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Modulated function of multidrug resistance-associated proteins in cisplatin-induced acute renal failure rats

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The effect of cisplatin-induced acute renal failure (ARF) on the function and expression of multidrug resistance-associated proteins (MRPs) was evaluated in rats. Rats received an intraperitoneal injection of cisplatin (9 mg/kg), and the induction of ARF state with high plasma concentrations of indoxyl sulfate and creatinine was observed 72 h after cisplatin treatment. The function of MRPs in the liver, kidney and brain was evaluated by measuring the tissue accumulation and biliary excretion of 2,4-dintrophenyl-S-glutathione (DNP-SG), a substrate for MRPs, after administration of 1-chloro-2,4-dintrobenzene (CDNB), a precursor of DNP-SG, in rats. The levels of MRP1-4 expression were evaluated by Western blot analysis. Effect of ARF plasma components on MRP function was also examined by using calcein acetoxymethyl ester (calcein-AM) in HepG2 cells. In ARF rats (72 h after cisplatin treatment), the accumulation of DNP-SG in the liver, kidney and brain was significantly higher than those in control and cisplatin-treated rats (1 h after treatment). In ARF rats, intrinsic biliary excretion clearance of DNP-SG, estimated by dividing the biliary excretion rate of DNP-SG with the liver concentration, was also significantly reduced, though the expression levels of MRP1-4 in the liver remained unchanged. ARF rat plasma (5%) significantly increased the accumulation of calcein, a MRP substrate, in HepG2 cells after application of calcein-AM. In conclusion, MRP function was found to be suppressed not only in the kidney but also in the liver and brain in cisplatin-induced ARF rats, possibly due to the accumulation of some MRP substrates/inhibitors in plasma.

1. Introduction

Cisplatin, an anti-cancer chemotherapy drug, is widely used to treat a variety of cancer types including neuroblastoma, sarcomas, melanoma, lung cancer, and lymphomas (Apps et al. 2015; Florea and Büsselberg 2011). As the clinical problem, however, the dosage of cisplatin is severely restricted because of the dose dependent induction of nephrotoxicity (Arany and Safirstein 2003). Nephrotoxicity is induced after cisplatin treatment in more than 20% of patients due to the high accumulation of cisplatin in the renal tubular cells (Peres and da Cunha 2013). The induction mechanism is as follows: cisplatin is a substrate for organic cation transporter 2 (OCT2), an uptake transporter expressed in the basolateral membrane of renal tubular cells, but not a substrate for multidrug and toxin extrusion protein 2-K (MATE2-K), an efflux transporter expressed in the apical membrane of renal tubular cells. Because of the substrate specificities, cisplatin is not efficiently excluded and retained in the renal tubular cells (Yokoo et al. 2007). Regarding the effects of cisplatin-induced acute renal failure (ARF) on the function and expression of transporters, many reports are available. For example, it is reported that multidrug resistance-associated protein2 (MRP2) and MRP4 levels were increased and (OAT1), OAT3, OCT1 and OCT2 levels were decreased in the kidney of cisplatin-induced ARF rats (Erman et al. 2014). An elevated urinary excretion of OAT5 in cisplatin-induced ARF rats is also reported (Bulacia and Torres 2015; Bulacio et al. 2015). However, there is little information on the effect of cisplatin-induced ARF on the function and expression of transporters in non-renal tissues, although many reports are available regarding the effect of chronic renal failure (CRF) on the expression and function of various transporters in various tissues (Naud et al. 2007, 2008, 2011, and 2012).

In the present study, the effect of cisplatin-induced ARF on the function and expression of multidrug resistance-associated proteins (MRPs) in kidney, liver and brain was evaluated in rats. MRPs superfamily contains 13 members, and some MRP members are ubiquitously expressed in various normal tissues (Sodani et al. 2012). Previously, our research group examined MRPs function by determining the tissue accumulation of 2,4-dintrophenyl-S-glutathione (DNP-SG), a typical MRPs substrate, after administration of 1-chloro-2,4-dintrobenzene (CDNB), a precursor of DNP-SG, in the absence and presence of MRP inhibitors such as probenecid and bilirubin in rats (Yokooji et al. 2010). Recently, the effect of glycerol-induced ARF on MRPs function in erythrocytes was also examined in rats using CDNB as a precursor of MRP substrate (Matsushima et al. 2017). CDNB, a lipophilic compound, is distributed into tissues by diffusion, immediately metabolized to DNP-SG by glutathione S-transferase (GST), and the glutathione-conjugated metabolite DNP-SG, a MRP substrate, is effluxed by MRPs-mediated transport from cells. Under suppression of MRP-mediated efflux transport, however, DNP-SG is accumulated in tissues. In the present study, the tissue accumulation and biliary excretion of DNP-SG, and expression levels of MRP1-4 were compared between normal and cisplatin-induced ARF rats. The effect of ARF plasma components on hepatic transporters was also examined by using HepG2 cells, a human liver cancer cell line expressing various solute carrier (SLC) and ATP binding cassette (ABC) transporters.

