Regular Article

Competitive Inhibition of Human Organic Anion Transporters 1 (SLC22A6), 3 (SLC22A8) and 4 (SLC22A11) by Major Components of the Medicinal Herb Salvia miltiorrhiza (Danshen)

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Summary: When herbal products are used in combination therapy with drugs, alterations in pharmacokinetics, pharmacodynamics, and toxicity can result. Many active components of herbal products are organic anions, and human organic anion transporter 1 (hOAT1, SLC22A6), hOAT3 (SLC22A8), and hOAT4 (SLC22A11) have been identified as potential sites of drug-drug interactions. Therefore, we assessed the effects of lithospermic acid (LSA), rosmarinic acid (RMA), salvianolic acid A (SAA), salvianolic acid B (SAB), and tanshinol (TSL), components of the herbal medicine Danshen, on the function of these transporters. Kinetic analysis demonstrated a competitive mechanism of inhibition for all five. $K_i$ values ($\mu$M) were estimated as 20.8 ± 2.1 (LSA), 0.35 ± 0.06 (RMA), 5.6 ± 0.3 (SAA), 22.2 ± 1.9 (SAB), and 40.4 ± 12.9 (TSL) on hOAT1 and as 0.59 ± 0.26 (LSA), 0.55 ± 0.25 (RMA), 0.16 ± 0.03 (SAA), 19.8 ± 8.4 (SAB), and 8.6 ± 3.3 (TSL) on hOAT3. No significant inhibition of hOAT4 activity by TSL was observed. Using published human pharmacokinetic values, unbound $C_{\text{max}}/K_i$ ratios were calculated as an indicator of $in vivo$ drug-drug interaction potential. Analysis indicated a strong interaction potential for RMA and TSL on both hOAT1 and hOAT3 and for LSA on hOAT3. Thus, herb-drug interactions may occur $in vivo$ in situations of co-administration of Danshen and clinical therapeutics known to be hOAT1/hOAT3 substrates.

Keywords: danshen; herb-drug interaction; kidney; organic anion transporter; renal transport; solute carrier

Introduction

Danshen, the dried root of Salvia miltiorrhiza, has been used as a traditional Chinese medicine for thousands of years in the treatment of angina, myocardial ischemia, and other cardiovascular diseases.\(^1\)\(^-\)\(^3\) In vitro and in vivo studies have demonstrated that Danshen could improve microcirculation, facilitate coronary vasodilatation, and increase blood flow.\(^4\)\(^-\)\(^5\) Danshen products are readily available throughout the world including Asia, Europe, and North America, and annual sales volume is in the hundreds of millions of dollars.\(^6\) According to the China State Food and Drug Administration database of drug manufacturing certificates, there are currently more than 980 commercial Danshen preparations, 289 of which are injectable dosage forms (http://www.sda.gov.cn/WS01/CL0001/). The oral dosage form, Fufang Danshen Dripping Pill, was approved for Phase II and Phase III clinical trials by the United States Food and Drug Administration in 1997 (IND No. 56956). In 2010, the Phase II clinical trials were successfully completed (http://clinicaltrial.gov/ct2/show/NCT00797953?term=taslly&rank=1), but no formal clinical trials for any injectable Danshen formulations have been reported. Use of the injectable products is of particular concern, as the spectra and amounts of the compounds they contain are not well standardized. More than 70 lipophilic and hydrophilic compounds have been separated and identified from Danshen extract.\(^8\) However, which component(s) is/are responsible for the beneficial pharmacological effects of Danshen is virtually unknown. Among hundreds of Danshen pharmaceutical products, lithospermic acid (LSA), rosmarinic acid (RMA), salvianolic acid A (SAA), salvianolic acid B (SAB), and tanshinol (TSL) exhibited markedly high content and were considered to be major components.\(^8\)\(^-\)\(^11\)

