Pharm Res (2014) 31:1120–1132
DOI 10.1007/s11095-013-1235-9

RESEARCH PAPER

MDR1 and OAT1/OAT3 Mediate the Drug-Drug Interaction between Puerarin and Methotrexate

Qi Liu • Changyuan Wang • Qiang Meng • Xiaokui Huo • Huijun Sun • Jinyong Peng • Xiaochi Ma • Pengyuan Sun • Kexin Liu

Received: 2 May 2013 / Accepted: 14 October 2013 / Published online: 16 November 2013

ABSTRACT

Purpose To conduct *in vivo* and *in vitro* experiments to investigate puerarin (PUR), an isoflavone C-glyoside, and elucidate its ability to alter methotrexate (MTX) transport and pharmacokinetics.

Methods *In vivo* absorption studies, *in vitro* everted intestinal sac preparation, kidney slices in rats and bi-directional transport assay with mock-/MDCK-MDR1 cells, uptake studies in HEK293-OAT1/3 cells were employed to evaluate the interaction.

Results *In vivo* and *in vitro* MTX absorption in rats were enhanced in combination with PUR. PUR inhibited digoxin efflux transport in MDCK-MDR1 monolayers with an IC_{50} value of 1.6 ± 0.3 μM, suggesting that the first target of drug interaction was MDR1 in the intestine during the absorption process. MTX renal clearance decreased significantly after simultaneous intravenous administration. MTX uptake in rat kidney slices and HEK293-OAT1/3 cells were markedly inhibited by PUR, suggesting that the second target of drug interaction was OATs located in the kidney. Moreover, concomitant administration of PUR reduced renal MTX accumulation and plasma levels of creatinine and BUN.

Conclusions Co-administration of PUR enhanced MTX exposure by inhibition of intestinal MDR1 and renal OAT1/3. Although the renal damage of MTX was improved by PUR, the high level exposure of MTX should be cautious in the clinical usage.

KEY WORDS drug-drug interaction · MDR1 · methotrexate · OATs · puerarin

ABBREVIATIONS

AUC	Area under the plasma concentration-time curve
BUN	Blood urea nitrogen
CL_P	Plasma clearance
CL_R	Renal clearance
CsA	Cyclosporin A
DDI	Drug-drug interaction
KRB	Krebs-Ringer buffer
MDR	Multidrug resistance
MDR1	Multidrug resistance 1
MTX	Methotrexate
OAT	Organic anion transporter
PAH	*p*-amino hippuric acid
PCG	Penicillin G
PUR	Puerarin
VER	Verapamil hydrochloride

INTRODUCTION

Chemotherapy regimens for cancer treatment typically consist of multidrug combinations designed to enhance desired effects

Electronic supplementary material The online version of this article (doi:10.1007/s11095-013-1235-9) contains supplementary material, which is available to authorized users.

Q. Liu • C. Wang • Q. Meng • X. Huo • H. Sun • J. Peng • X. Ma • P. Sun • K. Liu (✉)
Department of Clinical Pharmacology, College of Pharmacy
Dalian Medical University, 9 West Section, Lvshun South Road
Lvshunkou District, Dalian 116044, China
e-mail: kexinliu@dlmedu.edu.cn

Q. Liu • C. Wang • Q. Meng • X. Huo • H. Sun • J. Peng • X. Ma • P. Sun • K. Liu
Provincial Key Laboratory for Pharmacokinetics and Transport, Liaoning
Dalian Medical University, Dalian, China

K. Liu
Research Institute of Integrated Traditional and Western Medicine
Dalian Medical University, Dalian, China

while reducing toxicity (1–3). However, combination therapy is accompanied by a greater probability for drug-drug interactions (DDI) (4,5). DDI can influence pharmacokinetics and pharmacodynamics (positively or negatively) by affecting drug disposition (6). Transporters are now known to play major roles in determining drug disposition; adding another dimension of complexity to pharmacokinetics (7). DDI involving metabolism and/or excretion processes can prolong plasma elimination half-lives, leading to the accumulation of the drug in the body after repeated administration and potentiating pharmacological adverse effects (8). Induction or inhibition of drug transporters, such as MDR1, is an important mechanism underlying DDI (6). MDR1 has a broad substrate spectrum and commonly makes cancer cells to display cross-resistance to many different cytotoxic drugs. It is therefore called as multidrug resistance (MDR) (9). MDR1 is encoded by *ABCB1*, belongs to the ATP-binding cassette family and is highly expressed in the gastrointestinal (GI) tract and tumor cells and contributes to limited oral bioavailability and multidrug resistance (10). MDCK-MDR1 cells are constructed by stably transfecting MDCK cells with human MDR1. As a model MDR1 expression system, MDCK-MDR1 cells are commonly used to identify MDR1 substrates by monitoring their transport properties. Organic anion transporters (OATs) play a vital role in the renal excretion of various drugs by facilitating the cellular uptake of substrate drugs and allowing renal tubule cells to efflux drugs into the urine. Renal clearance may be reduced as a consequence of OAT inhibition (11).

MTX, an antifolate and anticancer agent with a narrow therapeutic window, is commonly used in anticancer chemotherapy, rheumatoid arthritis, and severe psoriasis (12). In recent years, several reports have indicated that MTX is a substrate of MDR1 (9). This effectively limits MTX oral bioavailability (9,13). High-dose-MTX-induced acute kidney injury and the secondary delay of MTX elimination can lead to prolonged elevation of systemic MTX concentrations and serious toxicity, including myelosuppression, mucositis, and dermatitis (12,14). Therefore, it is useful to find an advantageous drug that is not only hypotoxicity but also can affect the function of MDR1, which can improve the bioavailability and reduce the dosage of MTX.

In recent years, herbal medicines have been shown to exert a wide range of biological effects, including protective roles in the prevention of cancer, diabetes, and cardiovascular and neurodegenerative diseases (15). Unfortunately, many of the active compounds, such as Ginsenoside Rh2 and quercetin, have low water solubility and poor bioavailability and it is thought that MDR1 may be involved (16). PUR, an isoflavone component extracted from Kudzu (Pueraria lobata), has been demonstrated to exhibit liver and kidney protective effects (17). It was reported that the uptake of the PUR in an everted intestinal sac preparation was significantly increased by MDR1 inhibitors such as quercetin and verapamil (18). Therefore, PUR, a nontoxic MDR1 inhibitor, is a potential candidate for co-administration with anti-cancer drugs to improve their bioavailability (19).

The purpose of the present study is to elucidate the effect of PUR on the intestinal absorption and renal excretion of MTX, and to understand how PUR affects MTX pharmacokinetics. Pharmacokinetic changes following MTX and PUR co-administration were evaluated. Urinary excretion, kidney slices, everted small-intestinal sac preparations, and transfected-cell uptake and transport were used in the evaluation. The results suggest that PUR inhibits MDR1 and OATs. PUR improved the bioavailability of MTX by influencing intestinal absorption (MDR1) and renal excretion (OATs) without additional nephrotoxicity.

MATERIALS AND METHODS

Materials

MTX, PAH and PCG were purchased from Sigma (USA). PUR was obtained from Shanxi Bosen BioPharmaceutical Co., Ltd. (Xi'an, China). Digoxin was purchased from Nanjing ZeLang Medical Technology Co., Ltd. (Nanjing, China). Cilostazol and JBP485 (cyclo-*trans*-4-L-hydroxyprolyl-L-serine, internal standard) were provided by Zhejiang Kinglyuan Pharmaceutical Co., Ltd. (Shangyu, China) and Japan Bioproducts Industry Co. Ltd. (Tokyo, Japan), respectively. Cyclosporin A (CsA) and verapamil hydrochloride (VER) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals were of analytical grade and were commercially available.

Cell Culture

Mock-, hOAT1/3-expressing HEK293 cells were kindly provided by Professors Yuichi Sugiyama (Graduate School of Pharmaceutical Sciences, University of Tokyo) and Gong Likun (Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai, China). Mock-, hMDR1-expressing MDCK cells were kindly provided by Professor Zeng Su (College of Pharmacy, Zhejiang University, China). All of the cell lines were routinely maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen, USA);

supplemented with 10% fetal bovine serum (FBS, heat-inactivated), 1% non-essential amino acid solution, 100 U/ml penicillin, and 0.1 mg/ml streptomycin and maintained at 37°C with 95% relative humidity and 5% CO_2.

