A Pilot Study to Assess the Suitability of Riboflavin as A Surrogate Marker of Breast Cancer Resistance Protein (BCRP) in Healthy Participants

Hong Shen¹, Runlan Huo², Yueping Zhang¹, Linna Wang², Nian Tong³, Weiqi Chen³, Andrew J. Paris⁴, Kofi Mensah⁴, Min Chen², Yongjun Xue², Wenying Li⁵, Michael Sinz¹

Departments of Drug Metabolism and Pharmacokinetics¹, Clinical Pharmacology, Pharmacometrics and Bioanalysis², Development Biotransformation³, and Early Clinical Development⁴, Bristol Myers Squibb, Route 206 & Province Line Road, Princeton, NJ 08543

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Corresponding author:
Dr Hong Shen
Department of Drug Metabolism and Pharmacokinetics
Bristol Myers Squibb
Room F.3414C, Route 206 & Province Line Road, Princeton, NJ 08543
Tel: 609-252-3095
Facsimile: (609) 252-6802
E-mail: hong.shen1@bms.com
Abbreviations: AUC, area under the plasma concentration-time curve; AUCR, area under the plasma concentration-time curve ratio; BCRP, breast cancer resistance protein; CCK-8, cholecystokinin octapeptide; CI, confidence interval; C\textsubscript{max}, maximum plasma concentration; C\textsubscript{max,u}, unbound maximum plasma concentration; C\textsubscript{max,inlet,u}, predicted unbound maximum plasma liver inlet concentration; CPI, coproporphyrin I; DDI, drug-drug interaction; E3S, estrone-3-sulfate; E17\beta G, estradiol-17\beta-glucuronide; EMA, European Medicines Agency; FDA, the U.S. Food and Drug Administration; GMR, geometric mean ratio; HBSS, Hanks’ balanced salt solution; HEK, human embryonic kidney; IC\textsubscript{50}, concentration required to inhibit transport by 50%; I\textsubscript{gut}, estimated intestinal luminal concentration; IVIVE, in vitro-in vivo extrapolation; LLC-PK1, Lewis-lung cancer porcine kidney 1 cells; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MATE, multidrug and toxin extrusion protein; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; OAT, organic anion transporter; OATP, organic anion-transporting polypeptides; OCT, organic cation transporter; PAH, para-aminohippuric acid; P-gp, P-glycoprotein; PROB, probenecid; PYR, pyrimethamine; R-value, victim AUC in the presence and absence of inhibitor; RFV, riboflavin; RFVT, riboflavin transporter; RIF, rifampin; SD, standard division; T\textsubscript{max}, time to reach maximum plasma concentration.
RUNNING TITLE

Clinical Validity of Riboflavin as a Marker of BCRP
ABSTRACT

We recently showed that riboflavin is a selected substrate of BCRP over P-gp and demonstrated its prediction performance in preclinical DDI studies. The aim of this study was to investigate the suitability of riboflavin to assess BCRP inhibition in humans. First, we assessed the substrate potential of riboflavin towards other major drug transporters using established transfected cell systems. Riboflavin is a substrate for OAT1, OAT3, and MATE2-K with uptake ratios ranging from 2.69 to 11.6 but riboflavin is not a substrate of OATP1B1, OATP1B3, OCT2, and MATE1. The effects of BMS-986371, a potent in vitro inhibitor of BCRP ($IC_{50}$ 0.40 µM), on the pharmacokinetics of riboflavin, isobutyryl carnitine, and arginine were then examined in healthy male adults ($N = 14$ or $16$) following oral administration of methotrexate (MTX) (7.5 mg) and enteric coated (EC) sulfasalazine (SSZ) (1,000 mg) alone or in combination with BMS-986371 (150 mg). Oral administration of BMS-986371 increased the $AUCs$ of rosuvastatin and immediate-release (IR) SSZ to 1.38- and 1.51-fold, respectively, and significantly increased $AUC(0-4h)$, $AUC(0-24h)$, and $C_{max}$ of riboflavin by 1.25-, 1.14-, and 1.11-fold ($P$-values of 0.003, 0.009, and 0.025, respectively) compared to the MTX/SSZ EC alone group. In contrast, BMS-986371 did not significantly influence the $AUC(0-24h)$ and $C_{max}$ values of isobutyryl carnitine and arginine (0.96- to 1.07-fold, respectively; $P > 0.05$). Overall, these data indicate that plasma riboflavin is a promising biomarker of BCRP that may offer a possibility to assess drug candidate as a BCRP modulator in early drug development.
SIGNIFICANCE STATEMENT

Endogenous compounds that serve as biomarkers for clinical inhibition of BCRP are not currently available. This study provides the initial evidence that riboflavin is a promising BCRP biomarker in humans. For the first time, the value of leveraging the substrate of BCRP with acceptable prediction performance in clinical studies is shown. Additional clinical investigations with known BCRP inhibitors are needed to fully validate and showcase the utility of this biomarker.
INTRODUCTION

