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Renal tubular secretion of tanshinol: molecular mechanisms, impact on its systemic exposure, and propensity for dose-related nephrotoxicity and for renal herb-drug interactions

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Running Title: RENAL TUBULAR SECRETION OF TANSHINOL

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ABBREVIATIONS: $AUC_{0-\infty}$, area under the plasma concentration-time curve from zero to infinity; CL_{int}, intrinsic clearance; CL_R, renal clearance; $CL_{R\text{-}cr}$, renal clearance of endogenous creatinine; $CL_{R,c\text{-}u}$, renal clearance by the cellular efflux into urine across the apical brush border membrane; $CL_{tot,p}$, total plasma clearance; C_{5min}, concentration at 5 min after dosing; $Cum.A_{e\text{-}U}$, cumulative amount excreted into urine; $f_{e\text{-}U}$, fraction of dose excreted into urine; f_u, unbound fractions in plasma; GFR, glomerular filtration rate; HEK-293, human embryonic kidney cell line; IC_{50}, half maximal inhibitory concentration; i.v., intravenous; K_m, Michaelis constant; OAT, Organic anion transporter; PK, pharmacokinetic; $t_{1/2}$, elimination half-life; V_{max}, maximum velocity; V_{SS}, apparent volume of distribution at steady state.

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ABSTRACT

Tanshinol has desirable antianginal and pharmacokinetic properties and is a key compound of *Salvia miltiorrhiza* roots (Danshen). It is extensively cleared by renal excretion. This study was designed to elucidate the mechanism underlying renal tubular secretion of tanshinol and to compare different ways to manipulate systemic exposure to the compound. Cellular uptake of tanshinol was mediated by human OAT1 (K_m, 121 μM), OAT2 (859 μM), OAT3 (1888 μM), and OAT4 (1880 μM) and rat Oat1 (117 μM), Oat2 (1207 μM), and Oat3 (1498 μM). Other renal transporters (human OATP4C1, OCT2, OCTN1, MATE1, MATE2-K, MRP2, MRP4, and BCRP and rat Oct1, Oct2, Octn1, Octn2, Mate1, Mrp2, Mrp4, and Bcrp) showed either ambiguous ability to transport tanshinol or no transport activity. Rats may be a useful model, to investigate the contribution of the renal transporters on the systemic and renal exposure to tanshinol. Probenecid-induced impairment of tubular secretion resulted in a 3–5-fold increase in rat plasma $AUC_{0-\infty}$ of tanshinol. Tanshinol exhibited linear plasma PK properties over a large intravenous dose range (2–200 mg/kg) in rats; the dosage adjustment could result in increases, in plasma $AUC_{0-\infty}$ of tanshinol, of about 100-fold. Tanshinol exhibited very little dose-related nephrotoxicity. In summary, renal tubular secretion of tanshinol consists of uptake from blood, primarily by OAT1/Oat1, and the subsequent luminal efflux into urine mainly by passive diffusion. Dosage adjustment appears to be an efficient and safe way to manipulate systemic exposure to tanshinol. Tanshinol shows low propensity to cause renal transporter-mediated herb-drug interactions.

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Introduction

Herbal medicines normally contain many constituents. It is hypothesized that only a few constituents with favorable drug-like properties, rather than all the constituents present, are responsible for the pharmacological effects of an herbal medicine (Lu et al., 2008). An herbal constituent can be defined as drug-like if it possesses the desired pharmacological potency, a wide safety margin, appropriate pharmacokinetic (PK) properties, and adequate content in the medicine dosed. Recent multicompound PK studies have indicated that human subjects and laboratory animals are considerably exposed to only a few constituents of an herbal medicine after dosing (Lu et al., 2008; Liu et al., 2009; Li et al., 2012a; Chen et al., 2013; Cheng et al., 2013; Hu et al., 2013; Jiang et al., 2015; Li et al., 2015). Such PK studies provide information for pharmacologists regarding which herbal compounds merit further evaluation. Follow-up PK studies should focus on the potentially important herbal compounds that exhibit desired pharmacological properties and considerable body exposure after dosing. Understanding molecular mechanisms underlying the major elimination pathways of key herbal compounds is a goal of such studies. This helps identify factors influencing the compound concentration after dosing and predict potential for the compound-related herb-drug interactions.

Salvia miltiorrhiza roots (Danshen) are used extensively in the treatment of angina pectoris in China (Zhou et al., 2005; Cheng, 2007). The emerging antianginal therapies are facilitating long-term use because they appear to have low incidence of side effects (Jia et al., 2012). The Danshen therapies are given orally or intravenously. Polyphenols are believed to be a class of major pharmacologically relevant constituents of Danshen. A PK study of cardiotonic pills, a Danshen-based formulation, in human subjects and laboratory animals indicated that tanshinol was the only Danshen polyphenol that exhibited considerable systemic exposure after dosing (Lu et al., 2008). The other polyphenols, including salvianolic acids A, B, and D, rosmarinic acid, lithospermic acid, and protocatechuic aldehyde, were

either poorly absorbed from the gastrointestinal tract or extensively metabolized; this resulted in their poor detection in plasma after dosing. Tanshinol was found to be the most abundant Danshen polyphenol in clinically important Danshen-based intravenous injections (details pending publication elsewhere). In a recent PK study of DanHong injection, a Danshen-based intravenous formulation, in human subjects and laboratory animals, tanshinol exhibited the most significant systemic exposure, of the Danshen polyphenols, after dosing (Li et al., 2015). The preceding PK studies of Danshen polyphenols after dosing Danshen-based formulations suggest that tanshinol deserves prior attention in further investigation.

Studies have shown that tanshinol exhibited vasodilatory properties, elevated serum nitric oxide levels and action of endothelial nitric oxide synthase, protected endothelial cells from homocysteine-induced injury and H_2O_2-induced apoptosis, and exerted antioxidant effects (Lam et al., 2007; Wang et al., 2013; Zhao et al., 2008; Chan et al., 2004; Yang et al., 2009). Tanshinol also decreased blood pressure in spontaneously hypertensive rats and attenuated cardiac hypertrophy, venular thrombosis, and methionine-induced hyperhomocysteinemia in rats (Tang et al., 2011a,b; Wang et al., 2009; Yang et al., 2010; Cao et al., 2009). The preceding pharmacological studies were either cell- or isolated-tissue-based, which provided effective concentrations of tanshinol, or whole animal-based, which provided effective doses of tanshinol. The reported effective concentrations are higher (3–100 times) than human maximum plasma concentrations of tanshinol after dosing Danhong injection at clinical dose level; the reported effective doses in rats also exceed (5–10 times) the rat dose of tanshinol derived from the clinical dose.

Because tanshinol is an antianginal and major PK constituent in Danshen-based therapies, matching its levels of systemic exposure after dosing to its effective concentrations for the antianginal activities most likely results in better translation of its pharmacological properties to the overall antianginal effect of Danshen therapy. This requires having a way to manipulate

the post-dose concentration of tanshinol. Dosage adjustment and drug combination are commonly used, either alone or in concert, to change drug concentration in blood. These may, however, raise some safety concerns. A therapeutically useful method should be both effective (large potential to increase concentration) and safe (such as with very little or no dose-related toxicity). Elimination is often a major determinant of drug concentration after dosing. Renal excretion is the predominant route of elimination of tanshinol in humans and rats; it attributes mainly to active tubular secretion (Lu et al., 2008). The current study was designed to elucidate the molecular mechanisms underlying the renal tubular secretion of tanshinol, to compare different ways to manipulate systemic exposure to tanshinol, and to predict possible renal transporter-mediated herb-drug interactions related to tanshinol.

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Materials and methods

Materials. Tanshinol (sodiated form; >98.0%) were obtained from the National Institutes for Food and Drug Control (Beijing, China). *Para*-aminohippuric acid, prostaglandin $F_{2\alpha}$, estrone-3-sulfate, estradiol-17β-D-glucuronide, tetraethylammonium, methotrexate, probenecid, cimetidine, verapamil, indomethacin, novobiocin, creatinine, puromycin, and cisplatin were obtained from Sigma-Aldrich (St. Louis, MO). Inside-out membrane vesicles (5 mg protein/ml; prepared from insect cells expressing human multi-drug resistance associated protein (MRP) 2, human MRP4, human breast cancer resistance protein (BCRP), rat Mrp2, rat Mrp4, or rat Bcrp were purchased from Genomembrane (Kanazawa, Japan).

Cellular Transport Assays. HEK-293 cells (ATCC, Manassas, VA) were grown, at 37°C and 5% CO_2, in Dulbecoo's modified Eagle medium, which was fortified with 10% fetal bovine serum, 1% MEM non-essential amino acids, and 1% antibiotic-antimycotic solution. Full open reading frames of human organic anion transporter (OAT) 1-, human OAT2-, human OAT3-, human OAT4-, human organic anion-transporting polypeptide (OATP) 4C1-, human organic cation transporter (OCT) 2-, human carnitine/organic cation transporter (OCTN) 1-, human multi-drug and toxin extrusion protein (MATE) 1-, human MATE2-K-, rat Oat1-, rat Oat2-, rat Oat3-, rat Oct1-, rat Oct2-, rat Octn1-, rat Octn2-, and rat Mate1-cDNA were synthesized and subcloned into pcDNA 3.1(+) expression vectors. The inserts of the pcDNA 3.1(+)-transporter constructs were sequenced and aligned according to the GenBank accession numbers NM_004790, NM_006672, NM_004254, NM_018484, NM_180991, NM_003058, NM_003059, NM_018242, NM_152908, NM_017224, NM_053537, NM_031332, NM_012697, NM_031584, NM_022270, NM_019269, and NM_001014118, respectively (www.ncbi.nlm.nih.gov/genbank/). The pcDNA 3.1(+)-transporter constructs and the empty vector were introduced separately into the

HEK-293 cells with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). This produced transporter-expressing cells and mock-transfected cells, respectively. Before use, the transfected cells were validated functionally using positive substrates *para*-aminohippuric acid (for OAT1 and Oat1), prostaglandin $F_{2\alpha}$ (OAT2 and Oat2), estrone-3-sulfate (OAT3, OAT4, OATP4C1, and Oat3) and tetraethylammonium (OCT2, OCTN1, MATE1, MATE2-K, Oct1, Oct2, Octn1, Octn1, and Mate1) and using positive inhibitors probenecid (for OAT1, OAT2, OAT3, OAT4, OATP4C1, Oat1, Oat2, and Oat3), cimetidine (OCT2, MATE1, MATE2-K, Oct1, Oct2, and Mate1), and verapamil (OCTN1, Octn1, and Octn1).

