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RESEARCH PAPER

Cumulative Organic Anion Transporter-Mediated Drug-Drug Interaction Potential of Multiple Components in *Salvia Miltiorrhiza* (Danshen) Preparations

Li Wang • Jürgen Venitz • Douglas H. Sweet

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ABSTRACT

Purpose To evaluate organic anion transporter-mediated drug-drug interaction (DDI) potential for individual active components of Danshen (*Salvia miltiorrhiza*) *vs*. combinations using *in vitro* and *in silico* approaches.

Methods Inhibition profiles for single Danshen components and combinations were generated in stably-expressing human (h)OAT1 and hOAT3 cells. Plasma concentration-time profiles for compounds were estimated from *in vivo* human data using an *i.v.* two-compartment model (with first-order elimination). The cumulative DDI index was proposed as an indicator of DDI potential for combination products. This index was used to evaluate the DDI potential for Danshen injectables from 16 different manufacturers and 14 different lots from a single manufacturer.

Results The cumulative DDI index predicted *in vivo* inhibition potentials, 82% (hOAT1) and 74% (hOAT3), comparable with those observed *in vitro*, 72 ± 7% (hOAT1) and 81 ± 10% (hOAT3), for Danshen component combinations. Using simulated unbound C_{max} values, a wide range in cumulative DDI index between manufacturers, and between lots, was predicted. Many products exhibited a cumulative DDI index > 1 (50% inhibition).

Conclusions Danshen injectables will likely exhibit strong potential to inhibit hOAT1 and hOAT3 function *in vivo*. The proposed cumulative DDI index might improve prediction of DDI potential of herbal medicines or pharmaceutical preparations containing multiple components.

KEY WORDS Drug elimination · OATs · Solute carriers · Traditional Chinese medicine · Transporter modeling

L. Wang • J. Venitz • D. H. Sweet (✉)
Department of Pharmaceutics, Virginia Commonwealth University
410 N 12th Street, Richmond, Virginia 23298-0533, USA
e-mail: dsweet@vcu.edu

ABBREVIATIONS

CHO	Chinese hamster ovary
DDI	Drug-drug interaction
ES	Estrone sulfate
FDA	Food and drug administration
HEK	Human embryonic kidney 293
HOAT1	Human OAT1
HOAT3	Human OAT3
IC_{50}	Median maximal inhibitory concentration
K_i	Inhibitory constant
K_m	Michaelis constant
LSA	Lithospermic acid
OAT	Organic anion transporter
PAH	Para-aminohippuric acid
PBPK model	Physiologically-based pharmacokinetic model
RMA	Rosmarinic acid
SAB	Salvianolic acid B
TSL	Tanshinol

INTRODUCTION

Danshen, the dried root of *Salvia miltiorrhiza*, is used to treat patients as a part of traditional Chinese medicine in the therapy of cardiovascular disease (1, 2). Their major cardiovascular effects may include improvement of microcirculation, preventing platelet adhesion and aggregation, and inhibition of the formation of thromboxane (1, 2). Presently, there are more than 900 commercial Danshen preparations available in China, and injectables account for about 30% of total Danshen products (http://www.sda.gov.cn/WS01/CL0001/). Typical of herbal medicines, Danshen pharmaceutical products are actually a mixture of ingredients, including phenolic acids such as lithospermic acid (LSA), rosmarinic acid (RMA), salvianolic acid A (SAA), salvianolic acid B (SAB, also named

lithospermic acid B), and tanshinol (TSL, also named danshensu), each of which has been identified as a major component in these preparations (3–6). However, because of variations in cultivars, cultivation regions, manufacturing, and lack of standard quality control criteria, the final content of these components in commercial Danshen preparations exhibit wide variation among products from different sources. Such lack of standardization of the major Danshen components makes it difficult to predict systemic and peak exposure of these components between products, or to evaluate any exposure-related toxicity and drug-drug interaction (DDI) potential of products from various manufacturers (5, 7).

Recently, the number of reports about drug transporter-mediated DDIs observed in numerous preclinical and clinical studies has increased (8). Among these transporter families, the organic anion transporter (OAT; SLC22) family mediates the renal transcellular solute flux of a multitude of endogenous substances and xenobiotics that carry negative charge(s) at physiological pH (9). Because of their broad substrate specificity, antibiotics, antiviral agents, angiotensin-converting enzyme inhibitors, and many other clinically important therapeutics have been identified as notable OAT substrates and/or inhibitors (9). As a result, United States Food and Drug Administration (FDA) regulatory, industrial and scientific experts have developed recommendations under what circumstances DDIs with seven selected transporters, including OAT1 and OAT3, need to be investigated (8, 10).

As part of these recommendations, a DDI index is introduced as the (predicted) maximal unbound plasma concentration (C_{max} or C_{ss}) of a drug component of interest at the highest clinically relevant dose divided by the *in vitro* transporter inhibition potency of the compound (K_i or IC_{50}) as an indicator of DDI potential. Conservatively, follow-up clinical DDI investigations are recommended when this DDI index exceeds 0.1 (8). For the majority of drug products, the DDI index is affected by only one or a few ingredients with constant content, even between different manufacturers; however, for herbal medicines and natural products, the DDI potential assessment becomes more complicated as they may contain numerous components that can interfere with drug transporter activity with different potency (and different pharmacokinetic characteristics), whether identified as an active ingredient or not. These components, in most cases, show marked similarity in their chemical structure. This fact is quite important for the assessment of DDI potential, since drug transporters, including OATs, interact with an array of compounds with marked diversity in their chemical structure (8, 11). As a result, it is highly probable that more than one component of an herbal product may have a strong potential to interact with any specific transporter. Therefore, a new index to express this multifaceted inhibition potential on transporters, the cumulative DDI index, is proposed. This index, estimated as the sum of each component's individual DDI index, is intended to reflect the overall DDI potential for multiple-component herbal preparations at clinically relevant concentrations/doses.

Using Danshen as a prototypical herbal medicine, our previous work has demonstrated that LSA, RMA, SAA, SAB and TSL each showed significant competitive inhibitory *in vitro* effects on human (h)OAT1- and hOAT3-mediated substrate uptake, and their corresponding K_i values for hOAT1 and hOAT3 are listed in Table I (12). Since each of these Danshen components showed competitive inhibition on human OAT transport activity, it is likely that these compounds share the same binding site, such that cumulative inhibitory effects would emerge after administration of Danshen products with a combination of these ingredients. The first aim of the present study was to compare the inhibitory effects of individual Danshen components (LSA, RMA, and TSL) *vs.* time-dependent contribution of each component to the cumulative DDI index. Our results demonstrate that the cumulative DDI index, which addresses the overall inhibition potential of Danshen preparations, was significantly higher than the DDI indices from any single component, indicating that the DDI index for any single component in a complex mixture is likely to underestimate the true DDI potential of that product. Additionally, the estimated cumulative DDI indices are highly variable between Danshen injectables from different manufacturers as well as between different lots produced by the same manufacturer. Such information might be useful in guiding the clinical use and quality control in the manufacture of such pharmaceutical products.