2. Investigations and results

2.1. Induction of ARF in rats

To examine the induction of ARF and/or acute hepatic failure (AHF) states after cisplatin treatment, plasma concentrations of renal function markers such as indoxyl sulfate and creatinine and hepatic function markers such as glutamic oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), total

Table 1: Biological data for kidney and liver function in control and ARF rats

	Control	ARF
Indoxyl sulfate (μM)	4.02 ± 0.94	151.9 ± 29.6**
Creatinine (mg/dL)	0.33 ± 0.08	2.23 ± 0.41**
GOT (IU)	63.0 ± 5.19	70.1 ± 15.3
GPT (IU)	18.6 ± 0.62	17.6 ± 5.48
T-BIL (mg/dL)	0.11 ± 0.01	0.10 ± 0.01
D-BIL (mg/dL)	0.04 ± 0.004	0.04 ± 0.004

ARF state was induced by an intraperitoneal injection of cisplatin (9 mg/kg), and biological data were examined 72 h after treatment. GOT, glutamate oxaloacetate transaminase; GPT, glutamate pyruvate transaminase; T-BIL, total bilirubin; D-BIL, direct bilirubin. Each value represents the mean±S.E. of results from three rats to seven rats. $^{**}P < 0.01$: significantly different from the value for control.

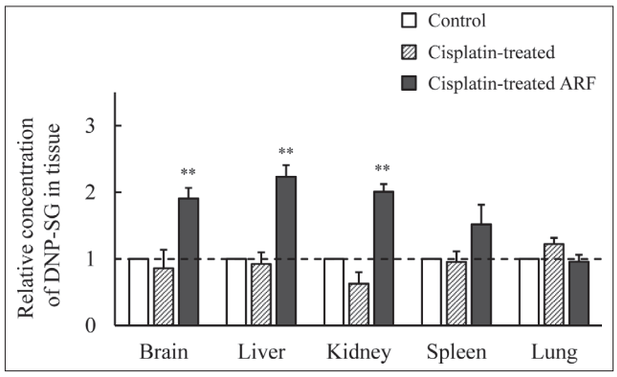

Fig. 1: Relative concentrations of DNP-SG in the brain, liver, kidney, spleen, and lung 30 min after intravenous administration of CDNB in untreated control, cisplatin-treated (1 h after treatment), and cisplatin-treated ARF (72 h after treatment) rats. The dose of CDNB was 30 μmol/kg. Each value represents the mean±S.E. of results from three to five rats. $^{**}P < 0.01$: significantly different from the value for control.

bilirubin (T-BIL), and direct bilirubin (D-BIL) were determined 72 h after treatment in rats (Table 1). Concentrations of indoxyl sulfate and creatinine in plasma were significantly increased in cisplatin-treated rats. On one hand, activities and/or concentrations of hepatic function markers such as GOT, GPT, T-BIL and D-BIL in plasma were remained unchanged. Thus, cisplatin-induced ARF state was not found to accompany AHF state in rats.

2.2. Effect of cisplatin-induced ARF on MRPs function in tissues

Accumulation of DNP-SG in the brain, liver, kidney, spleen, and lung after intravenous administration of CDNB were compared among three rat groups: untreated control rats, cisplatin-treated rats (1 h after treatment), and cisplatin-treated ARF rats (72 h after treatment) (Fig. 1). Cisplatin-treated rats (1 h after treatment) were used to examine the effect of cisplatin itself without causing ARF state on MRP function in tissues. The effect of cisplatin itself on rat MRPs function was not observed in all tissues examined. In cisplatin-induced ARF rats (72 h after treatment), concentrations of DNP-SG in the brain, liver, and kidney were significantly higher than those in control and cisplatin-treated rats (1 h after treatment).