Transport studies were carried out in 24-well poly-D-lysine-coated plates with cells 48 h after transfection. After washing twice with Krebs-Henseleit buffer (containing 118 mM NaCl, 4.83 mM KCl, 1.53 mM $CaCl_2$, 0.96 mM KH_2PO_4, 23.8 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 5 mM glucose, and 12.5 mM HEPES; pH 7.4; 500 µl per wash; the second wash involving 10-min preincubation at 37°C; pH 7.4), the transfected cells and the mock-transfected cells were incubated with tanshinol in the presence or absence of probenecid. After incubation for 10 min, the transport was terminated by removing the medium from the wells and rapidly rinsing the cells three times with ice-cold Krebs-Henseleit buffer (500 µL per rinse). Different from those with the other transporters, the transport studies with human MATE and rat Mate used a buffer (containing 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES, and 30 mM NH_4Cl; pH 7.4) for cell-washing and preincubation; the buffer (removing 30 mM NH_4Cl; pH 8.4) was used for incubation. The cells were lysed with water (150 µl) using a freeze-thaw and ultrasonication. Aliquots (100 µl) of the resulting lysates were precipitated with ice-cold acetonitrile (300 µl). After centrifugation at 21,100 × *g* for 10 min, the supernatants (5 µl) were analysed by LC-MS/MS. The total amount of protein in the lysate was measured using a method first described by Bradford (1976). The transport

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rate in pmol/mg protein/min was calculated using the following equation:

$$\text{Transport} = (C_C \times V_C)/T/W_C$$

where C_C, V_C, T, and W_C represent concentration of test compound in cellular lysates (μM), volume of the lysates (μl), incubation time (10 min), and measured cellular protein amount of each well (mg), respectively. Differential uptake between the transfected cells (TC) and the mock cells (MC) was defined as net transport ratio ($\text{Transport}_{TC}/\text{Transport}_{MC}$ ratio); a net transport ratio greater than three suggested a positive result.

The kinetics of human OAT1-, human OAT2-, human OAT3-, human OAT4-, rat Oat1-, rat Oat2-, and rat Oat3-mediated cellular uptake of tanshinol was assessed with respect to Michaelis constant (K_m), maximum velocity (V_{max}), and intrinsic clearance (CL_{int}). The incubation conditions were the same as those for the preceding transport study except for using an incubation time of 5 min. The incubation time was optimized to ensure that the assessment was performed under linear uptake conditions. The concentrations of tanshinol in the incubation medium were 25–1600 μM for OAT1 and Oat1 and 156–5000 μM for OAT2, OAT3, OAT4, Oat2, and Oat3. Background accumulation of tanshinol was also determined in the mock-transfected cells. The inhibitory effect of probenecid on the activity of OAT1-, OAT2-, OAT3-, OAT4-, Oat1-, Oat2-, and Oat3-mediated cellular uptake of tanshinol was measured with respect to half maximal inhibitory concentration (IC_{50}); the tanshinol concentrations were equal to the respective K_m values for the transporters. The IC_{50} values of tanshinol against *para*-aminohippuric acid (10 μM), prostaglandin $F_{2\alpha}$ (10 μM), estrone-3-sulfate (10 μM), and estrone-3-sulfate (10 μM) for OAT1, OAT2, OAT3, and OAT4, respectively, were also measured.

Vesicular Transport Assays. Membrane vesicles expressing one of the transporters human MRP2, human MRP4, human BCRP, rat Mrp2, rat Mrp4, and rat Bcrp were tested with tanshinol using a rapid filtration method. Before use, these membrane vesicles were

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functionally validated using estradiol-17β-D-glucuronide and methotrexate. To start the transport, preincubated membrane vesicle suspension (10 µl) was combined with preincubated tanshinol/ATP or tanshinol/AMP medium (50 µl). After incubation for 10 min, the transport was terminated by adding 200 µl of an ice-cold buffer (containing 40 mM MOPS and 70 mM KCl; adjusted to pH 7.0 with 1.7 M Tris-base) followed by immediate transfer of the mixture into a Millipore MultiScreen-FB filtration plate (0.65 µm; Billerica, MA, USA). Five washes of the membrane vesicles with the ice-cold terminating buffer (200 µl per wash) were performed and the filters that retained membrane vesicles were transferred to 1.5-ml polypropylene tubes. The membrane vesicles were lysed and extracted with 200 µl of 80% methanol per sample. After centrifugation at 21,100 × *g* for 10 min, the supernatants (5 µl) were analysed by LC-MS/MS. The transport rate in pmol/mg protein/min was calculated using the following equation:

$$\text{Transport} = (C_V \times V_V)/T/ W_V$$

where C_V, V_V, T, and W_V represent concentration of the compound in vesicular lysates supernatant (µM), volume of the lysates (µl), incubation time (10 min), and amount of vesicle protein amount per well (0.05 mg), respectively. Positive results for ATP-dependent transport were defined as net transport ratio ($Transport_{ATP}$/$Transport_{AMP}$ ratio) greater than two.

Rat Pharmacokinetic Studies. Use and treatment of rats were in compliance with the Guidance for Ethical Treatment of Laboratory Animals (The Ministry of Science and Technology of China, 2006). Three rat PK studies were conducted, all according to protocols approved by the Institutional Animal Care and Use Committee at the Shanghai Institute of Materia Medica (Shanghai, China). Male Sprague-Dawley rats (230–270 g) were obtained from Sino-British SIPPR/BK Laboratory Animal (Shanghai, China). The femoral arteries were cannulated for blood sampling and the rats were allowed to regain their preoperative body weight before use. The rats were euthanatized with CO_2 gas after use.

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The aim of the first rat PK study was to determine the impact of tubular secretion on systemic exposure to and renal excretion of tanshinol after i.v. administration of tanshinol. Four rats were individually housed in rat metabolic cages and the urine collection tubes were kept at −20°C. The study was conducted in a two-period, two-sequence crossover fashion with a two-day washout between periods 1 and 2. In sequence 1, two of the rats received an i.v. bolus dose of a tanshinol solution (0.4 mg tanshinol/ml) and an i.v. bolus dose of a probenecid-tanshinol solution (0.4 mg tanshinol/ml + 20 mg probenecid/ml) at 5 ml/kg through the tail veins in periods 1 and 2, respectively. In sequence 2, the other two rats received these solutions in reverse order. Before and after each dosing, serial blood samples (80 μl; 0, 5, 15, and 30 min and 1, 2, and 4 h) were collected, heparinized and centrifuged to produce the plasma fractions. Urine samples were also collected 0–4, 4–12, and 12–24 h after dosing and weighed. The rat study was repeated once.

The second rat PK study was designed to assess changes in tissue distribution of tanshinol caused by the probenecid-induced impairment of tubular secretion. Rats were randomized into two groups. They received an i.v. bolus dose of the tanshinol solution (0.4 mg tanshinol/ml) at 5 ml/kg or an i.v. bolus dose of the probenecid-tanshinol solution (0.4 mg tanshinol/ml + 20 mg probenecid/ml) at 5 ml/kg through the tail veins. The rodents (under isoflurane anesthesia) were killed by bleeding from the abdominal aorta at 0, 5, and 30 min and 1 and 2 h after dosing (three rats per point in time). The collected blood samples were heparinized and centrifuged to produce the plasma fractions. Selected tissues, including the heart, lungs, brain, liver, and kidneys, were excised, rinsed in ice-cold saline, blotted, weighed, and homogenized in fourfold volumes of ice-cold saline. The rat study was repeated once.

The third rat PK study was a single ascending dose study. It was performed to determine the influence of dose on level of systemic exposure to and renal disposition of tanshinol. Rats were randomly divided into five groups (12 rats per group) and each group received an i.v.

bolus dose of tanshinol solution (0.4, 1, 3, 10, or 40 mg tanshinol/ml) at 5 ml/kg through the tail veins. Three rats from each group were randomly selected and received an i.v. dose of tanshinol at their group dose level. Urine samples were collected 0–24 h after dosing. After the urine sampling was completed, the rats were returned to their group enclosures and received a two-day washout. Thereafter, all the rats in the different groups received an i.v. dose of tanshinol at the designated level. Each group of rats was further divided randomly and the rats were killed under isoflurane anesthesia by bleeding from the abdominal aorta at 5 and 30 min and 1 and 2 h after dosing (three rats per point in time). The blood samples were heparinized and centrifuged to prepare plasma fractions. The kidneys and livers were excised, rinsed and homogenized. All the rat samples were stored at −70°C until analysis.

According to dose normalization by body surface area (Reagan-Shaw et al., 2008), the i.v. dose of tanshinol 2 mg/kg given to rats was close to the clinical daily dose of tanshinol from Danshen injection (a solution prepared from Danshen extract available as a sterile, nonpyrogenic parenteral dosage form for intravenous injection for treatment of angina pectoris; the China Food and Drug Administration ratification no. GuoYaoZhunZi-Z33020177). Accordingly, the i.v. dose of tanshinol 200 mg/kg given to rats was 100 times as much as the clinical daily dose. Plasma PK and urine excretion profile of probenecid were evaluated in rats receiving an i.v. bolus dose at 100 mg/kg.

