasma CRE, BUN, and ALT levels were measured 24 hours after ZP administration. The data are shown as the mean ± S.E.M. (n = 5–7). Differences compared with the inhibitors only–treated group were considered significant at *P < 0.05 and **P < 0.01 (one-way analysis of variance followed by Dunnett’s test).
Results

Establishment of the ZP-Induced Kidney Injury Mouse Model.
In female BALB/c mice treated with ZP alone, the plasma levels of CRE, BUN, and ALT were not increased at 24 hours after ZP administration (Fig. 1). Considering the possibility that GSH conjugation and hydrolysis of ZP-AG might be involved in ZP-induced toxicity, the effects of cotreatment with BSO or TOTP, a GSH synthesis inhibitor (Shimizu et al., 2009), or an esterase inhibitor (Emeigh Hart et al., 1991), respectively, were investigated. Coadministration of BSO resulted in a slight increase in CRE and BUN (0.640 and 94.5 mg/dl, respectively) compared with the group treated with ZP alone (0.340 and 50.6 mg/dl, respectively). However, CRE and BUN were significantly elevated by coadministration of TOTP (1.08 and 160 mg/dl, respectively), although ALT was not affected. The highest increases of CRE and BUN were observed in mice cotreated with both TOTP and BSO (1.49 and 176 mg/dl, respectively). No increase in plasma CRE, BUN, and ALT levels in mice receiving TOTP or BSO only was confirmed. These results suggest that the inhibition of GSH conjugation and hydrolysis of ZP-AG contributes to the kidney injury caused by ZP administration.

Dose- and Time-Dependent Changes in ZP-Induced Kidney Injury. ZP was administered to mice at a dose of 50, 100, or 150 mg/kg with coadministration of TOTP and BSO. Plasma CRE and BUN levels were significantly and dose-dependently increased in mice receiving 100 mg/kg ZP (1.12 and 137 mg/dl, respectively) and 150 mg/kg ZP (1.43 and 155 mg/dl, respectively) compared with vehicle-treated (potassium phosphate buffer) control mice; thus, for subsequent experiments, we adopted a dose of ZP at 150 mg/kg (Fig. 2). As shown in Fig. 3, A–C, plasma CRE and BUN levels were time-dependently increased 12 hours (0.583 and 99.7 mg/dl, respectively) and 24 hours (1.15 and 158 mg/dl, respectively) after ZP administration, but plasma ALT levels were not.

Time-Dependent Changes in ZP and ZP-AG Concentration in Plasma, Kidney, and Liver. The concentrations of ZP and ZP-AG in plasma, kidney, and liver were measured in mice after ZP administration with or without coadministration of TOTP and BSO. Plasma CRE and BUN levels were significantly and dose-dependently increased in mice receiving 100 mg/kg ZP (1.12 and 137 mg/dl, respectively) and 150 mg/kg ZP (1.43 and 155 mg/dl, respectively) compared with vehicle-treated (potassium phosphate buffer) control mice; thus, for subsequent experiments, we adopted a dose of ZP at 150 mg/kg (Fig. 2). As shown in Fig. 3, A–C, plasma CRE and BUN levels were time-dependently increased 12 hours (0.583 and 99.7 mg/dl, respectively) and 24 hours (1.15 and 158 mg/dl, respectively) after ZP administration, but plasma ALT levels were not.
TOTP, BSO, and ZP compared with mice receiving ZP alone (Fig. 3E).

By coadministration with inhibitors, 4.9-, 1.9-, and 9.0-fold increases in the maximum concentration (C\text{max}) of ZP-AG were observed in plasma, liver, and kidney, respectively. The plasma C\text{max} of ZP-AG in mice receiving ZP and inhibitors was approximately 50-fold higher than that in humans after a 100-mg oral dose of zomepirac (Smith et al., 1985b). In groups coadministered with or without the inhibitors, the ZP concentrations in the kidney were almost the same as those in the liver, whereas the ZP-AG concentrations in the kidney were much higher than those in the liver. These results imply that ZP-AG is slowly eliminated from the kidney, resulting in a high accumulation in the kidney.

**Histopathological Examination in ZP-Induced Kidney Injury.**

To evaluate the tissue injury, histopathological examination of kidneys 24 hours after ZP administration (150 mg/kg) was performed. Vacuoles and the denaturation and aggregation of eosinophilic materials were observed in the kidney of mice cotreated with ZP and inhibitors in the renal tissue. Arrowheads indicate immune cell infiltration. The number of MPO-positive cells was counted and compared with the ZP only–treated group (C). The data are shown as the mean ± S.E.M. (n = 5–7). The difference between the ZP only–treated group and the ZP and inhibitor–treated group was considered significant at *P < 0.05 by Student’s t tests. KPB, potassium phosphate buffer; ND, not detected.

