ARTICLE

Assessing Transporter-Mediated Natural Product-Drug Interactions Via *In vitro-In Vivo* Extrapolation: Clinical Evaluation With a Probe Cocktail

James T. Nguyen[1], Dan-Dan Tian[1,9], Rakshit S. Tanna[1], Deena L. Hadi[1,2], Sumit Bansal[3], Justina C. Calamia[3], Christopher M. Arian[3], Laura M. Shireman[3], Bálint Molnár[4], Miklós Horváth[4], Joshua J. Kellogg[5,10], Matthew E. Layton[6], John R. White[7], Nadja B. Cech[2,5], Richard D. Boyce[2,8], Jashvant D. Unadkat[2,3], Kenneth E. Thummel[2,3] and Mary F. Paine[1,2,*]

The botanical natural product goldenseal can precipitate clinical drug interactions by inhibiting cytochrome P450 (CYP) 3A and CYP2D6. Besides P-glycoprotein, effects of goldenseal on other clinically relevant transporters remain unknown. Established transporter-expressing cell systems were used to determine the inhibitory effects of a goldenseal extract, standardized to the major alkaloid berberine, on transporter activity. Using recommended basic models, the extract was predicted to inhibit the efflux transporter BCRP and uptake transporters OATP1B1/3. Using a cocktail approach, effects of the goldenseal product on BCRP, OATP1B1/3, OATs, OCTs, MATEs, and CYP3A were next evaluated in 16 healthy volunteers. As expected, goldenseal increased the area under the plasma concentration-time curve (AUC_{0-inf}) of midazolam (CYP3A; positive control), with a geometric mean ratio (GMR) (90% confidence interval (CI)) of 1.43 (1.35–1.53). However, goldenseal had no effects on the pharmacokinetics of rosuvastatin (BCRP and OATP1B1/3) and furosemide (OAT1/3); decreased metformin (OCT1/2, MATE1/2-K) AUC_{0-inf} (GMR, 0.77 (0.71–0.83)); and had no effect on metformin half-life and renal clearance. Results indicated that goldenseal altered intestinal permeability, transport, and/or other processes involved in metformin absorption, which may have unfavorable effects on glucose control. Inconsistencies between model predictions and pharmacokinetic outcomes prompt further refinement of current basic models to include differential transporter expression in relevant organs and intestinal degradation/metabolism of the precipitant(s). Such refinement should improve *in vitro-in vivo* prediction accuracy, contributing to a standard approach for studying transporter-mediated natural product-drug interactions.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ The natural product (NP) goldenseal is a clinically relevant inhibitor of CYP2D6 and CYP3A, raising concerns for NP-drug interactions (NPDIs). Except P-glycoprotein, the effects of goldenseal on other clinically relevant transporters remain unknown.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ Do current recommended basic models successfully predict transporter-mediated NPDIs? What are the effects of goldenseal supplementation on the pharmacokinetics of transporter probe substrates?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ The current models successfully predicted some, but not all, NPDIs. An oral transporter probe cocktail helped elucidate potential transporter-mediated NPDIs. Goldenseal unexpectedly decreased metformin systemic exposure. The observed pharmacokinetic changes suggested goldenseal altered processes involved in metformin intestinal absorption.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ Results from this translational study prompt refinement of current models to improve *in vitro-in vivo* prediction accuracy of transporter-mediated NPDIs, which will contribute to establishing a standard approach for studying these complex interactions. The observed pharmacokinetic changes raise concern regarding goldenseal supplementation with standard antidiabetic pharmacotherapies, thus co-consuming goldenseal with metformin is not recommended.

[1]Department of Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Washington State University, Spokane, Washington, USA; [2]Center of Excellence for Natural Product Drug Interaction Research, Spokane, Washington, USA; [3]Department of Pharmaceutics, School of Pharmacy, University of Washington, Seattle, Washington, USA; [4]SOLVO Biotechnology, SZTE Biológiai Epület, University of Szeged, Szeged, Hungary; [5]Department of Chemistry and Biochemistry, University of North Carolina at Greensboro, Greensboro, North Carolina, USA; [6]Elson S. Floyd College of Medicine, Washington State University, Spokane, Washington, USA; [7]Department of Pharmacotherapy, College of Pharmacy and Pharmaceutical Sciences, Washington State University, Spokane, Washington, USA; [8]Department of Biomedical Informatics, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [9]Present address: Drug Disposition, Eli Lilly and Company, Indianapolis, Indiana, USA; [10]Present address: Department of Veterinary and Biomedical Sciences, College of Agricultural Science, Penn State University, University Park, Pennsylvania, USA. *Correspondence: Mary F. Paine (mary.paine@wsu.edu)

Botanical natural products (NPs), including herbal supplements, constitute a multibillion-dollar industry that has more than doubled in market sales since passage of the Dietary and Supplement Health Education Act in 1994.[1] Supplements containing goldenseal (*Hydrastis canadensis* L. (Ranunculaceae)), a perennial herb native to North America, have consistently ranked among the top 20 highest selling NPs during the last decade.[2] Goldenseal products are marketed as licensed natural health products in Canada and as dietary supplements in the United States.[3] NPs made from dried roots of the goldenseal plant are purported to have therapeutic value and are used to self-treat a range of medical complications, including the common cold, allergic rhinitis, and digestive disorders, such as diarrhea and constipation.[4,5] As more patients continue to seek goldenseal and other NPs to self-treat their medical conditions, there is an increasing need to characterize their safety profiles, especially when co-consumed with prescribed medications, which can lead to adverse NP-drug interactions (NPDIs).

The potential for goldenseal to precipitate clinically relevant pharmacokinetic NPDIs has long been suspected.[6,7] Mechanisms underlying these interactions have been attributed to inhibition of the cytochromes P450 (CYPs).[8] Clinical studies conducted by Gurley and colleagues have since confirmed that a goldenseal extract (900 mg) administered thrice daily for 28 days inhibited CYP2D6 and CYP3A activity by ~ 40%.[9–11] Regarding the effects of goldenseal on transporters, only one clinical study has been reported, which showed minimal effects on the pharmacokinetics of the P-glycoprotein (P-gp) probe drug digoxin after administration of a goldenseal extract (1.3 g) to healthy volunteers thrice daily for 14 days.[12] These observations contrast with an earlier *in vitro* study demonstrating that a major alkaloid in goldenseal, berberine, upregulated P-gp function in the human intestinal cell line Caco-2[13] and with a later *in vitro* study demonstrating goldenseal extracts and berberine to increase P-gp ATPase activity in a baculovirus system expressing P-gp.[14] The effects of goldenseal on the function of other clinically relevant transporters have not been evaluated. Likewise, the effects of berberine and the abundant alkaloids (−)-β-hydrastine and hydrastinine, all of which are time-dependent inhibitors of CYP3A and/or CYP2D6,[15] have not been tested on transporter function.

Numerous validated probe drug cocktails for CYPs are widely used to assess the drug interaction potential of new chemical entities. Validated cocktails covering a range of clinically relevant transporters that contain probe drug substrates for multiple uptake (OATs, OATPs, OCT1) and efflux (BCRP, MATEs, P-gp) transporters are emerging (**Table S1**). Selection of a given transporter cocktail, ideally guided by *in vitro* data, allows for rapid and robust clinical assessment of potential xenobiotic-drug interactions.