Animals

Male Wistar rats weighing 220–250 g were obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008–0002). Experiments were performed according to local institutional guidelines for the care and use of laboratory animals. Before the onset of each experiment, rats were fasted overnight with free access to water. In all cases, rats were anesthetized with pentobarbital (60 mg/kg, ip) prior to pharmacokinetic experiments.

Pharmacokinetic Interaction in Rats

Rats were divided randomly into three groups ($n=4$): (1) MTX (5 mg/kg), (2) PUR (50 mg/kg) and (3) MTX (5 mg/kg) + PUR (50 mg/kg). MTX was dissolved in 2% sodium bicarbonate and brought up to volume with 0.9% sodium chloride. PUR was dissolved in normal saline.

In Vivo Absorption in Rats

MTX and PUR, suspended in 0.5% sodium carboxymethyl cellulose solution, were administered orally with a gavage needle. Blood samples (0.2 ml) were collected via jugular vein at 1, 5, 15, 30, 60, 120, 240, 360, 480 and 600 min in heparin tubes and centrifuged at 1000 g for 10 min to obtain plasma for MTX and PUR determination as described below.

In Vivo Renal Excretion

Rat bladders were cannulated with polyethylene tubing, the distal end of which flowed into a tube for urine collection (4). Rats received MTX (dissolved in 2% sodium bicarbonate and brought up to volume with 0.9% sodium chloride) and/or PUR (dissolved in 50% propylene glycol) via the jugular vein. Blood samples were collected at indicated times and centrifuged at 1000 g for 10 min to immediately obtain plasma. Urine was collected directly from the bladder at 2, 4, 6, 8, 10 and 24 h after dosing. MTX and PUR plasma and urine concentrations were measured as described below.

In Vitro Everted Intestinal Sac Preparation

An *In vitro* everted intestinal sac model was performed as previously described (8). A 10-cm-long intestinal segment was removed (approximately 2 cm distal to the ligament of Treitz) and everted with one end ligated to make a sac. The other end was used as a sampler and the empty sac was filled with 1 ml KRB (Krebs-Ringer buffer, containing 0.5 mM $MgCl_2$, 4.5 mM KCl, 120 mM NaCl, 0.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 1.2 mM $CaCl_2$, 15 mM $NaHCO_3$, 10 mM glucose and 20 mg/l phenolsulfonphthalein as a non-absorbable marker, pH 7.4) as serosal solution. The entire sac was placed in pre-oxygenated incubation medium (mucosal solution) containing 10 μM MTX and/or 10 μM PUR at 37°C ($n=4$). At 15, 30, 60 and 90 min post dosing, a 50 μl aliquot of serosal solution was collected for MTX and PUR determination as described below.

In Vitro Uptake in Kidney Slices

Rat kidney was cut into slices using a ZQP-86 tissue slicer (Zhixin Co. Ltd., Shanghai, China; thickness 300 μm, surface area 0.15 cm^2) as previously described (20). After pre-incubation for 3 min in oxygenated (O_2/CO_2, 95%:5%) Krebs-bicarbonate slicing buffer (containing 120 mM NaCl, 16.2 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) at 37°C, kidney slices were placed in 1 ml fresh oxygenated (O_2/CO_2, 95%:5%) buffer containing MTX (10 μM) and/or PUR (10 μM) for further incubation (n=4). At 1, 10, 30 and 45 min, uptake was terminated by removing the buffer and washing the slices with ice-cold saline. The slices were then homogenized (IKA-T 10 homogenizer; IKA, Staufen, Germany) for MTX and PUR determination as described below.

Trans-Epithelial Transport Studies

Trans-epithelial transport studies were conducted as previously described (21). MDCK-mock/MDR1 cells (passage number 18–30) were seeded on 24-well trans-well inserts (12 mm diameter, 0.6 cm^2 growing surface area, 0.4 μm pore size; Corning Costar, Acton, MA) and grown for 3–5 days to form cell monolayers. TEER (trans-epithelial electrical resistance) measurements were used to evaluate the integrity of the cell layer (Millicell-ERS equipment; Millipore, MA, USA) and TEER ≥ 350 Ω·cm^2 was used as acceptance criteria for further experiments. The MDR1 probe substrate digoxin (10 μM) was assessed as a positive control. Trans-epithelial transport experiments were initiated by adding transport buffer containing digoxin (10 μM) or PUR (10 μM) to the apical (total volume of 400 μl) or basolateral (total volume of 600 μl) side of the monolayer, which served as donor compartments ($n=3$). The other side was filled with blank buffer; serving as receiver compartments. A 50 μL

aliquot was taken from the receiver compartments at 0.5, 1.0, 2.0 and 3.0 h for determination and replaced with fresh buffer. For the efflux inhibition assay, digoxin (10 μM) or PUR (10 μM) was incubated in the basolateral side, and inhibitors were present on both sides of the monolayer. At the conclusion of the transport assay, the buffer was removed and monolayers were rapidly washed on both sides with ice-cold HBSS to measure the intracellular accumulation of PUR (described below).

In Vitro Transporter Uptake Assays

Uptake studies were performed as previously described (22). HEK293-mock/OAT1/3 (passage number 18–30) monolayers were washed 3 times with transport buffer (containing 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose and 1.5 mM $CaCl_2$, pH 7.4) and then pre-incubated in transporter buffer for 15 min at 37°C. Uptake studies were initiated with the adding of 1 ml buffer containing PAH (10 μM), PCG (10 μM), MTX (10 μM) or PUR (10 μM) ($n=3$). For inhibition assays, various concentrations of PUR (0.01–100 μM) were simultaneously added to the buffer. After incubation for the designated times at 37°C with gentle shaking, the medium was removed to stop uptake, followed by 3 washes with 1 ml ice-cold buffer. The cell monolayers were subsequently lysed with 0.3 mL of 0.1% Triton X-100® for 2 h. Test drug concentrations in cell lysates were determined as described below and protein concentrations were measured by the bicinchoninic acid procedure using bovine serum albumin as the standard (BCA; Solarbio, Beijing, China).

Renal Accumulation and Damage Assay

Rats were divided randomly into 3 groups ($n=4$). Control groups received the vehicle alone for 1 or 3 days. The MTX group received a single dose or 3 consecutive daily intraperitoneal injections of MTX at a dose of 5 mg/kg body weight. The PUR group received a single dose or 3 consecutive daily intraperitoneal injections of MTX (5 mg/kg) and PUR (50 mg/kg). Rats were killed 3 h after the final dose and the plasma and kidney were collected for determination of MTX concentrations. The plasma concentrations of creatinine and BUN were determined using a test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histological Analyses

On day 1 or 3, rat kidney cortex in vehicle (MTX and MTX + PUR groups) was removed for histopathological examination. Renal cortex was fixed in 10% neutral buffered formalin immediately after the application of ether and left in the same fixative solution for 24 h before being embedded in paraffin. Specimens (3 μm) were prepared for hematoxylin - eosin (H&E) for morphological evaluation.

Sample Preparation

Preparation of various biological samples was conducted as previously described (4). An aliquot (50 μl) of rat plasma, urine, KRB, cell lysate or HBSS was mixed with 50 μl internal standard solution (500 nM cilostazol or JBP485) and 200 μl of methanol. After centrifugation at 16 099*g* for 10 min to remove the protein precipitate, the upper organic layer was transferred into a polyethylene tube and concentrated to dryness under a gentle stream of nitrogen at 37°C. The residue was dissolved in 200 μl of mobile phase solution. Ten microliters of each sample was injected into LC-MS/MS for analysis.

LC-MS/MS Analysis

The concentrations of MTX, PUR, PAH and PCG were determined (23–27) with a LC-MS/MS system (Agilent HP1200, Agilent Technology Inc., Palo Alto, CA, USA; API 3200 triple-quadrupole mass spectrometer, Applied Biosystems, Concord, Ont, Canada). Chromatographic separation was performed on a Hypersil BDS-C_{18} column (150 mm×4.6 i.d., 5 μm, Dalian Elite Analytical Instruments Co. Ltd, Dalian, China). The mobile phase consisted of acetonitrile and water with 0.1% formic acid (40:60, v/v) for both MTX and PUR. The mobile phase consisted of 3% methanol and 97% water with 0.1% formic acid for both PAH and PCG. The flow rate was 0.3 mL/min. Ionization was conducted using a TurboIonspray interface in positive ion mode. Multiple reaction monitoring (MRM) was employed for data acquisition. The selected transitions were m/z 455.2 → 308.2 for MTX, m/z 415.4 → 267.1 for PUR, m/z 370.4 → 288.1 for cilostazol, m/z 779.0 → 649.0 for digoxin, m/z 195.2 → 120.2 for PAH, m/z 335.1 → 176.0 for PCG and m/z 201.1 → 86.1 for JBP485. Analyst 1.4.1 software (Applied Biosystems) was used for the control of equipment, data acquisition and data analysis.

