

Smilax glabra Rhizoma affects the pharmacokinetics and tissue distribution of methotrexate by increasing the P-glycoprotein mRNA expression in rats after oral administration

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Abstract. Methotrexate (MTX) is a widely used immunosuppressant and anticancer agent with high toxicity. *Smilax glabra Rhizoma* (SGR) has the effect of detoxification and immunoregulation, and has been used as both food and folk medicine in many countries. Co-administration of MTX and SGR occurs in several diseases. However, whether they work synergistically or are incompatible remains unknown. In the present study, MTX was administered to rats alone or combined with SGR. Blood and tissue samples were collected at designated times. The concentrations of MTX were determined by high-performance liquid chromatography. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect the gene expression. SGR decreased the AUC_{0-t} and C_{max} of MTX by 44.5 and 48.2%, but in a tissue-dependent manner. The total exposure of MTX was significantly decreased in the small intestine, stomach, plasma, and kidney by 61.6, 34.7, 63.3 and 46.1%, respectively, but was increased in the lung and spleen by 82.9 and 21.0%, respectively. RT-qPCR demonstrated that SGR increased the mean P-glycoprotein (gp) mRNA expression in the small intestine 2.54 times, but had a marginal effect on the expression of organic anion transporting polypeptide 2, and organic anion transporter (OAT)1 and OAT2. These results suggested that SGR affects the pharmacokinetics of MTX in

a tissue-dependent manner by affecting P-gp, and the clinical effect of co-administration depended on the disease site.

Introduction

Methotrexate (MTX), an antifolate and anticancer agent with a narrow therapeutic window, is commonly used for refractory rheumatoid arthritis, psoriasis, breast carcinoma, lung and liver cancer and systemic lupus erythematosus (1,2). However, besides its affirmative effect in the clinic, MTX has many side effects including nausea, vomiting, mucositis, diarrhea, stomatitis, myelosuppression (3,4) and hepatotoxicity (5). All these side effects limit its use, and the toxicity is so severe that >50% patients give up the treatment (6).

Currently, methods such as folic acid supplementation for reducing the dosage and regularly checking the body functions are a clinical way to reduce the side effects of MTX. However, the remedial method of detoxification after MTX poisoning does not tend to be effective. Therefore, ethnomedicines may be of importance, particularly if they have detoxifying effects. For example, one of the most commonly used detoxicants is *Smilax glabra Rhizoma* (SGR; Tufuling in Chinese).

SGR is a natural dietary supplement widely used in food-making and health care in Southeast Asia, Arabian countries and North America (7,8). Various bioactivities of SGR have been demonstrated in *in vitro* and *in vivo* assays, including anti-cancer (9), antiviral (10), anti-inflammatory (11), immunomodulatory (12), detoxification and hepatoprotective effects (13). In the clinic, patients with rheumatoid arthritis, cancer, nephroma and even MTX-induced hepatotoxicity (14-16), receive treatment with a combination of MTX and SGR or SGR-containing herbal mixtures frequently (17,18).

Investigating the drug-drug interaction (DDI) or drug-herb interaction (DHI) using pharmacokinetic methods has become popular. DDI or DHI are usually induced by drug metabolism enzyme or drug transporters. MTX enters cells primarily by carrier-mediated uptake (19,20). Therefore, DDI or DHI induced by MTX may mainly associate with drug transporters. MTX is the substrate of P-glycoprotein (gp) (21), multi-drug-resistance-associated protein (MRP)1, 2 and 3 (22-25),

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and organic anion transporters (OAT)1, 2 (26,27). Anything which could change the activity of the above transporters may induce DDI or DHI of MTX.

Astilbin is the major active component of SGR, and the content in SGR usually >0.4%. Wang *et al* (28) reported that astilbin increased the expression of P-gp in rats. *In vivo*, astilbin are further metabolized into sulfates and glucuronides (29), which all are the substrate of MRP1, MRP2, and MRP3 (30-32). This indicates that DHI may happen with a combination of MTX and SGR, but this interaction requires further study.

Therefore, in the present study, the effect of SGR on the pharmacokinetics and tissue distribution of MTX was studied in rats, and the potential underlying mechanism was investigated.