Currently, the clinical pharmacokinetic literature on Danshen is extremely limited. One study assayed LSA, RMA, SAA, SAB, and TSL in human urine and plasma samples obtained after oral dosing and found TSL was the only compound detectable.\(^12\) However, this study examined just a single formulation and dose. In contrast, LSA, RMA, SAA, SAB, and TSL were all detected after administration of an injectable Danshen preparation.\(^10\) To improve the clinical safety of Danshen herbal therapies (especially injectables), better estimation of the composition and dose of active...
components, as well as greater knowledge of the processes affecting their pharmacokinetic and pharmacodynamic properties, including potential transporter-mediated herb-drug interactions, is required. Since these Danshen components are relatively small (198–718 Da) and exist as anions at physiological pH, it is possible that they are organic anion transporter (OAT; SLC22) family substrates or inhibitors. OATs are key mediators in the distribution and elimination of a multitude of endogenous compounds and xenobiotics, which include clinically important therapeutics such as antibiotics, antiviral and anticancer agents, statins, and angiotensin-converting enzyme inhibitors. A number of clinical and pre-clinical studies have demonstrated that concomitant administration of drugs that are eliminated from the body by renally expressed OATs may result in longer plasma half-life and reduced renal clearance.

For example, co-administration of probenecid, a known OAT inhibitor, has been demonstrated to diminish the renal clearance of benzylpenicillin and ciprofloxacin. In accord with these clinical findings, in vivo pharmacokinetic studies using organic anion transporter 3 (Oat3) knockout mice demonstrated that loss of murine Oat3 (mOat3) activity increased the plasma half-life and reduced the clearance of benzylpenicillin, ciprofloxacin, and methotrexate. Recent work with two cancer chemotherapy adjuvants, mesna and dimesna, demonstrated in vitro that hOAT1 and hOAT3 transport these compounds and in vivo that concomitant probenecid administration reduced their renal elimination in healthy human volunteers. In addition, concomitant gemfibrozil administration was found to influence the pharmacokinetic properties of pravastatin in patients, increasing pravastatin-related adverse effects, and it has been proposed that the mechanism may be due to competition for hOAT3-mediated tubular secretion.

Similarly, co-administration of the lipid-altering compound, gycabene, with the angiotensin-converting enzyme inhibitor, quinapril, resulted in a reduction in blood pressure in hypertensive humans and rats. In vitro experiments demonstrated dose-dependent inhibition of hOAT3- and rat Oat3-mediated quinaprilat (the pharmacologically active metabolite of quinapril) transport by gycabene, at clinically relevant levels, implicating drug-drug interaction on hOAT3 as the mechanism for increased pharmacological activity.

Further, co-administration of Danshen and warfarin resulted in an unexpected increase in the anticoagulation effects of warfarin and in vitro studies suggested that this interaction may be due to inhibitory effects of Danshen components on cytochrome P450 activity. However, we recently demonstrated LSA, RMA, and SAA are potent competitive inhibitors of murine Oat1 and Oat3 (mOat1 and mOat3), exhibiting Ki values ranging from 4.9–14.9 µM for mOat1 and from 4.3–31.1 µM for mOat3, indicating that interaction with transporters may also be an important component.

In response to the increase in known transporter-mediated drug-drug interactions such as these the United States Food and Drug Administration and the European Medicines Agency have issued guidance documents regarding circumstances under which drug interactions with specified transporters (including hOAT1 and hOAT3) need to be investigated (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf) and http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/document/document_detail.jsp?webContentId=WC500090112&murl=menus/document_library/document_library.jsp&mid=WC0b01ac05809a3de&jsenabled=true).

The affinities of Danshen compounds to human OATs remained unknown, and the potential for marked species differences between transporter orthologs has been well documented. Therefore, the aim of the present study was to characterize the inhibitory effects of LSA, RMA, SAA, SAB, and TSL on p-aminophenolic acid (PAH) transport mediated by hOAT1 or estrone sulfate (ES) transport mediated by hOAT3 or hOAT4. Further, studies designed to elucidate the mechanism of inhibition (competitive vs. non-competitive vs. uncompetitive) were also conducted. Using this information we derived inhibitory constants (Ki) values for each compound to allow direct comparison of their potencies between transporter paralogs and orthologs. Finally, using published human pharmacokinetic values, unbound Cmax/Ki (recommended by FDA Guidance for Drug Interaction Studies) were calculated as an indicator of the in vivo drug-drug interaction potential for these compounds.

Together, these data demonstrated that LSA, RMA, SAA, SAB, and TSL serve as potent competitive inhibitors of hOAT1 and hOAT3 and that there is strong potential for herb-drug interactions, such as altered pharmacokinetics and pharmacodynamics of co-administered clinical therapeutics that are hOAT substrates.