Data Analysis

Main pharmacokinetic parameters were calculated using the Practical Pharmacokinetic Program (3P97) program edited by the Chinese Mathematical Pharmacological Society. The 3P97 program computes all main pharmacokinetic parameters and C-t curves automatically. The quality of the fit was judged by evaluating the S.E. of parameter estimates and the coefficient of determination (r^2), as well as by visual inspection of the residual plots. The main pharmacokinetic parameters were calculated using equations 1–3. The plasma clearance (CL_p) was calculated by the following:

$$CL_p = Dose/AUC_{i.v} \quad (1)$$

where $AUC_{i.v.}$ is the area under the plasma concentration-time profile after intravenous injection. Oral availability (F) was calculated as follows:

$$F = AUC_{p.o.}/AUC_{i.v} \quad (2)$$

where $AUC_{p.o.}$ is the AUC after oral administration and calculated by the trapezoidal rule.

The renal clearance (CL_R) of MTX or PUR was calculated using the following equation:

$$CL_R = A_{total}/AUC_{i.v.} \quad (3)$$

where A_{total} is the total cumulative amount of MTX or PUR excreted in urine over 24 h.

The apparent permeability values (Papp) were calculated in all experiments according to the equation:

$$Papp = (dQ/dt)/(AC_0) \quad (4)$$

where dQ/dt is the slope of the cumulative amount transported during the time course of the period studied, A is the area of the inserts and C_0 is the starting concentration.

Efflux ratios were calculated according to the following equation:

$$\text{Efflux ratio(ER)} = Papp_{AP}/Papp_{BL} \quad (5)$$

where AP is the apical to basolateral transport and BL is the basolateral to apical transport.

Net efflux ratios were calculated according to the following equation:

$$\text{Net efflux ratios(NER)} = ER_{MDCK-MDR1}/ER_{MDCK-mock} \quad (6)$$

Statistical Analysis

Statistical analysis was carried out using the SPSS 11.5 package. Test results are expressed as mean ± S.D. Statistically significant differences among multiple treatments for a given parameter were determined using one-way analysis of variance (ANOVA). Statistical significance of differences between mean values was calculated using a non-paired *t*-test. Statistical significance was defined as p values <0.05 or <0.01.

RESULTS

The Effect of PUR on the Pharmacokinetics of MTX *In Vivo*

To understand the target of interaction between MTX and PUR, the two drugs were administered simultaneously intravenously or orally. When MTX (5 mg/kg) and PUR (50 mg/kg) were orally administered in combination, their plasma concentrations and pharmacokinetic parameters significantly increased compared with that of control groups (Table I). The C_{max} of MTX and PUR increased by 79% and 30% of their control groups, respectively. The AUCs of MTX and PUR increased approximately by 74% and 37% of their control groups, respectively. However, there was no significant statistical difference between PUR and PUR + MTX group. The data suggests an interaction between MTX and PUR, which results in an increase in intestinal absorption of MTX when the two drugs are co-administered orally. Moreover, when MTX and PUR were co-administered intravenously, both their plasma concentrations and pharmacokinetic parameters were significantly altered compared to corresponding control groups (Table I). Other kinetic data for MTX and PUR, including the $t_{1/2}$ and T_{max},

Table I Pharmacokinetic Parameters of MTX and PUR Following per o.p. or i.v. Administration

	Parameters		MTX (5 mg/kg)		PUR (50 mg/kg)	
			without PUR	with PUR (50 mg/kg)	without MTX	with MTX (5 mg/kg)
P.O.	C_{max}	ng/ml	108 ± 8	194 ± 21[a]	202 ± 26	263 ± 32
	T_{max}	min	60	60	45	60
	AUC	μg·min/ml	28.7 ± 2.6	50 ± 4[a]	133 ± 15	182 ± 21
	$t_{1/2}$	min	108 ± 10	184 ± 16[a]	655 ± 83	450 ± 39
	CL/F	ml/kg/min	172 ± 13	102 ± 9[a]	370 ± 29	264 ± 20
	F%		6.6 ± 0.7	11.5 ± 1.3[a]	1.01 ± 0.06	1.39 ± 0.09
I.V.	AUC	μg·min/ml	434 ± 37	689 ± 72[a]	13101 ± 1513	21953 ± 1800[b]
	$t_{1/2}$	min	81 ± 9	111 ± 13[a]	164 ± 21	135 ± 16
	CL_p	ml/kg/min	11.5 ± 2.3	7.25 ± 1.15[a]	3.82 ± 0.35	2.28 ± 0.33 [b]
	CL_R	ml/kg/min	9.43 ± 0.89	4.10 ± 0.47[a]	1.20 ± 0.23	0.48 ± 0.05 [b]

[a] $p < 0.05$ *vs* MTX group

[b] $p < 0.05$ *vs* PUR group

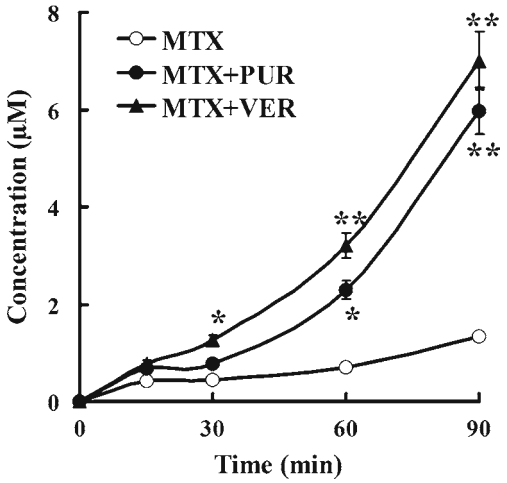

Fig. 1 Effect of PUR and VER on the absorption of MTX in everted small intestinal sac preparations *in vitro*. (mean ± S.D.; *, $p < 0.05$ *vs*. control, **, $p < 0.01$ *vs*. control; $n = 3$).

also changed when the drugs were co-administered. The $t_{1/2}$ of MTX was prolonged and the CLp decreased by 37% for MTX and 41% for PUR (Table I). The results indicate that clearance of the two drugs is delayed when they are co-administered.

The Effect of PUR on the Intestinal Absorption of MTX *In Vitro*

To exclude the impact of changes in physiological conditions and to further confirm the effect of PUR on the intestinal absorption of MTX, we employed a rat everted gut sac model. The serosal side concentration of MTX when combined with PUR increased and the AUC increased 2.24-fold compared to control (Fig. 1). The concentration of MTX in the serosal side also increased in the presence of verapamil, suggesting that the first target of the interaction between MTX and PUR is in the intestine.

The Effect of PUR on MTX Transport in MDCK-MDR1 Cells

To elucidate the target transporter involved in the DDI between MTX and PUR, an MDCK-MDR1 cell model was used to characterize the influence of PUR on the transport of digoxin, a probe substrate of MDR1. With the addition of CsA, the NER of digoxin was significantly reduced from 4.82 to 1.07 (Table II). Like CsA, PUR also inhibited the efflux transport of digoxin and in a concentration-dependent manner (Fig. 2) with an IC_{50} value of 1.6 ± 0.3 μM; an indication that PUR is an inhibitor of MDR1. The transcellular transport of PUR was also evaluated but no obvious vectorial transport was observed in MDCK-mock cells. The Papp of PUR from basolateral-to-apical was higher than that of the opposing direction. The NER was 3.2 and CsA and verapamil inhibited PUR efflux transport (Fig. 3). The results demonstrate that PUR is a substrate of MDR1 and that MDR1 mediates the efflux transport of PUR.

The Effect of PUR on the Renal Excretion of MTX

To further characterize the DDI between MTX and PUR, renal excretion following iv co-administration was analyzed. Cumulative urinary excretion over 24 h was 83% for MTX and 32% for PUR when MTX or PUR were administered alone. When MTX and PUR were co-administered, cumulative urinary excretion decreased by 32% for MTX and 36% for PUR (Fig. 4). MTX or PUR renal clearance decreased by 43% or 40% by each other after iv administration, respectively (Table I); indicating that PUR inhibits MTX renal excretion.