Materials and methods

Chemicals and reagents. MTX was purchased from Guangzhou Lubex Biological Technology Co., Ltd. (Guangdong, China; batch no. 100138-201104). SGR was purchased from Kangmei Pharmaceutical Co., Ltd., (Guangdong, China) in February 2013 (batch no. 130800051) and was verified by Chen Wen-liang in the Second Affiliated Hospital, Guangzhou University of Chinese Medicine (Guangzhou, China). SPE cartridges were supplied by Dikma Technologies, Inc. (Beijing, China). All other materials were analytical grade or better and used as received.

Preparation of SGR decoction. Ethanol (75%) was added to 450 g crude drug, soaked for 30 min and then heated on a reflux extraction device. After boiling, gentle heating was continued for 1 h, the mixture was filtered, and the filtrate was concentrated by rotary evaporators. The volume was kept to 150 ml and frozen at -20°C for later use. The chromatographic fingerprint of SGR has been analyzed in our previous study (33). The concentration of astilbin in the extract was ~0.48%.

Animal and drug administration. A total of 54 male Sprague-Dawley (SD) rats aged 7-8 weeks, weighing 300±50 g, were supplied by Southern Medical University (Guangzhou, China; certification no. 44002100003362). The rats were housed in cages at 21±3°C and 50% relative humidity on a 12-h light/dark cycle. They were acclimated to this environment for 7 days with standard diet until the night prior to the experiment. The rats were then fasted overnight with free access to water. The studies were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (Guangzhou, China), and conducted in strict accordance with the recommendations in the guide for the care and use of Laboratory Animals of the National Institutes of Health (Bethesda, MA, USA).

Pharmacokinetic study. Pharmacokinetic studies were carried out in SD rats. Animals were randomly divided into two groups (n=6 per group). MTX was diluted with deionized water to obtain concentration of 0.6 mg/ml. MTX (6.0 mg/kg) was given orally to rats with and without a concomitant oral dose of SGR (30 g/kg of crude drugs) at 5 min before. Blood

samples (0.6 ml) were collected from orbit venous plexus into heparinized 1.5 ml polythene tubes at predetermined time points (10, 20, 40, 60, 120, 240, 360, 480 and 540 min). The samples were immediately centrifuged at 3,500 x g for 15 min at room temperature, and the supernatant was gathered as plasma and stored at -80°C until analysis.

Tissues distribution study. Tissue distribution studies were carried out in 36 male SD rats. Animals were randomly divided into two groups (n=18 per group): MTX alone and MTX combined with SGR. MTX (6.0 mg/kg) was given orally to rats with and without a concomitant oral dose of SGR (30 g/kg of crude drug) at 5 min beforehand. Following intragastric administration, rats were anesthetized by 10% chloral hydrate (4 ml/kg; Tianjin Damao Chemical Reagent Factory, Tianjin, China) and sacrificed at predetermined time points (20, 60 and 240 min; 6 rats were sacrificed at each time point of each group). Blood samples were obtained from the abdominal aorta; the chest and abdominal cavity were opened and tissues of interest (heart, liver, spleen, kidney, lung, stomach, intestine and marrow) were harvested and rinsed with ice-cold 0.9% NaCl to remove the superficial blood. Following being blotted dry with filter paper, the tissue samples were stored at -80°C until analysis.

Sample preparation. The plasma was thawed and swirled for 10 sec, and then 100 µl plasma was added to a pre-conditioned SPE column. After washing with 1 ml deionized water, 1 ml washing solution [0.2% methanoic acid- methanol (v/v)] was used. The deionized water elution was discarded and washing solution elution was collected, then evaporated to dryness under a stream of nitrogen at 40°C. The residue was re-dissolved with 100 µl mobile phase and then filtered through a 0.22-µm disposable syringe filter, of which 10 µl was injected into the high-performance liquid chromatography (HPLC) system for analysis. Each tissue sample weight ~300 mg after thawing, and was homogenated in 0.9% NaCl [1:6 (w/v)]. Tissue homogenate samples were centrifuged at 3,500 x g for 15 min at 4°C, the supernatant was collected and conducted as plasma sample, and 10 µl supernatant was used for HPLC analysis.

HPLC analysis. The chromatographic analysis was performed on an Agilent 1200 system (Agilent Technologies, Inc., Santa Clara, CA, USA) with a VWD detector at 302 nm. Samples were analyzed on a Diamonsil C18 column (250x4.6 mm I.D, 5 µm; Dikma Technologies Inc.) at 30°C. The mobile phase consisted of 0.1% formic acid aqueous solution (A) and methanol (B), using a gradient elution (0 min: 10% B, 8 min: 60% B, 10 min: 100% B) at a flow rate of 1.0 ml/min, and the injection volume was 10 µl.

