European Journal of Pharmaceutical Sciences 101 (2017) 66–70

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

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

Effects of nonsteroidal anti-inflammatory drugs on the renal excretion of indoxyl sulfate, a nephro-cardiovascular toxin, in rats

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ARTICLE INFO

Article history:
Received 3 October 2016
Received in revised form 10 January 2017
Accepted 5 February 2017
Available online 7 February 2017

Keywords:
NSAIDs
Chronic kidney disease
Uremic toxin
Indoxyl sulfate
Organic anion transporters

ABSTRACT

Chronic kidney disease (CKD) is a health problem worldwide. Indoxyl sulfate (IS) is a nephro-cardiovascular toxin accumulated in CKD patients and cannot be removed through hemodialysis. The renal excretion of IS was mediated by organic anion transporters (OATs) OAT 1 and OAT 3. Because a number of nonsteroidal anti-inflammatory drugs (NSAIDs) have been reported to inhibit OATs, we hypothesize that NSAIDs might inhibit the renal excretion of IS. Rats were intravenously injected IS with and without diclofenac, ketoprofen or salicylic acid, individually. Blood samples were collected at predetermined time points and the concentrations of IS were determined by HPLC method. The results showed that diclofenac and ketoprofen at 10.0 mg/kg significantly decreased the systemic clearance of IS by 71% and 82%, and increased the MRT of IS by 106% and 105%, respectively, whereas salicylic acid did not exhibit significant effects. Cell studies indicated that diclofenac and ketoprofen inhibited the uptake of IS mediated by OAT 1 and OAT 3. In conclusion, diclofenac and ketoprofen inhibited the excretion of IS through inhibition on OAT 1 and OAT 3.

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1. Introduction

Chronic kidney disease (CKD) is becoming a major global health problem (Hernandez and Nasri, 2014). The occurrence of CKD is likely associated with uses of medications (Choudhury and Ahmed, 2006). It is well known that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) may lead to CKD so called analgesic nephropathy (Harirforoosh and Jamali, 2009; Whelton, 1999). It was hypothesized that NSAIDs increased the risk of CKD through recurrent episodes of acute kidney injury. Subsequently, chronic exposure to NSAIDs may worsen unrecognized acute interstitial nephritis that could develop chronic interstitial nephritis and papillary necrosis (Kleinknecht, 1995; Rossert, 2001; Segasothy et al., 1994). However, till now the underlying molecular mechanisms of analgesic nephropathy have not been fully understood.

In CKD patients, uremic toxins were accumulated in the body, and so far the known varieties of uremic toxins were >90 (Vanholder et al., 2003). Indoxyl sulfate (IS) is one of acidic uremic toxins highly bound to plasma protein (Lekawanvijit and Krum, 2015), and cannot removed through hemodialysis. Therefore, the IS in blood was also accumulated in those patients undergoing hemodialysis treatment (Herget-Rosenthal et al., 2009; Muting, 1965). Recent studies reported that the blood IS concentration was associated with the progression of CKD, cardiovascular disease (CVD) and mortality (Barreto et al., 2009; Lekawanvijit and Krum, 2015; Lin et al., 2012; Wu et al., 2011). Owing to the strong acidity, IS exists totally as anion under physiological pH in the systemic circulation, and the uptake transport of IS into the cells of renal proximal tubules was mediated by organic anion transporters (OAT) 1 and OAT 3 (Deguchi et al., 2004; Enomoto et al., 2002; Peng et al., 2015). On other hand, numerous NSAIDs were reported to interact with OAT 1 and OAT 3 (Rizwan and Burckhardt, 2007; VanWert et al., 2010). Therefore, we hypothesized that the renal excretion of IS might be inhibited by NSAIDs.