MATERIALS AND METHODS

Chemicals

The Danshen components LSA, RMA, and TSL (≥96% purity) were obtained from Tauto Biotech (Shanghai, China). Tritiated *para*-aminohippuric acid ([^{3}H]PAH) and estrone sulfate ([^{3}H]ES) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA), and unlabeled PAH, ES, and probenecid were purchased from Sigma-Aldrich (St. Louis, MO).

Tissue Culture

The derivation of stably transfected Chinese hamster ovary (CHO) cells expressing hOAT1 and stably transfected human embryonic kidney 293 (HEK) cells expressing hOAT3, and their corresponding empty vector transfected control cell lines,

Table I Estimated K_i values (μM) of Danshen components for hOAT1 and hOAT3

Compound	hOAT1	hOAT3
LSA	20.8 ± 2.1	0.59 ± 0.26
RMA	0.35 ± 0.06	0.55 ± 0.25
SAB	22.2 ± 1.9	19.8 ± 8.4
TSL	40.4 ± 12.9	8.6 ± 3.3

Values are reported as mean ± SE ($n = 3$). These values were obtained from reference 11

has been described previously (13, 14). CHO cell lines were cultured in phenol red-free RPMI 1,640 medium (Gibco Invitrogen., Grand Island, NY) containing 1 mg/mL G418. HEK cell lines were cultured in DMEM high glucose medium (Mediatech, Inc., Herndon, VA) containing 125 μg/ml hygromycin B. All cultures contained 10% FBS and 1% Pen/Strep, and were maintained at 37°C with 5% CO_2.

Cell Accumulation Assays

Cell transport assay procedures were adapted from those previously published (15). In brief, 2×10^5 cells/well were seeded in 24-well tissue culture plates and grown in the absence of antibiotics for 48 h. On the day of the experiment, cells were equilibrated with transport buffer for 10 min [500 μL of Hanks' Balanced Salt Solution containing 10 mM HEPES, pH 7.4]. Equilibration buffer was replaced with 500 μL of fresh transport buffer containing 1 μM [^{3}H]PAH (0.5 μCi/mL) or 1 μM [^{3}H]ES (0.25 μCi/mL) with or without inhibitors. After incubation, the cells were immediately rinsed three times with ice-cold transport buffer, lysed, and intracellular content measured *via* liquid scintillation counting. Substrate accumulation was reported as picomoles of substrate per milligram total protein. Total protein content was determined by the Bradford method. Results were confirmed by repeating all experiments at least three times with triplicate wells for each data point in every experiment.

Derivation of the Cumulative DDI Index

The effect of a single competitive inhibitor on transporter activity is described as follows;

$$\frac{V_0}{V_i} = 1 + \frac{I}{K_i} \tag{1}$$

where V_0, V_i, and I represent transport activity in the absence of inhibitor, transport activity in the presence of inhibitor and the relevant concentration of the inhibitor (usually unbound peak plasma concentration), respectively, while K_i denotes the binding affinity/potency of the inhibitor to the transporter. According to the "Guidance for Industry: Drug Interaction Studies" issued by the FDA the ratio I/K_i is proposed as the DDI index, an indicator for predicting the clinical DDI potential (10);

$$\text{DDI index} = \frac{I}{K_i} \tag{2}$$

However, assuming independent competitive inhibition by multiple (n) inhibitors, the combined effect from multiple inhibitors on transport activity becomes:

$$\frac{V_0}{V_i} = 1 + \frac{I^1}{K^1{}_i} + \frac{I^2}{K^2{}_i} + \cdots + \frac{I^n}{K^n{}_i} \tag{3}$$

where I^n and $K^n{}_i$ represent the relevant concentration and inhibition constant of the individual inhibitor (n), respectively. Thus, the cumulative DDI index for multiple (n) inhibitors becomes:

$$\text{Cumulative DD index} = \frac{I^1}{K^1{}_i} + \frac{I^2}{K^2{}_i} + \cdots + \frac{I^n}{K^n{}_i} \tag{4}$$

As illustrated, the sum of $I^n/K^n{}_i$ for n inhibitors plays an equivalent role on transporter function as I/K_i for a single competitive inhibitor. As a result, for Danshen preparations, which include multiple components known to be competitive OAT inhibitors (12), the sum of $I^n/K^n{}_i$, designated as the cumulative DDI index, was used to describe the DDI potential of these combinations. Note that I^n can be represented as a static value (observed or predicted maximal unbound plasma concentration $f_u{*}C_{max}$, as shown in Equ. 5) or dynamically predicted as a function of the plasma concentration-time profiles (C(t)) of each inhibitor (as shown in Equ. 6), provided that sufficient information is available to adequately model their disposition:

$$\text{Cumulative DDI index} = \frac{f_u^1 \times C_{\max}{}^1}{K^1{}_i} + \frac{f_u^2 \times C_{\max}{}^2}{K^2{}_i} + \cdots + \frac{f_u^n \times C_{\max}{}^n}{K^n{}_i} \tag{5}$$

$$\text{Cumulative DDI index} = \frac{f_u^1 \times C(t)^1}{K^1{}_i} + \frac{f_u^2 \times C(t)^2}{K^2{}_i} + \cdots + \frac{f_u^n \times C(t)^n}{K^n{}_i} \tag{6}$$

where $f_u{}^n$, $C_{max}{}^n$, and $C(t)^n$ represent the fraction unbound in human plasma, the maximum human plasma concentration, and the human plasma concentration of the individual inhibitor (n) at time t after administration, respectively.

This estimation of the cumulative DDI index involves several assumptions, namely, that all inhibitors are

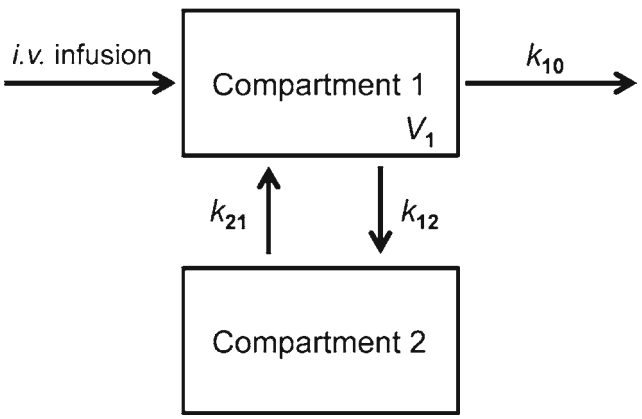
competitive by nature, the *in vivo* unbound plasma concentration of the victim drug (OAT substrate) is much lower than its K_m value for hOATs, inhibition by each inhibitor is independent from inhibition by the others, and all inhibitors follow dose-proportional pharmacokinetics.