The Effect of PUR on MTX Uptake in Kidney Slices

Rat kidney slices were used to investigate the effect of PUR on MTX uptake. PUR significantly inhibited MTX uptake in kidney slices. MTX uptake decreased by 35% compared with the control group. Probenecid (PRO, an OAT inhibitor) also inhibited the uptake of MTX (Fig. 5), suggesting that the second target of the DDI between PUR and MTX is located in the kidney.

The Effect of PUR on MTX Uptake in HEK293-OAT1/3 Cells

To identify the target transporter supporting the DDI between PUR and MTX, the effect of PUR on MTX uptake

Table II Papp Values of Digoxin Across MDCK-mock and MDCK-MDR1 Cell Monolayers

MDR1 substrate		Papp (mock-MDCK)			Papp (MDR1-MDCK)			NER
		A-B	B-A	ER	A-B	B-A	ER	
digoxin	control	1.26 ± 0.13	3.90 ± 0.51	3.1	0.57 ± 0.03	8.5 ± 1.42	15.0	4.82
	CsA[b]	1.42 ± 0.18	1.76 ± 0.22	1.24	0.78 ± 0.08	1.04 ± 0.21	1.33	1.07
	PUR[b]	1.24 ± 0.15	1.6 ± 0.05	1.29	0.72 ± 0.06	1.3 ± 0.18	1.81	1.4

[a] Papp: $\times 10^{-6}$ cm/s

[b] CsA: 10 μM, PUR: 10 μM

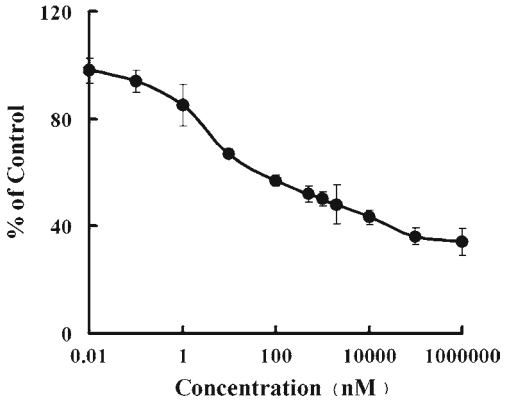

Fig. 2 Effect of PUR on the efflux transport of digoxin by MDCK-MDR1 cells. Data are expressed as mean ± S.D. ($n = 3$).

in HEK293-OAT1/3 cells was studied. PAH or PCG uptake was inhibited by PUR in HEK293-OAT1/3 cells (Fig. 6a and b). MTX uptake increased linearly over a period of 2 min in a time-dependent manner and was inhibited by both PAH and PCG. MTX uptake in the presence of PUR decreased significantly (Fig. 6c and d). The findings show that PUR is an inhibitor of OAT1/3.

The concentration-dependence of the inhibitory effect of PUR on the uptake of PAH and PCG was also investigated. PUR significantly inhibited PAH and PCG uptake in HEK293-OAT1/3 cells (Table III). PUR inhibited MTX uptake in HEK293-OAT1/3 cells in a concentration-dependent manner. The IC_{50} values were summarized in Table III. The results demonstrate that PUR inhibits MTX uptake via OAT1/3, resulting in delayed MTX renal excretion and prolonged residence time.

Finally, PUR uptake in HEK293-OAT1/3 cells and HEK293-mock cells was measured. No statistically significant difference between HEK293-OAT1/3 cells and HEK293-mock cells was found.

The Effect of PUR on MTX Renal Toxicity

To determine whether PUR-induced changes in MTX pharmacokinetics influence MTX renal toxicity, changes in renal morphology, MTX accumulation in rat kidney and creatinine and BUN levels in rat plasma were measured. Three hours following a single intraperitoneal dose of MTX (5 mg/kg), no pathological or histological changes were observed (Fig. 7a, b and c). After 3 consecutive daily intraperitoneal injections of MTX, tubular dilatation (arrow in Fig. 7) with cellular casts composed mostly of detached cells, mono and polynuclear infiltrating cells and tubulitis were visible in the MTX treatment group (Fig. 7e). When PUR was co-administered with MTX, the histological damage was significantly reduced (Fig. 7f). Moreover, PUR significantly decreased the concentration ratios of MTX in kidney and plasma (Kp) in groups receiving a single intraperitoneal dose and 3 consecutive daily intraperitoneal injections of MTX (Fig. 8a). MTX caused an increase in the plasma concentration of creatinine and BUN following intraperitoneal administration, which was partially reversed by co-administration with PUR (Fig. 8b and c). The results indicate that MTX renal toxicity is not enhanced by PUR co-administration.

DISCUSSION

Transporter-mediated DDI is now a prominent consideration in the evaluation of pharmaceutical therapies. Combination use of anticancer drugs with chemo-sensitizers is considered a beneficial strategy for reversing multidrug resistance (MDR) mediated by multidrug resistance transporters (9–11). Transporter-based DDI can cause untoward and even life-threatening side effects, as exemplified by the interaction between gemfibrozil and cerivastatin (28). It is therefore imperative to characterize and understand these phenomena.

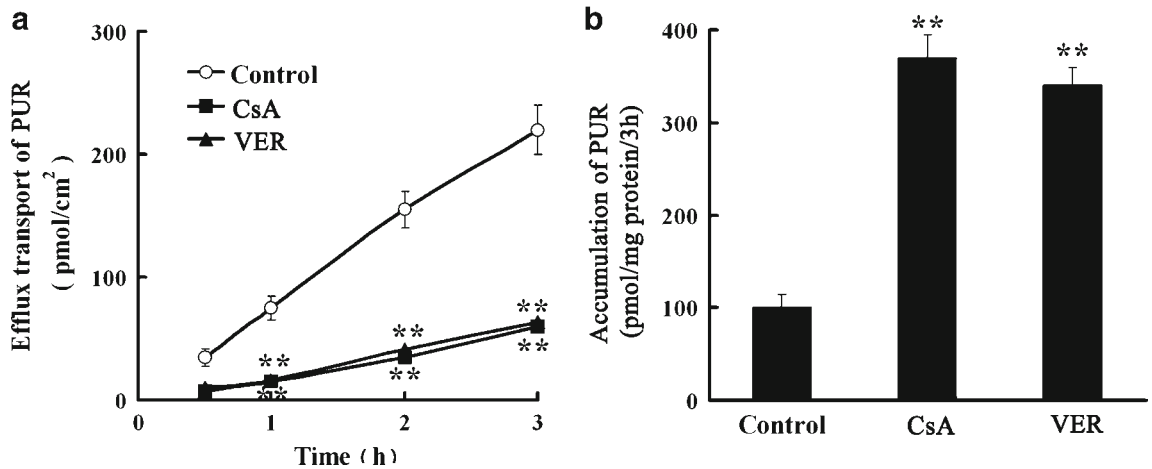

Fig. 3 Effects of MDR1 inhibitors on the efflux transport (**a**) and intracellular accumulation (**b**) of PUR in MDCK-MDR1 cells. (mean ± S.D.; **, $p < 0.01$ *vs.* control group; $n = 3$).

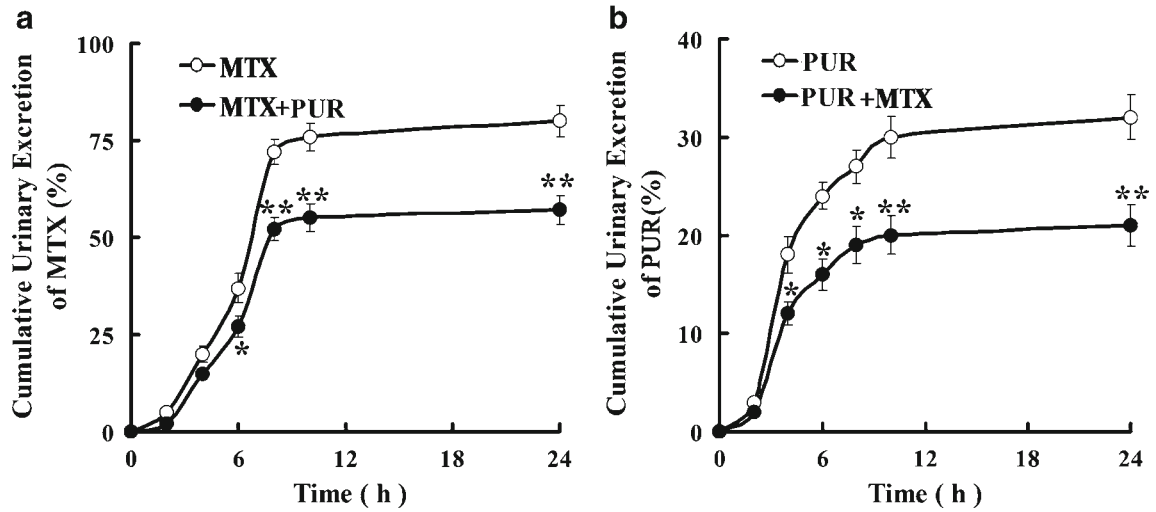

Fig. 4 Cumulative urinary excretion curves of MTX (**a**) and PUR (**b**) after intravenous injection. Data are expressed as mean ± S.D. (* $p < 0.05$ *vs*. control, ** $p < 0.01$ *vs*. control; $n = 4$).