Assessment of Tanshinol-induced Nephrotoxicity. Rats were randomly divided into four treatment groups (four or five rats per group) and given subchronic doses of tanshinol (i.v. through the tail veins; 200 mg/kg/day), saline (i.v. through the tail veins; 5 ml/kg/day), cisplatin (i.p. into the lower abdomen above the left leg; 1 mg/kg/day), and puromycin (i.p.; 40 mg/kg/day) for 14 consecutive days. On day 15, the rats were anesthetized with isoflurane and killed by bleeding from the abdominal aorta. The collected blood samples were centrifuged to produce serum fractions for measurement of levels of blood urea nitrogen

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(BUN), creatinine (sCr), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). The kidneys and livers were excised, weighed and placed in 10% neutral buffered formalin for histopathologic evaluation. The tissues were fixed for over 24 h, processed and embedded in paraffin. The embedded tissues were sectioned at 4–6 μm and stained with hematoxylin and eosin. Histopathologic examinations of the tissue sections were conducted by a veterinary pathologist and subjected to peer review.

Quantification of Tanshinol and Other Test Compounds in Biological Samples. Validated LC/MS/MS-based bioanalytical methods were used to measure the concentrations of tanshinol, *para*-aminohippuric acid, prostaglandin $F_{2\alpha}$, estrone-3-sulfate, estradiol-17β-D-glucuronide, methotrexate, tetraethylammonium, probenecid, and creatinine in biological samples. A Thermo Fisher TSQ Quantum mass spectrometer (San Jose, CA) was interfaced via an electrospray ionization-probe with an Agilent 1100 series liquid chromatograph (Waldbronn, Germany). Chromatographic separation was achieved on a 5 μm Phenomenex Gemini C_{18} column (50 mm × 2.0 mm I.D.; Torrance, CA). Tetraethylammonium and creatinine levels were analysed using a 3 μm Phenomenex Luna Hilic column (100 mm × 3.0 mm I.D.). The mobile phase, which consisted of solvent A (water/acetonitrile, 98:2, v/v, containing 1 mM formic acid) and solvent B (water/acetonitrile, 2:98, v/v, containing 1 mM formic acid), was delivered at 0.35 ml/min, except for analysis of creatinine at 0.7 ml/min. A pulse gradient elution method was used, in measurement of the compounds (except for creatinine), with an analyte-dependent start proportion (0%–50% solvent B) and analyte-independent elution proportion (100% solvent B), elution proportion segment (1.5 min), and column equilibrium segment (3.5 min) (Wang et al., 2007); for creatinine, the gradient parameters were 100% solvent B and 80% solvent B, 1 min and 7 min, respectively. MS/MS measurement was performed in the negative ion mode with the precursor-product ion pairs for multiple-reaction-monitoring of tanshinol, *para*-aminohippuric

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acid, prostaglandin $F_{2\alpha}$, estrone-3-sulfate, estradiol-17β-D-glucuronide, and probenecid at *m/z* 197→135, 193→93, 353→193, 349→269, 447→271, and 281→140, respectively. Measurement of tetraethylammonium, methotrexate, and creatinine in the positive ion mode was performed at *m/z* 130→86, 445→135, and 114→44, respectively. For measurement of tanshinol in the rat samples, 2 M hydrochloride acid (5 μl) was used to acidify the samples (15 μl) before extraction with ethyl acetate (500 μl). For measurement of creatinine in the rat samples, methanol (40 μl) was used to precipitate the samples (10 μl). Matrix-matched calibration curves were constructed using weighted (1/*X*) linear regression of the analyte area (*Y*) against the corresponding nominal analyte concentration (*X*, μM).

Data Processing. GraFit software (version 5; Surrey, UK) was used to determine K_m and V_{max} by nonlinear regression analysis of initial transport rates as a function of tanshinol concentration. The IC_{50} for inhibition of transport activity was obtained from a plot of percentage activity remaining (relative to control) versus $\log_{10}$ inhibitor concentration.

Plasma PK parameters were determined using noncompartmental analysis with a Thermo Kinetica software package (version 5.0; Philadelphia, PA). The renal clearance (CL_R) was calculated by dividing the cumulative amount excreted into urine ($Cum.A_e$) by the area under the plasma concentration-time curve from zero to infinity ($AUC_{0-\infty}$). Kidney clearance by the cellular efflux into urine across the apical brush border membrane ($CL_{R,c-u}$) was calculated by dividing the $Cum.A_e$ by kidney homogenate $AUC_{0-\infty}$ (Imaoka et al., 2007). The glomerular filtration rate (GFR) of the rats was estimated in terms of renal clearance of endogenous creatinine (CL_{R-cr}; Takahashi et al., 2007). Dose proportionality was assessed using the regression of log-transformed data (the Power model) with the criteria calculated according to a method by Smith et al. (2000). All data are expressed as means ± standard deviations. Statistical analysis was performed with IBM SPSS Statistics Software (version 19.0; Chicago, IL). $P < 0.05$ was considered to be the minimum level of statistical significance.

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Results

In Vitro Interactions between Tanshinol and Human Renal Transporters. There was significantly more uptake of tanshinol into human OAT1-expressing HEK-293 cells than into the mock-transfected cells, suggesting that tanshinol was a substrate of OAT1. Tanshinol was also taken up by human OAT2-, OAT3-, and OAT4-expressing cells. The relevant net transport ratios are shown in Table 1. OAT1-mediated uptake of tanshinol was saturable with K_m, V_{max}, and CL_{int} values shown in Table 2. OAT2, OAT3, and OAT4 exhibited lower affinity for tanshinol than OAT1 (Table2). The OAT1-, OAT2-, OAT3-, and OAT4-mediated uptake of tanshinol was considerably inhibited by probenecid and the IC_{50} values are shown in Table 2. Human OATP4C1, OCT2, OCTN1, MATE1, and MATE2-K did not exhibit any transport activities for tanshinol (Table 1). Human MRP2 and MRP4 had low in vitro transport activities for tanshinol; reliable kinetic parameters were difficult to find. Human BCRP had no significant transport activity. Tanshinol exhibited low inhibition potency towards the OAT transporters; its IC_{50} values against *para*-aminohippuric acid for OAT1, against prostaglandin $F_{2\alpha}$ for OAT2, against estrone-3-sulfate for OAT3, and against estrone-3-sulfate for OAT4 are shown in Table 2. Tanshinol (1 mM) did not exhibit any significant inhibitory activity towards OATP4C1, OCT2, OCTN1, MRP2, MRP4, BCRP, MATE1, and MATE2-K (Table 1).

In Vitro Interactions between Tanshinol and Rat Renal Transporters. Rat Oat1, Oat2, and Oat3 are orthologs of human OAT1, OAT2, and OAT3, respectively. To delineate and extrapolate the in vivo regulatory role of the human renal transporters in the plasma pharmacokinetics and renal disposition of tanshinol, the rat transporters were examined with respect to saturability and affinity. Tanshinol was taken up by rat Oat1-, Oat2-, and Oat3-expressing cells; the relevant K_m, V_{max}, and CL_{int} values are shown in Table 2. The results indicated that Oat1, Oat2, and Oat3 exhibited in vitro saturability and affinity for

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tanshinol similar to their human counterparts, except that the V_{max} of rat Oat3 was considerably greater than that of human OAT3 but comparable with that of human OAT2. Probenecid inhibited the Oat1-, Oat2-, and Oat3-mediated transport of tanshinol with IC_{50} values shown in Table 2. Rat Oct1, Oct2, Octn1, Octn2, and Mate1 exhibited no transport activities for tanshinol (Table 1). Rat Mrp2 and Mrp4 had low in vitro transport activities for tanshinol and rat Bcrp had no significant transport activity. Taken together, the rat renal transporters exhibited profiles of interactions with tanshinol similar to the human transporters.

Impact of Probenecid-impaired Tubular Secretion on Plasma Pharmacokinetics and Disposition of Tanshinol in Rats. To determine the impact of the renal transporter-mediated tubular secretion on systemic exposure to and renal disposition of tanshinol, i.v. bolus dose of probenecid (100 mg/kg) was used to impair the rat tubular secretion of tanshinol by inhibiting Oat1 and Oat3. Probenecid exhibited concentration-dependent binding to rat plasma protein; its unbound fractions in plasma (f_u) increased from 28% to 60% as the plasma concentrations increased from 200 μM to 2000 μM. Probenecid exhibited unbound plasma concentrations at 5 min (unbound C_{5min}) and 4 h after dosing (unbound C_{4h}), which were 100 and 14 times, respectively, as much as its IC_{50} against tanshinol for rat Oat1, respectively (Supplemental Table S1). The unbound C_{5min} and C_{4h} were 60 and 9 times, respectively, as much as the IC_{50} for rat Oat3. These data suggested that probenecid treatment could impair Oat1/Oat3-mediated tubular secretion of tanshinol in rats. Probenecid had a total plasma clearance ($CL_{tot,p}$) of 0.09 l/h/kg in rats. Its renal excretion was poor, with a CL_R of 0.001 l/h/kg; the fraction of dose excreted into urine ($f_{e\text{-}U}$) was only 1.5%.