Pharmacokinetic Modeling

In order to determine relevant plasma concentrations for the different Danshen components after their different doses in the various products, pharmacokinetic models had to be developed. Pharmacokinetic modeling was performed with WinNonlin (version 6.2.0, Pharsight, St. Louis, MO, USA) using plasma concentration-time profiles from a previously reported clinical pharmacokinetic study in human subjects after a single 60 min *i.v.* infusion of a Danshen injectable (4). The individual doses were, 5 mg for caffeic acid (CA), 3 mg for LSA, 160 mg for RMA, 65 mg for SAB, and 90 mg for TSL, respectively (4). All reported mean plasma concentrations were above the assay lower limit of quantification (8 ng/mL for all components), except for those of RMA at the last two time points (4). As a result, these two points were excluded from pharmacokinetic analysis.

Initially, different pharmacokinetic compartmental models were investigated during the model selection process. Based on the law of parsimony (simplest model), goodness of fit including Akaike Information Criterion (AIC), Schwarz Bayesian Criterion (SBC) and Weighted Sum of Square of Residuals (WSSR), visual inspection of weighted residuals *versus* observed concentrations, and fitted and observed plasma concentrations *versus* time, an open two-compartment body model with first-order distribution and elimination was chosen. This model exhibited markedly lower AIC, SBC, and WSSR values (Table II) compared to an open one-compartment body model, and a three-compartment body model failed to further improve the fit (similar values of AIC, SBC, and WSSR) over the two-compartment body model while increasing imprecision in parameter estimation due to overparameterization (data not shown).

The schematic representation of this model is as follows:



where k_{10} is the elimination rate constant from compartment 1, k_{12} is the distribution rate constant from compartment 1 to compartment 2, k_{21} is the distribution rate constant from compartment 2 to compartment 1, and V_1 represents volume of compartment 1. Compartments 1 and 2 represent central (plasma) and peripheral compartments, respectively. The four model parameters were estimated from the published profile using 1/y as weighting factor, secondary pharmacokinetic parameters (Cl_{tot}, Vd_{ss}, and $t_{½}^{terminal}$) were estimated, and pharmacokinetic simulations performed for estimation of the dynamic DDI potential for different products (see below).

Simulation of Plasma Concentrations and Cumulative DDI Index

The content of the Danshen components, LSA, RMA, SAB and TSL in Danshen injectables from different manufacturers, or from different lots produced by a single manufacturer, were obtained from the literature (4–6, 16, 17). In order to estimate the DDI indices, the plasma concentration-time profile of each Danshen component was simulated for administration of Danshen injectables under two clinically relevant scenarios, namely as *i.v.* bolus and 60 min *i.v.* infusion. The dose for *i.v.* infusion was 4-fold higher than *i.v.* bolus administration based on the package inserts of these formulations. Simulated plasma concentration-time profiles were generated, assuming that all four Danshen components follow dose-proportional pharmacokinetics. From the simulated plasma concentration-time profiles, C_{max} was obtained. In addition, Yang *et al.* (2007) determined that, for RMA, SAB, and TSL, the fraction unbound in human plasma (f_u) was 9.5%, 6.8%, and 100% (18). In the absence of information about LSA plasma protein binding, it was assumed that LSA is highly protein bound (90%), *i.e.*, f_u was set at 10%, for static DDI index estimation (Tables V and VI). However, a sensitivity analysis to this assumption was performed, where three values for f_u of LSA, namely 1%, 10% and 90%, were used to plot the cumulative DDI index in a dynamic manner in order to illustrate the impact that mischaracterization of f_u may have had on LSA's DDI index (Fig. 3).

Statistical Analysis

Experimental *in vitro* accumulation data in Fig. 1 are reported as mean ± SD; parameter estimates in Tables I and III are reported as point estimate ± standard error (SE). Statistical differences were assessed using one-way ANOVA followed by *post-hoc* analysis with Dunnett's *t*-test ($\alpha = 0.05$).

Table II *AIC*, Akaike information criterion; *SBC*, Schwarz bayesian criterion; *WSSR*, Weighted Sum of Square of Residuals values for a one-, two- or three-compartment body model with first-order distribution and elimination

Compound	One compartment model			Two compartment model			Three compartment model		
	AIC	SBC	WSSR	AIC	SBC	WSSR	AIC	SBC	WSSR
LSA	176	177	96,365	67	70	51	71	75	51
RMA	122	123	4,579	82	84	196	82	86	150
SAB	141	142	8,974	105	107	628	99	104	336
TSL	126	128	3,513	106	109	676	110	114	685

RESULTS

Cumulative Inhibitory *in Vitro* Effects of LSA, RMA, and TSL on hOAT1 and hOAT3

Accumulation of PAH in the CHO-hOAT1 cell line (4.6±0.3 pmol/mg protein/10 min) was markedly greater than that in the empty vector background CHO-EV cells (0.9±0.3 pmol/mg protein/10 min; Fig. 1a). LSA (10 μM), RMA (1 μM) and TSL (50 μM) produced 61±3%, 60±1%, and 52±6% inhibition of hOAT1-mediated PAH uptake, respectively. Application of these three compounds in combination (at their specified concentrations) yielded a significantly increased inhibition (72±7%) on hOAT1 transport activity, compared to the individual inhibitory effects of each compound (Fig. 1a). Stably transfected hOAT3-expressing (HEK-hOAT3) cells showed significantly enhanced accumulation of ES (~4 fold) relative to empty vector background HEK-EV cells (6.2±0.5 *vs.* 1.5±0.2 pmol/mg protein/10 min, respectively; Fig. 1b). Similarly, LSA (0.5 μM), RMA (0.5 μM) and TSL (10 μM) produced approximately 20±2%, 53±6%, and 45±2% inhibition of hOAT3-mediated ES uptake, respectively. When these compounds were included as a combination in the incubation solution at the above concentrations, the inhibition significantly increased to 81±10%. Therefore, LSA, RMA and TSL, known competitive inhibitors for hOAT1 and hOAT3, showed a cumulative inhibitory effect on both hOAT1- and hOAT3-mediated uptake.

Pharmacokinetic Modeling of Plasma Concentration-Time Profiles

The final, open two-compartmental body model with first-order distribution and elimination yielded the lowest values of AIC, SBC, and WSSR, and was used to fit the plasma concentration-time profile data for LSA, RMA, SAB and TSL (Fig. 2). Weighted residuals were randomly distributed (data not shown), and r^2 was greater than 0.96, indicating excellent fit. The observed and predicted concentration-time profiles for each Danshen component are shown in Fig. 2. The final pharmacokinetic model parameter estimates for each Danshen component are summarized in Table III. Overall, the final parameter values were estimated quite precisely. Data for CA, another component in Danshen preparations, failed to fit any of the tested models due to secondary peaks and was omitted from further analysis. RMA exhibited the highest total clearance (CL_{tot}), being 3.8 to 5.5 fold greater than that for TSL and SAB, respectively (Table III). In contrast, LSA had the lowest CL_{tot}, which was 128 fold lower than that for RMA and 23–35 fold lower than SAB and TSL. These differences in CL_{tot} were reflected as a similar trend in terminal half-life ($t_{1/2}^{terminal}$) with RMA, SAB and TSL exhibiting shorter terminal half-lives (0.8–1.6 h) compared to LSA (6 h). Compartment 1 distribution volume (V_1) and volume of distribution at steady-state (V_{dss}) showed similar rank order for the compounds as RMA>TSL>SAB>LSA (Table III).