Based on the knowledge gaps in transporter-mediated NPDIs, the objective of this work was to assess the effects of a model NP, goldenseal, on clinically relevant transporters using an *in vitro-in vivo* extrapolation approach. The aims were to (1) test the goldenseal alkaloids (berberine, (−)-β-hydrastine, and hydrastinine) and an extract prepared from a well-characterized goldenseal product as inhibitors of multiple transporters using established *in vitro* systems, (2) use recommended basic models to predict potential transporter-mediated interactions involving the goldenseal product, and (3) evaluate the effects of the goldenseal product on the pharmacokinetics of the probe substrates furosemide (OAT1/3), metformin (OCT1/2 and MATE1/2-K), rosuvastatin (OATP1B1/3 and BCRP), and midazolam (CYP3A; positive control) administered as a cocktail to healthy volunteers. Results from this comprehensive study provide a foundation to improve *in vitro-in vivo* prediction accuracy of transporter-mediated NPDIs.

METHODS
Chemicals and reagents

Goldenseal product selection and preparation. The goldenseal product (500 mg root extract capsules; Now Foods, Bloomingdale, IL) was chosen from a set of 35 commercial goldenseal supplements that were selected based on their popularity among US consumers (determined from Amazon sales data). The number of products selected was consistent with recommended approaches for research studies with botanical NPs.[16] One representative sample for the *in vitro* and clinical studies was selected from among the 35 products based on extensive analysis as described.[17] Liquid chromatography-mass spectrometry (LC-MS) metabolomics profiling ensured that the specific product chosen (arbitrarily coded as GS-16) was authentic *Hydrastis canadensis* (*H. canadensis*).[18] Composite scores analysis was used to compare the chemical composition of all 35 commercial *H. canadensis* samples, and GS-16 (dried *H. canadensis* root material) was chosen based on its similarity to reference standards and other commercially available goldenseal supplements.[19] LC-MS data comparing GS-16 to other commercial goldenseal products are freely accessible in the Center of Excellence for Natural Product Drug Interaction Research data repository.[20]

To prepare the extract from the goldenseal product, 200 mg of the goldenseal root material was removed from the capsule and combined with 20 mL methanol in a 20 mL scintillation vial. Mixtures were shaken overnight at room temperature, filtered, dried under a stream of nitrogen, and the resulting extract was stored at room temperature until analysis. Based on the calculated concentration of berberine (0.1926 ± 0.0060 μmol/mg; **Table S2**), a 10 mM standard stock was prepared by resuspending the extract (51.9 mg/mL) in DMSO. This diluted goldenseal extract containing a known concentration of berberine is referred to as extract "standardized to berberine." Berberine served as a marker for assessing potential transporter-mediated drug interactions of the select goldenseal product because the identity of the constituent(s) in the goldenseal extract responsible for transporter inhibition was unknown.

Alkaloid identification and quantification. Standards of berberine chloride (CAS number 633-65-8) and hydrastinine chloride monohydrate (CAS number 65945-18-8) were purchased from Sigma-Aldrich

(St. Louis, MO). (1R,9S)-(–)-β-Hydrastine (CAS number 118081) was purchased from Cayman Chemical (Ann Arbor, MI). For quantitative analysis by LC-MS, the alkaloid standards were prepared in spectrometric-grade MeOH and diluted in a 2-fold dilution series ranging from 0.1–200 µg/mL before injection and analysis using previously established methods.[17] Alkaloid concentrations were determined by $1/x^2$ weighted least-squares linear regression (**Table S2**).

Clinical study probe substrates. Furosemide (10 mg/mL solution; West-ward Pharmaceuticals, Eatontown, NJ), metformin (500 mg/5 mL solution; Ranbaxy Pharmaceuticals, Jacksonville, FL), rosuvastatin (10 mg tablet; Camber Pharmaceuticals, Piscataway, NJ), and midazolam (2 mg/mL syrup; West-ward Pharmaceuticals) were obtained from a local pharmacy.

In vitro transporter inhibition assays

Solubility of the test articles in buffer. The solubility of each test article (berberine, (–)-β-hydrastine, hydrastinine, and goldenseal extract) in relevant buffer (transporter, Krebs-Henseleit, or Hank's Balanced Salt Solution) was assessed to determine the appropriate concentration range for conducting the inhibition assays. In brief, a stock solution of each test article (extract standardized to berberine) was prepared in DMSO and diluted in a five-step, twofold dilution series to achieve five individual concentrations. Each test article was mixed with appropriate buffer in a 96-well plate to achieve the desired final concentrations (**Table S3**) and incubated at 37°C (or 32°C for BCRP and P-gp due to temperature compatibility) for 10–15 minutes. Final organic solvent concentration in the incubation mixture did not exceed 1.5% (v/v). Each solution was evaluated by optical microscopy to verify appropriate solubility necessary for transport inhibition assays.

Test articles as inhibitors of transporters. Inside-out membrane vesicles prepared from HEK293 cells overexpressing a single transporter (vesicular transport inhibition assay) or cell lines (HEK293 and MDCKII) stably expressing a single transporter (uptake transporter inhibition) were used to determine the inhibitory effects of each test article on the designated transporter as described[21–23] using established probe substrates and inhibitors (**Table S4**). Each alkaloid was tested at 10 and 100 µM, whereas the goldenseal extract, standardized to berberine concentrations (detailed above), was tested at 1.75 µM and 17.5 µM (based on solubility limits). Inhibition by each test article was then compared against reference inhibitors and negative control cell lines. If ≥ 50% inhibition was observed, the concentration that corresponded to 50% inhibition (IC_{50}) was determined using varying concentrations for each goldenseal constituent/transporter combination (**Table S5**) and recovered using Prism 7.0 (GraphPad, San Diego, CA). Each concentration of test article, reference inhibitor, and solvent control for a given transporter were tested in triplicate.

In vitro-in vivo prediction of goldenseal-drug interactions

Whether co-administration of the goldenseal product with the transporter probe cocktail would result in a clinical NPDI was predicted using basic models described per the US Food and Drug Administration (FDA) guidance.[24] The models were populated with IC_{50} values obtained from the goldenseal extract, which was standardized to berberine (detailed above), and estimated maximum berberine concentrations at relevant sites (i.e., intestinal lumen, inlet to the liver, and systemic plasma).