Intestinal drug absorption and metabolism can be governed by the efflux transporter breast cancer resistance protein (BCRP) in the brush-border (apical) enterocyte membrane (Maliepaard et al., 2001, Fetsch et al., 2006). Additional tissue sites of BCRP expression include endothelial cells of the brain and epithelial cells of the liver, kidney, and reproductive organs (e.g., placenta and lactating breast) (Maliepaard et al., 2001, Fetsch et al., 2006). This membrane transporter also crucially contributes to drug delivery and response when a pharmacological target is in cancer cells, in which BCRP is highly expressed (Natarajan et al., 2012). Variable BCRP activity resulting from perpetrators and generic mutations decisively contribute to interindividual variation in the pharmacokinetics, efficacy, and toxicity of certain drugs. The effect of an altered BCRP function on pharmacokinetics can be exemplified by sulfasalazine (SSZ). In humans, a BCRP variant with significantly decreased activity has been identified (i.e., \textit{ABCG2} c.421 C>A) (Urquhart et al., 2008). Consistently, subjects with \textit{ABCG2} c.421 AA genotype, had an area under the plasma concentration-time curve (\textit{AUC}) of SSZ 2.0- to 3.5-fold greater than those with \textit{ABCG2} c.421 CC (Yamasaki et al., 2008, Adkison et al., 2010, Gotanda et al., 2015). Additionally, clinical drug-drug interaction (DDI) studies indicated that administration of curcumin, pantoprazole and rolapitant, known BCRP inhibitors, resulted in 3.2-, 1.8- and 2.2-fold increases, respectively, in the \textit{AUC} of SSZ compared to the SSZ alone group, (Adkison et al., 2010, Kusuhara et al., 2012, Wang et al., 2018).

Furthermore, it is believed that an interaction at the level of the intestine may mainly contribute to observed SSZ and rosuvastatin DDIs (Taskar et al., 2022). Given the importance of BCRP and to unravel its role in drug therapy, there is an urgent heed to develop novel and feasible tools to monitor BCRP activity in vivo.

For many years, the usual approach to assess BCRP inhibition for an investigational drug has been using a dedicated clinical study with a BCRP drug substrate, such as SSZ and rosuvastatin, when there is potential for transporter inhibition based on in vitro inhibition
and basic static model analyses. Difficulties with this approach include the uncertainty in the translatability of in vitro inhibition data [i.e., concentration required to inhibit transport by 50% (IC\textsubscript{50})] and conservative cut-off values in the mechanistic static model method, resulting in high false-positive rates (i.e., DDI study triggered when estimated intestinal luminal concentration $I_{\text{gut}}/IC_{50} > 10$) (Costales et al., 2021). In the last decade, endogenous biomarkers have emerged as a tool to determine a phenotypic function of specific drug transport pathways in subjects, resulting in improved prediction over simple in vitro to in vivo extrapolation (IVIVE) (Chu et al., 2018, Muller et al., 2018, Rodrigues et al., 2018, Arya et al., 2022). Moreover, endogenous biomarkers have a great potential to assess altered transporter function in diseased and special populations (Mori et al., 2020, Tatosian et al., 2021, Takita et al., 2022, Lin et al., 2023). In the case of BCRP, biomarkers have not yet been discovered and established although it is envisioned that such tools will help predict BCRP-mediated DDI and quantify its contribution to a complex DDI (Chu et al., 2018, Lai et al., 2022, Rodrigues, 2023). The most promising biomarker candidate studied to date in relation to BCRP activity in vivo has been riboflavin that demonstrated prediction performance in animal DDI studies (Zhang et al., 2023).

BMS-986371 (previously CC-99677) is a covalent inhibitor of mitogen-activated protein kinase-activated protein kinase 2 targeted to be a safe and effective oral agent for the treatment of chronic inflammatory diseases, such as rheumatoid arthritis and ankylosing spondylitis (Gaur et al., 2022, Malona et al., 2022). In vitro studies demonstrated that BMS-986371 is an inhibitor of BCRP with an $IC_{50}$ value of 0.40 µM. The concentration of BMS-986371 in the gut was estimated to be sufficient to inhibit BCRP [i.e., $(\text{Dose} / 250 \text{ mL})/IC_{50} = 1277$] as per the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidance. We report the results of two clinical DDI studies here, and in agreement with the prediction, administration of 150-mg of BMS-986371 increased the clinical $AUC$ values of 1000-mg SSZ IR and 7.5-mg methotrexate (MTX), known BCRP substrates, by 1.5- and 1.2-fold respectively.
Herein, we studied the in vivo effects of BMS-986371, a potent BCRP inhibitor, on the pharmacokinetics of riboflavin, isobutyryl carnitine, and arginine as candidate markers for BCRP activity in humans. In addition, we examined the selectivity of riboflavin as a BCRP biomarker by assessing the in vitro transporter phenotyping profile.
MATERIALS AND METHODS

Riboflavin as a Substrate for Uptake Transporters:

The cellular uptake of riboflavin and probe substrates was performed as described previously (Shen et al., 2013a, Shen et al., 2013b, Shen et al., 2016). We utilized a previously-established cell system consisting of human embryonic kidney (HEK)-293 cells expressing human organic anion-transporting polypeptides (OATP) 1B1, OATP1B3, organic anion transporter (OAT) 1, OAT3, organic cation transporter (OCT) 2, multidrug and toxin extrusion protein (MATE) 1, or MATE2-K as well as the respective vector-transfected HEK-Mock control cells (Han et al., 2010a, Han et al., 2010b, Shen et al., 2013b). [3H]Estradiol-17β-D-glucuronide (E17βG), [3H] cholecystokinin octapeptide (CCK-8), [3H]para-aminohippuric acid (PAH), and [3H]estrone-3-sulfate (E3S) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [14C]Metformin (MFM) was purchased from Moravek Biochemicals, Inc. (Brea, CA). All cold compounds were purchased from Sigma-Aldrich (St. Louis, MO) or Toronto Research Chemicals Inc. (North York, ON, Canada). In brief, all transfected HEK cells were grown in 24-well poly-D-lysine coated plates (BD Biosciences, San Jose, CA) at a density of 500,000 cells per well for 2 or 3 days to obtain a confluent monolayer culture. The incubation medium consisted of Hanks' balanced salt solution (HBSS) containing 10 mM HEPES. The pH was 7.4 and 8.4 for the basolateral (OATP1B1, OATP1B3, OAT1, OAT3, and OCT2) and apical membrane transporters (MATE1 and MATE2-K), respectively. Riboflavin-[15N2] and radiolabelled probe substrate were dissolved in incubation medium.