Estimation of DDI Index and Evaluation of OAT-Mediated DDI Potential for Different Danshen Injectables

In clinical practice, Danshen injectables can be administered by *i.v.* bolus injection or *i.v.* infusion. As the plasma concentration of LSA, RMA, SAB or TSL did not reach steady-state (C_{ss}) after 60 min *i.v.* infusion, C_{max} values (plasma concentration at 1 h) were used for estimation of individual DDI indices and the cumulative DDI index.

As shown in Table IV, the content of Danshen components in injectables from different manufacturers has been determined (4–6, 16). Marked variation was observed between Danshen products from different manufacturers, with the content of LSA, RMA, SAB and TSL showing 2.3–, 50-, 172- and 4.6-fold variation, respectively (n=16). As a consequence, the predicted C_{max} values of LSA, RMA, SAB and TSL in these injectables exhibited the same relative variation due to dose-proportional pharmacokinetics. With this information, the DDI index (for individual components) and the cumulative DDI index (for combinations) were calculated (Tables V and VI). Most of these investigated Danshen injectables are predicted to have hOAT1- and hOAT3-mediated DDI potential (cumulative DDI index>0.1), and the majority

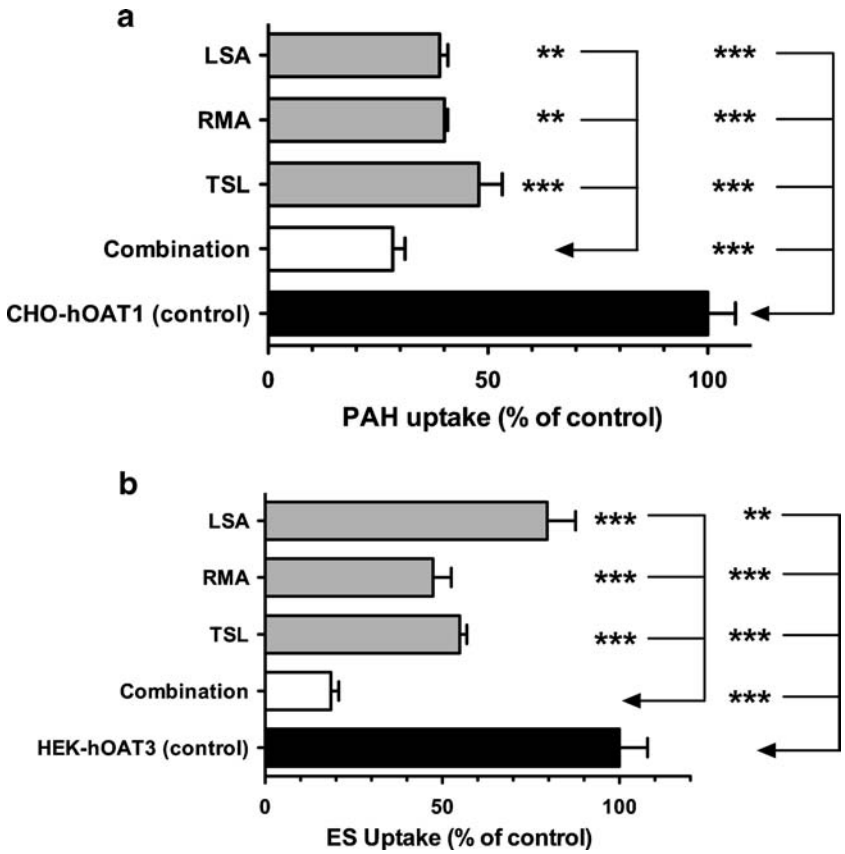


Fig. 1 Individual and combined inhibitory effects of LSA, RMA and TSL on hOAT1 and hOAT3. (**a**): Inhibition of hOAT1-mediated uptake (10 min) of [^{3}H]PAH (1 μM) by LSA (10 μM), RMA (1 μM), and TSL (50 μM) individually, and by a combination of these three components at these concentrations, was measured in CHO-hOAT1 cells. (**b**): Inhibition of hOAT3-mediated uptake (10 min) of [^{3}H]ES (1 μM) by LSA (0.5 μM), RMA (0.5 μM), and TSL (10 μM) individually, and as a combination of these three components at these concentrations, was determined in HEK-hOAT3 cells. All data were corrected for background substrate accumulation measured in corresponding empty vector control cells. Values are mean ± SD of triplicates. Single Danshen components and the combinations all produced significant inhibition of hOAT1 and hOAT3 transport activity ($p < 0.001$). Individual compounds were compared with their corresponding combined group by one-way ANOVA followed by Dunnett's *t*-test and results are indicated as ** denoting $p < 0.01$ and *** denoting $p < 0.001$.

of preparations exhibited higher interaction potential for hOAT3 than for hOAT1. Among Danshen components, TSL emerged as the largest contributor to DDI potential, while LSA and RMA showed substantial individual contribution only in some products. SAB failed to exhibit any DDI potential in most injectables.

In addition, similar variation in the content of Danshen components between different lots of an injectable product produced by the same manufacturer was also observed. The content of LSA, SAB and TSL showed 2.8–, 6.7- and 3.2-fold variation, respectively, between 14 lots from a single manufacturer (17). Therefore, the actual *i.v.* infusion doses (which are four-fold the maximal dose for *i.v.* bolus administration) for RMA, SAB and TSL were 2.53 ± 0.62 mg, 1.48 ± 0.45 mg and 24.3 ± 5.2 mg, respectively (17). The DDI indices for individual components and the cumulative DDI indices for the combinations were calculated for the fourteen lots of the Danshen injectable and are presented in Table VII. These

Table III Pharmacokinetic parameter estimates for a two-compartment body model with first-order distribution and elimination of four Danshen components after *i.v.* infusion

Compound	V_1 (L)	k_{10} (1/h)	k_{12} (1/h)	k_{21} (1/h)	V_{dss} (L)	CL_{tot} (L/h)	$t_{1/2}^{terminal}$ (h)	f_u (%)
LSA	1.9 ± 0.1	0.25 ± 0.03	0.29 ± 0.07	0.35 ± 0.15	3.4 ± 0.5	0.48 ± 0.04	6.0 ± 1.8	—[a]
RMA	18.9 ± 3.3	3.3 ± 0.3	0.28 ± 0.07	0.44 ± 0.09	23.4 ± 4.0	61.6 ± 2.8	0.81 ± 0.23	9.5
SAB	4.1 ± 0.6	2.7 ± 0.4	0.86 ± 0.34	0.60 ± 0.25	9.4 ± 1.9	11.2 ± 0.7	1.5 ± 0.5	6.8
TSL	10.4 ± 1.3	1.6 ± 0.2	0.32 ± 0.21	0.55 ± 0.35	16.4 ± 3.4	16.4 ± 1.1	1.6 ± 0.9	100