Among NSAIDs, diclofenac and ketoprofen were the commonly recommended and highly consumed NSAIDs in clinical practice (McGettigan and Henry, 2013; Sarzi-Puttini et al., 2010). Aspirin, the prodrug of salicylic acid, was widely prescribed for the prevention of heart attack and stroke in high-risk patients beyond the indications of

Abbreviations: CKD, chronic kidney disease; NSAIDs, non-steroidal anti-inflammatory drugs; IS, indoxyl sulfate; CVD, cardiovascular diseases; OAT, organic anion transporters; CHO, Chinese hamster ovary; HEK 293, human embryonic kidney 293; MDCKII, Madin-Darby canine kidney type II; MRPs, multidrug resistance-associated proteins; AUC_{0-t}, the areas under the curve from time zero to last; Cl, clearance; MRT, mean residence time; 6,7-DMC, 6,7-dimethoxycoumarin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modified eagle medium; HBSS, Hank's buffered salt solution.

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http://dx.doi.org/10.1016/j.ejps.2017.02.007
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pain reliever, fever reduction and anti-inflammation (Kim and Bang, 2013). This study investigated the effects of diclofenac, ketoprofen and salicylic acid, the major metabolite of aspirin, on the pharmacokinetics of intravenous IS in rats. Furthermore, cell models were used to verify the effects of NSAIDs on the uptake transport of IS into cells mediated by OAT 1 and OAT 3.

2. Materials and methods

2.1. Chemicals and reagents

Indoxyl sulfate (purity 97%) was obtained from Alfa Aesar (Lancaster, UK). Phosphoric acid (glacial, 85%), protocatechuic acid, probenecid and ketoprofen were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 6,7-dimethoxycoumarin (6,7-DMC, purity 98%) was supplied by Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Diclofenac was purchased from Yung Shin (Taichung, R.O.C.). Salicylic acid was supplied by Wako Pure Chemical Co. (Osaka, Japan). Acetonitrile and methanol were LC grade and obtained from Mallinckrodt Baker (Phillipsburg, NJ, U.S.A.). Fetal bovine serum was obtained from Biological Industries Inc. (Kibbutz, Beit Haemek, Israel). Penicillin/Streptomycin/Glutamine, Dulbecco's modified Eagle medium (DMEM), trypsin/EDTA, Hank's balanced salt solution (HBSS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Invitrogen (Carlsbad, CA, U.S.A.). Dulbecco's modified Eagle medium F12 (DMEM/F12) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). MK571 was purchased from Enzo Life Science (Farmingdale, NY, U.S.A.). Milli-Q plus water (Millipore, Bedford, MA) was used for all preparations.

2.2. Animals

The animal study adhered to "The Guidebook for the Care and Use of Laboratory Animals" published by the Chinese Society for the Laboratory Animal Science, Taiwan, R.O.C. The Institutional Animal Care and Use Committee of China Medical University approved this experimental protocol (Permit number: 104-339). Male Sprague-Dawley rats weighing 480–600 g were supplied by BioLASCO Taiwan Co., Ltd. (Yi-Lan, Taiwan) and kept in a 12 h light-dark cycle, constant temperature environment in the Animal Center of China Medical University (Taichung, Taiwan) prior to study.

2.3. Drug administration

Rats were randomly divided into four groups ($n = 5$ in each group), and drug administrations were conducted in a parallel design. Before experiment, rats were fasted overnight, but drinking water was allowed ad libitum. The control rats were intravenously injected a bolus of IS (10.0 mg/kg), and the other three groups were injected a bolus of IS (10.0 mg/kg) with a bolus of 10.0 mg/kg of diclofenac, ketoprofen and salicylic acid, individually, at 5 min prior to IS. Equal volume of saturated sodium bicarbonate solution was administered at 5 min prior to IS as blank vehicle for control rats. Food was withheld for another 3 h after dosing.

2.4. Blood collections

In pharmacokinetic study, blood samples (0.3 mL) were withdrawn at 5, 15, 30, 45, 60, 90, 120 and 180 min after dosing of IS. Blood samplings were conducted under anesthesia with 2–3% isoflurane to minimize the suffering and distress of rats. The blood specimens were centrifuged at 10,000 $\times g$ for 15 min to obtain serum and stored at −30 °C until analysis.