Uptake experiments were initiated by washing the cells twice with prewarmed incubation medium. Subsequently, incubation medium was exchanged against incubation medium containing riboflavin-[15N2] (0.5 and 2.5 µM), [3H]E17βG (1 µM; OATP1B1 substrate), [3H]CCK-8 (1 µM; OATP1B3 substrate), [3H]PAH (1 µM; OAT1 substrate), [3H]E3S (1 µM; OAT3 substrate), and [14C]MFM (2 µM; OCT2, MATE1, and MATE2-K substrate). Rifampin (RIF) was used as a positive control inhibitor of OATP1B1 and
OATP1B31 and to confirm the functional activities of each transporter in the test system. Probenecid (PROB) was used as a reference inhibitor of OAT1 and OAT3, and pyrimethamine (PYR) was used as a control inhibitor of OCT2, MATE1, and MATE2-K. The transport experiments were then stopped at 2 minutes (min) by removing the medium and immediately washing the cells three times with ice-cold incubation medium. Riboflavin-$^{15}$N$_2$ and radiolabelled compound amounts were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and liquid scintillation counting (LSC), respectively. Cellular uptake was normalized to the protein content of the HEK-293 cells measured by protein assay kit.

**Quantification of Riboflavin-$^{15}$N$_2$ in Transporter-Expressing HEK-293 Cells by LC-MS/MS:**

The bioanalytical analyses of riboflavin-$^{15}$N$_2$ were performed as described in detail by Zhang et al. (Zhang et al., 2023).

**Evaluation of BMS-986371 as An Inhibitor of Uptake and Efflux Transporters:**

All transporter inhibition experiments were performed by Sekisui Medical Co., Ltd. (Ibaraki, Japan) following the protocol described previously (Sasabe et al., 2016, Sasabe et al., 2021). The inhibition potential of BMS-986371 towards P-gp and BCRP was assessed using transporter-overexpressing Lewis-lung cancer porcine kidney 1 (LLC-PK1) cells whereas that towards OATP1B1, OATP1B3, OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K was evaluated using transporter-overexpressing HEK-293 cells. Two concentrations of BMS-986371 (2 and 20 μM) were initially used. If inhibition was observed in the initial testing, the $IC_{50}$ was determined using standard inhibition assays at multiple concentration (Supplementary Tables 1 to 10).

Porcine kidney LLC-PK1 cells were grown and seeded in medium M199 supplemented with 9% foetal bovine serum (FBS), 50 μg/mL gentamicin, and 100 μg/mL
hygromycin B in a CO\textsubscript{2} incubator (37°C and 5% CO\textsubscript{2}). Stably transfected LLCPK1-MDR1, LLCPK1-BCRP cells were seeded at 40,000 and 25,000 cells/inert on 3- and 0.4-µm pore size polycarbonate membranes for LLCPK1-MDR1 and LLCPK1-BCRP cells, respectively, in 24-well transwell plates (0.3 cm\textsuperscript{2} culture insert) (BD Falcon, Bedford, MA). The medium was changed every 2 or 3 days, and the cells were ready to use 7 to 9 days post-seeding. To assess the inhibition potential of BMS-986371 towards P-gp and BCRP, the bi-directional transport of radiolabelled probe substrates (1 µM $[^{3}\text{H}]$digoxin and 0.01 µM $[^{3}\text{H}]$prazosin for P-gp and BCRP, respectively) across cell monolayers in the presence and absence of BMS-986371 (0.05, 0.2, 0.5, 2.0, 5.0, or 20 µM) were determined ($N = 3$). Working solutions were freshly prepared in HBSS buffer containing 10 mM HEPES at pH 7.4. Prior to the transwell assay, medium in the apical and basolateral compartments of each well was aspirated and the compartments were washed twice with pre-warmed HBSS (pH 7.4). The HBSS buffer containing 10 mM HEPES (pH 7.4) containing 0.2% DMSO (i.e., vehicle) or inhibitor solution were then added to apical and basolateral compartments (P-gp assay: 100 and 600 µL, respectively; BCRP assay: 250 and 900 µL, respectively), and the cells were preincubated at 37°C for 60 min. To start the transport of a radiolabelled probe substrate in the presence and absence of various concentrations of BMS-986371, 100 and 250 µL testing solutions were added to the apical compartment [for apical-to-basolateral (A→B) transport] for P-gp and BCRP assays, respectively, or 600 and 900 µL testing solutions were added to the basolateral compartment [for basolateral-to-apical (B→A) transport]. The incubation was maintained for 120 min in a CO\textsubscript{2} incubator (37°C and 5% CO\textsubscript{2}) and samples were collected from both the apical and basolateral compartments after a 120-min incubation. To determine the amount of $[^{3}\text{H}]$digoxin or $[^{3}\text{H}]$prazosin in samples, 70 and 100 µL of samples from the apical and basolateral compartments of LLCPK1-MDR1 and LCCPK1-BCRP cell plates, respectively, were added to 5 mL Emulsifier-Safe (PerkinElmer Life Sciences, Boston, MA). Radioactivity was measured using LSC to calculate the permeability of each probe substrate in the A→B and B→A directions across cell monolayers.
Human embryonic kidney HEK-293 cells were grown and seeded in medium Dulbecco’s Modified Eagle Medium containing 9% FBS, 1% antibiotic-antimycotic solution, and 2 mM L-glutamine in a CO₂ incubator (37°C and 5% CO₂). Stably transfected HEK-OATP1B1, HEK-OATP1B3, HEK-OAT1, HEK-OAT3, HEK-OCT1, HEK-OCT2, HEK-MATE1, HEK-MATE2-K, and HEK-Mock cells were seeded at 250,000 to 300,000 cells/well in 24-well plates coated with collagen I (BD Falcon, Bedford, MA). The cells were ready to use 2 days post-seeding. To assess the inhibition potential of BMS-986371 towards the transporters, the uptake of radiolabelled probe substrates [0.05 µM [³H]E17βG (OATP1B1 and OATP1B3), 1 µM [³H]PAH (OAT1), 0.05 µM [³H]E3S (OAT3), and 10 µM [¹⁴C]MFM (OCT1, OCT2, MATE1, and MATE2-K)] into the transporter-overexpressing cells in the presence and absence of BMS-986371 (0.05, 0.2, 0.5, 2.0, 5.0, or 20 µM) were determined (N = 3). Working solutions were freshly prepared in HBSS buffer containing 10 mM HEPES at pH 7.4 (OATP1B1, OATP1B3, OAT1, OAT3, OCT1, and OCT2) or pH 8.5 (MATE1 and MATE2-K). Prior to cellular uptake assay, the medium in each well was aspirated and the cells were washed twice with pre-warmed HBSS (pH 7.4 or pH 8.5). The HBSS buffer (pH 7.4 or pH 8.5) containing 0.2% DMSO (i.e., vehicle) or inhibitor solution were then added to plates and the cells were preincubated at 37°C for 30 min (OATP1B1 and OATP1B3) or 15 min (OAT1, OAT3, OCT1, OCT2, MATE1, and MATE2-K). To start the uptake of a radiolabelled probe substrate in the presence and absence of various concentrations of BMS-986371, the preincubation buffer was replaced with 300 µL of testing solution after preincubation. Cells were incubated at 37°C for designated period [0.5 min (OAT1), 2 min (OAT3, OATP1B1, OATP1B3, and OCT2), and 5 min (OCT1, MATE1, and MATE2-K)]. The transport experiments were then stopped by removing the solution and immediately washing the cells three times with ice-cold HBSS buffer. Radiolabelled probe amounts were measured by LSC. Cellular uptake was normalized to the protein content of the HEK-293 cells measured by protein assay kit.
Clinical Study Design:

The initial DDI study (NCT04268394) was a phase I, open-label, fixed-sequence, cross-over design, and multi-part study in healthy adult participants to assess the safety, tolerability, and pharmacokinetics of multiple doses of BMS-986371 administered alone or in combination with either MTX and SSZ [immediate release (IR)]; itraconazole, rifampin, midazolam, or a cocktail of digoxin, metformin, and rosvastatin (N = 16) (Supplementary Figure 1). The study was conducted in London, United Kingdom, and the majority of the participants were white British. The second DDI study (NCT05445440) was a phase I, open-label, fixed-sequence, and cross-over study in fourteen healthy adult participants (N = 14) to evaluate the effect of BMS-986371 on the pharmacokinetics of MTX in the presence of SSZ and the effect of different SSZ formulations [IR and enteric coated (EC)] on the pharmacokinetics of MTX (Figure 1). The study was conducted in Miami, Florida, United States, and the majority of the participants were White, and demographics characteristics were generally balanced across period. Both studies were conducted in accordance with regulatory and Good Clinical Practice guidelines and Declaration of Helsinki principles. Ethics committee/institutional review board approval was obtained before study conduct. Signed, informed consent approving the use of blood samples for exploratory analysis was obtained from all participants in both studies. In both clinical trials, MTX was only administered to male participants because MTX is an abortifacient. Contraception requirements were specified in each study protocol per the prescribing label for MTX.

Prior clinical studies did not suggest a clinical DDI between SSZ and MTX, and the two drugs are co-administered without dose-adjustment as part of rheumatic disease therapy (Haagsma et al., 1996). In Part 1 of the first DDI study, NCT04268394, a single oral dose of 7.5-mg MTX was administered concurrently with a single dose of 1,000-mg SSZ IR on Day 1 to 16 male participants (N = 16) (Supplementary Figure 1A). The IR formulation of SSZ was chosen for the first DDI study in line with recommendations in the literature (Lee et al., 2015). From Day 3 through Day 7, participants received 150 mg oral BMS-986371. On Day
8, participants received 150 mg BMS-986371 followed 1 hour (h) later by the MTX and SSZ IR. Part 2 of the study, was to evaluate the pharmacokinetics of BMS-986371 when administered with cytochrome P450 (CYP) 3A inhibitor itraconazole and CYP3A inducer rifampin on the pharmacokinetics of BMS-986371 compared to BMS-986371 alone (N = 16) (Supplementary Figure 1B). Part 3 of the study consisted of Period 1 to evaluate the effect of BMS-986371 on the CYP3A substrate midazolam (N = 16). After a 7-day washout phase, Part 3, Period 2 was conducted to assess the effect of BMS-986371 on P-gp, OCT 1/2, and OATP1B1/3 (N = 16). On Day 19, participants received a cocktail containing single doses of oral digoxin (0.25 mg), metformin (500 mg), and rosuvastatin (10 mg) as probes for P-gp, OCT2/MATEs, and OATP1B1/3, respectively (Stopfer et al., 2016). Beginning on Day 22 through Day 29, participants received 150 mg of BMS-986371. On Day 27, the dose of BMS-986371 was followed 1 h later by the digoxin-metformin-rosuvastatin cocktail (Supplementary Figure 1C). All drugs in the first DDI study were administered under monitored conditions in the fasted state at a clinical research unit.