After an i.v. bolus dose of tanshinol (2 mg/kg), the systemic exposure to tanshinol in probenecid-treated rats was significantly enhanced as compared with that in the same rats when they were not given probenecid treatment (Fig. 1). As shown in Table 3, probenecid treatment resulted in 3–5-fold increases in plasma $AUC_{0\text{-}\infty}$ of tanshinol (P = 0.0001),

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1.6–2.3-fold elevations in C_{5min} (P = 0.00001), and 1.7–2.3-fold increases in elimination half-life ($t_{1/2}$) (P = 0.00001). Probenecid treatment led to a notable decrease in CL_R of tanshinol, i.e., only 20%–34% of those of the same rats when given no probenecid (P = 0.00004). To rule out differences in glomerular function as a confounding factor, endogenous creatinine excretion was measured in the rats during both the probenecid treatment period and the probenecid-free period. Probenecid treatment was not found to significantly change the renal clearance of endogenous creatinine (P = 0.158) or the f_u values of tanshinol (P = 0.795) (Table 3). Accordingly, the probenecid-induced changes in the CL_R of tanshinol may be caused predominantly by decreases in Oat-mediated tubular secretion. Noncompartmental PK analysis also revealed probenecid-induced abnormalities in total plasma clearance ($CL_{tot,p}$) of tanshinol, demonstrating 61%–78% reductions (P = 0.00004), and in apparent volume of distribution at steady state (V_{SS}), demonstrating 5%–38% reductions (P = 0.006). The effects of probenecid on rat tissue distribution of tanshinol were further determined by measuring tanshinol concentrations in tissue homogenates of rats after dosing. As with the systemic exposure, probenecid treatment led to heart, lung, brain, and liver C_{max} and $AUC_{0-\infty}$ levels of tanshinol that were higher than those in the normal rats not treated with probenecid (P = 0.000005–0.005) (Table 4). However, probenecid treatment did not cause a significant change in average kidney $AUC_{0-\infty}$ level (P = 0.309); the average maximum kidney concentration after dosing (C_{max}) in the probenecid-treated rats was markedly lower than that in the normal rats (P = 0.001). These data suggested that the reduced V_{SS} of tanshinol by probenecid treatment resulted, at least in part, from the change in kidney exposure to the compound.

Dose-dependent Changes in Levels of Systemic and Renal Exposure to Tanshinol in Rats. Changes in systemic and renal exposure to tanshinol were evaluated in a single ascending dose study in rats. As shown in Table 5 and Fig. 2A–C, systemic exposure to tanshinol increased as a function of the dose (i.v.; 2–200 mg/kg). Plasma C_{5min} of tanshinol

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exhibited a dose-proportional increase; the slope of ln(plasma $AUC_{0-\infty}$) versus ln(dose) was 1.04 (Table 6). There were dose-independent trends in plasma $t_{1/2}$ (P = 0.280–0.542), $CL_{tot,p}$ (P = 0.193–0.842), and V_{SS} (P = 0.196–0.568) of tanshinol (Table 5). Meanwhile, kidney C_{5min} and $AUC_{0-\infty}$ of tanshinol also increased as the dose increased (Table 5 and Fig. 2D–F). The slopes of ln(kidney C_{5min}) and ln(kidney $AUC_{0-\infty}$) versus ln(dose) were 0.98 and 1.07, respectively. Over the dose range, kidney $t_{1/2}$ of tanshinol was also dose-independent (P = 0.066–0.417). The kidney C_{5min} and $AUC_{0-\infty}$ of tanshinol at each dose level were 6.9–11.0 and 5.1–6.7 times, respectively, as high as the corresponding plasma data. It is worth mentioning that the rat Oat1/Oat3-mediated basolateral uptake is expected to result in the real concentration of tanshinol in the tubular epithelium considerably higher than the associated kidney homogenate concentration. Tanshinol exhibited concentration-independent renal clearance by luminal efflux into urine ($CL_{R,c-u}$; P = 0.548–0.956); no evidence of saturation of $CL_{R,c-u}$ suggested that the luminal efflux of tanshinol into urine probably did not involve transporter-mediated mechanism (Table 5). Taken together, tanshinol exhibited a linear plasma pharmacokinetics over a wide range of i.v. dose in rats and the change in systemic exposure to tanshinol by the dosage adjustment (about 100 times) was substantially greater than that by the probenecid-impaired tubular secretion (about 3–5 times). A similar scenario is expected to take place in humans. Oat1/Oat3-mediated tubular uptake resulted in a level of kidney exposure to tanshinol considerably higher than the level of systemic exposure. This raised concerns regarding the risk of dose-related nephrotoxicity of tanshinol.

Lack of Nephrotoxicity after Tanshinol Overdose in Rats. As with saline-treated rats (the negative controls), both the renal tubules and glomeruli of rats given 14 consecutive days of subchronic treatment with tanshinol at an i.v. dose of 200 mg/kg per day (equivalent to 100 times the clinical daily dose) were histologically normal on day 15 (Fig. 3). No evidence of toxicity to the liver was observed in tanshinol-treated rats (data not shown). Consistent with

these histopathological observations, these rats undergoing multiple-dose treatment with tanshinol showed serum markers of renal function (BUN, 4.6–6.3 mM; sCr, 20–24 μM) within the normal ranges (4.2–7.8 mM for BUN and 16–31 μM for sCr) (Fig. 3). In contrast, the positive controls cisplatin and puromycin caused considerable renal injury in rats. The lesions observed in cisplatin-treated rats were characterized by dilated tubules filled with necrotic tubular epithelial cells, cellular debris and proteinaceous casts, but the glomeruli were histologically normal. The histopathologic evaluation of the kidneys from the puromycin-treated rats demonstrated renal cellular degeneration, necrosis, and sloughing of proximal tubule epithelium and vacuolation of glomerular podocytes. In rats, cisplatin treatment led to the BUN (11–33 mM) and sCr levels (47–108 μM) that exceeded the normal ranges. Abnormally elevated BUN and sCr levels were also observed in the puromycin-treated rats, i.e., 103–131 mM and 179–266 μM, respectively. Collectively, tanshinol exhibited very little dose-related nephrotoxicity in rats. A similar scenario is expected to take place in humans.

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Discussion

Tanshinol is a carboxyl acid and cleared predominantly by renal excretion. Many organic anions are substrates of renal organic anion transporters (Masereeuw and Russel, 2010). Renal excretion of tanshinol mainly involves active tubular secretion. This suggests that the transporters influence the systemic exposure to and renal disposition of tanshinol via mediating the tubular secretion. To test this hypothesis, a comprehensive investigation of interactions between renal transporters and tanshinol was undertaken. This resulted in an understanding of the mechanistic tubular secretion of tanshinol, impact on its systemic exposure, and propensity for dose-related nephrotoxicity and renal transporter-mediated herb-drug interactions.

Cellular uptake of tanshinol could be mediated by human OAT1, OAT2, OAT4, and OAT3 (in decreasing order of affinity for tanshinol), rather than human OATP4C1, OCT2, OCTN1, MATE1, and MATE2-K. The transporters OAT1, OAT2, and OAT3 are expressed at the basolateral membrane of renal proximal tubules and play roles in uptake of tanshinol from blood in tubular secretion (Enomoto et al., 2002; Motohashi et al., 2002). OAT1 and OAT3 are major renal transporters and OAT2 probably expresses at a lower level. OAT2 and OAT3 exhibited in vitro CL_{int}, which were 39% and 1%, respectively, of the OAT1 efficiency. OAT4 has a major role in the tubular reabsorption of organic anions from urine (Ekaratanawong et al., 2004). OAT4 exhibited a CL_{int}, which was only 1% of the OAT1 value. The (i) low affinity of OAT4 for tanshinol, (ii) its relatively low expression in the kidney, and (iii) the short residence time of tanshinol in luminal filtrate (a couple of seconds), indicate that OAT4 had limited contribution to renal excretion of tanshinol. The apically located MRP2, MRP4, BCRP, MATE1, MATE2-K, and OCTN1 transporters that support luminal efflux from proximal renal tubules (Masereeuw and Russel, 2010) exhibited ambiguous or no in vitro ability to transport tanshinol. A similar scenario was observed with the rat orthologs at the

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apical membrane. Rats exhibited dose-independent trends in kidney $t_{1/2}$ and $CL_{R,c\text{-}u}$ of tanshinol over the i.v. dose range 2–200 mg/kg; its C_{5min} in kidney homogenate increased from 118 to 9671 μM as the dose increased (Table 5). A Caco-2 cell-based study revealed that tanshinol had favorable membrane permeability for intestinal absorption (Lu et al., 2008). The concentration of tanshinol in the rat epithelia of proximal tubules at the dose 200 mg/kg should be much higher than 10 mM. Such a high intracellular concentration is expected to exceed the K_m and results in saturation of possible transporter-mediated efflux. According to the Michaelis–Menten equation for membrane permeation, when a drug compound with high membrane permeability has a concentration significantly higher than K_m, concentration-gradient-driven passive transcellular transport can be the dominating mechanism (Sugano et al., 2010). Accordingly, the luminal efflux of tanshinol into urine was most likely based on a passive diffusion mechanism.