Preparation of standard solution and quality control samples. Standard solution of MTX was prepared by using 0.1% formic acid water containing 5% methanol; the working concentration was 200 mg/l. This was stored at 4°C in a dark place until use. Appropriate amounts of the standard solution were added to 100 µl blank rat plasma or tissue homogenates to prepare the calibration standards of MTX, the final calibration standard concentrations were 0.052, 0.104, 0.157, 0.313, 0.626, 1.252, 2.503 and 5.006 mg/l for plasma samples and 0.020,

Table I. Primer sequences used in reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Rat-P-gp	ATGAAACTGCCCCACAAATT	CTTTCTGTGTCCAAGGCTGA
Rat-OAT1	CCATGCTGTGGTTTGCCACTA	ACAAACTTGGCAGGCAGGTC
Rat-OAT2	TGGCCTATGCCATTCCAGAC	GGGATTCTGGGACAAACCAGT
Rat-GAPDH	ATGATTCTACCCACGGCAAG	CTGGAAGATGGTGATGGGTT
Rat-MRP1	AGGTTGGTATTGTGGGTCGTA	GATGGTGATCTTGAAGCGCAGGTT
Rat-MRP2	GGGACTCCAACCTGACTGTAT	CCTCTGGGCAAGGATTTGT
Rat-MRP3	TCAGCATCCTCATCAGGTTTATT	ATGATAGCAGTCCGTATCCTCAA

0.039, 0.078, 0.156, 0.3125, 0.625, 1.25, 2.5 and 5 mg/l for tissue samples. Quality control (QC) samples were prepared at the lower limit of quantification (LLOQ); low, medium and high concentrations of 0.052, 0.313, 1.252 and 5.006 mg/l for plasma samples and 0.020, 0.078, 0.3125 and 1.25 mg/l for tissue samples.

Method validation. The specificity of the assay was assessed by comparing the chromatograms of blank sample with the corresponding spiked samples, and each blank sample was tested to ensure that it had no interference on the elution of MTX. The calibration curves were constructed from the peak area of each standard solution against homographic concentrations using eight-level nonzero standards and a linearly weighed (1/x) least squares regression model. The accuracy and precision were determined by replicate analysis (n=6) of QC samples on three different time points. Relative standard deviation (RSD)% was used to evaluate the accuracy and the intra- and inter-day precision. The extract recovery of MTX at QC levels were evaluated by assaying the samples as described above and comparing peak areas with those obtained from the compounds dissolved in the supernatant of the processed blank plasma. The stability of plasma and tissues was investigated by analyzing of the samples at QC levels (n=6) under different conditions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tissues were homogenized in RNAiso Plus (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to extract total RNA according to the manufacturer's protocol. The quality and concentration of RNA were determined using the OD260/280 ratio measured by the Thermo NanoDrop 2000c (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stored at -80°C prior to use. cDNA was synthesized from total RNA (1 µg; 20 µl final reaction volume) with oligo(dT) priming using M-MLV platinum Green qPCR Super Mix-UDG (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, then performed on Veriti 96 Well Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.).

qPCR was performed on an ABI Prism TM 7500 Real Time qPCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the SYBR Select Mastermix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The experiment was carried out using optical 96-well reaction plates covered with plate sealers. Samples were treated following

the instructions of SYBR Select Mastermix (Applied Biosystems; Thermo Fisher Scientific, Inc.): Each tube of reaction mix consisted of 2 µl of RT cDNA, forward and reverse primers (each 0.4 µl), 10 µl SYBR Select Mastermix and 7.2 µl no enzyme water (20 µl final reaction volume). Primer sequences used for RT-qPCR are listed in Table I. Cycling parameters were as follows: 50°C for 2 min, 95°C for 1 min, then 40 cycles of 95°C for 15 sec and 60°C for 1 min. A melting temperature-determining dissociation step was performed at 96°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec, for a total of 45 cycles in a procedure.

The data were analyzed by the comparative Cq method (34). Cq was recorded for the expression of each gene assayed in the RT-qPCR. All the Cq values were means of triplicate samples tested. GAPDH was chosen as the reference gene for the internal control. Differences between the Cq values of the target gene and the internal control gene ($\Delta Cq = Cq_{\text{target}} - Cq_{\text{internal control}}$) were calculated to normalize the differences. Differences between ΔCq of each sample and calibrator $\Delta\Delta Cq = (Cq_{\text{calibrator}} - \Delta Cq)$ were calculated. The change in the gene expression was calculated as $2^{-\Delta\Delta Cq}$.