2.3. Effect of cisplatin-induced ARF on biliary excretion of DNP-SG after CDNB administration

The biliary excretion of DNP-SG is mediated by MRP2 expressed in the bile canalicular membrane. After intravenous administration of CDNB , biliary excretion of DNP-SG was significantly lower in ARF rats than in control rats, though plasma and hepatic concentrations of DNP-SG were significantly higher in ARF rats than in control rats (Figs. 1, 2).

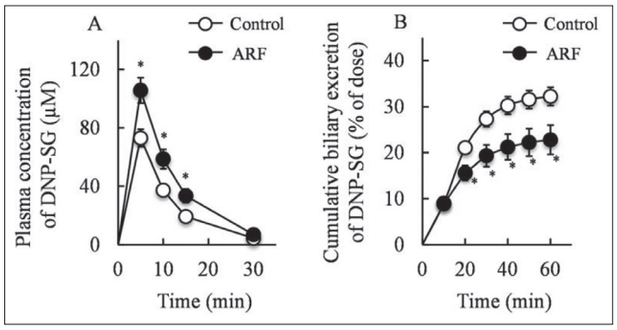

Fig. 2: Concentrations of DNP-SG in plasma (A) and cumulative biliary excretion of DNP-SG (B) after intravenous administration of CDNB in control and ARF rats. The dose of CDNB was 30 μmol/kg. Each value represents the mean±S.E. of results from five rats. $^{*}P < 0.05$: significantly different from the value for control.

Table 2: Total and biliary clearances of DNP-SG under a steady-state plasma concentration of DNP-SG in control and ARF rats

	Control	ARF
C_{pss} (μM)	27.1 ± 1.56	89.8 ± 4.82**
CL_{tot} (mL/min/kg)	55.3 ± 2.55	18.4 ± 1.19**
CL_{bile} (mL/min/kg)	8.89 ± 0.44	1.34 ± 0.46**
Concn. in liver (nmol/g tissue)	1.79 ± 0.51	3.28 ± 0.62
*CL_{bile} (mL/min/kg)	51.9 ± 10.1	10.2 ± 1.91*

DNP-SG was administrated by a constant rate infusion (30 μmol/h). CL_{bile} was estimated by dividing the biliary excretion rate with plasma concentration (C_{pss}) of DNP-SG. *CL_{bile} was estimated by dividing the biliary excretion rate with liver concentration of DNP-SG. Each value represents the mean±S.E. of results from three rats. $^{*}P < 0.05$, $^{**}P < 0.01$: significantly different from the value for control.

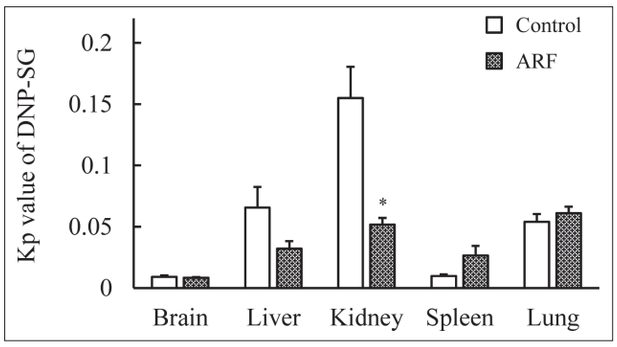

Fig. 3: Kp values of DNP-SG in the brain, liver, kidney, spleen, and lung under a steady-state plasma concentration of DNP-SG in control and ARF rats. DNP-SG was administrated by a constant rate infusion at a dose of 30 μmol/h. Each value represents the mean±S.E. of results from three rats. $^{*}P < 0.05$: significantly different from the value for control.

2.4. Clearance and tissue distribution of DNP-SG during a constant-rate infusion of DNP-SG

DNP-SG is also known to be a substrate for organic anion transporting protein 2 (OATP2) (Li et al. 2000). To evaluate the effect of cisplatin-induced ARF state on OATP2 function, total clearance, biliary clearance, and tissue distribution of DNP-SG during/after a constant rate infusion of DNP-SG were determined in control and ARF rats (Table 2, Fig. 3). The steady-state plasma concentrations of DNP-SG in ARF rats were 3.3-fold higher than those in control rats, indicating the decrease in total plasma clearance (CL_{tot}) of DNP-SG in ARF rats. The biliary clearance (CL_{bile}) of DNP-SG in ARF rats decreased to approximately 15% of control rats (Table 2). The tissue-to-plasma partition coefficient (Kp) of DNP-SG in the kidney of ARF rats was significantly lower than that in control rats.