In the second DDI study, NCT05445440, in Period 1, a 7.5-mg oral dose of MTX was administered concurrently with 1,000-mg SSZ EC under fasted conditions on Day 1 (MTX + SSZ EC alone) (Figure 1). In Period 2, starting on Day 7, subjects began receiving once a day oral doses of 150 mg BMS-986371 under fasted conditions and continued through Day 21. On Day 10, an oral dose of 7.5-mg MTX was administered concurrently with 1,000-mg SSZ EC and BMS-986371 under fasted conditions (MTX/SSZ EC + BMS-986371). In Period 3, on Day 17, participants were received a 7.5 mg oral dose of MTX and 1,000 mg SSZ IR administered concurrently with BMS-986371 under fasted conditions (MTX/SSZ IR + BMS-986371). Serial blood samples for measurement of MTX and SSZ as well as riboflavin, isobutyryl carnitine, and arginine were collected at predose and at 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12, 24, and 48 h post-dose in each study period. The blood samples were collected into dipotassium EDTA tubes and stored on ice until centrifugation. Plasma samples were stored at −70°C until analysis.
Quantification of Riboflavin, Arginine, and Isobutyryl Carnitine in Human Plasma Samples by LC-MS/MS:

**Chemicals and Reagents:**

Acetonitrile (HPLC grade), methanol (HPLC grade) and formic acid were purchased from Sigma-Millipore (St. Louis, MO). Dimethyl sulfoxide (DMSO) (HPLC grade) and phosphate buffered saline were purchased from Thermo Fisher Scientific (Waltham, MA). Ammonium formate and bovine serum albumin (BSA) were purchased from Sigma-Millipore (St. Louis, MO). Distilled water was generated in-house using a Barnstead water purification system (Lake Balboa, CA). Reference standards of L-arginine, isobutyryl-L-carnitine, (-)-riboflavin, and stable isotopic labeled L-arginine-$^{13}$C$_6$ hydrochloride, riboflavin-(dioxopyrimidine-$^{13}$C$_4$, $^{15}$N$_2$) were obtained from Sigma-Aldrich (St. Louis, MO). Reference standard of stable isotopic labeled isobutyryl-L-carnitine-d$_3$ chloride was obtained from Cayman Chemical (Ann Arbor, MI).

**LC–MS/MS Analysis Method for Riboflavin, Arginine, and Isobutyryl Carnitine:**

The plasma concentrations of (-)-riboflavin, L-arginine, and isobutyryl-L-carnitine were measured using a qualified LC-MS/MS method. The LC system used in this study consisted of Shimadzu Nexera LC-30AD ultra-HPLC (UHPLC) pumps and a Shimadzu Nexera SIL-30AC autosampler (Columbia, MD). The LC system was coupled to a 6500 Q-TRAP quadrupole linear ion trap hybrid mass spectrometer with an electrospray ionization (ESI) source (Toronto, ON, Canada with Analyst software version 1.7.1).

Chromatographic separation for riboflavin, arginine, and isobutyryl carnitine was conducted using an Acquity UPLC BEH Amide column (2.1 x 100 mm, 1.7 μm particle size) purchased from Waters Corporation (Milford, MA). The column was maintained at a temperature of 40°C using a column heater, and the mobile phase consisted of solvent A (10 mM ammonium formate in water) and solvent B (0.1% formic acid in acetonitrile).
gradient for the LC-MS method was as follows: hold %B at 85 % during 0-1.00 min, decrease %B from 85 % to 75 % during 1.01-2.50 min, decrease %B from 75 % to 50 % at 2.51 min and hold until 4.50 min, decrease %B from 50 % to 10 % at 4.51 min and hold until 6.00 min for column wash, increase %B from 10 % back to 85 % at 6.01 min to re-equilibrate column, and run stopped at 8.00 min. The flow rate was 0.40 mL/min and the sample analysis injection volume was 2 μL. The mass spectrometer was operated in electrospray positive ionization mode and the optimized operating parameters were as follows: curtain gas 40 units; ion source gas 1 and gas 2, 50 units; temperature 500 °C; ion spray voltage 5000 V; dwell time 150 ms; declustering potential 100 V; collision energy 28 eV for riboflavin and its internal standard riboflavin-dioxopyrimidine-$^{13}$C$_4$, $^{15}$N$_2$, collision energy 30 eV for arginine and its internal standard arginine-$^{13}$C$_6$, and collision energy 20 eV for isobutryl carnitine and its internal standard isobutryl carnitine-d$_3$. The multiple reaction monitoring (MRM) was used to monitor the analytes, with transitions m/z 377.1 → 243.1 for riboflavin, m/z 383.2 → 248.9 for riboflavin-dioxopyrimidine-$^{13}$C$_4$, $^{15}$N$_2$, m/z 175.1 → 69.9 for arginine, m/z 181.2 → 74.0 for arginine-$^{13}$C$_6$, m/z 231.9 → 173.0 for isobutryl carnitine, m/z 236.0 → 173.0 for isobutryl carnitine-d$_3$.

**Preparation of Calibration Standard and Quality Control Samples and Plasma Sample Processing:**

Standard stock solutions of riboflavin, arginine, and isobutryl carnitine were prepared at concentrations of 1.00 mg/mL, 10.0 mg/mL, and 1.00 mg/mL, respectively. Arginine and isobutryl carnitine were dissolved in water, while riboflavin was dissolved in DMSO. A standard working solution was prepared by diluting the stock solution of each analyte with methanol to obtain a concentration of 2000/5.00/5.00 μg/mL of arginine/isobutryl carnitine/riboflavin. This solution was then appropriately diluted with 2% BSA in phosphate buffered saline (PBS) as surrogate matrix (SM) to obtain standards with final concentrations of 200/0.500/0.500, 400/1.00/1.00, 800/2.00/2.00, 1600/4.00/4.00, 4000/10.0/10.0, 16000/40.0/40.0, 32000/80.0/80.0, 48000/120/120, 80000/200/200 and
100000/250/250 ng/mL for arginine/isobutyryl carnitine/riboflavin. Four levels of quality control samples (QCs) (Low, geometric mean [GM], mid, high) were prepared at final concentrations of 600/1.50/1.50, 1800/4.50/4.50, 18000/45.0/45.0, and 60000/150/150 ng/mL for arginine/Isobutyryl carnitine/riboflavin. QC Low and GM were prepared in the same surrogate matrix as calibration standards, while QC mid and high were prepared in authentic matrix, K²EDTA human plasma.