[a] Not available. Values are reported as mean ± SE (raw data from reference 4)

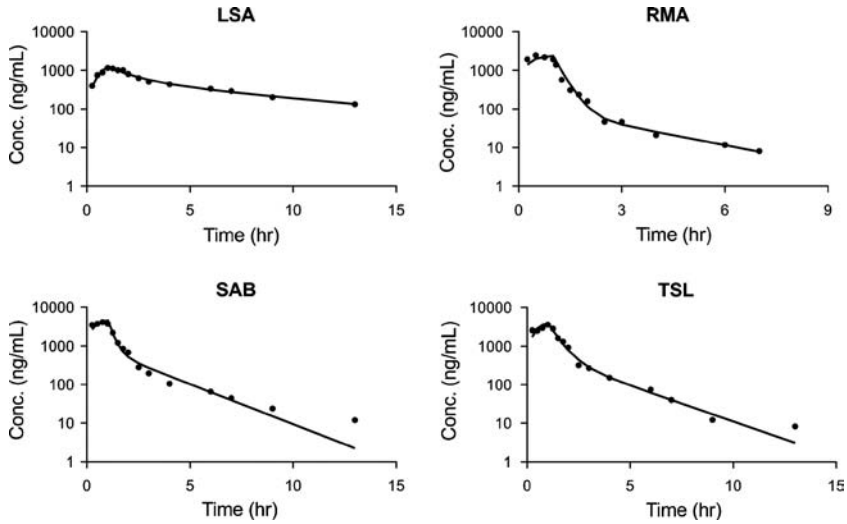


Fig. 2 Pharmacokinetic modeling of published human plasma concentration-time profiles of the four Danshen components, LSA, RMA, SAB and TSL, after *i.v.* infusion using an open two-compartment body model with first-order transfers. Original plasma concentration-time profiles (closed circles) were taken from reference 4. Solid lines represent the model-fit. The doses were 3 mg (LSA), 160 mg (RMA), 65 mg (SAB) and 90 mg (TSL), respectively.

Danshen products showed significant hOAT3-mediated DDI potential for both *i.v.* bolus injection and *i.v.* infusion use. In addition, the RMA and TSL content in some lots were high enough to cause hOAT1-mediated DDIs when administered as *i.v.* bolus or *i.v.* infusion.

Finally, the dynamic hOAT3-associated individual and cumulative DDI indices (time-dependent unbound plasma concentration over K_i) for Danshen components were plotted against time for a Danshen injectable (manufacturer 16 from Table IV) with *i.v.* bolus administration (Fig. 3). The individual doses were 0.75 mg (LSA), 40 mg (RMA), 15.8 mg (SAB) and 22.5 mg (TSL). If most of LSA in plasma is plasma protein bound ($f_u = 1\%$), the cumulative DDI index is largely driven by TSL and RMA (Fig. 3a, $t=0$). However, as the CL_{tot} of TSL is markedly lower than that of RMA (Table III), the dynamic DDI index *vs.* time profile is dominated by TSL (Fig. 3b), and both the individual DDI index for TSL and the cumulative DDI index exhibited similar time intervals (~1.6 h) above 0.1. Using an f_u value of 10% for LSA also indicated that the cumulative DDI index was largely driven by TSL and RMA at $t=0$ (Fig. 3c). However, under this condition the lower CL_{tot} of LSA *vs.* RMA allows the contribution of LSA to the cumulative DDI index to surpass that of RMA within 1 h, and increases the time period the cumulative DDI index stays above 0.1 to almost 2.5 h (Fig. 3d). Finally, assuming an f_u value of 90% for LSA, all three components contributed roughly equally to the cumulative DDI index at $t=0$ (Fig. 3e). However, in marked contrast to the previous two scenarios, within 1 h of administration LSA dominated the dynamic DDI index *vs.* time profile and the cumulative DDI index remained above 0.1 over the entire simulation period (Fig. 3f).

Table IV Reported content of four Danshen components in injectable dosage forms from different manufacturers

Manufacturer	Maximum Dose (mg)[a]				Reference
	LSA	RMA	SAB	TSL	
1	NR[b]	10.2	17.2	21.2	(5)
2	NR	4.2	13.8	41.4	(5)
3	NR	4.6	7.0	31.2	(5)
4	NR	5.6	16.2	29.8	(5)
5	NR	12	12.6	80.6	(5)
6	3.9	10.3	39.5	37.8	(6)
7	1.7	3.4	6.8	45.2	(6)
8	NR	6.6	ND[c]	98.4	(16)
9	NR	14.1	343	ND	(16)
10	NR	5.1	2.0	25.4	(16)
11	NR	18.1	50.2	42.9	(16)
12	NR	4.4	2.9	59.7	(16)
13	NR	3.2	2.5	49.0	(16)
14	NR	7.7	16.8	52.5	(16)
15	NR	3.5	10.0	51.5	(16)
16	3	160	63	90	(4)

[a] Used for *i.v.* infusion administration, which is four-fold of the maximum dose for *i.v.* bolus administration; [b] Not reported; [c] Not detectable (value set to 0 for DDI index calculations in Tables V and VI)

DISCUSSION

Botanical drug products are increasing in popularity because of renewed interest in complementary and alternative therapies. Herbal medicines, which are derived from plants, are considered "natural" and, therefore, are often presumed to be safer by patients and the general public. However, because of their wide use as complementary and alternative medicines, they are often used in combination therapy with other

Table V Estimates for individual and cumulative DDI indices on hOAT1 and hOAT3 for four Danshen compounds in injectable dosage forms from different manufacturers after *i.v.* bolus administration

Manufacturer	DDI index for hOAT1					DDI index for hOAT3				
	LSA[a]	RMA	SAB	TSL	Cumulative	LSA[a]	RMA	SAB	TSL	Cumulative
1	—[b]	*0.10*	<0.005	0.06	*0.16*	—	0.06	0.01	*0.30*	*0.37*
2	—	0.04	<0.005	*0.12*	*0.16*	—	0.03	<0.005	*0.58*	*0.61*
3	—	0.05	<0.005	0.09	*0.14*	—	0.03	<0.005	*0.44*	*0.47*
4	—	0.06	<0.005	0.09	*0.15*	—	0.04	<0.005	*0.42*	*0.46*
5	—	*0.12*	<0.005	*0.24*	*0.36*	—	0.08	<0.005	***1.14***	***1.22***
6	0	*0.10*	0.01	*0.11*	*0.21*	*0.16*	0.07	0.01	*0.54*	*0.78*
7	0	0.03	<0.005	*0.14*	*0.17*	0.07	0.02	<0.005	*0.64*	*0.73*
8	—	0.07	0	*0.30*	*0.37*	—	0.04	0	***1.40***	***1.44***
9	—	*0.14*	0.09	0	*0.23*	—	0.09	*0.10*	0	*0.19*
10	—	0.05	<0.005	0.08	*0.13*	—	0.03	0	*0.36*	*0.39*
11	—	*0.18*	0.01	*0.13*	*0.32*	—	*0.11*	0.01	*0.61*	*0.73*
12	—	0.04	<0.005	*0.18*	*0.22*	—	0.03	<0.005	*0.85*	*0.88*
13	—	0.03	<0.005	*0.15*	*0.18*	—	0.02	<0.005	*0.70*	*0.72*
14	—	0.08	<0.005	*0.16*	*0.24*	—	0.05	<0.005	*0.74*	*0.79*
15	—	0.03	<0.005	*0.15*	*0.18*		0.02	<0.005	*0.73*	*0.75*
16	<0.005	***1.60***	0.02	*0.27*	***1.89***	*0.13*	***1.02***	0.02	***1.27***	***2.43***