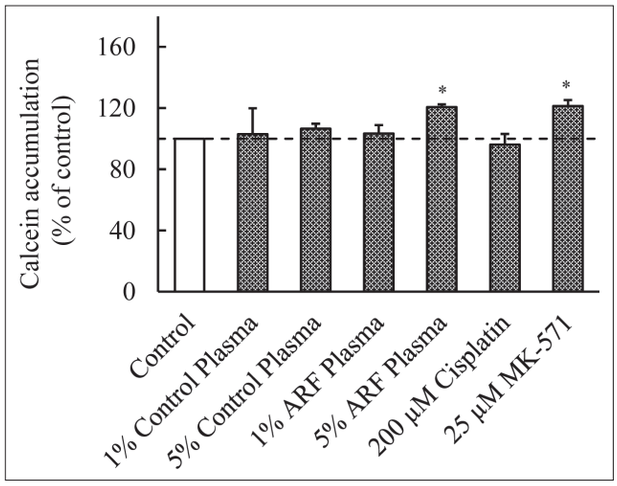

Fig. 4: Accumulation of calcein in HepG2 cells after application of calcein-AM (500 nM). HepG2 cells were pre-incubated with PBS containing 2 μM elacridar with or without plasma components, cisplatin or MK-571 at 37 °C for 30 min before the accumulation study of calcein. The concentration of intracellular calcein was measured 10 min after calcein-AM application. Each value represents the mean±S.E. of results from three trials. $^{*}P < 0.05$: significantly different from the value for control.

The liver of ARF rats also showed a lower Kp value, although it did not reach the significant level. To evaluate the MRP2 function expressed in the bile canalicular membrane by eliminating the effect of variation in tissue concentrations of DNP-SG between control and ARF rats, the intrinsic biliary clearance ($^{*}CL_{bile}$) of DNP-SG was estimated by dividing the biliary excretion rate with liver concentration of DNP-SG. Estimated $^{*}CL_{bile}$ of DNP-SG in ARF rats was approximately 20% of control rats (Table 2).

2.5. Effect of plasma components on MRPs function in HepG2 cells

Effect of plasma components and cisplatin on MRP function was evaluated by using HepG2 cells and calcein-AM (Fig. 4). Calcein-AM is uptaken by HepG2 cells by single diffusion and metabolized to calcein, a typical MRP substrate, in HepG2 cells. MK-571 was also used as a typical MRP inhibitor (positive control). Cisplatin alone and control plasma components exerted no significant effect on calcein accumulation. In contrast, 5% ARF plasma components, as well as 25 μM MK-571, increased the accumulation of calcein significantly, indicating the presence of some MRP inhibitors in cisplatin-induced ARF plasma.

2.6. Protein levels of MRP1-4 in control and ARF rats

The protein levels of MRP1, 2, 3, and 4 in control and ARF rats were determined by Western blot analysis (Fig. 5). There was no significant difference in the MRPs protein levels in the brain and liver between control and ARF rats, though MRP2 protein level in the liver of ARF rats showed increasing tendency (Fig. 5A, B). On one hand, protein levels of MRP2 and MRP4 in the kidney of ARF rats were significantly higher than those in the control rats (Fig. 5C).