The internal standard stock solutions of arginine-$^{13}$C₆ were prepared at a concentration of 1.00 mg/mL in methanol, while isobutyryl carnitine-d₃ and riboflavin-dioxopyrimidine-$^{13}$C₄ were prepared at 1.00 mg/mL in DMSO, respectively. The three internal standard stock solutions were combined and further diluted in acetonitrile: water (1:1) to obtain a working solution of 2000/100/200 ng/ml for arginine-$^{13}$C₆/isobutyryl carnitine-d₃/riboflavin-dioxopyrimidine-$^{13}$C₄. All stock solutions, internal standard solutions, calibration standards and quality control samples were stored at approximately -20°C until use.

The standard curves, quality control samples and plasma samples were prepared as follows: A volume of 25 μL of standard curves, quality control and plasma samples were mixed with 25 μL of internal standard working solution (2000/100/200 ng/ml of L-arginine-$^{13}$C₆/isobutyryl-L-carnitine-d₃/riboflavin-dioxopyrimidine-$^{13}$C₄ in acetonitrile: water (1:1, v:v)), followed by addition of 150 μL of acetonitrile with 0.1% formic acid. The samples were shaken for 5 min and then centrifuged at 4000 rpm for 10 min, after which, 150 μL of supernatant was transferred into a new 96-well plate for LC-MS/MS analysis of riboflavin, arginine, and isobutyryl carnitine.

All standard and quality control (QC) samples performed well within the acceptance criteria. The accuracy of the method was within ± 20% (± 25% for the lowest standard) of the nominal concentration for all analytes, and the precision was less than 20% CV for all QC samples.
Transport, Pharmacokinetic, and Statistical Analyses:

The apical-to-basolateral permeability ($P_{\text{app}, \text{A} \rightarrow \text{B}}$) and basolateral-to-apical permeability ($P_{\text{app}, \text{B} \rightarrow \text{A}}$) of radiolabeled probe substrates ([3H]digoxin and [3H]prazosin) across LLCPK1-MDR1 and LLCPK1-BCRP cell monolayers, and efflux ratio (ER) was calculated according to following equation:

$$ER = \frac{P_{\text{app}, \text{B} \rightarrow \text{A}}}{P_{\text{app}, \text{A} \rightarrow \text{B}}}$$

Furthermore, the ratio of $ER_{\text{MDR1}}$ or $ER_{\text{BCRP}}$ to $ER_{\text{control}}$ was calculated as net efflux ratio (NER) according to the following equation:

$$NER = \frac{ER_{\text{MDR1}} \text{ or } ER_{\text{BCRP}}}{ER_{\text{control}}}$$

The initial rate for transporter-mediated uptake of radiolabeled probe substrates was obtained by subtracting the uptake velocity in HEK-Mock cells from that of transporter-overexpressing HEK-293 cells. Uptake clearance ($CL$) was calculated according to the following equation:

$$CL = \frac{\text{Initial uptake rate}}{\text{Initial probe concentration}}$$

To determine the extent of efflux and uptake transporters inhibition, probe NER and V were plotted against BMS-986371 concentration, and an $IC_{50}$ value was then determined by fitting the $NER$ and $V$ versus concentration data by means of nonlinear least-squares regression analysis using pharmacokinetic software, Phoenix WinNonlin, version 6.4 (Certara, Princeton, NJ) according to following equations:

$$NER = NER_0 - \frac{I_{\text{max}} \times C^Y}{C^Y + IC_{50}^Y}$$

$$CL = CL_0 - \frac{I_{\text{max}} \times C^Y}{C^Y + IC_{50}^Y}$$
where \( NER_0 \) and \( CL_0 \) are the \( NER \) and \( CL \) in the absence of BMS-986371, \( C \) is BMS-986371 concentration, and \( \gamma \) is the sigmoidicity factor that determines the slope of the relationship.

Mechanistic static model predictions of intestinal (P-gp and BCRP), hepatic (OATP1B1, OATP1B3, and OCT1), and renal transporters (OAT1, OAT3, OCT2, MATE1, and MATE2-K) DDIs were performed using the equations recommended by FDA and EMA (https://www.fda.gov/media/161199/download).

Pharmacokinetic parameters of riboflavin, isobutyryl carnitine, and arginine were determined from individual plasma concentration-time profile using non-compartmental analysis (Phoenix WinNonlin, version 8.2.2; Certara, Princeton, NJ). Maximum plasma concentration (\( C_{max} \)) and time to reach maximum plasma concentration (\( T_{max} \)) were estimated directly from experimental observations. Area under the plasma concentration-time curve from zero to 4 hours [\( AUC(0-4h) \)] and area under the plasma concentration-time curve from zero to 24 hours [\( AUC(0-24h) \)] were calculated using the mixed log-linear trapezoidal method up to the measurable concentration at 4 and 24h, respectively.

Data are expressed in mean ± standard deviation (SD) in this study. The primary endpoints of this clinical study are the ratios of pharmacokinetic parameter [\( C_{max} \), \( AUC(0-4h) \), or \( AUC(0-24h) \)] in the coadministration treatment group (i.e., MTX/SSZ EC + BMS-986371) over that in the MTX/SSZ EC group for riboflavin, isobutyryl carnitine, and arginine. Geometric mean ratio (\( GMR \)) was back-transformed from log scale of the ratio of pharmacokinetic parameter in the MTX/SSZ EC + BMS-986371 group over that in MTX/SSZ EC alone group. The 90% distribution confidence interval (\( CI \)) of pharmacokinetic parameters is also presented.

