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Prioritizing pharmacokinetic drug interaction precipitants in natural products: application to OATP inhibitors in grapefruit juice

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Abstract

Natural products, including botanical dietary supplements and exotic drinks, represent an ever-increasing share of the health care market. The parallel ever-increasing popularity of self-medicating with natural products increases the likelihood of co-consumption with conventional drugs, raising concerns for unwanted natural product-drug interactions. Assessing the drug interaction liability of natural products is challenging due to the complex and variable chemical composition inherent to these products, necessitating a streamlined preclinical testing approach to prioritize precipitant individual constituents for further investigation. Such an approach was evaluated in the current work to prioritize constituents in the model natural product, grapefruit juice, as inhibitors of intestinal organic anion-transporting peptide (OATP)-mediated uptake. Using OATP2B1-expressing MDCKII cells and the probe substrate estrone 3-sulfate, IC_{50} s were determined for constituents representative of the flavanone (naringin, naringenin, hesperidin), furanocoumarin (bergamottin, 6',7'-dihydroxybergamottin), and polymethoxyflavone (nobiletin and tangeretin) classes contained in grapefruit juice. Nobiletin was the most potent (IC_{50} , 3.7 μ M); 6',7'-dihydroxybergamottin, naringin, naringenin, and tangeretin were moderately potent (IC_{50} , 20–50 μ M); and bergamottin and hesperidin were the least potent (IC_{50} , >300 μ M) OATP2B1 inhibitors. Intestinal absorption simulations based on physiochemical properties were used to determine ratios of unbound concentration to IC_{50} for each constituent within enterocytes and to prioritize in order of pre-defined cut-off values. This streamlined approach could be applied to other natural products that contain multiple precipitants of natural product-drug interactions.

Keywords

OATP2B1; grapefruit juice; drug interaction; transporter; simulation

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Conflict of Interest

The authors declare no conflicts of interest.

Introduction

Phytochemicals contained in botanical natural products, including citrus juices, teas, and herbal supplements, can precipitate pharmacokinetic interactions with conventional drugs by altering absorption and elimination processes, which can lead to altered systemic drug exposure and potentially, enhanced or reduced pharmacologic effect(s) [1–3]. Rigorous assessment of the drug interaction potential of specific constituents within natural products is challenging due to the complex and variable chemical composition inherent to these products [4]. There are no harmonized guidelines for assessing precipitant constituents within natural products, and pre-clinical and clinical testing of each constituent is neither practical nor efficient. These observations, combined with the ever-growing consumer and health care markets for botanical natural products, highlight the need for a streamlined approach to identify, screen, and prioritize high-risk constituents that can precipitate pharmacokinetic interactions with conventional drugs.

Grapefruit juice (GFJ) is an extensively studied natural product that inhibits the intestinal metabolism and/or transport of numerous drugs. The most rigorously studied mechanism underlying the GFJ effect is inhibition of the prominent intestinal drug metabolizing enzyme cytochrome P450 (CYP) 3A. A relatively less studied mechanism is inhibition of intestinal organic anion-transporting polypeptides (OATPs) [5], uptake transporters located on the apical membranes of enterocytes that act to facilitate substrate absorption. Such OATP-mediated interactions can lead to a significant decrease in systemic exposure to substrate drugs (*e.g.*, fexofenadine, fluoroquinolone antimicrobials, beta-blockers, and aliskiren), potentially leading to reduced therapeutic effect [6–10]. Natural product-drug interactions mediated via inhibition of intestinal OATPs are not limited to GFJ. For example, orange juice and apple juice have been shown to significantly reduce the systemic exposure to fexofenadine, some beta-blockers, and aliskiren by at least 50% [7, 11, 12]. More recently, constituents in a green tea beverage were reported to decrease systemic exposure to the beta-blocker, nadolol, by 85% in healthy volunteers by inhibiting intestinal OATP1A2, albeit the existence of this transporter is controversial [13, 14]; this reduction in systemic exposure was accompanied by an attenuated reduction in systolic blood pressure [9]. These observations prompted evaluation of a streamlined approach for pre-clinical testing of candidate intestinal OATP inhibitors using GFJ as an archetypal, well-characterized natural product with successful *in vitro-in vivo* extrapolated drug interaction liability.