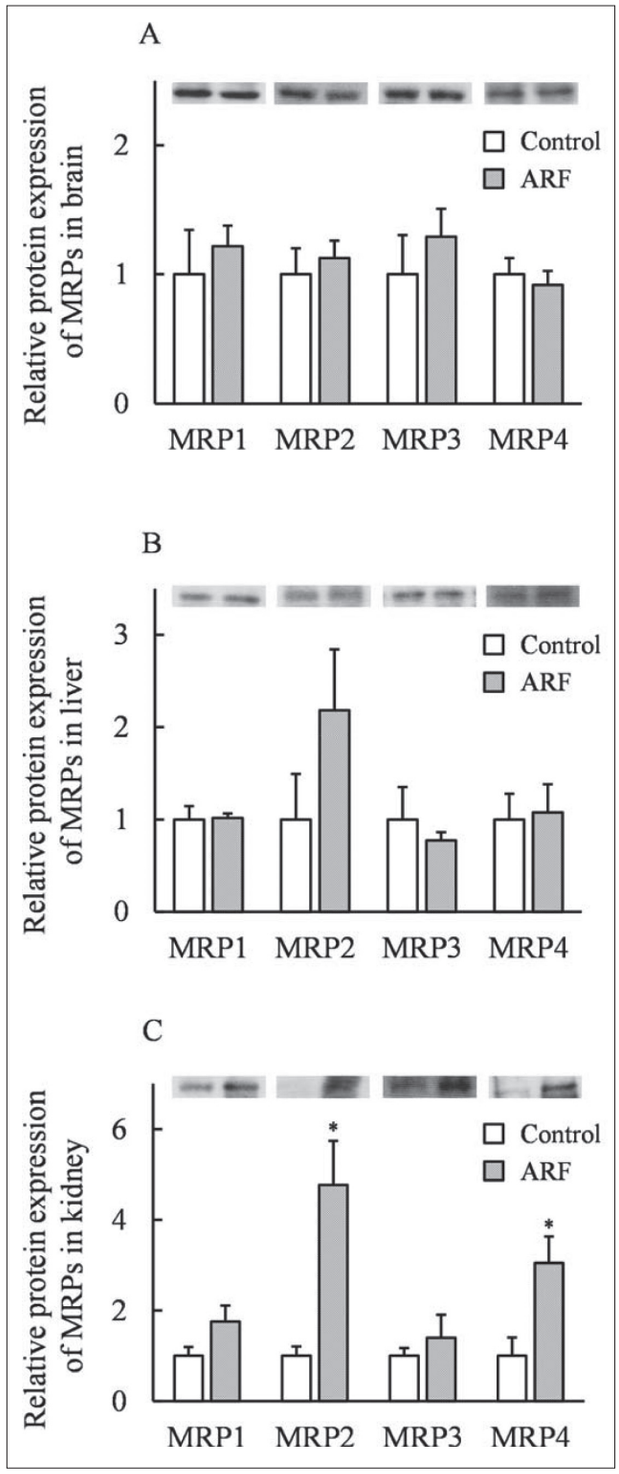

Fig. 5: Relative protein levels of rat MRP1, 2, 3, and 4 in the brain (A), liver (B), and kidney (C) in control and ARF rats. Each value represents the mean±S.E. of results from three rats. $^{*}P < 0.05$: significantly different from the value for control.

3. Discussion

In the present study, we examined the effects of cisplatin-induced ARF state on MRPs function and expression in various tissues in rats, since information on the effect of cisplatin-induced ARF state has been restricted only in the kidney so far, as described in the introduction section. After administration of CDNB, the accumulation of DNP-SG was significantly increased in the brain, liver, and kidney in cisplatin-induced ARF rats. In these tissues, MRPs are expressed, and the accumulation of DNP-SG was considered to be due to the suppression of MRP-mediated efflux transport of DNP-SG from tissues (Figs. 1, 5), because the uptake amount of CDNB to a certain tissue is considered to be the same extent between control and ARF rats, since CDNB is distributed to tissues by simple diffusion. Cisplatin is also known to be a substrate for MRP2 and OCT2 (Wen et al. 2014). However, no significant effect of cisplatin on MRPs function was found in cisplatin-treated rats (1 h after treatment) (Fig. 1), though it is reported that the highest plasma concentration of cisplatin is observed 1 h after intraperito-

neal injection in rats (Los et al. 1989). The effect of cisplatin on MRP2 function could be dependent on its dosage amounts. Our results indicated that MRPs function is modulated systemically under cisplatin-induced ARF state (72 h after cisplatin-treatment), possibly due to the accumulation of some MRP inhibitors in plasma of cisplatin-induced ARF rats. The suppressive effect of ARF plasma components on MRP function in the liver was also observed in HepG2 cells, in which 5% ARF plasma components significantly increased calcein accumulation at the similar extent with MK-571 (Fig. 4). Previously, we demonstrated that many endogenous MRP inhibitors such as p-cresol, hippuric acid, indoxyl sulfate, indole-3-acetic acid, kynurenic acid, and 3-carboxy-4-metyl-5-propyl-2-franpropanpic acid (CMPF) can modulate MRP function in rat erythrocytes using inside-out erythrocyte membrane vesicles (IOVs) *in vitro* (Matsushima et al. 2017). Some of them are considered to inhibit MRP function in tissues under cisplatin-induced ARF state *in vivo*, since various SLC uptake transporters for endogenous inhibitors are expressed in the liver and kidney. When DNP-SG was administered by a constant rate infusion, low Kp values of DNP-SG in the kidney and liver were observed in cisplatin-induced ARF rats (Fig. 3), in addition to the suppression of CL_{tot}, CL_{bile}, and $^{*}CL_{bile}$ of DNP-SG (Table 2). DNP-SG is also known to be a substrate for OATP2, in addition to a substrate for MRPs (Li et al. 2000), and OATP2 is expressed in the liver, brain and kidney (Guo and Jiang 2010).