[a] In the absence of available LSA plasma protein binding information, f_u was set at 10%. [b] — DDI index could not be calculated as no value was reported (NR in Table IV). Values in *italic* indicate DDI index values≥0.1. Values in ***bold italic*** indicate values≥1

prescribed and over-the-counter drugs. As a result, numerous clinical cases of phytomedicine-associated DDIs have been identified (19–21), making it necessary to explore the DDI potential between herbal medicines and co-administered

Table VI Estimates for individual and cumulative DDI indices on hOAT1 and hOAT3 for four Danshen compounds in injectable dosage forms from different manufacturers after *i.v.* infusion administration

Manufacturer	DDI index for hOAT1					DDI index for hOAT3				
	LSA[a]	RMA	SAB	TSL	Cumulative	LSA[a]	RMA	SAB	TSL	Cumulative
1	—[b]	*0.11*	0.01	*0.12*	*0.24*	—	0.07	0.01	*0.55*	*0.63*
2	—	0.05	<0.005	*0.23*	*0.28*	—	0.03	<0.005	***1.08***	***1.11***
3	—	0.05	<0.005	*0.17*	*0.22*	—	0.03	<0.005	*0.81*	*0.84*
4	—	0.06	<0.005	*0.16*	*0.22*	—	0.04	0.01	*0.77*	*0.82*
5	—	*0.13*	<0.005	*0.45*	*0.58*	—	0.08	<0.005	***2.09***	***2.17***
6	0.01	*0.11*	0.01	*0.21*	*0.33*	*0.51*	0.07	0.01	*0.98*	***1.57***
7	0.01	0.04	<0.005	*0.25*	*0.29*	*0.22*	0.02	<0.005	***1.17***	***1.41***
8	—	0.07	0	*0.54*	*0.61*	—	0.05	0	***2.56***	***2.61***
9	—	*0.15*	*0.10*	0	*0.26*	—	*0.10*	*0.12*	0	*0.22*
10	—	0.06	<0.005	*0.14*	*0.20*	—	0.04	<0.005	*0.66*	*0.70*
11	—	*0.20*	0.02	*0.24*	*0.46*	—	*0.13*	0.02	***1.11***	***1.26***
12	—	0.05	<0.005	*0.33*	*0.38*	—	0.03	<0.005	***1.55***	***1.58***
13	—	0.03	<0.005	*0.27*	*0.30*	—	0.02	<0.005	***1.27***	***1.29***
14	—	0.08	0.01	*0.29*	*0.38*	—	0.05	0.01	***1.36***	***1.42***
15	—	0.04	<0.005	*0.28*	*0.32*	—	0.02	<0.005	***1.34***	***1.36***
16	0.01	***1.76***	0.02	*0.50*	***2.28***	*0.39*	***1.12***	0.02	***2.34***	***3.87***

[a] In the absence of available LSA plasma protein binding information, f_u was set at 10%. [b] — DDI index could not be calculated as no value was reported (NR in Table IV). Values in *italic* indicate DDI index values≥0.1. Values in ***bold italic*** indicate values≥1

Table VII Estimates for DDI indices on hOAT1 and hOAT3 for fourteen different lots of an injectable Danshen dosage form from a single manufacturer after *i.v.* bolus administration and *i.v.* infusion

i.v. bolus administration			
	hOAT1		hOAT3
DDI index	RMA: 0.02 ± 0.01 (0.01–0.03)	DDI index	RMA: 0.02 ± 0.01 (0.01–0.02)
	TSL: 0.07 ± 0.02 (0.03–0.09)		TSL: 0.34 ± 0.07 (0.13–0.41)
	Cumulative: 0.10 ± 0.02 (0.04–0.12)		Cumulative: 0.36 ± 0.08 (0.14–0.43)
Number of batches with cumulative DDI ≥ 0.1	8	Number of batches with cumulative DDI ≥ 0.1	14
i.v. infusion			
	hOAT1		hOAT3
DDI index	RMA: 0.03 ± 0.01 (0.01–0.04)	DDI index	RMA: 0.02 ± 0.01 (0.01–0.02)
	TSL: 0.14 ± 0.03 (0.05–0.16)		TSL: 0.63 ± 0.14 (0.23–0.75)
	Cumulative: 0.16 ± 0.04 (0.06–0.20)		Cumulative: 0.65 ± 0.14 (0.24–0.77)
Number of batches with cumulative DDI ≥ 0.1	13	Number of batches with cumulative DDI ≥ 0.1	14

The DDI indices are shown as mean ± SD. Values in parentheses are the range of DDI index values obtained for the fourteen lots. DDI indices for SAB are not shown in the table as all values were below 0.001

drugs more systematically. Because herbal drugs contain mixtures of plant components or extracts, they are extraordinarily complex in their composition, particularly when compared with prescription medications that commonly contain only