Materials and Methods

Chemicals: Purified (>96.0%) lithospermic acid (LSA), rosmarinic acid (RMA), salvianolic acid A (SAA), salvianolic acid B (SAB), and tanshinol (TSL) were purchased from Tauto Biotech (Shanghai, China). The chemical structures are shown in Figure 1. Tritiated p-aminophenolic acid ([3H]PAH) and estrone sulfate ([3H]ES) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Unlabeled PAH, ES, and probenecid were obtained from Sigma-Aldrich (St. Louis, MO).

Tissue culture: The derivation and culture of stably transfected Chinese hamster ovary (CHO) cells expressing hOAT1, hOAT4, mOat1, and mOat3, human embryonic kidney 293 (HEK) cells expressing hOAT3, and their corresponding empty vector (ev) transfected background control cell lines, has been described previously.

Briefly, the CHO-hOAT1 and CHO-ev cell lines were maintained in DMEM F-12 (Mediatech, Inc., Herndon, VA) containing 10% serum, 1% Pen/Strep and 1 mg/mL G418 (Mediatech, Inc.); CHO-hOAT4 and CHO-ev4 cell lines were maintained in EMEM Alpha Modification media (Sigma-Aldrich) containing 10% serum (Lorna, Walkersville, MD), 1% Pen/Strep and 500 µg/mL G418; HEK-hOAT3 and HEK-ev cell lines were maintained in DMEM high glucose media (Mediatech, Inc.) containing 10% serum, 1% Pen/Strep and 125 µg/mL hygromycin B (Vitrogen, Carlsbad, CA); CHO-mOat1, CHO-mOat3, and CHO-FRT (ev) cell lines were maintained in DMEM F-12 containing 10% serum, 1% Pen/Strep and 125 µg/mL hygromycin B.

Cell accumulation assays: Accumulation assay protocols were adapted from those previously published. In brief, cells were seeded in 24-well tissue culture plates (2 × 105 cells/well) and grown in the absence of antibiotics for two days prior to uptake assays. Before initiation of transport experiments, the cells were equilibrated with 500 µL of transport buffer [Hanks’ balanced salt solution containing 10 mM HEPES (Sigma-Aldrich), pH 7.4] for 10 min. The equilibration buffer was replaced with 500 µL of fresh transport buffer containing 1 µM [3H]PAH or 1 µM [3H]ES (0.25 µCi/mL) in the presence or absence of inhibitors as indicated in the figure legends. Substrate concentration and accumulation

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time used for kinetic analysis of hOAT1 (1 µM PAH for 3 min, \( K_m = 15.4 \mu M \)) were based upon previous determinations in this cell line\(^{30}\)) and those for hOAT3 were determined in this study. After incubation for the indicated time, cells were immediately rinsed three times with ice-cold transport buffer, lysed with 1 M NaOH, and neutralized with 1 M HCl and 0.1 M HEPES. Aliquots were removed for liquid scintillation counting and total protein determination using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Substrate accumulation was calculated as picomoles of substrate per milligram of protein. Michaelis constant (\( K_m \)) and inhibition constants (\( K_i \)) were determined using nonlinear regression with the appropriate models in GraphPad Prism Software version 5.0 (GraphPad Software Inc., San Diego, CA). Mode of inhibition was identified by using mixed-model inhibition analysis as described in our previous work.\(^{27}\) The mode of inhibition was determined by the parameter \( \alpha \). A large value for \( \alpha (\alpha \gg 1) \) indicates competitive inhibition. Results were confirmed by performing experiments at least three times with triplicate samples.

**Statistics:** Statistical analysis was done with one-way ANOVA followed by post-hoc analysis with Dunnett’s \( t \)-test or using Student’s two-tailed unpaired \( t \)-test (\( \alpha \) for significance set at 0.05). Data are reported as mean ± S.D. or mean ± S.E.M., as indicated.