Statistical analysis. DAS software version 2.0 (DAS for eCDM; Shanghai University of Traditional Chinese Medicine, Shanghai, China) was used to calculate the pharmacokinetic parameters by non-compartmental model. Concentrations below the lower limit of quantify were treated as 0. Data analysis was performed using SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). One-way analysis of variance was used for multiple comparisons, and post hoc testing was performed using Tukey if necessary, or comparisons between two groups were made using Student's t-test. Data are expressed as the mean \pm standard deviation and mean \pm standard error of the mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Method validation (Tables II and III). No endogenous interference was observed at retention times of MTX. The calibration curves were linear over the concentration range of 0.052-5.006 mg/l with correlation coefficients of 0.9983 for plasma samples and 0.020-5.000 mg/l for tissue samples, with correlation coefficients over 0.9981. RSD in rat plasma and tissues were measured to be ranged between 2.3-6.3% for intra-day precision, and 3.1-9.4% for inter-day precision.

Table II. The lineal regression analysis of MTX in rat plasma and tissues.

Sample	Calibration curves	r
Plasma	$Y=21.621, X-0.0793$	0.9983
Heart	$Y=33.026, X+0.3935$	0.9981
Liver	$Y=28.034, X-0.0175$	0.9992
Spleen	$Y=23.367, X+0.1808$	0.9989
Lungs	$Y=28.265, X+0.3864$	0.9985
Kidney	$Y=31.839, X+0.5685$	0.9971
Marrow	$Y=23.288, X+0.2463$	0.9917
Small intestine	$Y=27.456, X+0.6947$	0.9990
Stomach	$Y=21.621, X-0.0793$	0.9978

MTX, methotrexate.

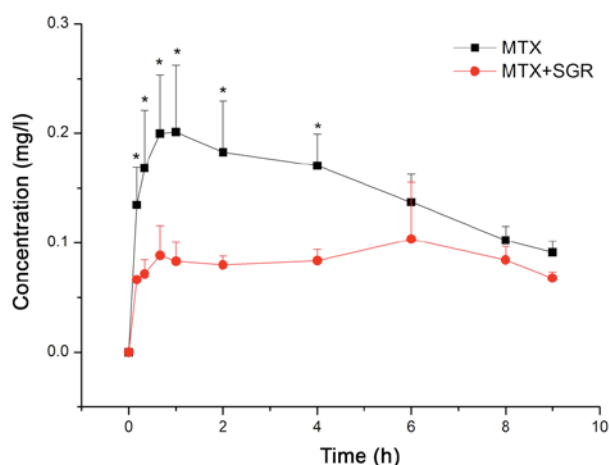


Figure 1. Mean plasma concentration-time curve of MTX treatment alone or combined with SGR, after oral administration to rats. Data are presented as the mean \pm standard deviation ($n=6$ per group). * $P<0.05$ compared with MTX+SGR group at each time point. MTX, methotrexate; SGR, *Smilax glabra Rhizoma*.

The accuracy obtained was between 87.3 and 109.8% with RSD $<8.6\%$. The extraction recoveries of MTX in plasma and various tissues were ranged from 77-102% with RSD $<8.1\%$. Stability study demonstrated that after 24 h at room temperature, three freeze-thaw cycles and 2 weeks of storage, the sample was stable and the RSD were within 9.8%.

SGR affects the pharmacokinetics of MTX significantly. The pharmacokinetics of mean concentration-time curves of MTX are presented in Fig. 1. In general, the maximal MTX concentration in serum occurred in 1 h and then gradually decreased to below detectable levels. This is consistent with previous studies (35,36). The plasma concentration profiles of MTX demonstrated nonlinear pharmacokinetics. The corresponding pharmacokinetic parameters are listed as the mean \pm standard deviation and presented in Table IV. When co-administrated with SGR, MTX demonstrated a much lower concentration than when used alone. The C_{max} was significantly decreased by 44.5% and the $T_{1/2}$, T_{max} were significantly prolonged by 92.8 and 398.9%, respectively. Although SGR significantly