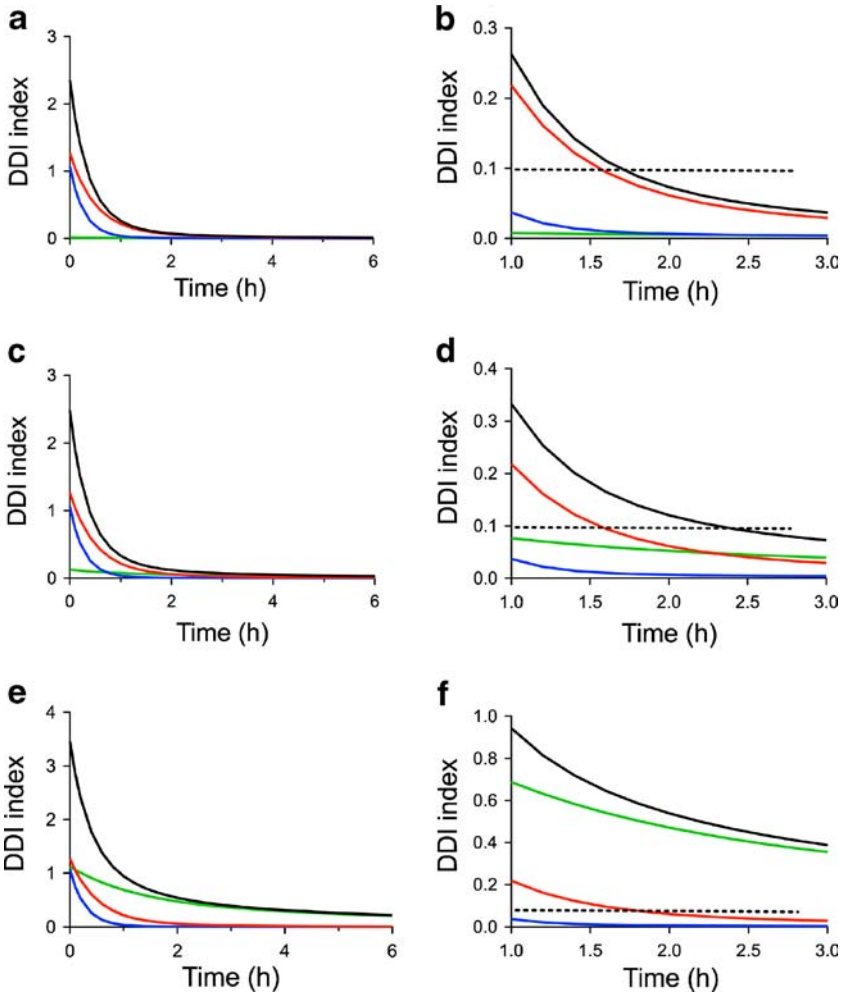


Fig. 3 Dynamic simulation of individual and cumulative DDI indices for LSA, RMA and TSL on hOAT3. Simulations were performed for LSA (green line), RMA (blue line), TSL (red line) and cumulative (black line) DDI indices using an open two-compartment body model with first-order transfer after *i.v.* bolus administration of the Danshen injectable produced by manufacturer 16 in Table III. The individual doses were 0.75 mg (LSA), 40 mg (RMA), 15.8 mg (SAB) and 22.5 mg (TSL). Left hand panels (**a**), (**c**), (**e**) depict simulation up to 6 h and right hand panels (**b**), (**d**), (**f**) focus on the initial window between 1 and 3 h post administration. Panels (**a**) and (**b**): Dynamic simulation using an f_u value for LSA of 1%. Panels (**c**) and (**d**): Dynamic simulation using an f_u value for LSA of 10%. Panels (**e**) and (**f**): Dynamic simulation using an f_u value for LSA of 90%. DDI indices for SAB were not shown as values were below 0.02 for this Danshen injectable. The dotted black line indicates the pre-specified threshold value of 0.1 for the DDI index.

one or a few well-characterized active ingredients. An important caveat to herbal medicines is that, regardless of any therapeutic benefits, they may still possess significant DDI potential due to high systemic/peak exposure *in vivo*. Furthermore, their components often share structural and physicochemical properties and, as such, might exert cumulative DDI effects. Currently, most transporter-mediated DDI studies focus on the interaction of each single component with the drug in question and, therefore, may underestimate the overall DDI potential for complex botanical drug products.

Presently, the individual DDI index, calculated as [unbound]C_{max}/K_i (or IC_{50}), is thought to predict *in vivo* DDI potential for a compound for a specific transporter reasonably well. However, for herbal medicines, it is necessary to consider the composite inhibitory effects induced by all of the components according to their inhibitory potency and pharmacokinetic characteristics. In addition, the content of major components in herbal products is often influenced by factors such as extraction methods, cultivars, cultivation region, manufacturing processes, and quality control criteria, which differ among manufacturers. Therefore, it might be more relevant to explore pharmaceutical product-specific DDI indices rather than compound-related indices.

In this study, the cumulative inhibitory effect of major Danshen components on OAT-mediated substrate uptake was assessed. Human OAT1 and hOAT3 transport activity was significantly lower when the components (LSA, RMA and TSL) were applied in combination as compared with being applied individually. These results suggest that in some situations the traditional DDI index might not reflect the true inhibition level of a multi-component system. Therefore, the cumulative DDI index, calculated as the sum of DDI indices for each individual component, was proposed in the present study to evaluate the DDI potential for such combinations.

As shown in Fig. 1, the individual DDI indices for the single compounds *in vitro*, LSA (10 μM), RMA (1 μM), and TSL (50 μM), as well as the cumulative DDI index for a combination at those concentrations, on hOAT1 were 0.45, 2.9, 1.2, and 4.6, respectively. Such DDI indices predict inhibition levels of 33%, 74%, 55%, and 82% for LSA, RMA, TSL, and the combination, respectively, which corresponded closely to the observed *in vitro* inhibition profiles, 61 ± 3%, 60 ± 1%, 52 ± 6%, and 72 ± 7% for RMA, TSL, and the combination, respectively. Similarly, for hOAT3, the individual DDI indices for LSA (0.5 μM), RMA (0.5 μM) and TSL (10 μM), as well as the cumulative DDI index for the combination, were 0.9, 0.9, 1.2, and 3.0, respectively, indicating that the predicted inhibition levels (46%, 48%, 54%, and 74% for LSA, RMA, TSL and the combination, respectively) were comparable with the observed *in vitro* values (20 ± 2%, 53 ± 6%, 45 ± 2%, and 81 ± 10% for LSA, RMA, TSL and the combination, respectively). These findings support use of the cumulative DDI index as a suitable indicator for evaluation of the *in vivo* DDI potential for combinations.

The same threshold value of DDI index≥0.1 (*i.e.*, transport activity decreased by 9%), as is currently recommended for further individual component assessment, was maintained for the cumulative DDI index. The estimated cumulative DDI indices for various Danshen injectables on hOAT1 and hOAT3 were greater than 0.1 in most cases. In addition, the cumulative DDI indices for hOAT3 were much greater than those for hOAT1 for most injectables. As shown in Tables V and VI, the cumulative DDI indices for hOAT3 exceeded unity for a number of Danshen injectables, after *i.v.* bolus injection and 60 min *i.v.* infusion, indicating that more than 50% of hOAT3 transport function could be inhibited at peak plasma concentrations. Only one product induced a cumulative DDI index exceeding 1 on hOAT1 due to a high RMA content. In general, the results demonstrate a strong potential that OAT function could be impaired *in vivo* after administration of these products at their recommended doses, which would manifest itself as reduced renal excretion and prolonged persistence of co-administered drugs that undergo significant OAT-mediated active renal tubular secretion (Tables V-VII).