2.5. Cell line and culture conditions

Chinese hamster ovary (CHO) cells expressing hOAT 1 (CHO-hOAT 1), human embryonic kidney 293 (HEK 293) cells expressing hOAT 3 (HEK 293-hOAT 3) and their corresponding empty vector-transfected control cell lines were used. CHO cells were maintained at 37 °C with 5% CO_2 in DMEM/F-12 media containing 10% serum, 1% penicillin/streptomycin and 1 mg/mL G418. HEK 293 cells were maintained at 37 °C with 5% CO_2 in DMEM high glucose media containing 10% serum, 1% penicillin/streptomycin and 50 μg/mL of hygromycin B. Cells were cultured in Poly-D-Lysine coated dishes.

Madin-Darby canine kidney type II (MDCK II) cells were kindly provided by Prof. Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam, Netherlands) and maintained at 37 °C with 5% CO_2 in DMEM media containing 10% serum and 1% penicillin/streptomycin/glutamine.

2.6. Effects of NSAIDs on the uptake transport of IS

CHO-hOAT 1 (2×10^5 cells/well) and HEK 293-hOAT 3 cells (2.5×10^5 cells/well) were cultured in a 12-well plate. Probenecid (200 μM) was used as a positive control for inhibitors of hOAT 1 and hOAT 3 (Wang and Sweet, 2012). After 48-h incubation of CHO-hOAT 1 or HEK 293-hOAT 3, the medium was removed and washed with PBS buffer. Before the transport study, CHO-hOAT 1 and HEK 293-hOAT 3 cells were pre-incubated with test agents (NSAIDs and probenecid) at 37 °C. After 30-min incubation, IS was added and incubated for another 5 min and 10 min, respectively. The plates were immediately placed on ice bath, and the supernatants were removed and the cells were washed with ice-cold PBS. Subsequently, 200 μL of 0.1% Triton X-100 was added to lyse the cells.

For the quantitation of IS, 50 μL of cell lysate was vortexed with 200 μL of methanol containing 2 μg/mL of 6,7-DMC (internal standard) and centrifuged to remove the precipitate, then 20 μL was subject to HPLC analysis. The HPLC apparatus included a pump (LC-10AT, Shimadzu, Kyoto, Japan), a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan) and automatic injector (SIL-10AF, Shimadzu, Kyoto, Japan). The Apollo C18 (5 μm, 4.6 mm × 250 mm, Alltech, Deerfield, IL, U.S.A.) column was equipped with a guard column (4.6 × 50 mm, 5 μm) (GL Science Inc., Tokyo, Japan). The mobile phase consisted of acetonitrile (A) – 0.1% phosphoric acid (B) and programmed in a gradient manner as follows: A/B: 17/83 (0–7 min), 40/60 (9–19 min) and 17/83 (19–25 min). Detector settings were E_x 280 nm/E_m 375 nm. The flow rates were 1.0 mL/min.

To quantitate the content of protein in each well, 10 μL of cell lysate was added to 200 μL of diluted protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.) and the optical density was measured at 570 nm. The relative intracellular accumulation of IS was calculated by comparing with that of controls after protein correction.

2.7. Data analysis

The areas under the curve from time zero to last ($AUC_{0\text{-}t}$), clearance (Cl) and mean residence time (MRT) were calculated by noncompartment model of Phoenix® WinNonlin® (version 6.3 Pharsight Corp., NC, U.S.A.). The half-lives were calculated from elimination constants, which were the slopes obtained through linear regression of the terminal portion of semi-log concentration - time curve. The differences among groups were analyzed by SPSS software using ANOVA with Scheffe test for in vivo study, and unpaired Student's *t*-test for in vitro studies, taking $P < 0.05$ as significant level.

3. Results

3.1. Effect of NSAIDs on the pharmacokinetics of intravenous IS in rats

The determination of serum IS concentrations followed our previous study (Peng et al., 2015). The mean serum concentration - time profiles of IS after intravenous injection of IS with and without diclofenac, ketoprofen and salicylic acid are shown in Fig. 1. The curves of IS after coadministrations of diclofenac and ketoprofen were well above the

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