**Fig. 4.** Histopathological examination of mouse kidney after ZP administration. TOTP, BSO, and ZP were administered as described in Materials and Methods. The kidneys were collected at 24 hours after ZP administration, and the kidney sections were stained with H&E (A) or immunostained with an anti-MPO antibody (B). Arrowheads indicate immune cell infiltration. The number of MPO-positive cells was counted and compared with the ZP only–treated group (C). The data are shown as the mean ± S.E.M. (n = 5–7). The difference between the ZP only–treated group and the ZP and inhibitor–treated group was considered significant at *P < 0.05 by Student’s t tests. KPB, potassium phosphate buffer; ND, not detected.

**Changes in mRNA Expression Levels in Immune-, Inflammation-, and Oxidative Stress–Related Genes in Kidney.** To investigate whether immune-, inflammation-, and oxidative stress–related factors are involved in ZP-induced kidney injury, time-dependent changes in the renal mRNA expression levels of IL-1α, IL-6, MIP-2, ICAM-1, S100A9, and HO-1 were measured (Fig. 5). IL-1α mRNA expression levels in mice treated only with ZP were significantly increased 12 hours after ZP administration. In mice treated with TOTP, BSO, and ZP, the levels increased not only at 12 hours but also 1 hour after ZP administration. IL-6 and ICAM-1 mRNA expression levels in mice receiving TOTP, BSO, and ZP were significantly increased in mice receiving TOTP, BSO, and ZP 1 and 12 hours after ZP administration, in contrast with the small increase in mice receiving only ZP. S100A9 mRNA expression levels in mice that were treated only with ZP were significantly increased 1 hour after ZP administration, but in TOTP, BSO, and ZP-treated mice, the levels were significantly higher 12 and 24 hours after ZP administration. In mice treated only with TOTP, BSO, and ZP, the mRNA expression levels of HO-1 were significantly increased 12 and 24 hours after ZP administration. However, in the mRNA expression levels of transcription factors for the T helper lineage in adaptive immunity, such as the T-box expressed in T cells, GATA-binding domain-3 retinoid-related orphan receptor-γt and forkhead box P3, no change was observed between any groups (data not shown).
Involvement of Oxidative Stress in ZP-Induced Kidney Injury.
To confirm the depletion of GSH by BSO treatment, and to investigate the involvement of oxidative stress in ZP-induced kidney injury, GSH and GSSG contents in kidney and liver were measured (Fig. 6, A–C). In the kidney, GSH depletion by BSO was confirmed in mice treated with TOTP and BSO. In addition, GSH levels in mice receiving TOTP, BSO, and ZP were significantly lower compared with mice receiving only inhibitors. Owing to the decrease in GSH levels, the GSH/GSSG ratio, a biomarker of oxidative stress, was significantly lower in mice treated with BSO, TOTP, and ZP compared with mice treated with BSO and TOTP. In the liver, GSH was depleted by BSO, but the GSH/GSSG ratio was not different between the TOTP and BSO–treated group and the TOTP, BSO, and ZP–treated groups.

In addition, MDA concentrations, a biomarker of lipid peroxidation, were measured in the kidney and liver (Fig. 6D). In the kidney, MDA concentrations were significantly higher in TOTP, BSO, and ZP–treated mice compared with TOTP and BSO–treated mice, but in the liver, there was no change in MDA concentrations between these groups.

Effect of Antioxidant Agents on ZP-Induced Kidney Injury. The changes in oxidative stress markers implied the involvement of oxidative stress in ZP-induced kidney injury. Next, the effect of tempol, an antioxidant agent, on ZP-induced kidney injury was investigated. The coadministration of tempol significantly decreased plasma CRE levels at 12 hours and BUN levels at 12 and 24 hours after ZP administration in BSO, TOTP, and ZP–treated mice (Fig. 7). These results also support the involvement of oxidative stress in ZP-induced kidney injury.

Discussion
AGs of drugs are generally unstable and are believed to be involved in drug-induced toxicity via the formation of covalent
adducts to endogenous proteins. Although there is increasing evidence that AGs form drug-protein adducts due to their chemical reactivity (Wang et al., 2001; Horng and Benet, 2013), cytotoxicity and genotoxicity of AGs have not been observed in vitro (Koga et al., 2011). Conversely, the AGs of withdrawn drugs and warning drugs that have idiosyncratic drug toxicity risks, such as zomepirac and diclofenac, induced the mRNA expression levels of immune- and inflammation-related genes in human PBMCs (Miyashita et al., 2014; Iwamura et al., 2015). Thus, these in vitro studies indicate that the toxicity of AGs remains controversial.