**Results**

**Inhibitory effects of hydrophilic Danshen components on hOAT1 and hOAT3 function:** The stably transfected hOAT1-expressing (CHO-hOAT1) cell line exhibited marked (~5-fold) accumulation of PAH relative to ev-transfected CHO-ev1 cells (Fig. 2A). CHO-ev1 cells exhibited a probenecid-insensitive background for PAH that was equal to ~20% of the total accumulation obtained in transporter-expressing cells (CHO-ev1: 0.9 ± 0.2 pmol/mg protein/10 min vs. CHO-hOAT1 (control): 4.4 ± 0.2 pmol/mg protein/10 min). This non-specific PAH signal was not significantly influenced by addition of the Danshen compounds (1 mM), indicating that the reduction in substrate accumulation in CHO-hOAT1 cells is attributable to inhibition of hOAT1 activity and this value represents an appropriate background correction factor (data not shown). Rudimentary assessment of the ability of the five Danshen components (1 mM), LSA, RMA, SAA, SAB, and TSL, to inhibit hOAT1 revealed that, similar to the OAT inhibitor probenecid, each of the compounds significantly inhibited (>83%) PAH uptake in CHO-hOAT1 cells (\( p < 0.001 \)) under these conditions (Fig. 2A).

ES uptake in hOAT3-expressing (HEK-hOAT3) cells was significantly greater (~4-fold) than that observed in ev-transfected HEK-ev (background) cells (HEK-ev: 2.5 ± 0.8 pmol/mg protein/10 min vs. HEK-hOAT3: 9.6 ± 1.4 pmol/mg protein/10 min, Fig. 2B). As with hOAT1, hOAT3-mediated uptake was significantly (\( p < 0.001 \)) inhibited by each Danshen component (>71% inhibition at 1 mM; Fig. 2B). LSA, RMA, SAA, SAB, and TSL (1 mM) failed to significantly influence non-specific uptake of ES in HEK-ev cells (data not shown), thus illustrating that hOAT3 expression correlates with ES accumulation and that HEK-ev ES level can be used for background correction.

The CHO-hOAT4 cells showed increased intracellular accumulation of ES (109 ± 11 pmol/mg protein/10 min) compared to ev-transfected cells (CHO-ev4: 2.8 ± 0.4 pmol/mg protein/10 min, Fig. 2C). This active transport was completely blocked (>99% inhibition) by ES at 1 mM (self-inhibition). TSL, the only Danshen component to undergo renal elimination as the unchanged parent form, failed to induce statistically significant inhibition of hOAT4 transport activity at a 500-fold excess concentration (Fig. 2C).

**Mode of inhibition:** In order to calculate the more universal kinetic parameter, \( K_a \), studies were conducted to determine the
values were much greater than 1, indicating that LSA, RMA, SAA, SAB, and TSL, non-competitive, and uncompetitive inhibition modes. Estimated $K_i$ values were estimated for SAB and TSL (10 µM) were based upon previous determinations in this cell line.30 For HEK-hOAT3 cells, ES accumulation was linear with time through at least the first minute and $K_m$ was estimated as 14.5 ± 4.5 µM (mean ± S.E.M.). The $K_m$ for ES on hOAT3 was estimated as 14.5 ± 4.5 µM (mean ± S.E.M.). The graph shown is from a representative experiment with values plotted as mean ± S.D. ($n = 3$).

Table 1. Estimated $\alpha$ values from mixed-model inhibition analysis

<table>
<thead>
<tr>
<th>Compound</th>
<th>hOAT1</th>
<th>hOAT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSA</td>
<td>$1.5 \times 10^{11}$</td>
<td>$9.6 \times 10^{13}$</td>
</tr>
<tr>
<td>RMA</td>
<td>$2.1 \times 10^{14}$</td>
<td>$3.2 \times 10^{17}$</td>
</tr>
<tr>
<td>SAA</td>
<td>$3.1 \times 10^{13}$</td>
<td>$4.2 \times 10^{16}$</td>
</tr>
<tr>
<td>SAB</td>
<td>$6.8 \times 10^{19}$</td>
<td>$8.2 \times 10^{17}$</td>
</tr>
<tr>
<td>TSL</td>
<td>$8.8 \times 10^{14}$</td>
<td>$3.5 \times 10^{15}$</td>
</tr>
</tbody>
</table>

Values are reported as mean. The S.E.M. is not applicable in this analysis.

SAAs, SABs, and TSL inhibition of hOAT1- and hOAT3-mediated transport was competitive in nature (Table 1).