In rats, Oat1 and Oat3 are highly expressed at the basolateral membrane of renal proximal tubules, and Oat2 is at the apical membrane (Kojima et al., 2002). The roles of Oat1 and Oat3 are to support basolateral uptake of organic anions from blood. Rat Oat2 has a major role in tubular reabsorption of organic anions from urine. Tanshinol was a substrate of Oat1, Oat2, and Oat3 but not of Oct1 and Oct2. The CL_{int} values of Oat2 and Oat3 were 29% and 14% of that of Oat1, respectively. Rat Mrp2, Mrp4, Bcrp, and Mate1 are expressed at the apical membrane. The apical membrane efflux transporters exhibited limited (Mrp2 and Mrp4) or no (Bcrp, Octn1, Octn2, and Mate1) affinity for tanshinol. Based on these results, rats were used to investigate the impact of renal transporters on systemic and renal exposure to tanshinol. For the purpose, probenecid was used to impair Oat1/Oat3-mediated tubular secretion of tanshinol. However, it also exhibits inhibitory activity against rat Oatp1a1/Oatp1a4, Mrp2/Mrp3, and UDP-glucuronosyltransferases (Sugiyama et al., 2001; Horikawa et al., 2002; Uchaipichat et al., 2004). For tanshinol, transport mediated by rat

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Mrp2 and human MRP3 is very poor; metabolism via glucuronidation is limited (details pending publication elsewhere). Tanshinol (MW, 198 Da) is not a substrate of rat Oatp1a1 and Oatp1b2. Therefore, inhibition of Oatp1a1/Oatp1a4, Mrp2/Mrp3, and UDP-glucuronosyltransferases by probenecid probably had negligible effect on rat pharmacokinetics of tanshinol.

Probenecid-induced impairment of tubular secretion resulted in 61%–78% reductions in $CL_{tot,p}$ of tanshinol and the $CL_R/(GFR \times f_u)$ ratio of tanshinol was reduced from 6.6 to 1.7. Although probenecid treatment led to 3–5 times enhancement of systemic exposure to the compound ($AUC_{0-\infty}$), the kidney exposure to tanshinol was reduced. It is worth mentioning that probenecid treatment also resulted in decrease in nonrenal clearance ($CL_{tot,p}-CL_R$; from 1.32 to 0.51 l/h/kg). This probably resulted, at least in part, from inhibition of hepatic Oat2 by probenecid, unbound plasma C_{5min} and C_{4h} of which were 6 and 0.8 times, respectively, of its IC_{50} against tanshinol.

Tanshinol exhibited a linear plasma pharmacokinetics over the i.v. dose range 2–200 mg/kg in rats; dosage adjustment could cause about 100-fold increases in plasma $AUC_{0-\infty}$ and C_{5min} of tanshinol. For tanshinol, Oat1 exhibited higher affinity and CL_{int} than Oat3. When the doses were 2–15 mg/kg, all the unbound plasma C_{5min} of tanshinol (15–97 μM) was lower than the K_m for Oat1. This suggested that the basolateral uptake for tubular secretion of tanshinol was mainly mediated by Oat1. As the dose increased, the initial unbound plasma concentrations of tanshinol, particularly at 200 mg/kg, exceeded the K_m for Oat1 (Fig. 2A). Tanshinol pharmacokinetics remained linear with increasing dose and CL_R was not markedly saturated over 2–200 mg/kg, suggesting that, at higher concentration, Oat3 supplements Oat1 in mediating renal uptake. This was evidenced by the K_m for Oat3, which was higher than the unbound plasma concentrations of tanshinol at the 200 mg/kg dose. The preceding linear pharmacokinetics mainly depended on Oat1/3-mediated tubular secretion. For matching levels

of systemic exposure to tanshinol after dosing to its effective concentrations for the antianginal activities, dosage adjustment was a more effective way to manipulate exposure, because the change in systemic exposure to tanshinol by the dosage adjustment (about 100 times) was significantly greater than that by the probenecid-induced drug interaction (about 3–5 times). Despite the Oat-mediated tubular secretion mechanism, tanshinol exhibited very little dose-related nephrotoxicity. This suggested that dosage adjustment probably was also a safe way to manipulate exposure to tanshinol and did not need to be used in concert with drug combination. Gao et al. (2009) and Li et al. (2009) reported that tanshinol exhibited very little dose-related toxicity in rats, mice, and dogs. Tanshinol is expected to have a similar linear plasma PK property over a large i.v. dose range in humans. This is because, like the rat Oat transporters, the human OAT transporters also exhibited low affinities and high transport capacities for tanshinol. Similar to rat Oat1, human OAT1 played a key role in mediating renal uptake of tanshinol at low concentration; like rat Oat3, human OAT2 and OAT3 supplemented OAT1 to mediate the tanshinol uptake at high concentration.

Herb-drug interactions are an important safety concern (Li et al., 2012b). Rat Oat transporters was found to influence systemic exposure to tanshinol; human OAT transporters are expected to be clinically important. Human OAT1, OAT3, and OCT2 are major renal transporters with a broad range of substrates; renal drug interactions often occurred in relation to their actions (Giacomini et al., 2010). Both the K_m and IC_{50} data shown in Table 2 indicated that tanshinol had low affinity for OAT1. After an i.v. infusion daily dose of DanHong injection (40 mL containing around 55 mg of tanshinol) in human subjects, average maximum plasma concentration of tanshinol was measured as about 2.5 μM (Li et al., 2015). Tanshinol exhibits a f_u of 85% in human plasma and a short $t_{1/2}$ of 1.1–1.3 h (Lu et al., 2008). According to the equation (drug-drug interaction index = unbound C_{max}/IC_{50}), the OAT1-mediated drug-drug interaction index was calculated for tanshinol as 0.02. This suggests tanshinol has

low propensity to act as inhibitory perpetrator in OAT1-mediated drug interactions when DanHong injection is used at a clinically relevant dose. Compared with OAT1, the renal transporters OAT2, OAT3, and OAT4 exhibited higher K_m and IC_{50} values for tanshinol (Table 2), suggesting lower potential for these transporter-mediated herb-drug interactions. In addition, tanshinol had no inhibition potency towards human OATP4C1, OCT2, OCTN1, MRP2, MRP4, BCRP, MATE1, and MATE2-K. In rats, the probenecid-impaired tubular secretion resulted in 1.6–4.5-fold elevations in systemic exposure to tanshinol, suggesting tanshinol could be a substrate victim on Oat transporters. A similar scenario is expected to take place in humans. However, the change in systemic exposure is probably not clinically relevant, because tanshinol exhibits very little dose-related toxicity. Wang and Sweet (2013) reported that the Danshen polyphenols rosmarinic acid, lithospermic acid, and salvianolic acid A exhibited strong inhibitory activities against human OAT1 or OAT3 (K_i, 0.16–0.59 μM). Like probenecid, these Danshen polyphenols, concurrently present in Danshen-based i.v. injections, may influence systemic and renal exposure to tanshinol after dosing. Studying the PK matrix effects will help more accurately define and predict the exposure level and pharmacokinetics of tanshinol.

Understanding the mechanisms governing systemic exposure to tanshinol helps with matching the exposure levels after dosing to the effective concentrations for its antianginal activities; this most likely results in enhanced efficacy of Danshen-based therapy. In summary, renal tubular secretion of tanshinol involves basolateral uptake from blood primarily by human OAT1 and rat Oat1 and subsequent luminal efflux into urine, mainly by passive diffusion (Fig. 4). Human OAT2/OAT3 and rat Oat3 are also important for the basolateral uptake at high tanshinol concentration in blood. Human OAT4- and rat Oat2-mediated tubular reabsorption of tanshinol may have limited contribution to the renal excretion. Tanshinol shows low propensity to cause renal transporter-mediated herb-drug interactions. Tanshinol

exhibits linear pharmacokinetic properties over a large i.v. dose range and very little dose-related nephrotoxicity in rats; dosage adjustment appears to be an efficient and safe way to manipulate its systemic exposure. Additional safety studies are under way to define risk of hyperhomocysteinemia related to dose-dependent tanshinol methylation.

Authorship Contributions

Participated in research design: C. Li, Jia

Conducted experiments: Jia, Du, Liu, Jiang, Xu, Wang, Olaleye, Dong

Performed data analysis: C. Li, Jia, Yang, L. Li

Wrote or contributed to the writing of the manuscript: C. Li, Jia

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Footnotes

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Legends to figures

Fig. 1. Plasma concentrations (A) and urinary excretion (B) of tanshinol over time after an i.v. bolus dose of tanshinol at 2 mg/kg in rats not treated with probenecid (open circles) and in the same rats treated with probenecid (solid circles). The details of the rat PK study are described in Materials and methods Section (the first rat PK study). The plasma PK and renal excretion parameters of tanshinol are shown in Table 3. The data represent means and standard deviations from two independent rat experiments, each of which was performed in tetraplicate.

Fig. 2. Plasma concentration (**A**) and kidney concentration (**D**) of tanshinol over time in rats receiving an i.v. bolus dose of tanshinol at 2 (open square), 5 (open circle), 15 (solid triangle), 50 (solid square), and 200 mg/kg (solid circle). Correlations of plasma C_{5min}, plasma $AUC_{0-\infty}$, kidney C_{5min}, and kidney $AUC_{0-\infty}$, of tanshinol with the dose are also shown in panels **B**, **C**, **E**, and **F**, respectively. The details of the rat PK study are described in Materials and methods Section (the third rat PK study). The plasma and kidney PK parameters of tanshinol are shown in Table 5.

Fig. 3. Comparative kidney histology (**A**–**F**) and serum biochemistry (**G** and **H**) in rats receiving subchronic treatment of 14 consecutive days of saline (negative control), tanshinol (i.v., 200 mg/kg/day), puromycin (i.p., 40 mg/kg/day; positive control), and cisplatin (i.p., 1 mg/kg/day; positive control). The rat blood samples were collected before (open bars) and after 15 days of treatment (solid bars) for assessment of blood urea nitrogen (BUN) and serum creatinine (sCr). The rat kidney tissues were sampled and processed for hematoxylin and eosin (H&E) staining to evaluate tubular damage, glomerular damage, and histology. *, P <0.05 versus the negative control. H&E, ×200.

Fig. 4. Tubular secretion of tanshinol mediated by human (h) and rat (r) organic anion transporters (OAT/Oat).