The flavanone naringin is the only isolated GFJ constituent that has been tested as an intestinal OATP inhibitor in human subjects. When administered to healthy volunteers as an aqueous naringin solution (~1.2 mM) or an equimolar concentration in GFJ, naringin alone did not fully reproduce the decrease in the area under the concentration-time curve of the OATP substrate fexofenadine, suggesting that other constituents contribute to the effect of whole juice [15]. A second clinical study involving a modified GFJ devoid of furanocoumarins and polymethoxyflavones showed similar effects as the original GFJ on the pharmacokinetics of fexofenadine [16], suggesting that flavanones are the major OATP inhibitors. Based on these clinical observations, constituents representative of the three chemical classes (Fig. 1) were tested to determine whether the proposed method could accurately identify and exclude clinically relevant OATP inhibitors. Results ultimately could

contribute to the generation of a decision tree for systematically identifying and prioritizing intestinal OATP inhibitors for further investigation.

Materials and Methods

Materials and chemicals

[³H]Estrone 3-sulfate ammonium salt (54.3 Ci/mmol, purity >97%) was purchased from Perkin Elmer (Waltham, MA). Estrone 3-sulfate potassium salt, D-glucose, 6',7'-dihydroxybergamottin (DHB), bergamottin, naringin, hesperidin, tangeretin, and nobiletin were purchased from Sigma-Aldrich (St. Louis, MO). Hanks' balanced salt solution with calcium and magnesium was purchased from Mediatech Inc. (Hendon, VA). Phosphate-buffered saline (PBS); fetal bovine serum; trypsin-EDTA; HEPES; and Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose, 2 mM L-glutamine, and 110 mg/L sodium pyruvate were purchased from Invitrogen (Carlsbad, CA). MDCKII parental cells and stably transfected MDCKII-OATP2B1 cells were kindly provided by Dr. Markus Grube (Ernst-Moritz-Arndt University, Greifswald, Germany). All other chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA). Ethical approval was not required for the following *in vitro* and *in silico* research activities.

Cell culture

Parental MDCKII and stably transfected MDCKII-OATP2B1 cells were cultured and maintained as described previously [17]. Cells were seeded onto 24-well plates at a density of 5×10^4 cells per well and grown to confluence for 2–3 days prior to experimentation.

IC₅₀ determination

Cells were washed and preincubated for 30 min at 37°C in uptake buffer (125 mM NaCl, 48 mM KCl, 5.6 mM D-glucose, 1.2 mM CaCl₂, 1.2 mM KH₂PO₄, 12 mM MgSO₄, and 25 mM MES, pH 6). Buffer was replaced, and cells were treated with a solution (200 µL) consisting of radiolabeled (0.27 µCi/well) plus unlabeled estrone 3-sulfate (total concentration, 1 µM) and GFJ constituent (0 to 316 µM). After 3 min at 37°C, cells were washed three times with ice-cold PBS and lysed with 0.1 N sodium hydroxide/0.1% SDS. Cell lysate aliquots (200 µL) were added to liquid scintillation cocktail (5 mL), and radioactivity was counted. Protein concentrations were determined with a BCA assay kit (Thermo Fisher Scientific, Waltham, MA). Estrone 3-sulfate uptake was linear up to 5 min (data not shown).

Data analysis

Uptake rates were normalized to protein content. Net OATP2B1-mediated estrone 3-sulfate uptake was calculated by subtracting uptake in parental cells (OATP2B1-negative) from uptake in OATP2B1-expressing cells. Percent control activity was calculated relative to vehicle (3% methanol), which was set at 100% uptake. Initial estimates of apparent IC₅₀s were determined from linear regression of net substrate uptake vs. natural logarithm of GFJ constituent concentration data. IC₅₀s were recovered by fitting the inhibitory E_{max} model (equation 1 or 2) with untransformed data using Phoenix® WinNonlin® (v7.0, Certara, Princeton, NJ):

$$E = E_0 - \frac{I_{\max} \cdot C}{IC_{50} + C} \quad (1)$$

$$E = E_0 - \frac{I_{\max} \cdot C^{\gamma}}{IC_{50} + C^{\gamma}} \quad (2)$$

where E and E_0 denote maximum (*i.e.*, observed) and baseline effects, respectively; $I_{\max}$ denotes the maximum inhibitory effect; C denotes inhibitor concentration; and γ denotes the Hill coefficient. Robustness of model fits was assessed from visual check of observed and predicted data, distribution of residuals, Akaike information criteria, and standard errors. Experimental data are presented as mean $\pm$ standard deviation of triplicate determinations. IC_{50} s are presented as estimate $\pm$ standard error of the estimates.