Determination of inhibition potencies of LSA, RMA, SAA, SAB, and TSL: Using increasing concentrations of unlabeled test compounds (1 × 10^{-5} to 2 × 10^{-3} M), inhibition of hOAT1- and hOAT3-mediated transport of [3H]PAH or [3H]ES, respectively, was measured (Figs. 4 and 5). As indicated above, LSA, RMA, SAA, SAB, and TSL were determined to be competitive inhibitors of hOAT1 and hOAT3; thus, $K_i$ rather than IC$_{50}$ values could be calculated by selecting competitive inhibition during analysis (Table 2). For all of these analyses the coefficient of determination ($r^2$) was >0.9. The $K_i$ values for LSA and SAA on hOAT3 were ~1—2 orders of magnitude lower than those for hOAT1 ($p < 0.001$, $K_i$ ratio = 35 for each), whereas the $K_i$ values for RMA and SAB were similar for the two transporters (Table 2).

We previously reported $K_i$ values for LSA, RMA, and SAA on mOat1 and mOat3.29 In the present study, dose-response experiments were performed on mOat1 and mOat3 with unlabeled SAB and TSL (1 × 10^{-5} to 5 × 10^{-3} M). IC$_{50}$ values were estimated as 255 ± 97 µM for SAB and 146 ± 18 µM for TSL on mOat1-mediated PAH transport and as 914 ± 311 µM for SAB and 2101 ± 524 µM for TSL on mOat3-mediated ES transport (Fig. 6).

With previous work demonstrating LSA, RMA, and SAA to be competitive inhibitors of mOat1 and mOat3, and this study confirming competitive inhibition of hOAT1 and hOAT3 by LSA, RMA, SAA, SAB, and TSL, $K_i$ values were estimated for SAB and
Fig. 4. $K_i$ determination for LSA, RMA, SAA, SAB, and TSL on hOAT1
One-minute uptake of $[^{3}H]$PAH (1 µM) in CHO-hOAT1 cells was measured in the presence of increasing concentrations ($1 \times 10^{-7}$ to $2 \times 10^{-3}$ M) of LSA, RMA, SAA, SAB, and TSL. Data were corrected for non-specific background measured in the CHO-ev1 (background) cells prior to kinetic analysis. $K_i$ values were determined with non-linear regression and the “one-site competition” model using GraphPad Prism software. Experiments were repeated three times with the mean $K_i \pm$ S.E.M. reported in Table 2. Graphs shown are from representative experiments with values plotted as mean $\pm$ S.D. ($n=3$).

Fig. 5. $K_i$ determination for LSA, RMA, and SAA, SAB, and TSL on hOAT3
One-minute uptake of $[^{3}H]$ES (1 µM) in HEK-hOAT3 cells was measured in the presence of increasing concentrations ($1 \times 10^{-7}$ to $2 \times 10^{-3}$ M) of LSA, RMA, SAA, SAB, and TSL. Data were corrected for non-specific background measured in the HEK-ev (background) cells prior to kinetic analysis. $K_i$ values were determined with non-linear regression and the “one-site competition” model using GraphPad Prism software. Experiments were repeated three times with the mean $K_i \pm$ S.E.M. reported in Table 2. Graphs shown are from representative experiments with values plotted as mean $\pm$ S.D. ($n=3$).
TSL on mOat1 and mOat3 assuming competitive inhibition and are reported in Table 3.

**Discussion**

The popularity of natural products as dietary supplements and/or alternative medicines has been increasing. Indeed, a recent survey found that ~20% of the adult population in the United States reported using one or more natural products.31) Because herbal medicines are produced from plants they are considered “natural” and, therefore, are often perceived as having no toxic effects by patients. However, many different side effects/adverse events are known to be associated with natural products, including herb-drug interactions.32,33) A number of herb-drug interactions involving metabolic enzymes (e.g., CYP450s) are well established; however, herb-drug interactions at the level of transporter proteins are not as well characterized.