TABLE 1

Comparative net transport ratios for a variety of human and rat renal transporters mediating in vitro transport of tanshinol

The concentrations of methotrexate for BCRP and Bcrp were 100 μM. The concentration of probenecid to inhibit OAT2- and Oat2-mediated transport of prostaglandin $F_{2\alpha}$ was 10 mM. Net transport ratios represent the means ± standard deviations (n = 3). When the net transport ratio was greater than three (for the SLC transporters) or two (for the ABC transporters), there were statistically significant differences between $Transport_{TC}$ and $Transport_{MC}$ or between $Transport_{ATP}$ and $Transport_{AMP}$ (P <0.05).

Transporter	Positive substrate (10 μM)				Tanshinol (100 μM)	
	Substrate	Not treated with any inhibitor	Treated with 1 mM positive inhibitor	Treated with 1 mM tanshinol as inhibitor	Not treated with any inhibitor	Treated with 1 mM positive inhibitor
Human renal SLC transporters						
OAT1	*Para*-aminohippuric acid	146.9 ± 22.0	1.7 ± 0.1 (Probenecid)	15.1 ± 2.7	164.0 ± 4.3	4.6 ± 0.4 (Probenecid)
OAT2	Prostaglandin $F_{2\alpha}$	68.0 ± 4.6	7.6 ± 0.8 (Probenecid)	23.9 ± 1.6	80.7 ± 3.1	6.7 ± 0.7 (Probenecid)
OAT3	Estrone-3-sulfate	23.9 ± 1.8	1.9 ± 0.3 (Probenecid)	10.3 ± 2.8	4.6 ± 0.4	1.2 ± 0.1 (Probenecid)
OAT4	Estrone-3-sulfate	14.1 ± 1.0	3.0 ± 0.6 (Probenecid)	8.3 ± 1.5	7.3 ± 1.5	0.9 ± 0.3 (Probenecid)
OATP4C1	Estrone-3-sulfate	5.7 ± 0.7	2.3 ± 0.4 (Probenecid)	5.4 ± 0.2	1.2 ± 0.2	—
OCT2	Tetraethylammonium	103.2 ± 10.7	46.1 ± 4.7 (Cimetidine)	105.7 ± 8.9	1.4 ± 0.2	—
OCTN1	Tetraethylammonium	7.9 ± 0.2	0.5 ± 0.1 (Verapamil)	7.4 ± 0.2	1.5 ± 0.2	—
MATE1	Tetraethylammonium	60.9 ± 2.0	0.5 ± 0.1 (Cimetidine)	63.2 ± 1.0	0.8 ± 0.2	—
MATE2-K	Tetraethylammonium	3.9 ± 0.4	0.6 ± 0.1 (Cimetidine)	5.3 ± 0.6	1.9 ± 0.6	—
Human renal ABC transporters						
MRP2	Estradiol-17β-D-glucuronide	24.2 ± 0.5	3.7 ± 0.1 (indomethacin)	17.1 ± 0.5	2.3 ± 0.4	—
MRP4	Estradiol-17β-D-glucuronide	20.2 ± 0.7	4.4 ± 0.8 (indomethacin)	15.9 ± 0.3	1.7 ± 0.2	—
BCRP	Methotrexate	17.7 ± 0.1	4.1 ± 0.3 (novobiocin)	17.9 ± 0.3	1.0 ± 0.3	—
Rat renal SLC transporters						
Oat1	*Para*-aminohippuric acid	58.8 ± 4.1	1.7 ± 0.1 (Probenecid)	7.8 ± 0.9	198.2 ± 22.0	3.4 ± 0.1 (Probenecid)
Oat2	Prostaglandin $F_{2\alpha}$	22.1 ± 1.6	1.8 ± 0.4 (Probenecid)	20.0 ± 0.7	17.9 ± 3.8	2.5 ± 0.7 (Probenecid)
Oat3	Estrone-3-sulfate	27.0 ± 7.3	1.3 ± 0.6 (Probenecid)	9.7 ± 1.9	5.0 ± 0.3	0.9 ± 0.2 (Probenecid)
Oct1	Tetraethylammonium	102.1 ± 10.5	46.9 ± 3.2 (Cimetidine)	99.1 ± 9.8	1.6 ± 0.7	—
Octn1	Tetraethylammonium	6.1 ± 0.1	0.7 ± 0.1 (Verapamil)	6.4 ± 0.2	1.3 ± 0.3	—
Octn2	Tetraethylammonium	59.2 ± 3.0	1.3 ± 0.1 (Verapamil)	61.6 ± 1.1	2.3 ± 0.6	—
Oct2	Tetraethylammonium	95.2 ± 10.1	41.2 ± 7.4 (Cimetidine)	93.3 ± 8.3	1.3 ± 0.3	—
Mate1	Tetraethylammonium	22.1 ± 0.3	0.6 ± 0.1 (Cimetidine)	19.3 ± 0.5	1.0 ± 0.2	—
Rat renal ABC transporters						
Mrp2	Estradiol-17β-D-glucuronide	10.2 ± 3.2	—	—	1.8 ± 0.1	—

Mrp4	Estradiol-17β-D-glucuronide	11.4 ± 1.3	—	—	2.2 ± 0.2	—
Bcrp	Methotrexate	43.4 ± 10.2	—	—	1.0 ± 0.2	—

TABLE 2

Comparative kinetic parameters for human OAT and rat Oat transporters mediating in vitro transport of tanshinol

Values represent the means ± standard deviations (n = 3).

Transporter	K_m (μM)	V_{max} (pmol/mg protein/min)	CL_{int} (μl/mg protein/min)	IC_{50} of probenecid against tanshinol (μM)	IC_{50} of tanshinol against positive substrate (μM)
Positive substrate (in parentheses)					
Human OAT1 (*Para*-aminohippuric acid)	60.3 ± 5.5	1379 ± 39	22.9	—	98 ± 7
Human OAT2 (Prostaglandin $F_{2\alpha}$)	45.5 ± 12.4	360 ± 54	7.91	—	1528 ± 51
Human OAT3 (Estrone-3-sulfate)	28.1 ± 3.7	73.1 ± 4.4	2.60	—	2803 ± 150
Human OAT4 (Estrone-3-sulfate)	49.9 ± 5.3	232 ± 9	4.64	—	4079 ± 410
Rat Oat1 (*Para*-aminohippuric acid)	102 ± 17	2299 ± 138	22.5	—	—
Rat Oat2 (Prostaglandin $F_{2\alpha}$)	27.0 ± 10.3	511 ± 113	18.9	—	—
Rat Oat3 (Estrone-3-sulfate)	17.1 ± 4.1	161 ± 11	9.41	—	—
Tanshinol as substrate					
Human OAT1	121 ± 13	1414 ± 40	35.4	4.57 ± 0.30	—
Human OAT2	859 ± 70	4555 ± 146	5.30	393 ± 118	—
Human OAT3	1888 ± 395	182 ± 17	0.0963	10.9 ± 2.9	—
Human OAT4	1880 ± 555	169 ± 22	0.0899	134 ± 50	—
Rat Oat1	117 ± 24	599 ± 35	5.12	6.19 ± 0.18	—
Rat Oat2	1207 ± 470	1751 ± 297	1.45	108 ± 31	—
Rat Oat3	1498 ± 225	1030 ± 63	0.688	9.51 ± 1.52	—

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TABLE 3

Comparative plasma pharmacokinetics and renal excretion of tanshinol after an i.v. bolus dose of tanshinol at 2 mg/kg in rats not treated with probenecid and in the same rats treated with probenecid

The details of the rat PK study are described in Materials and methods Section (the first rat PK study). $AUC_{0-\infty}$, area under the plasma concentration-time curve from zero to infinity; CL_R, renal clearance; CL_{R-cr}, renal clearance of endogenous creatinine; $CL_{tot,p}$, total plasma clearance; C_{5min}, concentration at 5 min after dosing; $Cum.A_e$, cumulative amount excreted; f_{e-U}, fraction of dose excreted into urine; f_u, unbound fractions in plasma; MRT, mean residence time; $t_{1/2}$, elimination half-life; V_{SS}, apparent volume of distribution at steady state. No obvious effect of tanshinol was observed on the plasma pharmacokinetics or renal disposition of probenecid. The data represent means ± standard deviations from two independent experiments where each rat group was performed in tetraplicate (total n = 8). *, P <0.05 when compared with the rat group not treated with probenecid.

PK parameter	Rats not treated with probenecid	The same rats treated with probenecid
Plasma data		
C_{5min} (μM)	11.8 ± 1.9	23.2 ± 3.6*
$AUC_{0-\infty}$ (μM·h)	3.19 ± 0.57	10.6 ± 3.1*
$t_{1/2}$ (h)	0.196 ± 0.055	0.441 ± 0.062*
MRT (h)	0.173 ± 0.015	0.450 ± 0.052*
$CL_{tot,\ p}$ (l/h/kg)	3.26 ± 0.64	1.01 ± 0.24*
V_{SS} (l/kg)	0.538 ± 0.087	0.428 ± 0.077*
Urine data		
$Cum.A_e$ (μmol)	1.51 ± 0.09	1.28 ± 0.14*
CL_R (l/h/kg)	1.94 ± 0.38	0.503 ± 0.096*
f_{e-U} (%)	59.7 ± 3.8	50.7 ± 5.7*
$CL_R/(GFR \times f_u)$	6.56	1.72
Plasma protein binding data		
f_u (%; at 0.5–50 μM)	98.6 ± 1.1	96.5 ± 2.5
GFR data		
CL_{R-cr} (l/h/kg) (Before dosing)	0.295 ± 0.043	0.315 ± 0.036
CL_{R-cr} (l/h/kg) (6 h after dosing)	0.314 ± 0.060	0.288 ± 0.037

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TABLE 4

Comparative tissue distribution of tanshinol after an i.v. bolus dose of tanshinol at 2 mg/kg in rats not treated with probenecid and in rats treated with probenecid

The rat blood and tissue samples were collected at 0, 5 and 30 min and 1 and 2 h after dosing. The details of the rat PK study are described in Materials and methods Section (the second rat PK study). $AUC_{0-\infty}$, area under the plasma concentration-time curve from zero to infinity; C_{5min}, concentration at 5 min after dosing; MRT, mean residence time; $t_{1/2}$, elimination half-life; No obvious effect of tanshinol was observed on the plasma pharmacokinetics or renal disposition of probenecid. The data represent means ± standard deviations from two independent experiments where each rat group was performed in triplicate (total n = 6). *, P <0.05 when compared with the rat group not treated with probenecid.