Clinical study

Clinical protocol and study participants. The clinical protocol was approved by the Washington State University institutional review board (IRB #16620), and the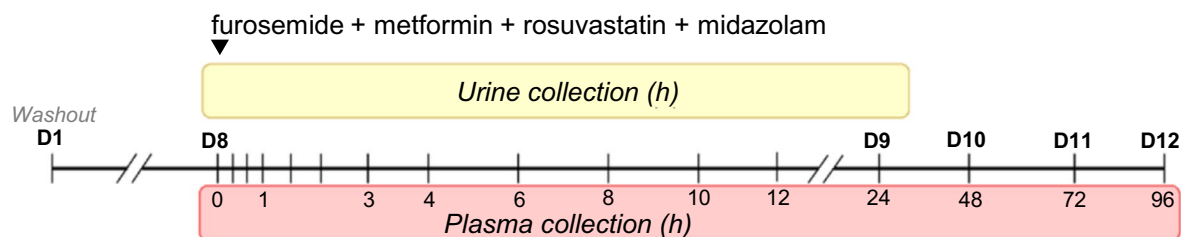 study was conducted at the Washington State University Clinical Research Unit on the Health Sciences Campus in accordance with the Code of Federal Regulations on the Protection of Human Subjects (45 CFR Part 46). The study is registered with the ClinicalTrials.gov database (NCT03772262). Healthy adult volunteers underwent screening that consisted of medical history, physical examination, and clinical laboratory testing (complete blood count with differentials and platelets, complete metabolic panel, and urinalysis) to determine eligibility; all women underwent serum pregnancy testing prior to enrollment and inpatient study visits. Participation eligibility was based on the screening evaluation and inclusion/exclusion criteria (**Table S6**). Written informed consent and Health Insurance Portability and Accountability Act authorization were obtained from all participants prior to screening.

Study design and procedures. Eight men and eight nonpregnant, non-lactating women participated in an open label, two-arm, fixed sequence crossover study (**Figure 1**). During arm 1 (baseline), participants were administered the oral transporter probe cocktail consisting of 1 mg furosemide, 50 mg metformin, 10 mg rosuvastatin, and 2.5 mg midazolam. During arm 2 (goldenseal exposure), participants were administered 1 g goldenseal thrice daily, evenly spaced during the 8-hour work interval (~ 8:00 AM, 12:00 PM, and 4:00 PM), for 5 consecutive days. On the morning of day 6, subjects were administered the oral probe cocktail with 1 g goldenseal; an additional 2 doses of goldenseal (1 g) followed, in 4-hour intervals. Blood (8 mL) was collected into BD K_3 EDTA-containing vacutainer collection tubes (Fisher Scientific, Pittsburg, PA) from an arm vein via an indwelling venous catheter before and 0.33–12 hours post-cocktail administration. Participants returned for single venous blood draws at 24, 48, 72, and 96 hours after cocktail administration. Urine was collected from 0–12 hours. Upon discharge, subjects were instructed to collect their urine until returning for the 24-hour blood draw; vital signs (blood pressure, oxygen saturation, and pulse) were recorded prior to oral cocktail administration and during every outpatient visit.

Bioanalytical methods. Plasma was isolated from whole blood via centrifugation (1,600 g × 10 minutes), and 0.5 mL was transferred to a cryovial containing ammonium acetate (1 M, pH 5, 25 µL) to stabilize rosuvastatin lactone; the remaining plasma was transferred to a second cryovial. Samples were stored at −80°C pending analysis for furosemide, metformin, rosuvastatin acid, rosuvastatin lactone, and midazolam by ultra-high-performance liquid chromatography-tandem accurate mass spectrometry (**Table S7**). Total urine volume and weight from 0–12 and 12–24 hours were recorded, and 2 aliquots (~ 5 mL) for each period were stored in 5-mL conical tubes at −80°C pending ultra-high-performance liquid chromatography-tandem accurate mass spectrometry analysis, allowing for robust calculation of analytes excreted in the urine.

Pharmacokinetic analysis. The pharmacokinetics of each analyte were determined via noncompartmental analysis using Phoenix WinNonlin version 7.0 (Certara, Princeton, NJ). Maximum concentration (C_{max}) and time to C_{max} (T_{max}) were recovered directly from the plasma concentration-time profile. Terminal slope (λ_z) was extrapolated via linear regression of at least three of the last data points. Terminal half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$ when the following criteria were satisfied: the plasma concentration time curve spanned at least three $t_{1/2}$, and the regression about λ_z (r^2) was ≥ 85%. Area under the plasma concentration-time curve (AUC) from time zero to the time of the last measurable concentration (C_{last}) was determined using the trapezoidal method with linear up/log down interpolation. Total AUC from zero to infinity (AUC_{0-inf}) was calculated as the sum of AUC_{0-last} and C_{last}/λ_z. Oral clearance (Cl/F) was calculated as the ratio of the administered dose to AUC_{0-inf}. Renal clearance (Cl_R) was calculated as the ratio of the mass of analyte recovered in the urine from time zero to the time of C_{last} to AUC_{0-last}.

Baseline

furosemide + metformin + rosuvastatin + midazolam

Goldenseal exposure

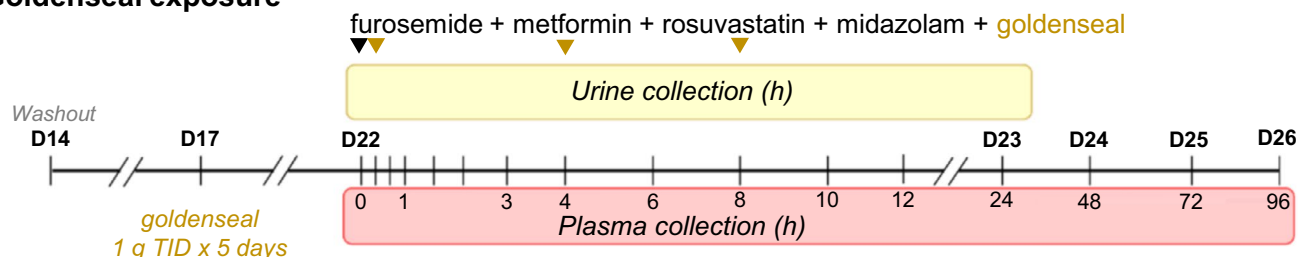furosemide + metformin + rosuvastatin + midazolam + goldenseal

Figure 1 Healthy adult volunteers were enrolled in a two-arm, open-label, fixed sequence, crossover study. Arm 1 (baseline) entailed administration of a transporter probe cocktail containing 1 mg furosemide, 50 mg metformin, 10 mg rosuvastatin, and 2.5 mg midazolam (black inverted triangle). A minimum of 9 days of washout ensured no residual analytes. Arm 2 (goldenseal exposure) entailed administration of 1 g goldenseal thrice daily for 5 consecutive days to ensure berberine reached steady state (terminal half-life ($t_{1/2}$) 28 hours).[49] On day 6 of arm 2, participants were administered the oral probe cocktail with 1 g goldenseal; 2 additional doses of goldenseal (1 g) were administered in 4-hour increments (gold inverted triangles). During both arms, plasma was collected at designated times (shaded in red) up to 96 hours post-cocktail administration; urine was collected in 12-hour intervals (0–12 and 12–24; shaded in yellow) immediately following cocktail administration.