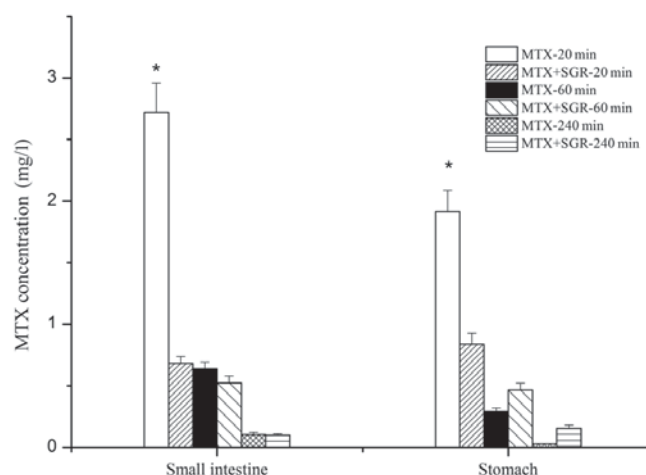


Figure 2. Mean concentration of MTX in the stomach and small intestine at 20, 60 and 240 min after oral administration to rats. Data are presented as the mean \pm standard error ($n=6$ per group). * $P<0.05$ compared with MTX+SGR group at 20 min. MTX, methotrexate; SGR, *Smilax glabra Rhizoma*.

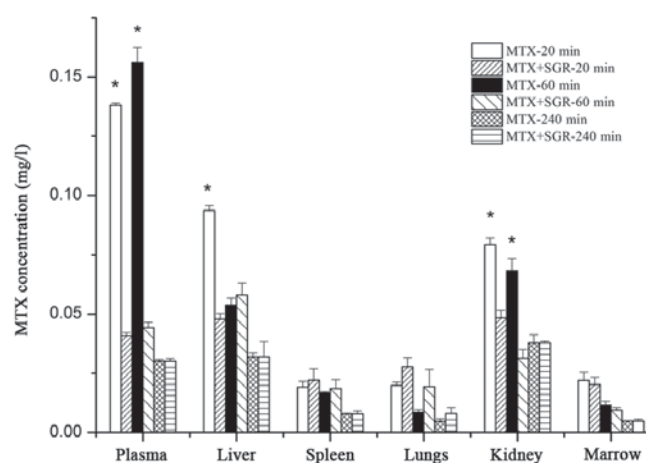


Figure 3. Mean concentration of MTX in the plasma, liver, kidney, lung, spleen and marrow tissues at 20, 60 and 240 min after oral administration to rats. Data are presented as the mean \pm standard error. * $P<0.05$ compared with MTX+SGR group at each time point. MTX, methotrexate; SGR, *Smilax glabra Rhizoma*.

decreased AUC_{0-t} of MTX ($P<0.001$), it still had marginal effect on $AUC_{0-\infty}$, demonstrating an elimination inhibiting effect. The concentration-time profile of MTX in the combination group remained level, and very similar to the profile of sustained release pharmaceuticals, in which the flat profile is as a result of continuous slow absorption. Concentration levels peaked at 6 h, indicating that there may be circulation between the gut and liver (Fig. 1).

SGR affects tissue distribution of MTX with a tissue dependent manner. The concentration of MTX in tissue homogenate at 20, 60 and 240 min after oral administration are presented in Figs. 2 and 3. When MTX was used alone, the C_{max} in majority tissues were 20 min, indicating that MTX underwent a rapid and wide distribution from blood to tissues, and this is in accordance with its wide curative effect to different diseases. The highest concentration of MTX was observed in the small intestine and stomach

Table III. The accuracy, precision, recovery, and stability of cisplatin in nude mouse plasma and tissues samples (% , mean \pm standard deviation, n=6 per group).