Taken together, the presented results suggest that both functions of MRPs and OATP2 were modulated systemically in the cisplatin-induced ARF state. It is reported that the increase in serum concentration of indoxyl sulfate is correlated with the increase in other uremic toxins such as blood urea nitrogen (BUN) and serum creatine in ischemic rats (Matsuzaki et al. 2007). For example, the hepatic clearance of erythromycin, a substrate for OATP2, was significantly reduced in patients with end stage renal disease, and CMPF directly inhibits the uptake of erythromycin by inhibiting OATP2 (Sun et al. 2004). It was also reported that conditions typical of uremia can cause inhibition of brain-to-blood transport mediated rat OATP2 and OAT3, leading to the accumulation of endogenous metabolites and drugs in the brain (Deguchi et al. 2006). Thus, it is hypothesited that OATP2, in addition to MRPs, was suppressed systemically in cisplatin-induced ARF rats.

In the present study, the protein levels of MRPs in ARF in the brain and liver were not significantly affected by ARF, though the level of MRP2 in the liver showed increasing tendency in ARF rats. In contrast, protein levels of MRP2 and MRP4 in the kidney were significantly increased in ARF rats (Fig. 5A, B). Regarding the increase in protein levels of ABC efflux transporters such as P-glycoprotein, MRP2 and MRP4 under renal failure states, many reports are available (Erman et al. 2014; Haung et al. 2000; Laouari et al. 2001; Liu et al. 2012; Masereeuw et al. 2000; Mutsaers et al. 2011). The increase in protein levels of MRPs in the kidney may be related to a compensation of decreased renal function. Further study is necessary to understand the effect of the cisplatin-induced ARF state on function and expression of various transporters.

In conclusion, the present study demonstrated that the MRPs in the brain, liver, and kidney were functionally modulated in cisplatin-induced ARF rats, not only due to the modulation of expression levels, but also due to the accumulation of some MRP substrates/inhibitors in plasma. In pharmacotherapy with MRP substrate drugs for patients with renal failure, special attention is necessary to prevent side effects.

4. Experimental

4.1. Materials

CDNB, reduced glutathione (GSH), LabAssay™ Creatinine, and Transaminase CII-test Wako were purchased from Wako Pure Chemicals (Osaka, Japan). 1-Fluoro-2,4-dinitrobenzene (FDNB) was obtained from Tokyo Kasei (Tokyo, Japan). Indoxyl sulfate potassium salt was purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). QuantiChrom™ Bilirubin Assay Kit was purchased from BioAssay Systems (Hayward, CA, USA). Rabbit Anti-MRP1 Polyclonal antibody and Rabbit Anti-MRP4 Polyclonal antibody were obtained from Bioss Antibodies (Woburn, MA, USA). Mouse Anti-MRP2 Monoclonal antibody was obtained from Chemicon international (Billerica, MA, USA). Goat Anti-MRP3 Polyclonal antibody and Donkey anti-goat IgG, HRP conjugated, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Affinity purified antibody peroxidase labeled goat anti-mouse IgG (H+L) and affinity purified antibody peroxidase labeled goat anti-rabbit IgG (H+L) were obtained from Kirkegaard & Perry Labolatories (Gaithersburg, MD, USA). All other chemicals used were of the highest purity available.

4.2. Synthesis of DNP-SG

DNP-SG was synthesized using FDNB as a lead compound in the same manner as reported previously (Yokooji et al. 2010). The DNP-SG synthesized was chromatographically pure, as well as in previous studies (Yokooji et al. 2010; Matsushima et al. 2017).

4.3. Animals

Male Sprague–Dawley (SD) rats aged 7–9 weeks were used. Experiments with animals were performed in accordance with the "Guide for Animal Experimentation" from the Committee of Research Facilities for Laboratory Animal Sciences, Hiroshima International University, which is in accordance with the guidelines for proper conduct of animal experiments" from Science Council of Japan. The license number of this animal study was AE15-005.