Simulations

GFJ constituent absorption into enterocytes was simulated to assess relative OATP-mediated interaction liability according to the decision trees set forth by the International Transporter Consortium [18]. Simulations were used to estimate $[I]_{\text{ent}}/IC_{50}$ and $[I]_{\text{GFJ}}/IC_{50}$, where $[I]_{\text{ent}}$ is the maximum unbound constituent concentration in the simulated enterocyte compartment; $[I]_{\text{GFJ}}$ is the maximum reported constituent concentration in GFJ (*i.e.*, the theoretical maximum concentration in the gastrointestinal lumen) (Table 1); and IC_{50} is the half-maximal inhibitory concentration measured for OATP2B1 in the current work.

Structure-based physicochemical properties for each GFJ constituent were predicted using MedChem Designer™ 4.0 (Simulations Plus, Inc., Lancaster, CA) (Table 1). These values were used to populate the Advanced Compartmental Absorption and Transit (ACAT) model within GastroPlus™ (v9.0.0007, Simulations Plus). The ACAT model expands upon the traditional compartmental absorption and transit model [19] by predicting dissolution, ionization, and pH-dependent solubility in simulated gastrointestinal fluid, as well as binding parameters and partition coefficients for multiple enterocyte compartments along the intestinal tract. The model was populated using the default physiology for a fasted 35-year-old healthy man. Specifying the physiology was necessary to create a simulated enterocyte compartment within GastroPlus™; however, neither age nor sex substantially alter intracellular xenobiotic concentrations within the enterocyte compartment produced by the ACAT model. Absorption of a single dose of each constituent was simulated as an immediate-release solution (250 mL) at $[I]_{\text{GFJ}}$ (Table 1). Gastric transit time was set at 0.1 h as appropriate for a liquid dosage form. Constituent particle density and mean precipitation time were set at the default values of 1.2 g/mL and 900 s, respectively. OATP2B1 expression and activity were assumed to be homogenous throughout the intestinal tract [13]; thus, $[I]_{\text{ent}}$ values represent the average for the entire intestinal compartment. Regulatory guidelines for intestinal transporters specify that $[I]_1/IC_{50} \geq 0.1$ (in this case, $[I]_{\text{ent}}/IC_{50} \geq 0.1$) or $[I]_2/IC_{50} \geq 10$ (in this case, $[I]_{\text{GFJ}}/IC_{50} \geq 10$) merits a clinical interaction study [18, 20]; as such, ratios are presented in reference to these cutoff values (Fig. 3).

Results

Nobiletin was the most potent OATP2B1 inhibitor, with an IC_{50} of $<5 \mu M$ (Fig. 2A). Naringenin was moderately potent, with an IC_{50} of $20 \mu M$ (Fig. 2B). DHB (Fig. 2C), naringin (Fig. 2D), and tangeretin (Fig. 2E) were approximately equipotent, with IC_{50} s ranging from 34 to $40 \mu M$. Bergamottin (Fig. 2F) and hesperidin (Fig. 2G) were the least potent OATP2B1 inhibitors, with IC_{50} s $>300 \mu M$. With the exception of nobiletin and bergamottin, all of the constituents stimulated estrone 3-sulfate uptake by as much as 20% at concentrations ranging from <1 to $3 \mu M$. The absorption simulation-predicted $[I]_{ent}$ for naringin, naringenin, hesperidin, DHB, bergamottin, nobiletin, and tangeretin were, respectively, 560, 63.9, 16.8, 2.28, 2.78, 3.71, and $8.85 \mu M$. The $[I]_{ent}/IC_{50}$ for naringenin, naringin, nobiletin, and tangeretin exceeded 0.1 (Fig. 3). The $[I]_{GFJ}/IC_{50}$ for naringenin and naringin exceeded 10.