Among these Danshen components, TSL is the main contributor to the cumulative DDI index because of its relatively high content in preparations and low degree of plasma protein binding. Collectively, TSL exhibited higher DDI indices for hOAT1 and hOAT3 compared to the other components. In contrast, the results show that the contribution of SAB can be ignored. This is due to its high degree of protein binding (f_u = 6.8%) and lower inhibitory potency (K_i = 22.2 and 19.8 μM for hOAT1 and hOAT3, respectively). However, the impact of RMA, which has a similar level of fraction unbound in human plasma compared to SAB (f_u = 9.5%), on DDI potential cannot be ignored, as it showed 56- and 36- fold greater inhibitory potency for hOAT1 and hOAT3 than SAB, respectively. In some Danshen injectables, RMA showed equal or greater contribution to the cumulative DDI index for hOAT1 (Tables V and VI). The content of LSA was available only for three investigated products, and its f_u value has not been reported in the literature. Nevertheless, LSA's high affinity for hOAT3 yielded an individual DDI index for LSA that accounts for a marked portion of the cumulative DDI index (Tables V and VI), as long as f_u exceeds 10%. In other words, considering only single component DDI assessment would suggest no concern for Danshen-mediated *in vivo* DDIs, whereas the cumulative DDI index indicates a major concern.

As for other herbal medicines, there is great variation in the content of major constituents of Danshen injectables. The content of LSA, RMA, SAB, and TSL, showed 2.3–, 50-, 172- and 4.6-fold variation between Danshen products from different manufacturers (Table IV). Additionally, RMA, SAB

and TSL showed 2.8–, 6.7- and 3.2-fold variation in content between different lots of a product produced by a single manufacturer (17). As a result, such variation leads to marked differences in the cumulative DDI indices for these products. As shown in Tables V and VI, Danshen products from different manufacturers exhibited 14.5- and 11.4-fold difference in the hOAT1-associated cumulative DDI index for *i.v.* bolus and *i.v.* infusion administration, respectively. Such discrepancy was even higher for the hOAT3-associated cumulative DDI index, which exhibited 12.8- and 17.6-fold differences for *i.v.* bolus and *i.v.* infusion administration, respectively. Variability in composition between lots led to an approximately 3-fold range in cumulative DDI indices (Table VII). Clearly, estimating a general DDI potential for the range of Danshen injectables is difficult. In order to address this problem, standardization of content and strict quality control measures should be established.

Danshen also is used in clinical therapy as tablets and "dripping pills" (1). The phenolic acid components investigated in the present work are also major components in these oral dosage forms. Unlike injections, TSL was the only component detectable in plasma after oral administration of Danshen products (3, 22). Therefore, TSL might be the only component with the potential for causing OAT-mediated DDIs *in vivo* after oral administration of Danshen preparations. However, there is a marked discrepancy in plasma concentration of TSL in clinical pharmacokinetic studies. Pei *et al.* reported the C_{max} of TSL was ~7.5 μg/mL (38 μM) after oral administration of 250 mg Danshen pills (22), while another independent investigation revealed that the C_{max} of TSL was much lower (~50 ng/mL or 0.25 μM) despite using a higher dose (750 mg Danshen pills) (3). Knowing that Danshen pills used in these studies were produced by the same manufacturer, the large difference in TSL C_{max} is likely due to variation in the content of TSL between different lots. Unfortunately, the actual content of TSL in the pills was not reported (22). Without further studies assessing the peak exposure of TSL after oral administration, it is impractical to estimate DDI potential for Danshen oral dosages forms. However, a TSL C_{max} of 7.5 μg/mL may be sufficient to cause significant DDI (DDI index = 0.9 and 6 for hOAT1 and hOAT3, respectively), while a C_{max} of 50 ng/mL is not (DDI index < 0.1 for both hOAT1 and hOAT3).

There are some limitations to the present study. Currently, there is a lack of clinical pharmacokinetic data for the Danshen components CA and SAA, which are also potent *in vitro* OAT inhibitors (12, 23). Potential phase 2 metabolites (glucuronides, sulfate conjugates) of Danshen components formed by hepatic metabolism may also impact OAT function, especially after oral administration. Since any contribution of these metabolites to OAT-mediated DDIs cannot be estimated, the cumulative DDI potentials reported herein may actually underestimate the true DDI potential of Danshen injectables. Additionally, the plasma protein binding for LSA is unknown, making it difficult to accurately predict the LSA-associated DDI potency. Furthermore, DDIs between each compound likely may occur, especially for TSL, which itself could undergo OAT-mediated *in vivo* renal elimination. Therefore, the presence of CA, LSA, SAA and SAB in Danshen preparations could inhibit renal elimination of TSL, and the plasma concentration of TSL might be elevated depending on the achieved systemic exposures of CA, LSA, SAA and SAB, which, in turn, might influence TSL-associated DDIs. Also, in the present study we used a basic model, which is simple and practical, to evaluate DDI potency – based on available *in vitro* inhibition data combined with available *in vivo* systemic disposition information. Recently, some DDI investigations have applied the more comprehensive dynamic approach of physiologically-based pharmacokinetic (PBPK) modeling. PBPK models offer several advantages including evaluation of the entire pharmacokinetic profile of a victim drug, of concurrent DDI caused by interacting metabolites, and of DDI when multiple intrinsic and/or extrinsic factors are involved; population-based PBPK models are further capable of predicting inter-individual variability in DDI (24). Thus, while PBPK modeling may yet prove to be a valuable tool for further DDI investigations of Danshen products, additional, currently not available PBPK properties/parameters (*e.g.*, tissue binding, rates and routes of metabolism, *etc.*) will be required to perform PBPK.

Finally, the existing DDI indices, based on C_{max}, are static indices of DDI potential and are recognized as being conservative in nature. As shown in Table III, the four Danshen compounds, despite their chemical similarity, very quite a bit in their CL_{tot} (from 0.5 to 60 L/h) and Vd_{ss} (3.4 to 23 L), as well as f_u (9.5 to 100%). As a result their terminal half-lives vary between < 1 h (for RMA) and ~6 h (for LSA). In addition to the intrinsic differences in inhibitory potency for hOAT1 and hOAT3 (Table I), considering the exposure profiles of the inhibitors (Fig. 2), these differences in pharmacokinetic behavior translate into dynamic differences in the time course of hOAT3-associated individual and cumulative inhibition (Fig. 3, time profile of unbound plasma concentration over K_i using the data for manufacturer 16 in Table IV). Such information should prove useful when considering combination therapy involving Danshen injectables and other therapeutics that interact with OATs by identifying windows of high likelihood of DDIs.

CONCLUSION

In summary, the major Danshen components, LSA, RMA, and TSL, were demonstrated to elicit cumulative inhibitory effects on hOAT1- and hOAT3-mediated substrate uptake. The cumulative DDI index was introduced as a more

comprehensive index to evaluate the DDI potential for multiple-component Danshen injectables. Even though TSL appears to exert a dominant contribution to the cumulative DDI index, the potential impact of LSA and RMA cannot be ignored. The large cumulative DDI indices (particularly those exceeding 1) suggested that Danshen injectables have a strong potential to cause OAT-mediated DDIs *in vivo* and underscores the need for improved manufacturing standards to eliminate such broad product variability.

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