4.3.1. Cisplatin-induced ARF state in rats

Cisplatin-induced ARF was induced by an intraperitoneal injection of cisplatin to rats at a dose of 9 mg/kg (Liu et al. 2012). The induction of ARF and/or AHF states was examined by determining plasma concentrations of indoxyl sulfate, creatinine, GOT, GPT, T-BIL, and D-BIL 72 h after the cisplatin treatment. Control rats received the same volume of saline.

4.3.2. Determination of DNP-SG concentrations in plasma, bile, and tissue after intravenous administration of CDNB in control and ARF rats

Rats were anesthetized with pentobarbital (40 mg/kg), and cannulation was made with polyethylene tubing (PE30) at the bile duct for the bile sampling. CDNB was administrated intravenously into the jugular vein at a dose of 30 µmol/kg, and blood and bile were sampled periodically. Blood samples were centrifuged at 1,500 g for 10 min to obtain plasma samples. At 30 min after administration of CDNB, rats were killed by decapitation and 30 mL of ice-cold saline was injected from the portal vein to remove blood in blood vessels systemically, except the brain. The brain, liver, kidney, spleen, and lung were isolated, and each tissue was washed with ice-cold saline.

4.3.3. Determination of DNP-SG concentrations in plasma, bile, and tissues under a constant rate infusion of DNP-SG in control and ARF rats

Steady state plasma concentrations of DNP-SG in plasma, bile, and tissues were estimated in control and ARF rats. Rats were anesthetized with pentobarbital (40 mg/kg), and cannulation was made at the bile duct (PE-30) for bile sampling and at the femoral vein (PE50) for the infusion of DNP-SG solution. DNP-SG was dissolved at a concentration of 15 mM in Tris-HCl buffer (100 mM Tris/HCl, 100 mM NaCl, pH 7.4). DNP-SG was administrated through the cannulated femoral vein at a dose of 30 µmol/2 mL/h, and blood and bile were sampled. Blood samples were centrifuged at 1,500 g for 10 min to obtain plasma samples. At 100 min after the initiation of constant rate infusion of DNP-SG, rats were killed by decapitation and immediately 30 mL of ice-cold saline was injected from the portal vein to remove the blood from the vessels. The brain, liver, kidney, spleen, and lung were isolated, and each tissue was washed in a short time with ice-cold saline.

4.4. Western blot analysis

The protein levels of MRP1-4 in the brain, liver, and kidney of rats were evaluated by Western blot analysis after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using crude membrane (CM) fraction of these tissues in the same manner as reported previously with a minor modification (Oda et al. 2011; Yokooji et al. 2014). Briefly, rats 72 h after injection of saline or cisplatin were anesthetized with pentobarbital (40 mg/kg), and killed by decapitation and 30 mL of ice-cold saline were injected from the portal vein. Brain, liver, and kidney were immediately isolated and washed in a short time with ice-cold Tris-HCl buffer (150 mM NaCl, 10 mM Tris/HCl, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4). The tissue was homogenized for 2 min with a Polytron tissue homogenizer (IKA, Labortechnik, Staufen, Germany) and subsequently with a glass-Teflon Potter homogenizer (1,000 rpm, 10 strokes). The homogenates were centrifuged at 8,000 g for 10 min, the supernatants were centrifuged at 15,000 g for 15 min, and then the supernatants were centrifuged at 100,000 g for 60 min. The pellet was suspended in Tris-HCl buffer containing surfactants (150 mM NaCl, 10 mM Tris/HCl, 5 mM EDTA, 1 mM PMSF, 1% Triton-X 100, 0.1% SDS, 1% sodium deoxycholate, pH 7.4). The suspension was dispersed by sucking 50 times through a fine needle (0.40 × 19 mm) attached with plastic syringe. After suspensions were centrifuged at 10,000 g for 15 min, the supernatants including CM fractions were stored at -80 °C. All procedures were performed on ice or at 4 °C. The concentration of protein was measured by the Lowry method using bovine serum albumin as the standard (Lowry et al. 1951). The CM samples were applied to a gel at an amount of 20 µg protein, and SDS-PAGE was performed. For the detection of MRP1-4, Rabbit Anti-MRP1 Polyclonal antibody (1:500 dilution), Mouse Anti-MRP2 Monoclonal antibody (1:1000 dilution), Goat Anti-MRP3 Polyclonal antibody (1:1000 dilution) and Rabbit Anti-MRP4 Polyclonal antibody (1:200 dilution) were used as the primary antibodies. As the secondary antibodies, affinity purified antibody peroxidase labeled goat anti-mouse IgG (H+L) (1:50000 dilution), donkey anti-goat IgG, HRP conjugated (1:5000 dilution), and affinity purified antibody peroxidase labeled goat anti-rabbit IgG (H+L) (1:5000 dilu-