Discussion

An increasing number of intestinal and hepatic OATP inhibitors have been identified in botanical natural products, including GFJ, other fruit juices, and green tea [21–26]. Systematic evaluation of the inhibitory effect of each of the many constituents contained in a given natural product using a single *in vitro* system, and especially human subjects, in a time- and cost-efficient manner is impossible. Because harmonized guidelines do not exist for prioritizing precipitant natural product constituents, an *in vitro-in silico* streamlined approach was developed using GFJ as a test natural product. IC_{50} s for representative GFJ constituents from three distinct chemical classes were determined using an established OATP2B1-transfected cell system and probe substrate (Fig. 2). These data, combined with results from simulations of intestinal absorption, were used to prioritize constituents based on the unbound concentration/ IC_{50} ratio. The IC_{50} s recovered for DHB, naringenin, naringin, nobiletin, and tangeretin were below the maximum reported concentration in grapefruit juice ($[I]_{GFJ}$) (Table 1), supporting these constituents as potential clinically relevant intestinal OATP inhibitors. However, simple comparison of the IC_{50} with $[I]_{GFJ}$ is theoretically not as robust for predicting clinical interactions as a comparison with the unbound concentration at the site of enterocyte uptake ($[I]_{ent}$). Such an approach has been suggested for hepatic transporters [27] and should be more predictive of the likelihood of an interaction *in vivo*. A similar strategy was taken in the current work to evaluate the drug interaction liability of GFJ constituents at the site of intestinal absorption.

As mentioned earlier, flavanones are likely the major OATP inhibitors in GFJ, which was confirmed by both metrics ($[I]_{GFJ}/IC_{50}$ and $[I]_{ent}/IC_{50}$). Hesperidin was the only flavanone not identified by either metric, probably due to having a relatively low $[I]_{GFJ}$ [28–31]; however, hesperidin may pose an interaction risk when consumed in orange juice, in which $[I]_{GFJ}$ approaches 2 mM [32]. Both metrics correctly excluded the furanocoumarins as OATP inhibitors in GFJ. All of the constituents except nobiletin and bergamottin stimulated estrone 3-sulfate uptake by as much as 20% at the lower concentrations tested (<1 to $3 \mu M$). OATP stimulation has been observed with other natural product constituents [21, 24, 25] and may be attributed to multiple binding sites or conformational changes, which could lead to enhanced uptake by OATP.

The IC₅₀s recovered in the current work differed somewhat from those recovered using OATP2B1-overexpressing *Xenopus laevis* oocytes [33]. For example, hesperidin showed no inhibition at the concentrations tested in MDCKII cells (current work), but was a potent inhibitor in oocytes (IC₅₀, >300 vs. 1.92 μM). Differences in membrane permeability, intra- and extracellular non-specific binding, and/or transporter localization and trafficking between the two model systems could account for this discrepancy. Because the mechanism of OATP inhibition by flavonoids is not completely known, studies comparing OATP2B1 function between model systems are needed to clarify the ability of each to appropriately rank these intestinal OATP2B1 inhibitors.

Unlike the [I]_{GFJ}/IC₅₀ ratio, the [I]_{ent}/IC₅₀ ratio additionally identified the polymethoxyflavones nobiletin and tangeretin as possible precipitants of OATP-mediated GFJ-drug interactions, highlighting two areas for further research. First, it is unclear which ratio is more predictive of the likelihood of intestinal OATP2B1 inhibition *in vivo*. Although both ratios were adapted from decision trees set forth by the International Transporter Consortium [18], studies elucidating mechanisms of OATP2B1 inhibition should aid in selection of the most appropriate ratio and refinement of the cutoff criteria. Second, studies of drug interaction liability of individual constituents within the native environment of the natural product are warranted to determine synergistic, additive, and/or antagonistic effects. For example, it is possible that the inhibitory effects of the polymethoxyflavones are overwhelmed by those of the more abundant flavanones in GFJ. Studies comparing juices enriched in polymethoxyflavones relative to flavanones would provide further insight.

Conclusion

A streamlined approach for prioritizing intestinal OATP inhibitors in a complex natural product was demonstrated using the model natural product GFJ, an established OATP2B1-expressing cell system, and candidate inhibitors representative of three chemical classes. Using *in silico*-predicted rather than experimentally-measured physicochemical properties contributed to the high-throughput nature of this approach. Further refinement of this approach, including addressing uncertainties in calculated physicochemical properties, may contribute to the development of a decision tree for intestinal uptake transporter-based interactions, similar to that used for interactions mediated by inhibition of the efflux transporter P-glycoprotein [34]. This approach may be applied to natural products for which candidate precipitants are unknown, in which case high throughput analytical chemistry procedures [35–37] can be used *a priori* to identify bioactive constituents. Collaborations between clinical pharmacologists and natural products chemists would expedite this process.