Table 2. Estimated $K_i$ values (µM) and DDI indices for hOAT1 and hOAT3

<table>
<thead>
<tr>
<th>Compound</th>
<th>hOAT1</th>
<th>hOAT3</th>
<th>$K_i$ ratio (hOAT1/hOAT3)</th>
<th>$f_u$ (%)</th>
<th>DDI indexa</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSA</td>
<td>20.8 ± 2.1</td>
<td>0.59 ± 0.26***</td>
<td>35</td>
<td>—</td>
<td>0.01c</td>
</tr>
<tr>
<td>RMA</td>
<td>0.35 ± 0.06</td>
<td>0.55 ± 0.25</td>
<td>0.6</td>
<td>9.5c</td>
<td>1.6</td>
</tr>
<tr>
<td>SAA</td>
<td>5.6 ± 0.3</td>
<td>0.16 ± 0.03***</td>
<td>35</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SAB</td>
<td>22.2 ± 1.9</td>
<td>19.8 ± 8.4</td>
<td>1.1</td>
<td>6.8c</td>
<td>0.02</td>
</tr>
<tr>
<td>TSL</td>
<td>40.4 ± 12.9</td>
<td>8.6 ± 3.3</td>
<td>4.7</td>
<td>100f</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Values are reported as mean ± S.E.M. ($n=3$). ***Significant difference of $K_i$ values between hOAT1 and hOAT3 is $p<0.001$ as determined by two-tailed Student’s unpaired t-test. $f_u$: fraction unbound in plasma, aclinical data taken from Li et al.,10) c—: unknown; dassuming LSA is highly protein bound ($f_u=10\%$), efraction unbound in plasma from Yang et al.42)

Table 3. Comparison of $K_i$ values (µM) between mOat1 and mOat3 and their human orthologs

<table>
<thead>
<tr>
<th>Compound</th>
<th>mOat1a</th>
<th>mOat3a</th>
<th>$K_i$ ratio (mOat1/mOat3)</th>
<th>$K_i$ ratio (mOat3/hOAT3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSA</td>
<td>14.9 ± 4.9</td>
<td>31.1 ± 7.0</td>
<td>0.7</td>
<td>53a</td>
</tr>
<tr>
<td>RMA</td>
<td>5.5 ± 2.2</td>
<td>4.3 ± 0.2</td>
<td>16</td>
<td>7.8***</td>
</tr>
<tr>
<td>SAA</td>
<td>4.9 ± 2.2</td>
<td>21.3 ± 7.7</td>
<td>0.9</td>
<td>133</td>
</tr>
<tr>
<td>SAB</td>
<td>226 ± 90c</td>
<td>845 ± 287b</td>
<td>11</td>
<td>43c</td>
</tr>
<tr>
<td>TSL</td>
<td>136 ± 17c</td>
<td>1,940 ± 486b</td>
<td>3.4a</td>
<td>226a</td>
</tr>
</tbody>
</table>

Values are reported as mean ± S.E.M. ($n=3$). *Significant difference between murine and human $K_i$ values $p<0.05$ and **significant difference between murine and human $K_i$ values $p<0.001$ as determined by two-tailed Student’s unpaired t-test. aData for LSA, RMA and SAA were taken from Wang and Sweet.27) cCorresponding $K_i$ values assuming competitive inhibition (see text for explanation).
Despite this, there is a growing literature base concerning the interaction of natural products with OATs. For example, the dietary polyphenol ellagic acid was found to potently inhibit hOAT1 (IC50 = 207 nM) and hOAT4.\textsuperscript{34} The aglycone of the natural sweetener stevioside was identified as an inhibitor of both hOAT1 (Kᵢ = 2.0 µM) and hOAT3 (Kᵢ = 5.4 µM).\textsuperscript{35} Similarly, dietary flavonoid and hydroxycinnamic acid aglycones, as well as their Phase II glucuronide and sulfate conjugates, produced pronounced inhibition of hOAT1- and hOAT3-mediated transport.\textsuperscript{36,37} Finally, the nephrotoxin aristolochic acid, the causative agent for “Chinese herbs nephropathy,” which is found in herbal products containing \textit{Aristolochia} sp., exhibited significant interaction with hOAT1 (Kᵢ = 600 nM), hOAT3 (Kᵢ = 500 nM), and hOAT4 (Kᵢ = 20.6 µM).\textsuperscript{38} Thus, herb-drug interactions involving OATs and components of natural products that impact the pharmacokinetics and pharmacodynamics of administered drugs may occur.

In the present study, we sought to determine the inhibitory effects of five major Danshen components, LSA, RMA, SAA, SAB, and TSL, on hOAT1-, hOAT3-, and hOAT4-mediated transport (Figs. 2, 4 and 5). In order to calculate Kᵢ values on hOAT3, the Kₘ value for ES (test substrate) needed to be determined in the HEK-hOAT3 cell system (Fig. 3). Saturation analysis revealed an estimated Kₘ of 14.5 ± 4.5 µM, which is similar to other reported values of 6.3–9.5 µM.\textsuperscript{39,41} LSA, RMA, SAA, SAB, and TSL were found to be potent competitive inhibitors of hOAT1- and hOAT3-mediated transport (Figs. 4 and 5, Tables 1 and 2). Their calculated Kᵢ values are similar to reported Kₘ and Kᵢ values for endogenous steroid and prostaglandin hormones on hOAT1 and hOAT3.\textsuperscript{14} Therefore, LSA, RMA, SAA, SAB, and TSL may affect the disposition and elimination of not only co-administered drugs, but also of endogenous substances that are OAT substrates.