PK parameter	Rats not treated with probenecid	Rats treated with probenecid
Heart data		
C_{5min} (μM)	4.18 ± 1.06	7.38 ± 1.49*
$AUC_{0-\infty}$ (μM·h)	1.16 ± 0.10	3.64 ± 0.50*
$t_{1/2}$ (h)	0.235 ± 0.027	0.429 ± 0.138*
MRT (h)	0.353 ± 0.05	0.588 ± 0.115*
Lung data		
C_{5min} (μM)	2.80 ± 0.29	4.25 ± 0.54*
$AUC_{0-\infty}$ (μM·h)	0.72 ± 0.09	2.05 ± 0.12*
$t_{1/2}$ (h)	0.15 ± 0.00	0.32 ± 0.02*
MRT (h)	0.26 ± 0.01	0.50 ± 0.03*
Brain data		
C_{5min} (μM)	0.212 ± 0.101	0.309 ± 0.068*
$AUC_{0-\infty}$ (μM·h)	0.0729 ± 0.0177	0.173 ± 0.055*
$t_{1/2}$ (h)	0.223 ± 0.116	0.363 ± 0.077*
MRT (h)	0.343 ± 0.19	0.543 ± 0.091*
Liver data		
C_{5min} (μM)	0.601 ± 0.298	1.35 ± 0.32*
$AUC_{0-\infty}$ (μM·h)	0.227 ± 0.143	0.644 ± 0.206*
$t_{1/2}$ (h)	0.124 ± 0.025	0.293 ± 0.018*
MRT (h)	0.201 ± 0.065	0.442 ± 0.037*
Kidney data		
C_{5min} (μM)	127 ± 18	68.0 ± 16.7*
$AUC_{0-\infty}$ (μM·h)	23.6 ± 3.2	26.5 ± 5.7
$t_{1/2}$ (h)	0.225 ± 0.032	0.286 ± 0.032*
MRT (h)	0.216 ± 0.012	0.378 ± 0.031*
Plasma data		
C_{5min} (μM)	13.3 ± 2.5	28.3 ± 5.1*
$AUC_{0-\infty}$ (μM·h)	4.17 ± 0.62	12.6 ± 1.5*
$t_{1/2}$ (h)	0.287 ± 0.057	0.396 ± 0.095*
MRT (h)	0.238 ± 0.020	0.416 ± 0.034*

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TABLE 5

Comparative plasma and kidney pharmacokinetics of tanshinol in rats receiving an i.v. bolus dose of tanshinol solution (2–200 mg/kg)

The rat blood and kidney tissue samples were collected at 5 and 30 min and 1 and 2 h after dosing. Urine samples were collected 0–24 h after dosing. The details of the rat PK study are described in **Materials and methods** Section (the third rat PK study). $AUC_{0-\infty}$, area under the plasma concentration-time curve from zero to infinity; $CL_{R,c-u}$, renal clearance by the cellular efflux into urine across the apical brush border membrane; CL_R, renal clearance; $CL_{tot,p}$, total plasma clearance; C_{5min}, concentration at 5 min after dosing; $Cum.A_e$, cumulative amount excreted; MRT, mean residence time; $t_{1/2}$, elimination half-life; V_{SS}, apparent volume of distribution at steady state.

PK parameter	Dosage				
	2 mg/kg	5 mg/kg	15 mg/kg	50 mg/kg	200 mg/kg
Plasma data					
C_{5min} (μM)	15.5 ± 1.5	36.7 ± 2.9	98.7 ± 17.9	388 ± 48	1403 ± 98
$AUC_{0-\infty}$ (μM·h)	4.33 ± 0.45	11.0 ± 1.0	31.2 ± 5.5	125 ± 23	504 ± 61
$t_{1/2}$ (h)	0.278 ± 0.017	0.285 ± 0.010	0.260 ± 0.027	0.269 ± 0.022	0.259 ± 0.014
MRT (h)	0.272 ± 0.030	0.252 ± 0.004	0.237 ± 0.031	0.288 ± 0.002	0.328 ± 0.009
V_{SS} (l/kg)	0.635 ± 0.023	0.582 ± 0.049	0.578 ± 0.052	0.595 ± 0.097	0.662 ± 0.067
$CL_{tot,p}$ (l/h/kg)	2.35 ± 0.25	2.31 ± 0.20	2.48 ± 0.45	2.07 ± 0.34	2.02 ± 0.26
Urine data					
$Cum.A_e$ (μmol)	1.48 ± 0.06	3.75 ± 0.29	12.3 ± 1.1	44.2 ± 2.9	192 ± 18
f_{e-U} (%)	61.5 ± 3.6	59.7 ± 6.8	66.3 ± 7.5	73.3 ± 5.7	77.9 ± 6.7
CL_R (l/h/kg)	1.44 ± 0.10	1.37 ± 0.05	1.62 ± 0.11	1.50 ± 0.15	1.56 ± 0.06
Kidney data					
C_{5min} (μM)	118 ± 37	278 ± 79	1083 ± 410	3199 ± 1046	9671 ± 1536
$AUC_{0-\infty}$ (μM·h)	22.7 ± 6.9	67.9 ± 18.0	268 ± 97	899 ± 309	3552 ± 518
$t_{1/2}$ (h)	0.277 ± 0.010	0.250 ± 0.031	0.238 ± 0.003	0.267 ± 0.016	0.256 ± 0.007
MRT (h)	0.232 ± 0.011	0.197 ± 0.028	0.172 ± 0.008	0.214 ± 0.010	0.254 ± 0.011
$CL_{R,c-u}$ (l/h/kg)	0.289 ± 0.075	0.282 ± 0.064	0.261 ± 0.061	0.269 ± 0.062	0.272 ± 0.017

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TABLE 6

Summary of results from dose proportionality assessment of a single ascending dose study in rats receiving an i.v. bolus dose of tanshinol solution (2–200 mg/kg)

Critical intervals were 0.952–1.048 for the systemic exposure data of tanshinol from the single ascending dose study of rat given an i.v. bolus dose of tanshinol (2–200 mg/kg). The term "*r*" denotes the correlation coefficient. Correlations were statistically significant with a "*P*" value of <0.05. The term "linear" was concluded statistically if the 90% confidence interval (90% CI) for slope was contained completely within the critical interval; "inconclusive" was concluded statistically if the 90% CI lay partly within the critical interval; "nonlinear" was concluded statistically if the 90% CI was entirely outside the critical interval.

PK parameter	*r*	*P*	Slope (90% CI)	Conclusion
Plasma C_{5min}	0.998	2.31×10^{-16}	0.989 (0.955–1.023)	Linear
Plasma $AUC_{0-\infty}$	0.997	2.57×10^{-16}	1.038 (1.002–1.075)	Inconclusive
Kidney C_{5min}	0.985	2.73×10^{-11}	0.978 (0.893–1.062)	Inconclusive
Kidney $AUC_{0-\infty}$	0.990	1.66×10^{-12}	1.070 (0.996–1.144)	Inconclusive

Figure 1

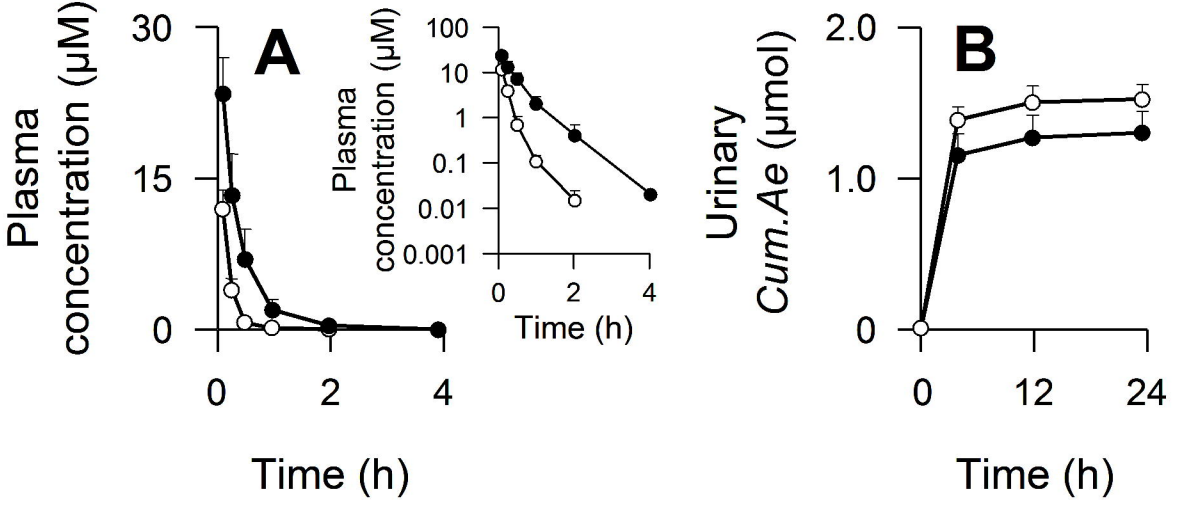

A
Plasma concentration (μM)
30
15
0
0
2
4
Time (h)
Plasma concentration (μM)
100
10
1
0.1
0.01
0.001
0
2
4
Time (h)
B
Urinary Cum.Ae (μmol)
2.0
1.0
0
0
12
24
Time (h)