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Nonstandard abbreviations

CYP	cytochrome P450
DHB	6',7'-dihydroxybergamottin
GFJ	grapefruit juice
MDCKII	Madin-Darby canine kidney type II
OATP	organic anion-transporting polypeptide

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Class	R Group	Constituent
Furanocoumarins		
		Bergamottin
		6',7'-Dihydroxybergamottin
Flavanones		
	-OH	Naringenin
		Naringin
		Hesperidin
Polymethoxyflavones		
	$R_1 = H$ $R_2 = OCH_3$	Tangeretin
	$R_1 = OCH_3$ $R_2 = OCH_3$	Nobiletin

Fig. 1.
Structures of constituents from representative chemical classes contained in grapefruit juice.

Fig. 2.

Concentration-dependent modulation of OATP2B1-mediated estrone-3-sulfate uptake by grapefruit juice constituents representative of three chemical classes (A–G). Percent control activity is relative to vehicle condition. Symbols and error bars denote means and standard errors, respectively, of triplicate incubations. Curves denote nonlinear least-squares regression of observed data.

Fig. 3.

Forest plot of the $[I]_{ent}/IC_{50}$ and $[I]_{GFJ}/IC_{50}$ ratios for grapefruit juice (GFJ) constituents as inhibitors of intestinal OATP2B1. $[I]_{ent}/IC_{50}$ (gray), ratio of the unbound concentration in simulated enterocyte compartments to IC_{50} . $[I]_{GFJ}/IC_{50}$ (orange), ratio of maximum constituent concentration in GFJ to IC_{50} . The absorption simulation predicted $[I]_{ent}$ s of 560 (naringin), 63.9 (naringenin), 16.8 (hesperidin), 2.28 (DHB), 2.78 (bergamottin), 3.71 (nobiletin), and 8.85 (tangeretin) μ M. Gray and orange lines denote ratios at which a clinical interaction study is suggested for each metric: $[I]_{ent}/IC_{50}$, 0.1 (gray); $[I]_{GFJ}/IC_{50}$, 10 (orange). DHB, 6',7'-dihydroxybergamottin. Red circles indicate constituents whose ratios exceeded these threshold values.

Table 1

Absorption model inputs

	Naringin	Naringenin	Hesperidin	DHB	Bergamottin	Nobiletin	Tangeretin
	[I] _{GFJ} (μM)						
	6540 [38]	595 [39]	117 [30]	52.5 [40]	36.6 [40]	28.4* [41]	78.4* [42]
	Predicted physicochemical properties						
Molar mass (daltons)	580.55	272.257	610.57	372.42	338.397	402.39	370.36
pK _a	9.94; 8.10	9.81; 9.12 7.38	9.71; 8.10	-	-	-	-
Diffusion coefficient	0.56	0.86	0.54	0.67	0.68	0.66	0.71
O:W partition	-0.26	2.42	-0.33	2.79	5.85	2.77	2.92
O:W distribution	-0.34	2.10	-0.40	2.79	5.85	2.77	2.92
P _{eff} (cm/s × 10 ⁴)	0.12	2.99	0.13	1.08	2.12	2.55	2.75
SW (mg/mL)	4.37	0.31	4.89	0.02	0.00	0.00	0.00
SL _{gastric fast} (mg/mL)	1.27	0.13	1.09	0.05	0.00	0.03	0.04
SL _{gut fast} (mg/mL)	1.46	0.36	1.31	0.03	0.00	0.04	0.03
SL _{gut fed} (mg/mL)	2.26	0.58	1.96	0.07	0.05	0.12	0.13
	Simulated f _a (%)						
	27.27	99.65	44.58	98.62	99.83	99.90	99.94

[I]_{GFJ}, maximum reported concentration in grapefruit juice. O:W partition, octanol-water partition coefficient. O:W distribution, octanol-water distribution coefficient. P_{eff}, effective human jejunal permeability coefficient. SW, native water solubility. SL_{gastric fast}, solubility in GastroPlus^{TM} simulated gastric fluid in the fasted state. SL_{gut fast}, solubility in simulated intestinal fluid in the fasted state. SL_{gut fed}, solubility in simulated intestinal fluid in the fed state. DHB, 6',7'-dihydroxybergamottin. f_a, fraction of the dose absorbed into enterocytes.

* Concentration in flavedo (peel).