The rank order of their potencies was different between the two transporters with RMA > SAA > LSA ≈ SAB > TSL for hOAT1 and SAA > LSA ≈ RMA > TSL > SAB for hOAT3. Regardless, LSA and SAA, exhibited significantly higher affinity (lower Kᵢ values) for hOAT3 than for hOAT1, with each compound showing ~35-fold preference, and TSL showed a marked ~5-fold preference for hOAT3 over hOAT1 (Table 2). RMA and SAB exhibited similar affinity for both transporters. hOAT4 is exclusively expressed on the apical side of human proximal tubules and it functions in reabsorption; thus, substances present in the urinary space might inhibit hOAT4 transport activity.\textsuperscript{45} Of the Danshen components investigated in this study, only TSL undergoes renal elimination as the unchanged parent form. Therefore, we investigated the potential interaction of TSL on hOAT4. TSL, even at 500 µM, a concentration that is much greater than that observed clinically (and experimentally a 500-fold excess vs. 1 µM substrate), failed to produce significant inhibition of hOAT4-mediated ES uptake, indicating that TSL was unlikely to block hOAT4 transport activity \textit{in vivo} (Fig. 2C).

In the draft guidance for the industry, “Drug Interaction Studies—Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations”, re-issued by the Food and Drug Administration in February 2012, it was proposed that a DDI index, indicative of a compound’s potential to cause marked \textit{in vivo} drug-drug interactions when its value exceeds 0.1, can be calculated as the [unbound] Cₘₚ/Kᵢ (or IC₅₀) ratio.\textsuperscript{28} In 2005, an \textit{in vivo} pilot study conducted in healthy humans reported Cₘₚ values for LSA, RMA, SAB, and TSL of 2.23, 5.55, 5.69 and 18 µM after i.v. administration of a Danshen product, in which the dose of LSA, RMA, SAB, and TSL were 3, 160, 65, and 90 mg, respectively.\textsuperscript{10} Further, Yang \textit{et al.} determined that, for RMA, SAB, and TSL the fraction unbound in plasma was 9.5%, 6.8%, and 100%, respectively.\textsuperscript{42} Thus, using the published Cₘₚ and plasma protein binding values for RMA, SAB, and TSL, as well as the Kᵢ values determined in this study (Table 2), DDI indices for each compound were calculated for each compound. DDI indices for RMA were 1.6 and 1.0, and for TSL were 0.5 and 2.1, for hOAT1 and hOAT3, respectively, indicating a strong potential for clinical herb-drug interactions. DDI indices for SAB were much smaller than 0.1 for both transporters (Table 2). The plasma protein binding for LSA, which showed high affinity to hOAT1 and hOAT3, is unknown. Therefore, to provide a conservative analysis it was assumed that LSA is highly plasma-protein bound (fₚ = 10%). Even under this conservative condition the DDI index for LSA is 0.4 for hOAT3, suggesting inhibition of hOAT3 \textit{in vivo}. The pharmacokinetic profile of SAA is virtually unknown. Clearly, greater understanding of the pharmacokinetic properties of LSA, RMA, SAA, and TSL, and the transporters that help determine them, is necessary to avoid potential herb-drug interactions. For Danshen pharmaceutical products this would be particularly relevant in coronary patients, where they may be taken in combination with drugs, such as angiotensin converting enzyme inhibitors, which are known to be hOAT1/hOAT3 substrates.