Statistical and power analyses. The sample size of 16 evaluable subjects was based on an 80% power to detect a 20% change in the primary end point (geometric mean ratio of midazolam AUC_{0-inf} in the presence to absence of goldenseal) with a type I error of 0.05, assuming a 25% intra-individual variability in midazolam AUC.[25] As recommended by FDA guidance,[26] the two one-sided testing procedure was used to determine whether a pharmacokinetic interaction was evident, with a predefined no effect range of 0.80–1.25. Secondary pharmacokinetic end points included AUC_{0-last}, $t_{1/2}$, C_{max}, and Cl_R for midazolam and, when recoverable, AUC_{0-inf}, AUC_{0-last}, $t_{1/2}$, C_{max}, and Cl_R for the remaining probe drugs. Secondary end points were evaluated using a paired Student's t-test or Wilcoxon signed-rank test when appropriate; a P value < 0.05 was considered statistically significant. Statistical Analysis System STAT version 14.3 (SAS, Cary, NC) was used for all statistical analysis procedures.

RESULTS
Inhibition assays and *in vitro-in vivo* predictions
The efflux transporters MATE1, MATE2-K, and BCRP were the most sensitive to inhibition by the goldenseal extract, with IC_{50} values < 1 µM (**Table 1**). The uptake transporters OAT1, OAT3, OCT1, OCT2, and OATP1B1/3 were the next most sensitive, with IC_{50} values of 1–12 µM. MATE1 and MATE2-K were sensitive to inhibition by berberine (IC_{50} < 1 µM), and OCT1 was sensitive to inhibition by β-hydrastine (IC_{50} < 10 µM), whereas hydrastinine had negligible inhibitory effects (IC_{50} ≥ 80 µM) against all transporters tested. Using current FDA-recommended basic models,[24] the goldenseal product was predicted to inhibit the intestinal efflux transporter BCRP and the hepatic uptake transporters OATP1B1 and OATP1B3 (**Table 2**).

Clinical study

Participants, safety, and tolerability. Of the 29 subjects screened, 19 were enrolled in the study, and 16 completed both arms. One male subject was discontinued due to poor venous access and was replaced with another eligible male subject. Participants who completed the study self-identified as white (7 men and 6 women) or Asian (1 man and 2 women). The median (range) age was 27 (23–42) and 26 (23–35) for men and women, respectively. None of the participants reported taking concomitant medications or NPs known to modulate the metabolism of midazolam and disposition of the remaining probe drugs. The goldenseal product and probe drugs were well-tolerated. No subject experienced a severe adverse event. Three subjects experienced an adverse event during at least one arm (abdominal cramping or intravenous site bruising) that did not result in study discontinuation.

Pharmacokinetics. As anticipated, midazolam systemic exposure increased in the presence of goldenseal (**Figure 2a**). The geometric mean ratio of the primary end point, midazolam AUC_{0-inf}, lay outside the predefined no effect range in the presence of goldenseal (1.43 (1.35–1.53); **Figure 3**, **Table 3**). The geometric mean C_{max} increased by 31%. The significant increases in Cl_R and $t_{1/2}$ were not clinically relevant because urinary excretion is a minor elimination pathway for midazolam ($Cl_R \sim 0.05$ L/h), and the observed change in $t_{1/2}$ was within the no effect range (0.80–1.25). Plasma concentrations of furosemide were quantifiable for up to 2–6 hours post-cocktail administration in the absence and presence

Table 1 Inhibitory effects of key goldenseal alkaloids and goldenseal extract against selected transporters

Transporter	Berberine	(−)-β-Hydrastine	Hydrastinine	Goldenseal extract[a]
		IC$_{50}$ (µM)		
NTCP	–	–	–	–
OAT1			–	7.3 [1.9–28]
OAT3	79 [47–134]	203 [169–243]	–	4.6 [2.6–8.0]
OATP1B1	–	120 [85–165]	–	8.0 [6.8–9.5]
OATP1B3	–		–	1.3 [0.5–3.7]
OATP2B1	–	–	–	
OCT1	19 [11–31]	6.6 [5.3–8.1]		2.6 [1.4–4.7]
OCT2	45 [17–117]	90 [38–214]	95 [70–129]	12 [5.6–25]
BSEP	–	–	–	–
BCRP	180 [53–624]	86.2 [19–391]	–	0.59 [0.43–0.78]
MATE1	0.73 [0.37–1.44]	110 [90–131]	82 [44–154]	0.45 [0.27–0.76]
MATE2-K	0.77 [0.41–1.48]		–	0.47 [0.24–0.92]
MRP2	–	–	–	–
MRP3	–	–	–	–
P-gp	–	–	–	–

The goldenseal alkaloids berberine, (−)-β-hydrastine, and hydrastinine were screened as inhibitors of each transporters at 10 and 100 µM; the extract was tested at 1.75 and 17.5 µM (based on berberine concentration and solubility limit). –, inhibition not observed; ▢, 20–50% inhibition; ▢, 50–70% inhibition; or ▢, >70% inhibition at the highest concentration tested. Subsequently, IC$_{50}$ values were determined for test articles demonstrating ≥ 50% inhibition. Values denote mean IC$_{50}$ [95% confidence interval] of three technical replicates.
BCRP, breast cancer resistant protein; BSEP, bile salt export pump; IC$_{50}$, half-maximal inhibitory concentration; MATE, multidrug and toxin extrusion protein; MRP, multidrug resistance-associated protein; NTCP, sodium/taurocholate co-transporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; P-gp, P-glycoprotein.
[a]Based on the concentration of berberine quantified in the extract.

Table 2 *In vitro-in vivo* predictions of transporter-mediated goldenseal-drug interactions using established basic models for clinically relevant transporters per FDA guidance

Transporter	Basic model[a]	Goldenseal extract R-value	Predicted effect
BCRP	$R = \frac{I_{gut}}{IC_{50}} \geq 10$	601	↑ rosuvastatin AUC
MDR1		–	No interaction
OAT1	$R = \frac{I_{max,u}}{IC_{50}} \geq 0.1$	< 0.001	No interaction
OAT3		< 0.001	No interaction
OCT2		< 0.001	No interaction
MATE1		0.002	No interaction
MATE2-K		0.002	No interaction
OATP1B1	$R = 1 + \frac{f_{u,p} \times I_{in,max}}{IC_{50}} \geq 1.1$	1.5	↑ rosuvastatin AUC
OATP1B3		4.2	↑ rosuvastatin AUC

$f_{u,p}$, fraction unbound of inhibitor (berberine) in plasma, estimated to be 0.75 using GastroPlus version 9.7 (Simulations Plus, Lancaster, CA). I_{gut}, intestinal luminal concentration of inhibitor, estimated as the ratio of the mass of berberine (29.8 mg) in a 1-gram capsule of goldenseal to 250 mL. $I_{max,u}$, maximum unbound plasma concentration of berberine at steady state, estimated as the product of $f_{u,p}$ and the maximum reported plasma concentration of berberine (0.44 ng/mL),[49] adjusted to dose (0.033 ng/mL), and scaling to 0.20 ng/mL (0.59 nM) based on an accumulation ratio of 5.95, calculated using the Eq. 1/ (1-e^{-k*T}), where k and T are the elimination rate constant[32] (0.023/hour) and dosing interval (8 hours), respectively. For simplicity, a dosing interval of 8 hours was used. $I_{in,max}$, estimated maximum plasma concentration of inhibitor (berberine) at the inlet of the liver, calculated as $I_{max} + (F_a \times F_g, \times k_a \times dose)/(Q_h/R_B)$, where F_a is the fraction of berberine absorbed after oral administration of a 1-gram capsule of goldenseal, F_g is the fraction of berberine that escapes intestinal metabolism, k_a is the first-order absorption rate constant for berberine, Q_h is hepatic blood flow (97 L/hr),[50] and R_B is the blood-to-plasma concentration ratio. Because the data were not available, F_a, F_g, and R_b were set to 1, and k_a was set to 0.1/minute per FDA guidance.[24] Light green indicates an interaction with the goldenseal product is predicted, whereas light red indicates an interaction is not predicted.
–, No interaction predicted based on low inhibition potential in transporter assay (IC$_{50}$ > 100 µM); AUC, area under the plasma concentration-time curve; FDA, US Food and Drug Administration.
[a]IC$_{50}$ values for the goldenseal extract, depicted in **Table 1**, were used in basic models.