Figure 2

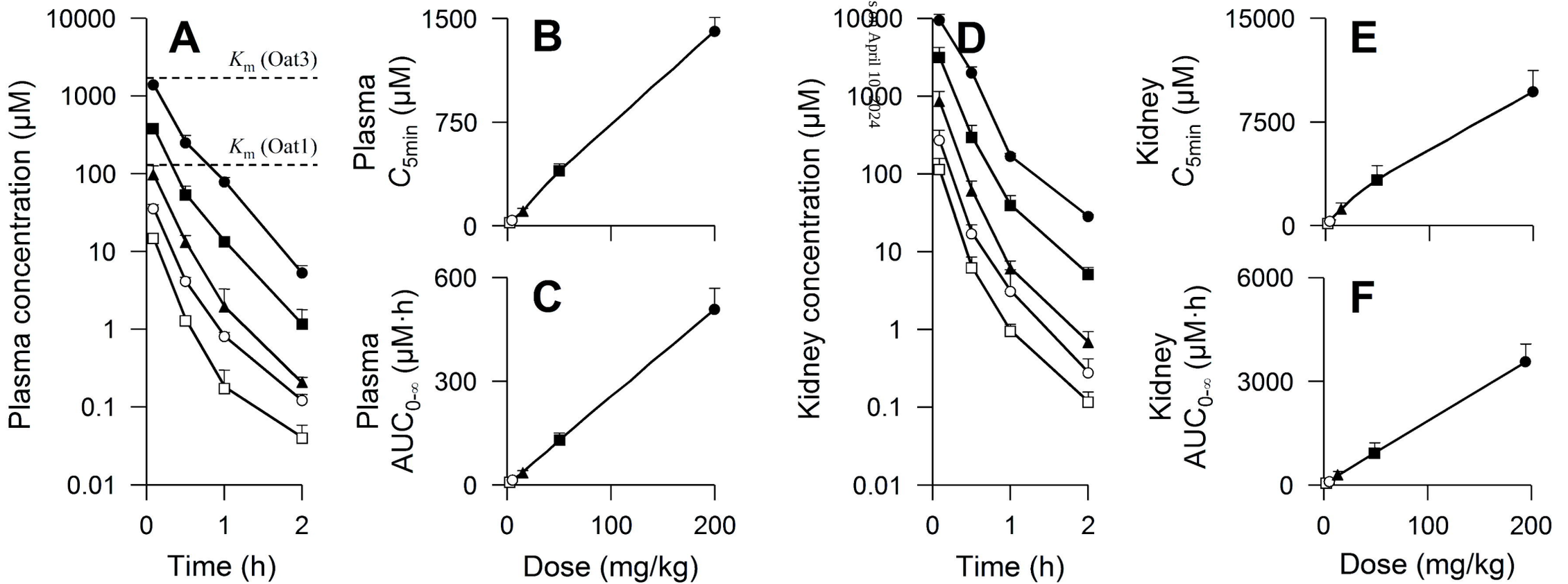


Figure 3

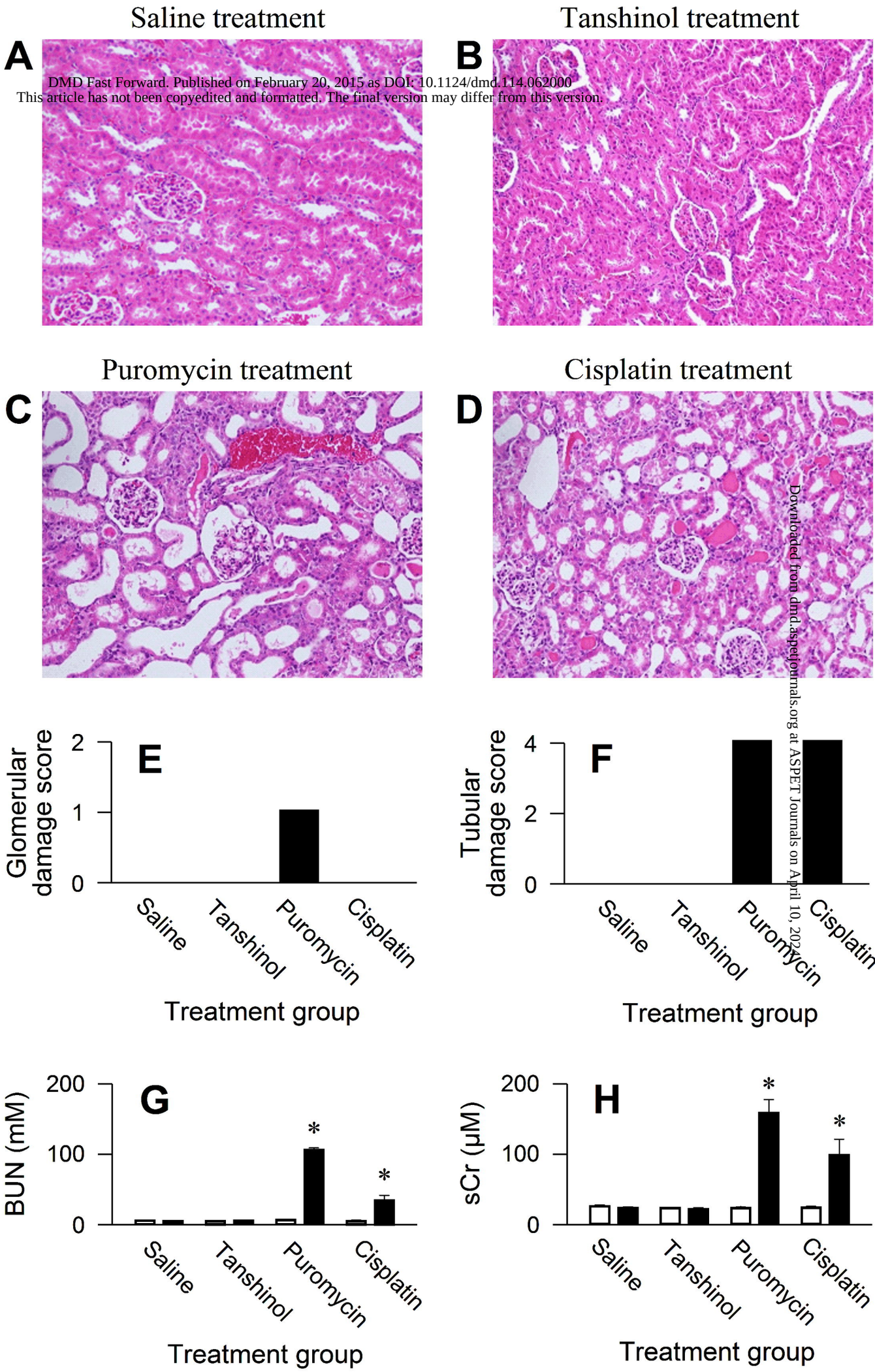
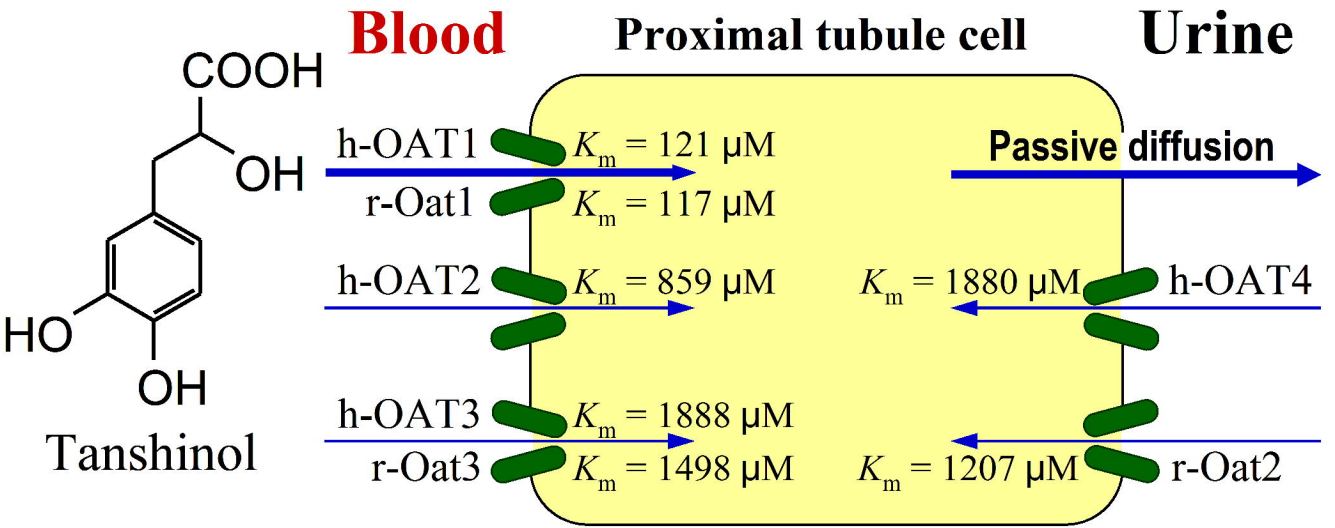


Figure 4


COOH
OH
HO
OH
Tanshinol
Blood
Proximal tubule cell
Urine
h-OAT1
K_m = 121 μM
r-Oat1
K_m = 117 μM
Passive diffusion
h-OAT2
K_m = 859 μM
K_m = 1880 μM
h-OAT4
h-OAT3
K_m = 1888 μM
r-Oat3
K_m = 1498 μM
K_m = 1207 μM
r-Oat2