Statistical differences in basal plasma riboflavin concentrations between subjects and between various timepoints within subject were determined using one-way analysis of variance (Version 9.4.0, GraphPad; San Diego, CA). Statistical comparison in pharmacokinetic parameters between the periods were performed using a paired two-tailed Student’s \( t \)-test, and the analysed pharmacokinetic parameters of biomarkers were
logarithmically transformed before the analyses (Version 9.4.0, GraphPad; San Diego, CA).

A $P$-value $< 0.05$ was considered statistically significant).
RESULTS

In Vitro Assessment of Riboflavin Drug Transporter Selectivity:

The uptake rates of 0.5 and 2.5 µM riboflavin in the HEK-293 cells stably transfected with major hepatic and renal drug uptake transporters are summarized in Figure 2. According to the FDA’s Guidance on the in vitro drug interaction studies for transporter-mediated drug interactions, a tested compound is determined to be a drug transporter substrate if the uptake rate in transporter-expressed cells is ≥2-fold that in controls (https://www.fda.gov/media/134582/download). In all experiments, the uptake rates of radiolabelled probe substrates were 5.49- to 85.8-fold higher in transporter-expressing HEK cells compared to those obtained in HEK-Mock cells, demonstrating proper expression and activity of transporters in the cells and suitability of the test systems for OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1, and MATE2-K substrate identification.

As indicated in Figure 2, less than 2-fold differences were observed with the uptake rates of riboflavin in human OATP1B1, OATP1B3, OCT2, and MATE1-expressing HEK cells compared to HEK-Mock cells (0.60- to 1.09-fold). These results indicate that riboflavin is not a substrate for these transporters. In contrast, the riboflavin uptake rates measured in HEK-OAT1, HEK-OAT3, and HEK-MATE2-K cells were 2.69- to 11.6-fold higher than its uptake rates in HEK-Mock cells (Figure 2). In addition, transporter inhibitors PROB (1 mM) (OAT1 and OAT3) and PYR (100 µM) (MATE2-K) reduced the uptake ratio of riboflavin to 1.00- to 1.23-fold (i.e., > 65% inhibition) in in transporter-expressing HEK cells. These data indicate that riboflavin is a substrate for human OAT1, OAT3, and MATE2-K.

Assessment of Transporter Inhibition for BMS-986371 Using In Vitro Data and Using Probe Substrates in a Clinical DDI Study:

To assess the potential for BMS-986371 to inhibit the major human intestinal (P-gp and BCRP), hepatic (OATP1B1, OATP1B3, and OCT1), and renal drug transporters (OAT1, OAT3, OCT2, MATE1 and MATE2-K) in vitro, a transporter inhibition study was performed using stably transfected LLC-PK1 and HEK-293 cells that individually overexpressed the...
transporters (Supplementary Tables). The experiments were performed using the methods described in the previous publications by Sekisui Medical Co., Ltd. (Ibaraki, Japan) (Sasabe et al., 2016, Sasabe et al., 2021). The assessments showed that BMS-986371 was a potent inhibitor of intestinal efflux transporters P-gp and BCRP. BMS-986371 inhibited P-gp- and BCRP-mediated transcellular transport of digoxin and prazosin with \( IC_{50} \) of 2.58 and 0.40 µM, respectively, using LLC-PK1-MDR1 and LLC-PK1-BCRP cells (Table 1; Supplementary Tables 1 and 2). In addition, BMS-986371 was a strong inhibitor of hepatic uptake transporters OATP1B1, OATP1B3, and OCT1 with \( IC_{50} \) values of 2.24, 3.56, and 0.13 µM, respectively, using HEK-OATP1B1, HEK-OATP1B3, and HEK-OCT1 cells (Table 1; Supplementary Tables 3, 4, and 7). Moreover, the uptake of a probe substrate by HEK-293 cells transfected with renal transporters OAT1, OAT3, OCT2, MATE1, or MATE2-K in the presence of various concentrations of BMS-986371 was measured, and derived \( IC_{50} \) values were less potent for OAT1 (4.90 µM), OAT3 (> 20 µM), OCT2 (1.45 µM), MATE1 (2.44 µM), and MATE2-K (6.03 µM) compared with intestine and liver drug transporters (Table 1; Supplementary Tables 5, 6, 8, 9, and 10). Collectively, these results indicated that BMS-986371 might affect the multiple transport clearance of drug substrates, necessitating further transporter investigations in accordance with FDA and EMA guidance (https://www.fda.gov/media/161199/download).

The multiple ascending dose part of the first-in-human trial with BMS-986371 showed that 150 mg was well tolerated over 14 days with sustained inhibition of inflammatory disease-relevant cytokines (Gaur et al., 2022). Therefore, this dose level was planned for proof-of-concept studies in inflammatory disease patients, and it was used, for that reason, in the two clinical DDI studies reported here. Consequently, the analysis using mechanistic static model method suggested the risk for P-gp and BCRP inhibition by 150 mg BMS-986371 in humans based on in vitro data. The ratios of estimated intestinal luminal concentration (\( I_g \)) over \( IC_{50} \) are 495 and 3216 for P-gp and BCRP, respectively (Table 1). However, in the first clinical DDI study (NCT04268394), digoxin area under the plasma concentration-time curve ratio (\( AUCR \)) was 1.22 (1.10, 1.36), whereas SSZ IR AUCR was
1.51 (1.09, 2.10) (N = 16) (Table 1). Since the cutoff value of $AUCR$ was defined as ≥ 1.25-fold (bioequivalence) by the FDA (https://www.fda.gov/media/87219/download; https://www.fda.gov/media/134582/download), this suggests that administration of oral 150 mg BMS-986371 had a minimal effect on P-gp but significant inhibition of BCRP in humans. Of note, NAT2 genotype was assessed in the study participants, and it did not influence the observed BCRP inhibition (data not shown).