Additionally, there is marked variation in the amount of each active component in plants cultivated in different regions and in Danshen products prepared by different manufacturing processes.\textsuperscript{9,10,43} For example, the content of RMA in raw plant material varied as much as 16 fold between different cultivation regions and the dose of RMA ranged from 4.1–160 mg (~40 fold) in Danshen injectables produced by different manufacturers.\textsuperscript{9,10} Moreover, the dose of RMA ranged from 1.16–3.47 mg from different batches of a Danshen injectable from the same pharmaceutical manufacturer.\textsuperscript{45} Large differences were also observed for TSL content (0.36–4.03 mg/mL) among different Danshen injectables.\textsuperscript{9,10} These data illustrate that clinically significant herb-drug interactions would or would not occur depending upon which manufacturer’s product was administered and/or the diligence of the quality control protocol utilized by a single manufacturer. Therefore, although tanshinone II A, SAB, and protocatechuic aldehyde have been chosen as quality control markers for Danshen pharmaceutical dosage forms in accordance with the Chinese Pharmacopeia, our data suggest that the content of other Danshen components, \textit{e.g.}, LSA, RMA, and TSL should be considered for monitoring and control as well.

Recently, we investigated the interaction between mOat1 and mOat3 and Danshen’s hydrophilic components (Fig. 6).\textsuperscript{27} As with the human orthologs, the rank order of potencies was slightly different between the two transporters with RMA ≈ SAA > LSA > SAB ≈ TSL for mOat1 and RMA > SAA ≈ LSA > SAB > TSL for mOat3. On comparing the mouse and human data, notable species differences appear to exist in terms of inhibitory potency (Table 3). Human OAT1 exhibited 16-, 11-, and 3.4-fold higher affinity for RMA, SAB, and TSL as compared to mOat1, while LSA and SAA showed similar inhibition potencies across species. In contrast, all five compounds exhibited much higher affinity, by 1–2 orders of magnitude, for hOAT3 over mOat3. Interestingly, for LSA, SAA, and TSL the two species are reversed, with hOAT3 having higher affinity for all three over hOAT1 vs. mOat1 having higher affinity for all three over mOat3 (Tables 2 and 3). While the
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human orthologs had ~10-fold lower $K_i$ values for RMA than the murine transporters, within species the values were similar for each transporter. SAB showed comparable affinity with hOAT1 and hOAT3, while it exhibited preferential interaction potency with mOat1 compared to mOat3. These species differences indicate that any interactions observed in vivo in mice at clinically relevant doses of LSA, RMA, SAA, SAB and TSL would potentially be of even greater magnitude in humans. Therefore, the mouse may not be the most suitable animal model to investigate OAT-mediated DDIs by Danshen components. This highlights the importance of delineating species differences in affinity in vitro prior to interpreting results from in vivo pharmacokinetic studies in pre-clinical models and attempting to extrapolate them to humans. Future studies should investigate whether other animal models (e.g., dogs or monkeys) show similar affinities for Danshen components as hOATs and potentially represent a more appropriate in vivo model system.

As discussed earlier, additional Danshen components also might show significant inhibitory effects on OATs. For example, caffeic acid was demonstrated to be an OAT inhibitor, with reported IC₅₀ values of 16.6 µM for hOAT1 and 5.4 µM for hOAT3. However, because of low plasma concentration (0.56 µM) and modest plasma protein binding (66%), the calculated DDI index is less than 0.1, indicating caffeic acid was not likely to cause significant OAT inhibition at clinically relevant concentrations.10) Protocatechuic acid was demonstrated to be an OAT inhibitor, with reported IC₅₀ values of 16.6 µM for hOAT1 and 5.4 µM for hOAT3. However, again, the extremely low content in Danshen products renders them unlikely to contribute to OAT-mediated Danshen-drug interactions in vivo.

In summary, the active Danshen components, LSA, RMA, SAA, SAB, and TSL, were demonstrated to elicit significant competitive inhibition on hOAT1- and hOAT3-mediated substrate uptake at clinically relevant concentrations. DDI indices for RMA and TSL on hOAT1 and hOAT3, and for LSA on hOAT3, suggest a strong potential for drug-drug interactions in patients when co-administered with drugs that are known hOAT1 and/or hOAT3 substrates. In addition, notable species differences were observed between human and murine OAT orthologs with human OATs showing higher affinity. All of these Danshen components preferentially interacted with hOAT3 compared to mOat3, while RMA, SAB, and TSL showed higher affinity with hOAT1 (vs. mOat1). This information should improve interpretation and extrapolation of pharmacokinetic data generated in pre-clinical studies. Finally, these components might serve as effective quality control markers to be monitored during the manufacturing process to improve product consistency, efficacy, and safety.

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