Sample	Level ng/ml	Accuracy %	Precision (RSD %)		Recovery %	Stability %		
			Intraday	Interday		Short term	Long term	Freeze-thaw
Plasma	0.052	87.5	5.3	9.4	78.6 \pm 4.3	2.3	6.7	2.9
	0.313	88.9	4.2	5.9	79.9 \pm 5.9	3.6	2.9	4.8
	1.252	91.6	5.5	4.4	91.0 \pm 8.1	1.6	3.3	7.6
	5.006	89.5	6.2	3.7	82.9 \pm 6.3	2.6	4.6	3.5
Marrow	0.020	88.0	2.5	4.7	91.3 \pm 0.9	1.3	3.4	4.9
	0.078	89.1	2.3	5.1	97.2 \pm 7.6	4.1	3.9	4.3
	0.313	88.3	4.6	6.8	83.1 \pm 8.5	1.9	3.9	5.2
	1.252	92.9	1.2	3.9	93.9 \pm 5.9	2.6	5.6	4.3
Liver	0.020	87.3	4.3	9.4	78.7 \pm 9.1	1.3	6.9	7.9
	0.078	88.6	4.9	6.4	97.4 \pm 7.2	2.1	3.1	3.8
	0.313	98.1	3.8	5.8	96.8 \pm 0.9	3.6	4.1	7.1
	1.252	105.9	2.5	4.9	91.6 \pm 4.1	1.7	4.5	4.5
Spleen	0.020	88.5	5.6	6.8	87.1 \pm 4.3	0.9	6.8	3.2
	0.078	91.7	5.1	9.3	81.8 \pm 4.7	3.6	6.6	4.1
	0.313	97.8	4.8	5.2	95.8 \pm 1.5	3.4	7.3	4.6
	1.252	102.3	3.2	6.8	101.6 \pm 7.5	4.1	8.2	6.5
Lung	0.020	92.6	5.8	7.9	79.4 \pm 5.6	4.3	8.9	2.1
	0.078	94.3	5.3	9.2	99.7 \pm 8.5	3.8	7.6	3.8
	0.313	108.3	4.1	6.6	98.0 \pm 5.7	2.7	4.3	5.6
	1.252	97.7	3.8	4.4	102.3 \pm 0.9	2.9	4.3	3.7
Kidney	0.020	100.1	4.1	6.4	88.4 \pm 5.7	2.8	4.7	2.3
	0.078	98.5	5.8	4.3	80.2 \pm 6.8	1.6	9.8	4.7
	0.313	101.5	3.4	3.8	85.4 \pm 0.8	0.9	9.3	5.6
	1.252	109.8	2.7	3.5	86.8 \pm 1.6	1.6	5.1	3.9
Small intestine	0.020	89.3	2.6	5.7	80.0 \pm 6.9	4.1	9.1	2.5
	0.078	90.3	3.8	4.9	89.5 \pm 4.4	7.1	6.9	6.1
	0.313	101.6	5.9	4.5	94.4 \pm 3.2	3.4	3.4	5.1
	1.252	105.7	3.5	6.6	101.8 \pm 7.4	5.8	4.4	3.9
Stomach	0.020	87.9	2.9	7.6	101.0 \pm 2.7	2.3	7.1	2.5
	0.078	101.8	5.3	6.6	88.9.5 \pm 2.4	4.4	3.5	3.6
	0.313	103.7	4.2	3.9	95.4 \pm 4.9	5.0	7.8	6.5
	1.252	104.3	5.1	4.2	96.8 \pm 6.1	6.6	6.3	7.3

(Fig. 2), followed by the plasma, liver, and kidney (Fig. 3). MTX concentrations were relatively low in the lung, spleen and marrow (Fig. 3), and the concentration of MTX in the heart was too low to test, which is roughly consistent with previous studies (37,38).

When co-administered with SGR, the concentration of MTX in tissues changed in a tissue- and time-dependent manner. Although the T_{\max} was at 20 min, the C_{\max} decreased significantly compared with MTX used alone. C_{\max} in the small intestine, stomach, plasma, liver and kidney were decreased by 74.9, 56.2, 70.4, 48.9 and 40.0%, respectively, at 20 min. Following that, the effect of SGR seemed to weaken; at the 1-h time point, SGR decreased the concentration of MTX in the plasma and kidney markedly, but increased the concentration of MTX in the stomach and lung; no obvious differences of MTX concentration were observed between the two groups

at 240 min. This indicated a much slower elimination in most tissues when combined with SGR, and which is accorded with the pharmacokinetic study.

On the whole, the total exposure of MTX was also significantly reduced in the small intestine, stomach, plasma and kidney by 61.6, 34.7, 63.3 and 46.1%, respectively. Notably, the total exposure of MTX in the lung and spleen were increased by 82.9 and 21.0%, respectively. However, was no obvious change was observed in the marrow.

SGR enhances P-gp gene expression in intestine but has a marginal effect on other transporters. As the effect of SGR on the *in vivo* behavior of MTX mainly focused on absorption and excretion, the gene expression of transporters which all transport MTX such as P-gp, MRP3, organic anion transporting polypeptide (OATP2), MRP2 in the intestine and P-gp,

Table IV. Pharmacokinetic parameters of MTX in rats orally administered MTX alone and in combination with SGR (n=6 per group).