of goldenseal. Because the descending concentrations did not meet the predefined criteria to obtain a robust estimate of λ_z for more than half of the subjects, AUC$_{0–inf}$, Cl/F, and terminal $t_{1/2}$ for

furosemide could not be determined. Goldenseal had no effect on the remaining evaluable pharmacokinetic end points (**Figure 2b**). The geometric mean AUC$_{inf}$ and C$_{max}$ of metformin decreased by
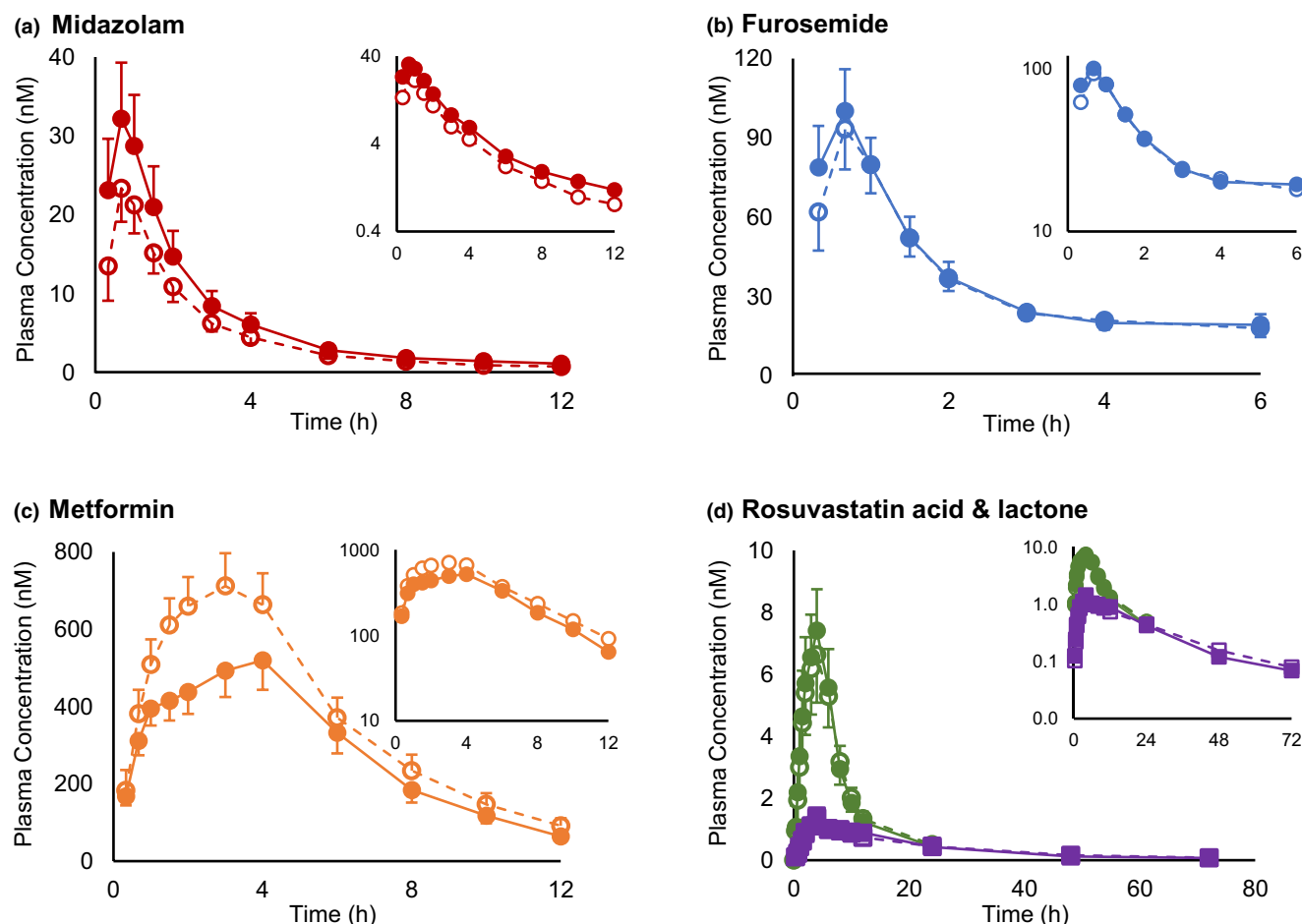
Figure 2 Plasma concentration vs. time profiles for (**a**) midazolam, (**b**) furosemide, (**c**) metformin, and (**d**) rosuvastatin acid (●) and rosuvastatin lactone (■) following oral administration to 16 healthy volunteers alone (open symbols) or upon five-day exposure to oral goldenseal (solid symbols). Symbols and error bars denote geometric means and 90% confidence intervals, respectively.

23% and 27%, respectively, in the presence of goldenseal, whereas Cl_R and $t_{1/2}$ were unaffected (**Figure 2c**). The AUC_{0-last} and Cl_R of both rosuvastatin acid and rosuvastatin lactone were unaffected by goldenseal (**Figure 2d**). The AUC_{inf}, Cl/F, and $t_{1/2}$ for both rosuvastatin acid and rosuvastatin lactone could not be determined based on the inability to obtain a robust λ_z.

DISCUSSION

This work represents the first comprehensive *in vitro-in vivo* assessment of a botanical NP to precipitate transporter-mediated pharmacokinetic drug interactions. Using goldenseal as a model NP, the following three-step approach was taken: (i) the alkaloids berberine, (−)-β-hydrastine, and hydrastinine and an extract prepared from a well-characterized product[17] were tested as inhibitors of clinically relevant transporters using established *in vitro* systems, (ii) basic models were populated with the resulting extract IC_{50} values and other relevant parameters to predict the likelihood of a clinical interaction, and (iii) model predictions were evaluated via a powered clinical study involving a transporter probe cocktail.

Consistent with previous clinical observations, goldenseal increased systemic exposure to the positive control probe substrate

midazolam by ~ 40%.[10] The negligible change in $t_{1/2}$ further indicated that goldenseal inhibited CYP3A primarily in the intestine. Goldenseal constituents, including the extensively studied major alkaloids berberine and (−)-β-hydrastine, are known time-dependent inhibitors of CYP3A,[15] suggesting goldenseal could have prolonged inhibitory effects *in vivo* similar to grapefruit juice.