ZP is a nonsteroidal anti-inflammatory drug that was withdrawn from the market because of severe adverse effects, such as anaphylaxis and renal toxicity (Smith, 1982; Miller et al., 1983; Heintz, 1995). ZP-AG, a glucuronide conjugate metabolite of ZP, covalently binds proteins such as microtubular protein and dipeptidyl peptidase IV in vitro and in vivo, suggesting the involvement of ZP-AG in ZP-induced toxicity (Bailey et al., 1998; Wang et al., 2001). However, an animal model for the toxicity induced by ZP-AG has never been reported. To establish a mouse model of the toxicity induced by ZP-AG, we attempted to increase the exposure to ZP-AG in mice by inhibiting its hydrolysis by using an esterase inhibitor. A previous study reported that ZP-AG is hydrolyzed by esterases (Smith et al., 1990b). TOTP, a nonselective esterase inhibitor, successfully inhibited esterase activity in mice and rats in vivo (Silver and Murphy, 1981; Kobayashi et al., 2012). In the present study, in addition to TOTP, BSO was used to reduce the GSH conjugation of ZP-AG.

Renal and hepatic toxicity was not observed after the administration of only ZP. Coadministration of TOTP significantly increased the plasma CRE and BUN levels, and that of BSO moderately increased them. Coadministration of TOTP and BSO with ZP led to the severest renal toxicity, suggesting that hydrolysis and GSH conjugation of ZP-AG play a role in the detoxification of ZP-AG. In contrast to plasma CRE and BUN levels, plasma ALT levels were not elevated in any group, which corresponds to the fact that acute kidney injury was frequently reported in ZP therapy in humans (Smith, 1982; Miller et al., 1983). The present study succeeded in establishing an animal model of ZP-induced kidney injury by coadministration of TOTP and BSO.

To examine the relationship between the extent of kidney injury and the exposure to ZP and ZP-AG, the concentrations of ZP and ZP-AG in the plasma, kidney, and liver were measured. ZP-AG concentrations in plasma, kidney, and liver were significantly higher in mice receiving TOTP, BSO, and ZP compared with mice receiving ZP alone. The opposite changes in the levels of ZP and ZP-AG by TOTP and BSO treatment indicate that these inhibitors inhibit the hydrolysis of ZP-AG to ZP rather than the efflux of ZP-AG from the kidney. A particularly high concentration of ZP-AG was observed in the kidney, suggesting that ZP-AG had the potential to lead to kidney injury. ZP-AG concentrations in the liver were much lower than those in the kidney, although the ZP concentrations in the liver were almost the same as those in the kidney. These results indicated that ZP-AG was more toxic than ZP because no hepatotoxicity was observed after ZP dosing. ZP and its metabolites are primarily excreted into the urine, and the urinary principal metabolite is ZP-AG in both mice and humans, although the urinary excretion ratio of ZP-AG in humans is higher than that in mice (57% and 19–28% of dose, respectively) (Grindel et al., 1980). The plasma concentration of ZP-AG in mice was approximately 50-fold higher than that in humans (100 mg/body, oral), corresponding to the ratio of dose (Smith et al., 1985b). In the present study, ZP-AG was highly accumulated in the kidney, suggesting that ZP-AG was not equilibrated between plasma and kidney and might be actively transported to the kidney. Moreover, the expression patterns of esterases and UGTs in the kidney and other tissues were different between mice and humans (Paul and Fottrell, 1961; Oda et al., 2015). Therefore, further investigations of interspecies differences in transporters, esterases, and UGTs are needed. Of course, the uncertainty of interspecies sensitivity to toxicity should also be considered.
Differences compared with the no tempol treatment group were considered significant (*P < 0.05 and ***P < 0.001 by Student’s t tests).

In general, glucuronides in hepatocytes are eliminated into the bile and blood mediated by multidrug-resistance proteins 2 and 3, respectively (Trauner and Boyer, 2003). In human hepatocytes, the AGs of nonsteroidal anti-inflammatory drugs (diclofenac, naproxen, ketoprofen, and ibuprofen) were rapidly excreted and did not accumulate in the cell (Koga et al., 2011). ZP-AG is immediately excreted from the liver into the blood rather than bile because excretion of ZP and its metabolites into bile is a minor route in humans and laboratory animals (Grindel et al., 1980). Thus, these reports support the finding that ZP-AG accumulated in the kidney but not in the liver.

In histopathological examination, our mouse model of ZP-induced kidney injury displayed vacuoles, denatured cytoplasm, and aggregated eosinophilic materials, probably reflecting cellular necrosis. In ZP-induced kidney injury in the clinic, renal cortical necrosis was observed (Darwish et al., 1984). Thus, the results observed in the present study are consistent with the clinical findings. In preclinical studies, almost all of the nonsteroidal anti-inflammatory drugs produced papillary necrosis in experimental animal models (Whelton and Hamilton, 1991). A possible mechanism of papillary necrosis is ischemic injury through the direct inhibition of cyclooxygenase-mediated production of prostaglandins (Brix, 2002). However, the inhibition of cyclooxygenase mediated by ZP might not contribute to ZP-induced kidney injury because papillary necrosis was not observed in this injury. The increased number of MPO-positive cells suggested the contribution of immune cell infiltration to ZP-induced kidney injury.