Parameters	MTX	MTX + SGR
AUC _{0-t} /mg/h/l	1.353±0.169	0.751±0.163 ^c
AUC _{0-∞} /mg/h/l	2.698±0.403	2.871±1.488
MRT _{0-t} /h	4.008±0.309	4.551±0.274
T _{1/2} /h	9.884±3.283	19.058±10.616 ^a
C _{max} /mg/l	0.218±0.059	0.113±0.047 ^b
CL/L/h/kg	2.271±0.370	2.663±1.440
T _{max} /h	0.902±0.683	4.5±2.665 ^a

Data are expressed as the mean ± standard deviation. ^aP<0.05, ^bP<0.01, ^cP<0.001 vs. MTX. MTX, methotrexate; SGR, *Smilax glabra Rhizoma*; T_{max}, time of peak serum level; C_{max}, concentration of peak serum level; T_{1/2}, elimination half-life; AUC_{0-t}, area under serum concentration-time curve to the last point; CL/L, clearance over absolute bioavailability; MRT_{0-t}, mean residence time.

OAT1, OAT2, MRP1 and MRP2 in the kidney, were studied. The results are presented in Figs. 4 and 5.

The effect of SGR on gene expression demonstrated a time-, transporter- and tissue-dependent manner. SGR increased P-gp mRNA expression in the small intestine by 2.54 times (P<0.05) at both 20 and 60 min time points, but had marginal effects on the gene expression of OATP2, MRP2 and MRP3 in the intestine at all the time points (Fig. 4), indicating that upregulation of P-gp expression is the main effect of SGR-induced MTX absorption decreasing. However, different from the results in intestine, SGR had a marginal effect on P-gp, OAT1 and OAT2 expression in the kidney, but increased the expression of MRP2 and MRP3. The result is coincidence with the concentration decreasing of MTX in kidney (Fig. 5). This indicated that SGR prolonged the retention time of MTX in plasma by increasing the expression of MRP1 and MRP2. This may be one of the reasons of the flat profile and low elimination rate of MTX in plasma.

Discussion

SGR was originally recorded by 'Diannan Materia Medica' in the Ming Dynasty of China (1368-1644), and officially listed in the Chinese Pharmacopeia (2015 edition) (39). The stipulated prescription dose is 15-60 g, and when SGR is generally consumed in tea or soup, the dosage often exceeds the prescribed dose. MTX and SGR are frequently used combination in the clinic; however, the benefits remain unclear (40). To the best of our knowledge, the present study was the first to examine the *in vivo* effects of SGR on the fate of MTX, and the results demonstrated that SGR affected the absorption, distribution and elimination significantly, and may induce significant DHI.

Drug concentration in plasma is significantly reduced in the intestines, stomach, liver and kidney, and it was hypothesized that SGR may affect the MTX treatment effect when used in the treatment of psoriasis, digestive tract cancer and systemic lupus erythematosus. Additionally, when patients

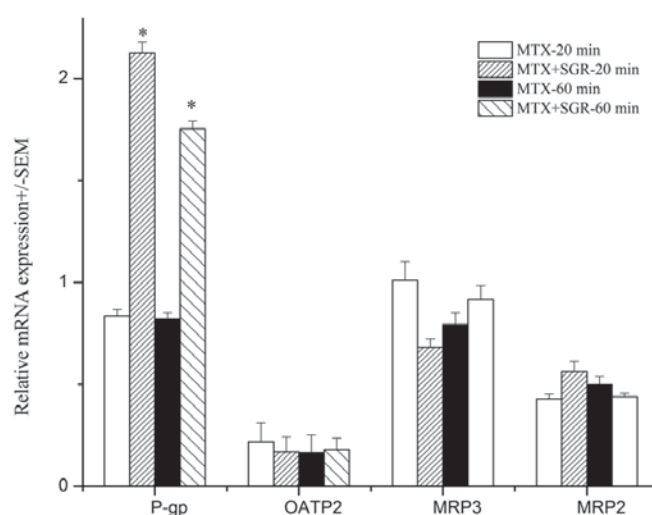


Figure 4. Relative gene expression of P-gp, OATP2, MRP3 and MRP2 in the small intestine at 20 and 60 min. Data are presented as the mean ± standard error. *P<0.05 compared with MTX alone at each time point. MTX, methotrexate; SGR, *Smilax glabra Rhizoma*; P-gp, P-glycoprotein; OATP2, organic anion transporting polypeptide; MRP, multidrug resistance-associated protein.