Prior to *in vitro* testing, the goldenseal extract was standardized to contain a fixed concentration of berberine based on typical high abundance in commercial goldenseal products,[17] including the product used in the current work (**Table S2**), and documented modulatory, albeit inconsistent, effects on transporter activity (i.e., P-gp).[13,14] Compared with berberine and the other tested alkaloids, (−)-β-hydrastine and hydrastinine, the extract showed the strongest inhibition toward all transporters examined (**Table 1**), indicating that other uncharacterized constituents in the goldenseal product contributed to the observed effects of the extract or that one or more constituents act synergistically. A systematic analysis using traditional bioassay-guided fractionation or biochemometric approaches of inhibitory activity of goldenseal extracts, fractions, and isolated constituents are needed to identify these constituents.[27,28] Nevertheless, with appropriate assumptions (detailed below), the goldenseal extract
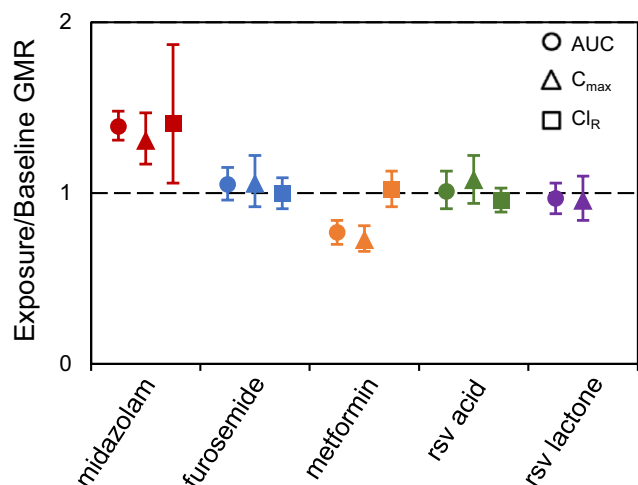
Figure 3 Goldenseal exposure-to-baseline geometric mean ratios (GMRs), with 90% confidence intervals, of the area under the plasma concentration-time curve (AUC), maximum concentration (C_{max}), and renal clearance (Cl_R) for each probe substrate. The AUC ratios for furosemide, rosuvastatin (rsv) acid, and rsv lactone were calculated using plasma concentration-time curves up to the last quantifiable time point (AUC_{0-last}), whereas AUC ratios for midazolam and metformin were calculated using plasma concentration-time curves to time infinity (AUC_{0-inf}). ■ – midazolam; ■ – furosemide; ■ – metformin; ■ – rsv acid; ■ – rsv-lactone. [Correction added on 6 January 2021, after first online publication: The colours defined in figure caption was incorrect and has been corrected in this version.]

IC_{50} values were used for the *in vitro* to *in vivo* predictions of potential berberine-mediated goldenseal-drug interactions.

The goldenseal extract showed relatively potent inhibition of the uptake transporters OAT1 and OAT3 ($4 < IC_{50} < 10 \mu M$; **Table 1**), which are expressed primarily on the basolateral membrane of kidney proximal tubule cells.[29,30] As such, precipitant constituents must overcome both intestinal and hepatic first-pass processes before entering the systemic circulation and reaching the target site. These processes contributed to the low anticipated berberine C_{max} (~ 0.59 nM).[31–33] By using the extract IC_{50} that is standardized to berberine, the assumption is that all other alkaloids and uncharacterized constituents in the goldenseal product have no bioavailability concerns, thus contributing to the increased inhibition potential of the extract compared with single alkaloids. Regardless, a berberine-mediated clinical interaction between the goldenseal product and the OAT1/3 probe furosemide was unlikely, as predicted by the basic model, due to the low systemic concentration of berberine (**Table 2**). Results from the clinical study confirmed these *in vitro-in vivo* predictions, as goldenseal co-administration had no effect on furosemide renal clearance and systemic exposure (**Table 3**, **Figure 3**).

The goldenseal extract showed potent *in vitro* inhibition ($IC_{50} \sim 0.6 \mu M$; **Table 1**) of the apical efflux transporter BCRP, which was unlikely caused by any of the tested alkaloids ($IC_{50} > 80 \mu M$). The basic model predicted a berberine-mediated interaction between intestinal BCRP, located on the luminal membrane of enterocytes, and the goldenseal product (increased rosuvastatin AUC predicted; **Table 2**) because the estimated concentration of berberine in the gut (~ 350 μM) greatly exceeded

the observed IC_{50}. Although BCRP is expressed in other tissues, the intestinal lumen represents the first target and the highest risk for a BCRP-mediated interaction involving orally administered xenobiotics. In addition, the IC_{50} was obtained using inside-out BCRP-expressing vesicles in compatible assay solution, whereas the berberine gut luminal concentration was calculated using the administered dose and intestinal fluid volume, the latter of which assumes complete dissolution of all goldenseal constituents in intestinal fluids. However, in the clinical study, the pharmacokinetics of rosuvastatin acid and lactone were unchanged in the presence of goldenseal. Two potential mechanisms could explain this *in vitro-in vivo* disconnect: (1) incomplete dissolution led to insufficient luminal concentrations, and/or (2) if enterocyte concentrations drive the interaction, low permeability or extensive enterocyte metabolism or efflux would lead to a discordance between luminal and enterocyte concentrations (current basic model incorporates luminal concentration).

Once absorbed into the portal circulation, rosuvastatin is taken up into hepatocytes primarily by OATP1B1 and NTCP and to a minor extent by OATP1B3.[34,35] The goldenseal extract inhibited OATP1B1 (IC_{50}, 8.0 μM) and OATP1B3 (IC_{50}, 1.3 μM) but not NTCP *in vitro*. Similar to BCRP, the inhibitory effects on OATP1B1/3 were unlikely due to any of the tested alkaloids ($IC_{50} \geq 120 \mu M$). Despite the relatively high IC_{50} for OATP1B1, interactions between both transporters and the probe substrate rosuvastatin were predicted (**Table 2**) because, theoretically, the precipitants could reach the target, basolateral membrane of hepatocytes, in high concentrations immediately following intestinal first-pass processes. As with BCRP, these predictions contradicted *in vivo* results, as the pharmacokinetics of both rosuvastatin acid and lactone were unaltered by goldenseal co-administration. This misprediction can be attributed to an overestimated hepatic inlet berberine concentration, which could reflect incomplete or impaired intestinal absorption and/or extensive metabolism in the gut.

The goldenseal extract showed the most potent inhibition against the apical efflux transporters MATE1 and MATE2-K ($IC_{50} \sim 0.5 \mu M$), major mediators of metformin renal excretion, with berberine representing a potential key precipitant ($IC_{50} \sim 0.75 \mu M$). However, based on the low anticipated berberine C_{max}, the basic model predicted no clinical interaction with these transporters in the kidneys (**Table 2**). The goldenseal extract was also a modest inhibitor of the basolateral uptake transporter OCT2 (IC_{50}, 12 μM), localized in kidney proximal tubules,[36] which was unlikely caused by any of the tested alkaloids. As with MATE1/2-K, the basic model predicted no interaction with OCT2, again due to the low anticipated berberine C_{max}. The clinical study demonstrated successful *in vitro-in vivo* predictions, as co-administration of the goldenseal extract had no effect on metformin Cl_R.