MTX is used widely for tumor treatment in clinical medicine and is particularly prone to DDI when co-administered with other drugs. Prominent examples are omeprazole and penicillin for which the underlying DDI mechanism is the competitive inhibition of MDR1 and OATs (29). As with other chemotherapy drugs, multi-drug resistance (MDR) is a major obstacle in MTX therapy. MDR1 plays a critical role in MDR and it has been found that inhibiting MDR1 increases the intestinal absorption of PUR (9,18). PUR exhibits a MDR reversal effect through the down-regulation of MDR1 (30). Furthermore, increasing evidence shows that PUR protects the kidneys from injury induced by nephrotoxins (17).

PUR/MTX co-administration could potentially be used to improve MTX bioavailability while at the same time reducing its renal toxicity. The present study investigated the effects and molecular mechanism of PUR's influence on the pharmacokinetics of MTX both *in vivo* and *in vitro*. Pharmacokinetic studies in rats, a rat everted gut sac model, rat fresh kidney slices and human transfected cell systems were employed to give insights into the potential for the clinical use of this drug combination.

MTX exhibits low oral bioavailability and large individual differences (9). A possible underlying mechanism could involve ABC transporter-mediated efflux transport in the gut (31). Many efforts have been made to develop new drug delivery systems to improve MTX bioavailability (32). MDR1, localized at the luminal membrane of enterocytes and responsible for exporting substrates back into the intestinal lumen, is abundantly expressed in the gut and limits the oral bioavailability of its substrates. MDR1 has been identified as a target to improve oral bioavailability (33). In the present study, when MTX and PUR were orally co-administered, changes in the pharmacokinetics of MTX were observed. Increased C_{max}, AUC and F (Table I) indicated that intestinal absorption of MTX was enhanced. It has been reported that MDR1 contributes to an increased AUC of MTX when omeprazole or pantoprazole are concomitantly administered (29). Moreover, evidence for a DDI between PUR and MDR1 has been found and PUR was also shown to reverse MDR via the down-regulation of MDR1 (30). On the other hand, when given with MDR1 inhibitors, PUR absorption improved in Caco-2 cells (18). Therefore, it is plausible that MDR1 mediates the DDI between PUR and MTX within the intestine.

To exclude the impact of changes in physiological conditions, uptake studies with an everted gut sac model were performed and similar results were obtained (Fig. 1). MTX intestinal absorption was significantly increased by PUR and verapamil, an inhibitor of MDR1 (Fig. 1), clearly illustrating that an interaction occurs in the intestine.

The mechanism underlying this inhibition was investigated by transport studies using MDCK-MDR1 cells. PUR

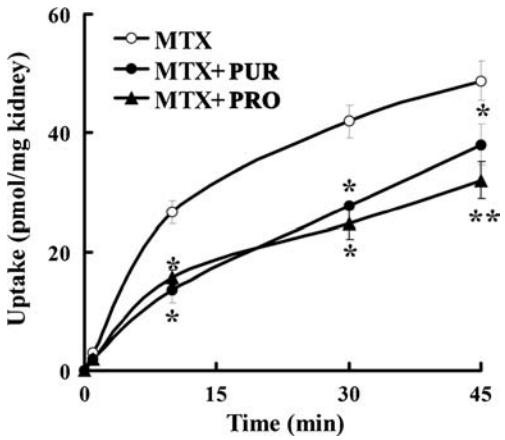

Fig. 5 Inhibition effects of PUR and PRO on MTX uptake in kidney slices. Data are expressed as mean ± S.D. (* $p < 0.05$ *vs*. control, ** $p < 0.01$ *vs*. control; $n = 3$).

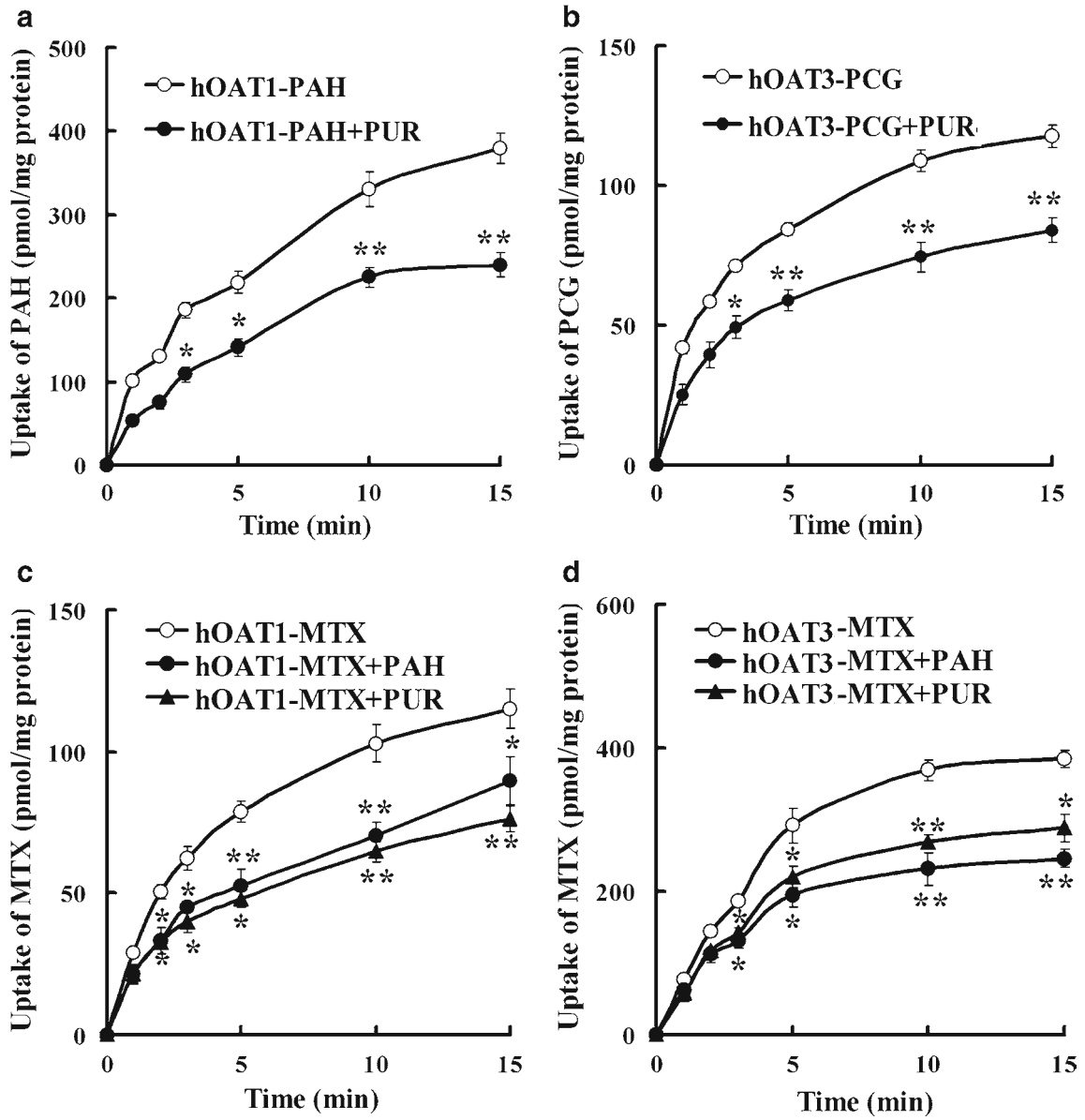

Fig. 6 Time profiles of the uptake of PAH (**a**), PCG (**b**) and MTX (**c**, **d**) by HEK293-OAT1/3 cells. Data are expressed as mean ± S.D. (*, $p < 0.05$; **, $p < 0.01$) ($n = 3$).

inhibited the efflux transport of digoxin, a probe substrate of MDR1 (IC_{50} = 1.6 μM, Table II, Fig. 2). The clinical plasma concentration of PUR was 2.4 μg/ml (4.8 μM) at 5 h after intravenous infusion at a dosage of 5 mg/kg (34). This was higher than the IC_{50} value, suggesting that a clinically significant DDI might be induced when MTX and PUR are co-administered. Consequently, PUR has been shown to cause a systemic DDI and decrease in the elimination of MDR1 substrate drugs (35). Conversely, the C_{max} of PUR in human plasma is 60 ng/ml following oral administration (36), potentially causing an intestinal rather than a systemic MDR1 mediated DDI *in vivo* (35).