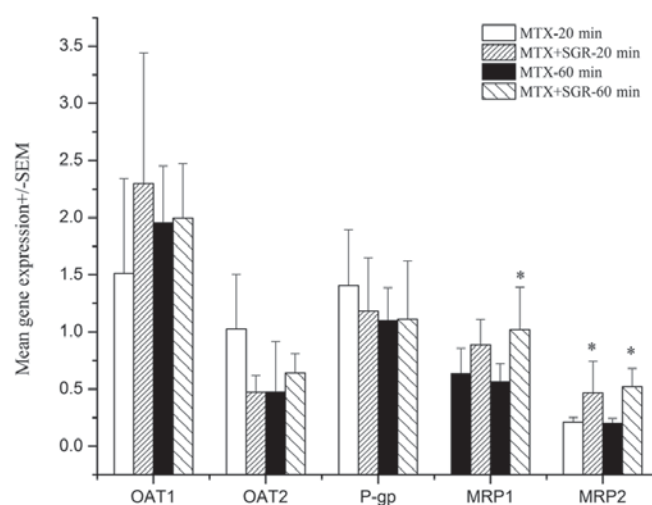


Figure 5. Relative gene expression of OAT1, OAT2, P-gp, MRP1 and MRP2 in the kidney at 20 and 60 min. Data are presented as the mean ± standard error. *P<0.05 compared with MTX alone at each time point. MTX, methotrexate; SGR, *Smilax glabra Rhizoma*; P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein; OAT, organic ion transporter.

are receiving MTX therapy and taking SGR at the same time, it is suggested that the dosing interval should be extended to prevent unnecessary toxic effect, due to the significant inhibition of the elimination of MTX by SGR.

In the present study, although SGR significantly decreased the concentration of MTX in most tissues, its concentration in the lung, spleen and marrow were not reduced; it increased the drug concentration of MTX in lung and spleen significantly. Therefore, in the treatment of various types of cancer, such as splenic carcinoma, osteosarcoma, acute leucocythemia, malignant lymphoma and multiple myeloma (41), MTX co-administration with SGR may be helpful to enhance its tumoricidal effect while decreasing the toxicity such as nausea, vomiting, diarrhea and stomatitis.

MTX is the substrate of many transporters, as previously described. The efficacy and toxicity of MTX may be changed drugs used in combination affect transporter activity (42). Transporters have different expression levels in different tissues, and our previous study demonstrated that the effect of drugs on the activity of transporters depends on the protein amount (data not shown) (43,44). The RT-qPCR results indicated that SGR increased P-gp expression by 2.5 times in the intestine; thus, it was hypothesized that SGR-induced gene expression of P-gp may be the reason for MTX low absorption in intestine, and much lower C_{max} in blood. This is consistent with a previous report that P-gp is associated with clinical responses to MTX (45,46). On the other hand, in the present study, SGR increased the expression of MRP1 and MRP2 in the kidney, but had a marginal effect on P-gp. This may be because P-gp had much higher expression in the intestine than in the kidney, and vice versa. This may partially explain the flat profile of MTX concentration over time. Elimination of drugs from blood is the total effect of all tissues, so the mechanism requires further study.

In recent years, flavonoids-rich botanical products have been used as dietary supplements worldwide. However, their safety remains unanswered (47). Additionally, the pharmacokinetics of other acidic pharmaceuticals such as probenecid, ciprofloxacin and penicillin, which were putative substrates of P-gp, may also be affected by concurrent use of SGR (48,49). As the side effect of drugs raised concern, herbal treatment has attracted increasing attention, particularly a combination of herbs and drugs. Therefore, investigations into herb-drug interactions HDI are urgently required.

In conclusion, to the best of our knowledge, the present study demonstrated for the first time the potential benefits of co-administration of MTX with SGR. Co-administration of SGR may be beneficial for therapy of lung cancer and splenic carcinoma, osteosarcoma, and liver diseases, but may reduce the therapeutic effect of treating cancer in the digestive tract, systemic lupus erythematosus and psoriasis (50-54). The herb-drug interaction may be associated with the activity change of transporters.

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