Although metformin Cl_R was unaffected by the goldenseal product, metformin AUC_{inf} and C_{max} were significantly reduced by 23–27% (**Table 3**). These observations, coupled with no change in half-life, suggested that goldenseal decreased metformin oral bioavailability by altering intestinal permeability, transport, and/or other processes involved in metformin absorption. Multiple uptake transporters contribute to the intestinal absorption of metformin, including OCT1/3, ThTR-2, PMAT, and SERT (**Figure 4**).[37–39] OCT3,

Table 3 Pharmacokinetics of furosemide, metformin, rosuvastatin acid, rosuvastatin lactone, and midazolam ($n = 16$ subjects) in the absence and presence of goldenseal co-administration

	Geometric mean (90% confidence interval)		
	Baseline	Goldenseal exposure	Goldenseal exposure/baseline ratio
Furosemide			
$AUC_{0\text{-inf}}$ (µM*h)	NC	NC	NC
$AUC_{0\text{-6}}$ (µM*h)	0.17 [0.14–0.20]	0.18 [0.15–0.20]	1.05 [0.96–1.15]
$t_{1/2}$ (h)	NC	NC	NC
C_{max} (µM)	0.10 [0.09–0.12]	0.11 [0.10–0.12]	1.06 [0.92–1.22]
Cl_R (L/h)	7.3 [6.3–8.6]	7.3 [6.4–8.3]	1.00 [0.91–1.09]
Metformin			
$AUC_{0\text{-inf}}$ (µM*h)	5.0 [4.5–5.6]	3.8 [3.3–4.4][a]	0.77 [0.71–0.83]
$AUC_{0\text{-24h}}$ (µM*h)	4.7 [4.2–5.3]	3.6 [3.1–4.2][a]	0.77 [0.70–0.84]
$t_{1/2}$ (h)	3.2 [2.9–3.5]	3.0 [2.6–3.5]	0.94 [0.83–1.06]
C_{max} (µM)	0.75 [0.67–0.82]	0.54 [0.48–0.62][a]	0.73 [0.66–0.81]
Cl_R (L/h)	41 [36–47]	42 [38–47]	1.02 [0.92–1.13]
Rosuvastatin acid			
$AUC_{0\text{-inf}}$ (nM*h)	NC	NC	NC
$AUC_{0\text{-24}}$ (nM*h)	59 [48–72]	60 [51–70]	1.01 [0.91–1.13]
$t_{1/2}$ (h)	NC	NC	NC
C_{max} (nM)	7.4 [5.9–9.1]	7.9 [6.7–9.3]	1.08 [0.94–1.22]
Cl_R (L/h)	15.4 [13.8–17.2]	14.8 [13.6–16.0]	0.96 [0.89–1.03]
Rosuvastatin lactone			
$AUC_{0\text{-inf}}$ (nM*h)	NC	NC	NC
$AUC_{0\text{-72}}$ (nM*h)	28 [24–32]	27 [23–32]	0.97 [0.88–1.06]
$t_{1/2}$ (h)	NC	NC	NC
C_{max} (nM)	1.5 [1.3–1.8]	1.5 [1.3–1.7]	0.96 [0.84–1.10]
Cl_R (L/h)	NC	NC	NC
Midazolam			
$AUC_{0\text{-inf}}$ (nM*h)	67 [55–82]	96 [78–119][a]	1.43 [1.35–1.53]
$AUC_{0\text{-12}}$ (nM*h)	62 [51–74]	86 [70–105][a]	1.39 [1.31–1.48]
$t_{1/2}$ (h)	3.9 [3.4–4.6]	4.8 [4.2–5.6][a]	1.23 [1.07–1.42]
C_{max} (nM)	26 [22–31]	35 [28–42][a]	1.31 [1.17–1.47]
Cl_R (L/h)	0.05 [0.03–0.07]	0.07 [0.05–0.09][a]	1.47 [1.14–1.88]

Colors correspond with those shown in **Figures 2** and **3**.
$AUC_{0\text{-inf}}$, area under the plasma-concentration time curve from time zero to infinity; $AUC_{0\text{-last}}$, area under the plasma-concentration time curve from time zero to the time of the last measured concentration; $t_{1/2}$, terminal half-life; C_{max}, maximum plasma concentration; Cl_R, renal clearance. NC, not calculable based on inability to obtain robust estimate of terminal slope.
[a]$P < 0.05$ compared to baseline.

ThTR-2, PMAT, and SERT are expressed on the luminal membrane, whereas the reported localization of OCT1 is conflicting.[40–42] An interaction with one or more of these transporters could explain the observed decrease in metformin systemic exposure, and follow-up studies are ongoing to address these potential mechanisms.

The goldenseal extract was a relatively potent inhibitor of OCT1 (IC_{50}, 2.6 µM). Because there is currently no recommended basic model for predicting OCT1-mediated drug interactions, a standard *in vitro-in vivo* prediction was not conducted. However, if OCT1 is localized on the luminal membrane of enterocytes, inhibition of uptake could explain the observed goldenseal-metformin interaction, as the estimated