tion) were used, respectively. The optical densities of immuno-reactive proteins were detected by Light-Capture AE-6971/2 (ATTO, Tokyo, Japan).

4.5. Extraction of uremic plasma components

Plasma obtained from rats 72 h after treatment with saline or cisplatin was deproteinized by mixing with the same volume of acetonitrile. After centrifugation at 1,500 g for 10 min, the supernatant containing acetonitrile was collected and evaporated to dryness with an evaporator (CC-105, Tomy Seiko, Tokyo, Japan). The residue was stored in a freezer kept at -20 °C until use.

4.6. Cell culture and accumulation study

HepG2 cells (6 and 8 passages), a hepatocellular carcinoma cell line, was maintained in culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. The intracellular accumulation of calcein after application of calcein-AM was examined in HepG2 cells in the same manner as reported previously with a minor modification (Yokooji et al. 2014). Briefly, HepG2 cells were seeded at a density of 5×10^4 cells/mL on the 24-well plates and cultured for 7 days. The culture medium was exchanged with fresh medium every 2-3 days. The calcein-AM was dissolved in phosphate-buffered saline (PBS) (137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5 mM D-glucose, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) at a concentration of 500 nM with 2 µM elacridar, a P-gp inhibitor. A small amount of DMSO was used to dissolve calcein-AM, elacridar, and plasma components, at which the final concentration of DMSO was adjusted at less than 1% in all procedures. After removal of the culture medium, each well was washed twice with 1 mL of PBS, and the cells were pre-incubated with 300 µL of PBS containing 2 µM elacridar with or without 1% or 5% plasma components, 200 µM cisplatin or 25 µM MK-571, a MRP inhibitor, at 37 °C for 30 min. Then, 300 µL of PBS containing 500 nM calcein-AM and 2 µM elacridar was added to each well and the cells were incubated at 37 °C. After incubation for 10 min, the medium was removed by aspiration and the cells were washed two times with 1 mL of ice-cold PBS. The cells were collected in 400 µL of 1 mM HEPES/Tris containing 0.1% (v/v) Triton X-100 (pH 7.4). The cells were lysed by vortex-mixing and the homogenate was allowed to stand for 1 h. After centrifugation at 9,560 g for 5 min, the supernatant was used to measure fluorescence intensity of calcein and protein contents. The concentration of calcein in cells was determined by microplate fluorometer (Perkin Elmer, Waltham, MA, USA) at wavelengths of 485 nm for excitation (Ex) and 535 nm for emission (Em), respectively.

4.7. Sample analysis

Biological fluid samples containing DNP-SG or indoxyl sulfate were mixed with perchloric acid solution to adjust a final concentration of perchloric acid of more than 4% or with a two-fold volume of methanol for deproteinization. The deproteinized samples were kept on ice at least for 30 min and centrifuged at 8,000 g for 10 min. Concentrations of DNP-SG and indoxyl sulfate were determined by HPLC using a column of J-Pak Wrapsil RP-18 (Jasco Engineering, Tokyo, Japan). Mobile phases used were a mixture of 1% acetic acid and acetonitrile in a ratio of 85:15 (v/v) for DNP-SG and a mixture of pH 4.5, 0.2 M acetate buffer and acetonitrile in a ratio of 93:7 (v/v) for indoxyl sulfate, respectively. Detection of DNP-SG was made at UV 365 nm and indoxyl sulfate was detected by fluorometer at Ex. 280 nm and Em. 375 nm, respectively. The flow rate of mobile phase was 1 mL/min for both compounds. The concentration of protein was measured by the Bradford method using bovine serum albumin as the standard (Bradford 1976).

4.8. Statistical analysis

Statistical analysis was performed by Student's t-test, or by one-way analysis of variance followed by the tukey Test for multiple comparisons. A difference of $P<0.05$ was considered statistically significant.

Conflicts of interest: None declared

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