Overall, PUR inhibits MDR1 substrate efflux transport, resulting in the increased intestinal absorption of MTX when MTX and PUR are co-administered orally (Fig. 1). Furthermore, the transport characteristics of PUR are similar to those of digoxin and the efflux transport of PUR is significantly inhibited by MDR1 inhibitors (Fig. 3). This suggests that PUR is a substrate of MDR1 and that the action of PUR could be changed with a concomitant intake of

Table III IC_{50} Values of PUR for MTX, PAH and PCG Uptake by OAT1/3

Substrate	OAT1 (μM)	OAT3 (μM)
MTX	16.9 ± 2.6	27.9 ± 3.4
PAH	6.7 ± 1.2	–
PCG	–	25.6 ± 4.2

MDR1 substrate drugs. It also explains the change in pharmacokinetics of PUR when PUR is co-administered with MTX (Table I). Oral co-administration of PUR and MTX improves the bioavailability of two drugs due to the competitive inhibition of MDR1 in the intestine. MTX at dose of 500 mg/m^2 or higher over 6–24 h was needed to treat high-grade lymphoma, osteogenic sarcoma and acute leukaemia (29). However, the patients who take such a high dose of MTX are at high risk for developing significant toxicity. Our findings indicated that PUR enhanced MTX bioavailability. It is predictable that, in the presence of PUR, MTX at a lower dose can reach the same level of plasma concentration compared with MTX alone at normal dose. The results provided a new idea for the clinical use of MTX.

Renal excretion is the major elimination pathway for MTX, in which uptake transporters in the basolateral membrane (e.g., OATs) and export transporters in the luminal membrane (e.g., MDR1) of renal tubule cells play important roles (29,37). It has been reported that concomitant administration of inhibitors for these transporters decreases MTX renal clearance, resulting in a higher plasma concentration and a longer $t_{1/2}$ (29). In our study, plasma concentration and $t_{1/2}$ increased whereas the CL_p and CL_R of MTX and PUR decreased when co-administered intravenously (Table I). About 1/3 of the PUR dosage was recovered from urine following intravenous administration (38), possibly indicating a DDI in the kidney that delays the renal elimination of the two drugs. MTX concentration was also found to be decreased in the urine (Fig. 4). According to previous results, co-administration of PUR can decrease the elimination of MDR1 substrate while the clearance of PUR is also weakened. MDR1 inhibition thereby provides an explanation for the DDI occurring in the intestine. Additionally, renal excretion mediated by OATs is important for MTX elimination (12).

The relevance of an interaction between PUR and OATs was confirmed by the results of uptake studies in kidney slices. PRO, an inhibitor of OATs, inhibited MTX uptake in kidney slices. PUR exhibited a similar inhibitory effect (Fig. 5). The results suggest that PUR is an inhibitor of OAT. It is well known that OATs in the kidneys play a major role in the drug excretion process. OAT inhibition could consequently reduce elimination (22,37). Indeed, PUR inhibited the uptake of MTX and specific substrates (PAH for OAT1 and PCG for OAT3) in HEK293-OAT1/3 cells (Fig. 6).

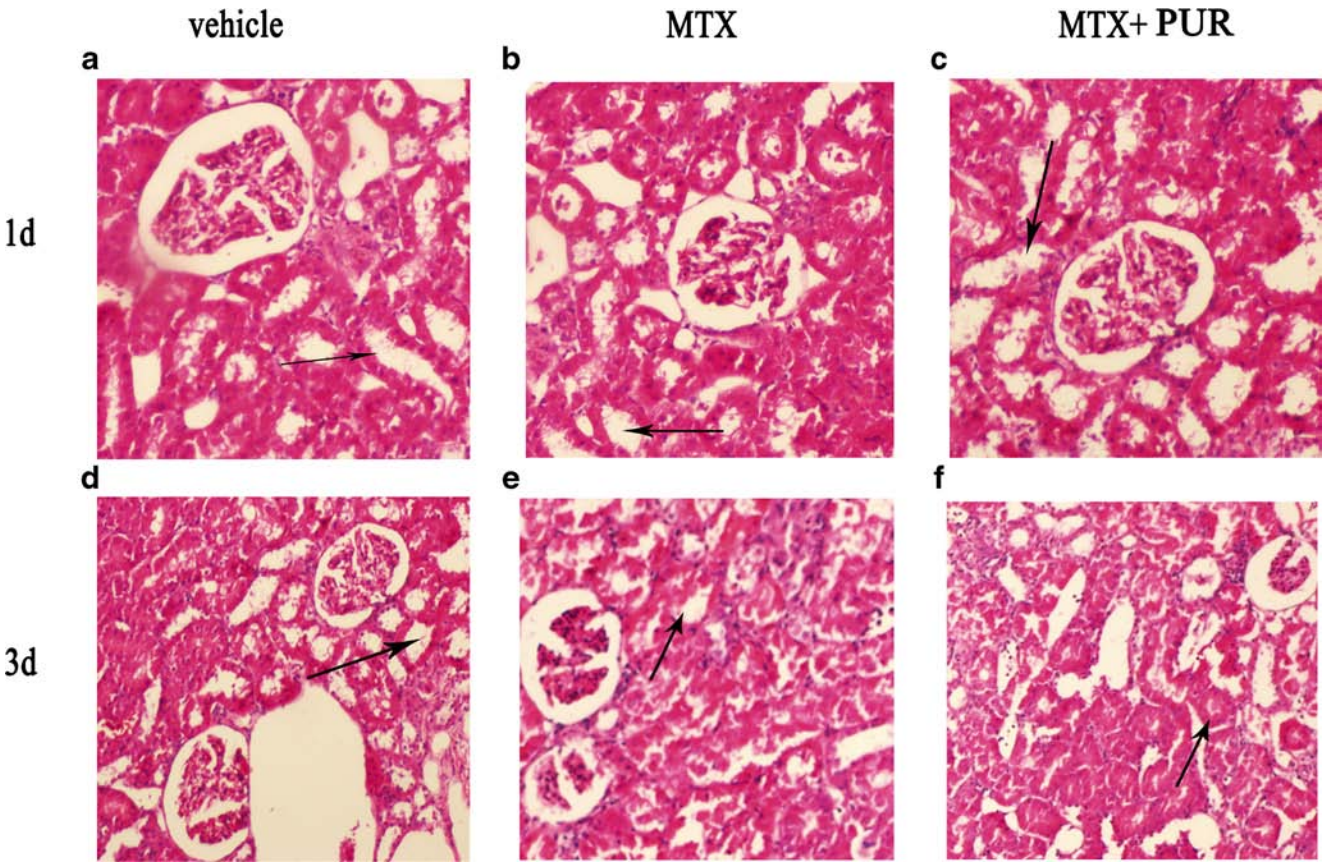

Fig. 7 The effect of MTX and PUR on renal morphology. (**a** and **d**) Normal architecture of kidney in control group receiving the vehicle alone for 1 or 3 days. (**b** and **e**), MTX group receiving a single intraperitoneal dose or 3 consecutive daily intraperitoneal injections of MTX (5 mg/kg). (**c** and **f**), MTX + PUR group receiving a single intraperitoneal dose or 3 consecutive daily intraperitoneal injections of MTX (5 mg/kg) and PUR (50 mg/kg). The tubular dilatation was markedly alleviated compared with the MTX group after 3 days. The tubular dilatation is indicated by black arrowhead. Magnification = 100 ×.