From our previous findings, AGs of warning and withdrawn drugs induced immune- and inflammation-related genes such as IL-6 and IL-8 in human PBMCs (Miyashita et al., 2014; Iwamura et al., 2015). Hence, the changes in the renal mRNA expression levels of immune- and inflammation-related genes were measured. The mRNA expression of IL-1α and IL-6 was induced in mice highly exposed to ZP-AG, followed by ICAM-1 and S100A9 mRNAs. IL-1α, a trigger of chemokine cascades, other cytokines, and inflammatory mediators, is synthesized in the first few hours after injury or the ischemic event (Dinarello et al., 2012). IL-6 is also rapidly induced as a lymphocyte-stimulating factor and leads to innate and adaptive immune activation (Hunter and Jones, 2015). ICAM-1, an adhesion molecule, is involved in infiltration of inflammatory cells (Ley et al., 2007). S100A9, a damage-associated molecular pattern, is released from activated or necrotic neutrophils and monocytes/macrophages and promotes innate immunity and inflammation (Schiopu and Cotoi, 2007). S100A9 induces ICAM-1 and S100A9 (Aziz and Wakefield, 1996; Zreiqat et al., 2010), and so does stimulation of IL-6 (Wang et al., 2005; Lee et al., 2012). Because the injection of recombinant IL-1α accelerates renal injury and mortality in mice (Brennan et al., 1989), and IL-6- or ICAM-1-deficient mice show protective effects against acute kidney injury, these factors could trigger and promote kidney injury (Kelly et al., 1996; Nechemia-Arbely et al., 2008). Taken together, it is conceivable that IL-1α and IL-6 induced by ZP-AG at the onset promoted the infiltration of immune cells via the induction of ICAM-1 and MIP-2, and then the infiltrating cells caused kidney injury.

Of particular note was the potent induction of HO-1 mRNA in mice cotreated with TOTP and BSO, suggesting that ZP-AG was involved in ZP-induced kidney injury via the induction of oxidative stress. A decrease in the GSH/GSSG ratio and an increase in MDA concentration were observed in the kidney, but not in the liver, consistent with each tissue injury. Partial involvement of oxidative stress was also demonstrated by tempol treatment. In accordance with the results of the GSH/GSSG ratio and the MDA concentration, the antioxidant tempol suppressed ZP-induced kidney injury, suggesting that oxidative stress is involved in renal toxicity. It was reported that the decrease in the GSH/GSSG ratio and the increase in the MDA concentrations were also observed in cisplatin-induced acute renal failure in rats (Santos et al., 2007). Tempol attenuated oxidative stress–mediated renal injury in rats (Chatterjee et al., 2000). These results were similar to the results observed in the present study.
In conclusion, a mouse model for ZP-induced kidney injury was established by using TOTP and BSO in consideration of the metabolic pathway of ZP. The pharmacokinetics of ZP and ZP-AG showed that the hydrolysis of ZP-AG by esterases contributed considerably to their pharmacokinetics, and ZP-AG could be responsible for ZP-induced kidney injury in vivo. In addition, it was demonstrated that renal toxicity was mediated via oxidative stress and immune cell infiltration (Fig. 8). The model using TOTP can be used to evaluate the toxicity of AGs in preclinical settings, and the present study sheds light on understanding the toxicological potential of AGs.

Acknowledgments

The authors greatly appreciate the valuable suggestions of Miki Nakajima.

Authorship Contributions

Participated in research design: Iwamura, Yokoi.

Conducted experiments: Iwamura, Watanabe, Akai, Nishinosono, Tsuneyama.

Contributed new reagents or analytic tools: Iwamura, Watanabe, Nishinosono.

Performed data analysis: Iwamura, Watanabe, Tsuneyama.

Wrote or contributed to the writing of the manuscript: Iwamura, Oda, Yokoi, Kume.

References


Smith PC, McDonagh AF, and Benet LZ (1990b) Effect of esterase inhibition on the disposition of zomepiric acid glucuronide and its covalent binding to plasma proteins in the guinea pig. J Pharmacol Exp Ther 252:218–223.


Address correspondence to: Atsushi Iwamura, Drug Metabolism and Pharmacokinetics Research Laboratory, Mitsubishi Tanabe Pharma Corporation, 2-2-50 Kawagishi, Toda, Saitama 335-8505, Japan. E-mail: iwamura.atsushi@mt-pharma.co.jp