luminal concentration of berberine was ~ 100× higher than the IC_{50}. This theory is further supported by applying the current basic model for other gut luminal membrane transporters (i.e., BCRP and P-gp) to OCT1, which would predict an interaction (R-value, ~ 125). In contrast to BCRP, the basic model would successfully predict an interaction for OCT1, suggesting that intestinal luminal concentrations drive apical uptake, whereas enterocyte concentrations drive apical/basolateral efflux. Based on the multiple dose design of this clinical study and the bi-directionality of OCTs localized in enterocytes, other potential mechanisms that could explain the decreased metformin exposure include induction of OCT1/3 efflux on the luminal
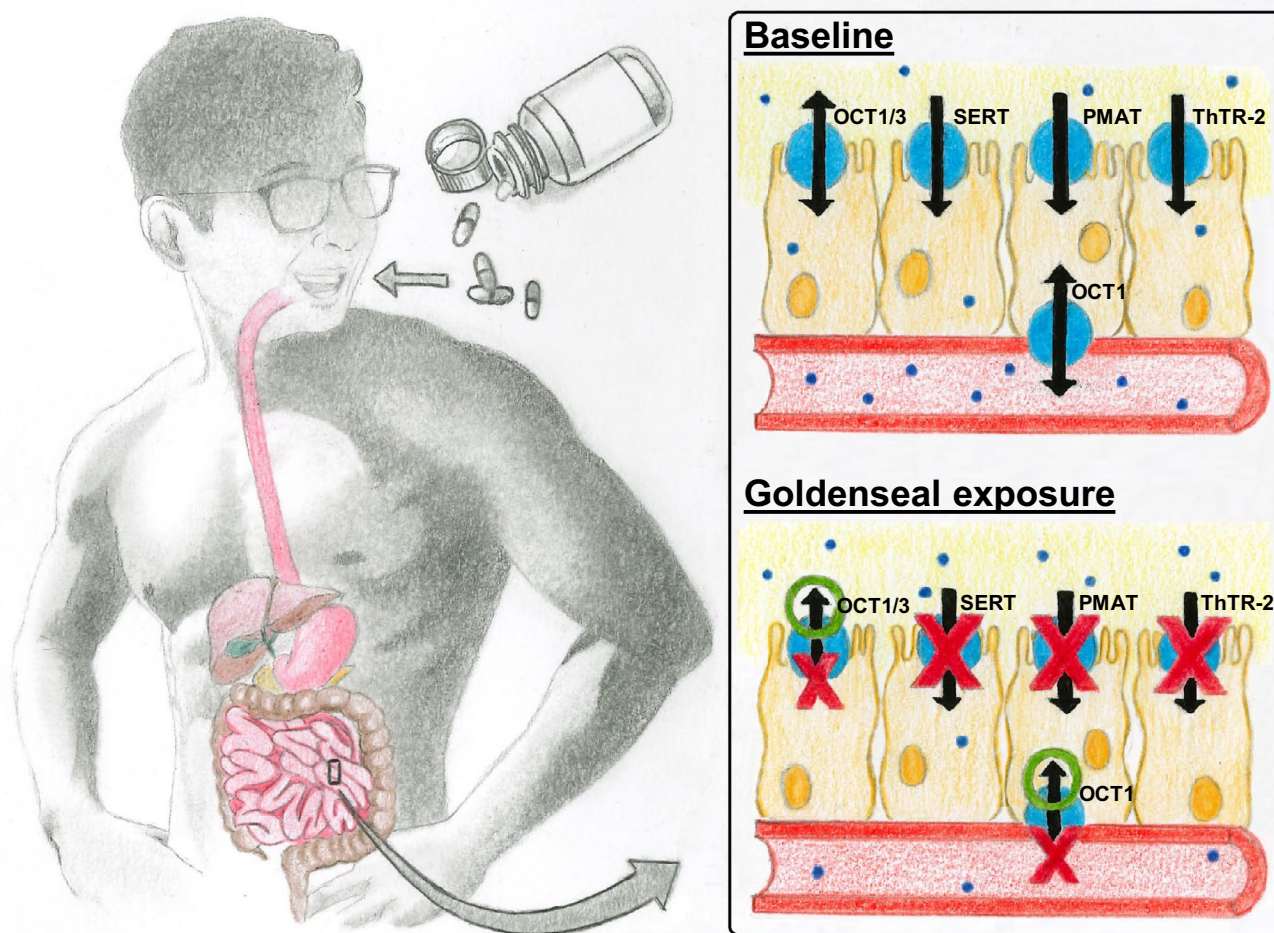
Figure 4 Proposed transporter-mediated mechanism underlying the decreased metformin systemic exposure upon goldenseal exposure. The indicated transporters have been reported to facilitate intestinal absorption of metformin.[37–42] Arrows represent uptake or efflux; double-headed arrows represent bidirectional transport. Red Xs represent inhibition of indicated transporters by goldenseal; green Os represent induction of indicated transporters by goldenseal. Solid blue circles represent metformin molecules.

membrane, induction of OCT1-mediated uptake on the basolateral membrane, or inhibition of OCT1-mediated efflux on the basolateral membrane (**Figure 4**). However, because goldenseal was administered orally, the high luminal to basolateral concentration gradient would favor modulation of net uptake, suggesting that the observed clinical results (decreased AUC with no change in half-life) were largely due to inhibition of luminal uptake. Additional studies are needed to determine the precise mechanism underlying the observed pharmacokinetic effects.

The current work demonstrated a comprehensive *in vitro-in vivo* approach involving established transporter inhibition assays, recommended basic models, and clinical pharmacokinetic evaluation to elucidate transporter-mediated goldenseal-drug interactions. Although several nuances regarding the basic models have been addressed, there are additional limitations to consider. First, as mentioned earlier, a confounding factor with the *in vitro* assays was that the IC_{50} of a single constituent was not always predictive of the goldenseal extract, suggesting that an uncharacterized constituent(s) in the extract contributed to the inhibitory effects. Second, disconnects between basic model predictions and pharmacokinetic outcomes for some, but not all, of the tested transporters were observed. As noted in a recent publication, refining the relevant models to include potential degradation and metabolism of the NP constituent in the gut could improve prediction accuracy.[43] Such refinement may distinguish the false positive prediction for BCRP from the successful prediction for P-gp (i.e., no interaction based on an R value < 10 and lack of clinical interaction with digoxin[12]). Physiologically-based pharmacokinetic models could further improve prediction accuracy by accounting for the variable expression of different transporters in relevant tissues. Third, the lack of pharmacokinetic changes for some transporter probes do not rule out the potential for interactions with competing pathways given that goldenseal was administered for multiple consecutive days. For example, hepatic OATP1B1/3 induction could have counteracted intestinal BCRP inhibition, resulting in the lack of change in systemic exposure to rosuvastatin. However, induction of transporters other than P-gp has become a topic of debate that requires further investigation.[44] Finally,

multiple transporters mediate the disposition of current clinical probe substrates. Endogenous transporter biomarkers are emerging, including coproporphyrins for OATP1Bs, which could help pinpoint specific transporters mediating a given interaction.[45,46]

There are potential clinical implications with the experimental observations. Although the decrease in metformin systemic exposure caused by goldenseal co-administration was modest, such a change could impact glucose control in patients with type 2 diabetes. Given that metformin is the most prescribed antidiabetic medication,[47] there are widespread concerns because this large patient population has been reported to self-treat their medical condition with goldenseal, as well as berberine.[48] Accordingly, supplementing a metformin-based pharmacotherapeutic regimen with goldenseal is not recommended for patients with type 2 diabetes.

In summary, the current work represents the first comprehensive translational approach to assess potential transporter-mediated NPDIs. An appropriately designed probe cocktail enabled rapid clinical assessment of the effects of a model NP on multiple transporters. Refinement of current recommended basic models, development of PBPK models, and incorporation of endogenous transporter probes will help establish a standard approach for elucidating transporter-mediated NPDIs. Results will provide additional guidance for consumers and healthcare providers regarding the drug interaction risk, or lack thereof, of goldenseal co-administered with certain drugs.

SUPPORTING INFORMATION
Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

CONFLICT OF INTEREST
The authors declared no competing interest for this work.

AUTHOR CONTRIBUTIONS
J.T.N. and M.F.P. wrote the manuscript. D.D.T., D.L.H., M.H., J.D.U., K.E.T., and M.F.P. designed the research. J.T.N., D.D.T., R.S.T., D.L.H., S.B., J.C.C., C.M.A., L.M.S., B.M., M.H., J.J.K., M.E.L., J.R.W., and N.B.C. performed the research. J.T.N., D.D.T., R.S.T., S.B., J.J.K., N.B.C, C.M.C., L.M.S., R.D.B., J.D.U., K.E.T., and M.F.P. analyzed the data. J.J.K. and N.B.C. contributed new reagents.

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