When PUR is administered intravenously in the clinic, its plasma concentration can be up to 28 μg/ml (67 μM) (34). This is above the IC_{50} values for OATs according to our findings (Table III). Though our results indicate that DDI mediated by OATs can occur when PUR is administered in combination with substrate drugs, PUR was not recognized by OATs as a substrate according to the results of uptake studies utilizing HEK293-OAT1/3 cells. Possible mechanism of puerarin of inhibition of OATs may involve in interference metabolism (e.g. cyanide) or blocking the Na^+,K-ATPase (e.g. ouabain) (39), which we will do further research .

As described above, MTX exposure can be increased and its elimination delayed through co-administration with PUR. A concern is whether increased exposure to MTX by PUR causes additional renal damage. Nephrotoxicity is an adverse effect of MTX and treatment at high doses may cause renal failure (12,14). Indeed, pathological and histological changes, and an increase in plasma creatinine and BUN, both markers of renal damage, were observed in MTX-treated rats (Figs. 7e and f and 8b and c). This is consistent with the results of Abraham *et al*. (40).

Here, we report that histological damage is improved and the accumulation of MTX reduced when PUR is co-administered (Figs. 7f and 8a). The results are rationalized by considering that OAT1 and OAT3 are localized on the basolateral membrane and contribute to the uptake of drugs into renal epithelial cells from the blood side (9,37). The inhibition of OATs consequently decreases the cellular uptake and renal accumulation of substrate drugs (37), resulting in reduced exposure of the kidney to MTX and a subsequent lowering of nephrotoxicity (Figs. 7e and f and 8b and c).

However, attention should be paid to the increased exposure of MTX after combination with PUR, which increased the risk of adverse reactions. Although the renal damage was improved by PUR, the gastrointestinal toxicity, liver damage and other systemic toxicity should be cautious in

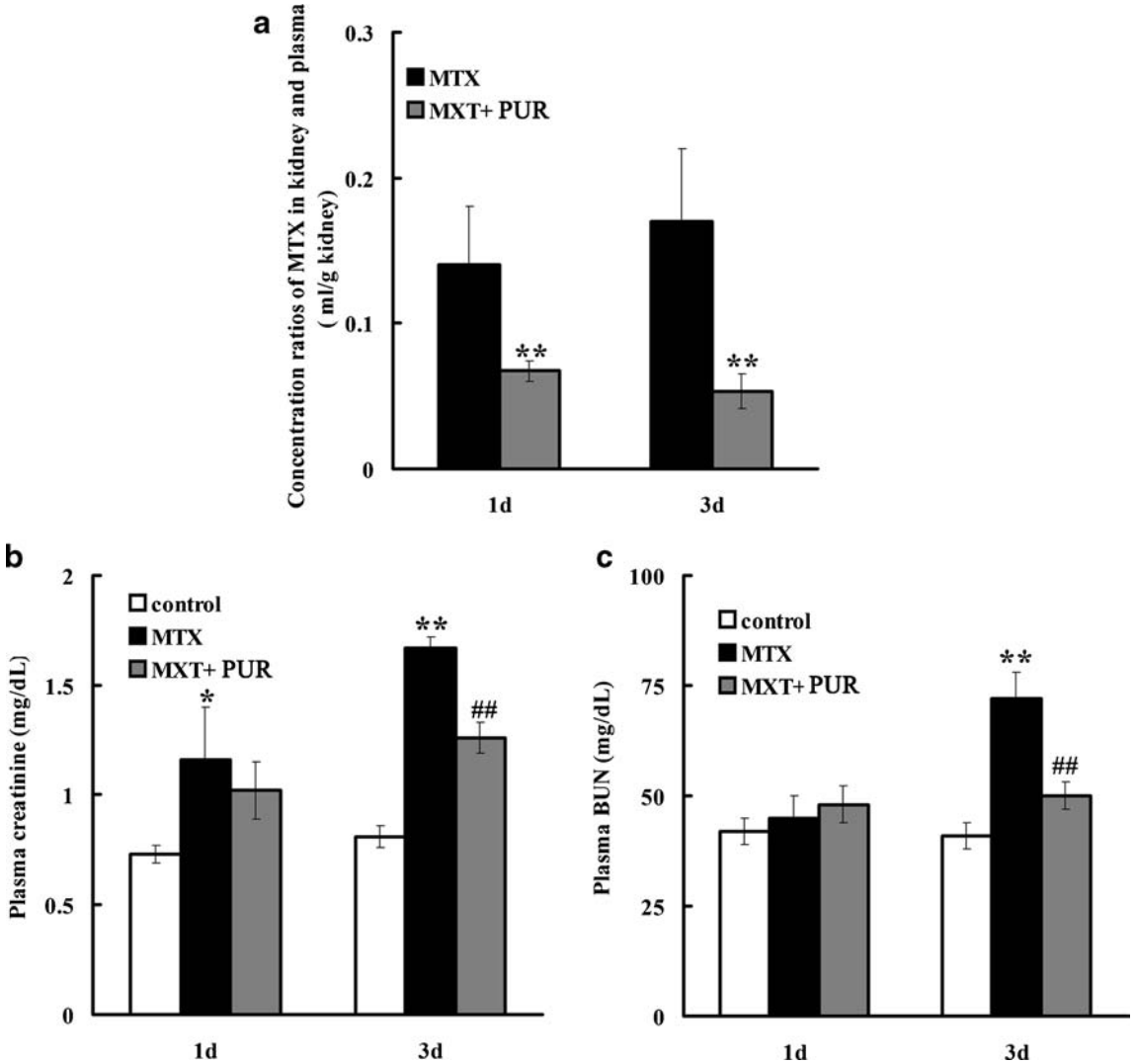

Fig. 8 Effects of PUR on the accumulation of MTX in rat kidney (**a**) and the levels of creatinine (**b**) and BUN (**c**) in rat plasma. (mean ± S.D.; *, $p < 0.05$ *vs*. control, **, $p < 0.01$ *vs*. control; ##, $p < 0.01$ *vs*. MTX group; $n = 4$).

the clinical usage. Pharmacokinetic monitoring is an important way to improve MTX therapy. In order to decrease the systemic toxicity, the interaction indicates the need for dosage adjustments (6). According to the results in the present study, when PUR was co-administered, MTX dose should be decreased so that the systemic exposure of MTX was within the therapeutic window and no additional toxicity.

CONCLUSION

(1) When MTX and PUR are co-administered, a DDI mediated by MDR1 and OAT1/3 may occur. (2) PUR is a potential candidate for clinical use as an efficiency and hypotoxicity supplement to enhance the bioavailability of MTX without creating further renal toxicity.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by a grant from the National Natural Science Foundation of China (No. 81273580, 81072694) and the Dalian Government (No. 2010E12SF060). We wish to express our deep gratitude to Professor Zeng Su (College of Pharmacy, Zhejiang University, China) for providing MDCK-MDR1 cells and Professors Yuichi Sugiyama (Graduate School of Pharmaceutical Sciences, University of Tokyo) and Gong Likun (Shanghai Institute of Materia Medica, Chinese Academy of Science, Shanghai, China) for providing HEK293-OAT1/3 cells.

REFERENCES

1. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, *et al*. ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010;376(9742):687–97.
2. Cruz-Munoz W, Man S, Kerbel RS. Effective treatment of advanced human melanoma metastasis in immunodeficient mice using combination metronomic chemotherapy regimens. Clin Cancer Res. 2009;15(15):4867–74.
3. Lee JO, Lee KW, Oh DY, Kim JH, Im SA, Kim TY, *et al*. Combination chemotherapy with capecitabine and cisplatin for patients with metastatic hepatocellular carcinoma. Ann Oncol. 2009;20(8):1402–7.
4. Guo X, Meng Q, Liu Q, Wang C, Mao Q, Sun H, *et al*. Peptide cotransporter 1 in intestine and organic anion transporters in kidney are targets of interaction between JBP485 and lisinopril in rats. Drug Metab Pharmacokinet. 2012;27(2):232–41.
5. Ye J, Liu Q, Wang C, Meng Q, Peng J, Sun H, *et al*. Inhibitory effect of JBP485 on renal excretion of acyclovir by the inhibition of OAT1 and OAT3. Eur J Pharm Sci. 2012;47(2):341–6.
6. US Food and Drug Administration. Draft guidance for industry: Drug interaction studies-study design, data analysis, and implications for dosing and labeling. 2012 (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf).
7. Zhang L, Zhang YD, Zhao P, Huang SM. Predicting drug-drug interactions: an FDA perspective. AAPS J. 2009;11(2):300–6.
8. Zhang J, Wang C, Liu Q, Meng Q, Cang J, Sun H, *et al*. Pharmacokinetic interaction between JBP485 and cephalexin in rats. Drug Metab Dispos. 2010;38(6):930–8.
9. Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev. 2003;55(1):3–29.
10. Breedveld P, Beijnen JH, Schellens JH. Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs. Trends Pharmacol Sci. 2006;27(1):17–24.
11. Masereeuw R, Russel FG. Therapeutic implications of renal anionic drug transporters. Pharmacol Ther. 2010;126(2):200–16.
12. Maeda A, Tsuruoka S, Kanai Y, Endou H, Saito K, Miyamoto E, *et al*. Evaluation of the interaction between nonsteroidal anti-inflammatory drugs and methotrexate using human organic anion transporters 3-transfected cells. Eur J Pharmacol. 2008;596(1–3):166–72.
13. Hoekstra M, Haagsma C, Neef C, Proost J, Knuif A, van de Laar M. Bioavailability of higher dose methotrexate comparing oral and subcutaneous administration in patients with rheumatoid arthritis. J Rheumatol. 2004;31(4):645–8.
14. Christensen AM, Pauley JL, Molinelli AR, Panetta JC, Ward DA, Stewart CF, *et al*. Resumption of high-dose methotrexate after acute kidney injury and glucarpidase use in pediatric oncology patients. Cancer. 2012;118(17):4321–30.
15. Yang G, Li X, Li X, Wang L, Li J, Song X, *et al*. Traditional chinese medicine in cancer care: a review of case series published in the chinese literature. Evid Based Complement Alternat Med. 2012;2012:751046.
16. Zhang J, Zhou F, Wu X, Gu Y, Ai H, Zheng Y, *et al*. 20(S)-ginsenoside Rh2 noncompetitively inhibits P-glycoprotein in vitro and in vivo: a case for herb-drug interactions. Drug Metab Dispos. 2010;38(12):2179–87.
17. Liu CM, Ma JQ, Sun YZ. Puerarin protects rat kidney from lead-induced apoptosis by modulating the PI3K/Akt/eNOS pathway. Toxicol Appl Pharmacol. 2012;258(3):330–42.
18. Liang XL, Zhao LJ, Liao ZG, Zhao GW, Zhang J, Chao YC, *et al*. Transport properties of puerarin and effect of Radix Angelicae Dahuricae extract on the transport of puerarin in Caco-2 cell model. J Ethnopharmacol. 2012;144(3):677–82.
19. Yang AK, He SM, Liu L, Liu JP, Wei MQ, Zhou SF. Herbal interactions with anticancer drugs: mechanistic and clinical considerations. Curr Med Chem. 2010;17(16):1635–78.
20. Miao Q, Liu Q, Wang C, Meng Q, Guo X, Sun H, *et al*. Inhibitory effect of 1α,25-dihydroxyvitamin D(3) on excretion of JBP485 via organic anion transporters in rats. Eur J Pharm Sci. 2012;48(1–2):351–9.
21. Liu Z, Wang C, Liu Q, Meng Q, Cang J, Mei L, *et al*. Uptake, transport and regulation of JBP485 by PEPT1 in vitro and in vivo. Peptides. 2011;32(4):747–54.
22. Zhu Y, Meng Q, Wang C, Liu Q, Sun H, Kaku T, *et al*. Organic anion transporters involved in the excretion of bestatin in the kidney. Peptides. 2012;33(2):265–71.
23. Guo P, Wang X, Liu L, Belinsky MG, Kruh GD, Gallo JM. Determination of methotrexate and its major metabolite 7-hydroxymethotrexate in mouse plasma and brain tissue by liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2007;43(5):1789–95.
24. Luo CF, Yuan M, Chen MS, Liu SM, Huang BY, Liu XW, *et al*. Determination of puerarin in rat plasma by rapid resolution liquid chromatography tandem mass spectrometry in positive ionization mode. J Chromatogr B Anal Technol Biomed Life Sci. 2011;879(19):1497–501.

25. Smalley J, Marino AM, Xin B, Olah T, Balimane PV. Development of a quantitative LC-MS/MS analytical method coupled with turbulent flow chromatography for digoxin for the in vitro P-gp inhibition assay. J Chromatogr B Anal Technol Biomed Life Sci. 2007;854(1–2):260–7.
26. Fan HY, Lin CC, Pao LH. Determination of p-aminohippuric acid in rat plasma by liquid chromatography-tandem mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2010;878(19):1643–6.
27. Liu C, Wang H, Jiang Y, Du Z. Rapid and simultaneous determination of amoxicillin, penicillin G, and their major metabolites in bovine milk by ultra-high-performance liquid chromatography-tandem mass spectrometry. J Chromatogr B Anal Technol Biomed Life Sci. 2011;879(7–8):533–40.
28. Shitara Y, Hirano M, Sato H, Sugiyama Y. Gemfibrozil and its glucuronide inhibit the organic anion transporting polypeptide 2 (OATP2/OATP1B1:SLC21A6)-mediated hepatic uptake and CYP2C8-mediated metabolism of cerivastatin: analysis of the mechanism of the clinically relevant drug-drug interaction between cerivastatin and gemfibrozil. J Pharmacol Exp Ther. 2004;311(1):228–36.
29. Joerger M, Huitema AD, van den Bongard HJ, Baas P, Schornagel JH, Schellens JH, *et al*. Determinants of the elimination of methotrexate and 7-hydroxy-methotrexate following high-dose infusional therapy to cancer patients. Br J Clin Pharmacol. 2006;62(1):71–80.
30. Hien TT, Kim HG, Han EH, Kang KW, Jeong HG. Molecular mechanism of suppression of MDR1 by puerarin from Pueraria lobata via NF-kappaB pathway and cAMP-responsive element transcriptional activity-dependent up-regulation of AMP-activated protein kinase in breast cancer MCF-7/adr cells. Mol Nutr Food Res. 2010;54(7):918–28.
31. Vlaming ML, van Esch A, van de Steeg E, Pala Z, Wagenaar E, van Tellingen O, *et al*. Impact of abcc2 [multidrug resistance-associated protein (MRP) 2], abcc3 (MRP3), and abcg2 (breast cancer resistance protein) on the oral pharmacokinetics of methotrexate and its main metabolite 7-hydroxymethotrexate. Drug Metab Dispos. 2011;39(8):1338–44.
32. Paliwal R, Rai S, Vyas SP. Lipid drug conjugate (LDC) nanoparticles as autolymphotrophs for oral delivery of methotrexate. J Biomed Nanotechnol. 2011;7(1):130–1.
33. Li Y, Yuan H, Yang K, Xu W, Tang W, Li X. The structure and functions of P-glycoprotein. Curr Med Chem. 2010;17(8):786–800.
34. Li N, Lu M, Chen H, Meng D, Chen Y. Pharmacokinetics of puerarin in patients with diabetic nephropathy. Chin J New Drugs Clin Rem. 2002;21(1):51–3.
35. Elsby R, Gillen M, Butters C, Imisson G, Sharma P, Smith V, *et al*. The utility of in vitro data in making accurate predictions of human P-glycoprotein-mediated drug-drug interactions: a case study for AZD5672. Drug Metab Dispos. 2011;39(2):275–82.
36. Ma Z, Wu Q, Lee DY, Tracy M, Lukas SE. Determination of puerarin in human plasma by high performance liquid chromatography. J Chromatogr B Anal Technol Biomed Life Sci. 2005;823(2):108–14.
37. Morrissey KM, Stocker SL, Wittwer MB, Xu L, Giacomini KM. Renal transporters in drug development. Annu Rev Pharmacol Toxicol. 2013;53:503–29.
38. Yan B, Xing D, Ding Y, Tao J, Du LJ. HPLC method for the determination and pharmacokinetic studies on puerarin in cerebral ischemia reperfusion rat plasma after intravenous administration of puerariae radix isoflavone. J Pharm Biomed Anal. 2005;37(2):297–301.
39. Pritchard JB, Sweet DH, Miller DS, Walden R. Mechanism of organic anion transport across the apical membrane of choroid plexus. J Biol Chem. 1999;274(47):33382–7.
40. Abraham P, Kolli VK, Rabi S. Melatonin attenuates methotrexate-induced oxidative stress and renal damage in rats. Cell Biochem Funct. 2010;28(5):426–33.