The assessment using the mechanistic static model method by comparing the ratio of unbound hepatic inlet concentration over $IC_{50}$ to cutoff of 0.1 suggested that the hepatic transporters OATP1B and OCT1 drug interaction potential for BMS-986371 was significant (Table 1). The ratios of victim $AUC$ in the presence and absence of inhibitor ($R$-values), based on 150 mg BMS-986371 and predicted unbound maximum plasma liver inlet concentration ($C_{\text{max,inlet,u}}$) of 0.44 µM in humans (Table 1), were estimated to be 1.20, 1.12, and 4.40, respectively. In the first clinical DDI study, the $AUC$ of rosuvastatin following the co-administration of 150 mg BMS-986371 was approximately 1.38-fold greater than that of rosuvastatin alone in healthy human participants (N = 16) [$AUCR$ 1.38 (1.23, 1.56); Table 1].

Based on in vitro $IC_{50}$ and unbound maximum plasma concentration ($C_{\text{max,u}}$), predicted DDI involving renal transporter inhibition for 150 mg BMS-986371 coadministration using the mechanistic static model approach were not significant. The ratios of $C_{\text{max,u}}$ to $IC_{50}$ for OAT1, OAT3, OCT2, MATE1, and MATE2-K were 0.004, < 0.001, 0.014, 0.008, and 0.003, respectively (i.e., < the FDA and EMA cut-off value of 0.10 or 0.02) (Table 1). The extent of change in systemic exposure to MTX and metformin, the known drug substrates of OAT1/3 and OCT2/MATEs, respectively, following pre-dosing with BMS-986371 150 mg in the first clinical DDI study confirmed the lack of in vivo inhibition on these renal transporters assessed using the mechanistic static model. When 150 mg BMS-986371 was co-administered in the DDI study, the MTX and metformin $AUC$ values were not significantly changed [$AUCR$ of 1.20 (0.93, 1.54) and 0.95 (0.89, 1.02), respectively] (N = 16) (Table 1).

Clinical Study NCT05445440 Participants:
A total of 14 healthy male volunteers with a mean age (SD) of 36.2 (9.28) years, weight of 85.9 (10.1) kg, and body mass index of 27.4 (2.37) kg/m$^2$ were enrolled in this study (N = 14). 13 of the subjects were White with 13 among them identifying themselves as Hispanic. Only 1 subject was African American. None of the participants reported taking concomitant medications known to alter BCRP activity. All subjects completed 3 periods of the study (Figure 1). Mild adverse events were observed and resolved quickly during the study.

**Effects of BMS-986371 on Plasma Concentrations of Riboflavin, Isobutryl Carnitine, and Arginine in Healthy Adults:**

The effects of BMS-986371, an inhibitor of BCRP, on the pharmacokinetics of riboflavin, isobutryl carnitine, and arginine, candidates of biomarkers of BCRP, were evaluated in plasma after oral administration of MTX (7.5 mg) and SSZ EC (1,000 mg) alone (MTX/SSZ EC alone) or in combination with BMS-986371 (150 mg) (MTX/SSZ EC + BMS-986371) in 14 healthy male adults in the second clinical DDI study (NCT05445440) (Figure 3 and Table 2). BMS-986371 co-administered with MTX/SSZ EC caused small but significant and consistent increases in the plasma concentration-time profiles of riboflavin (Figures 3A and 4A, 4B, and 4C), resulting in 1.25-, 1.14-, and 1.11-fold increases of $AUC_{(0-4h)}$, $AUC_{(0-24h)}$, and $C_{max}$, respectively [$AUC_{(0-4h)}$: 15.5 ± 20.5 versus 17.4 ± 19.2 ng•h/mL, 1.25 (1.11-1.35), $P = 0.003$; $AUC_{(0-24h)}$: 94.1 ± 118 versus 99.2 ± 106 ng•h/mL, 1.14 (1.05-1.21), $P = 0.009$; and $C_{max}$: 4.93 ± 6.19 versus 5.27 ± 5.88 ng/mL, 1.11 (1.03-1.18), $P = 0.025$] (Table 2). These within-subject increases in riboflavin systemic exposure by BMS-986371 treatment were consistent as the increases were observed in almost all subjects (Figures 4A, 4B, and 4C).

To evaluate the intra- and inter-individual variability of basal plasma concentration versus time profile of riboflavin, we measured the basal plasma riboflavin concentrations ($T = 0$ h prior to dose) over a 15-day period on Days 1-6 and 10-15 in 14 healthy male adults (Figure 5). The basal plasma riboflavin concentrations between individuals varied widely,
and significant differences in the concentrations between subjects were observed ($P = 0.0001$). The maximal inter-individual difference in the mean riboflavin level was 20.4-fold. However, the basal plasma riboflavin concentrations over a 15-day period were not significantly different between different days for each subject ($P = 0.336$).

BMS-986371 did not change isobutyl carnitine $C_{\text{max}}$ and $AUC(0-24\text{h})$ significantly [$C_{\text{max}}$: $36.7 \pm 13.4$ versus $38.1 \pm 13.5$ ng/mL, 1.06 (0.97-1.13); $P = 0.323$; and $AUC(0-24\text{h})$: $692 \pm 240$ versus $725 \pm 231$ ng$\cdot$h/mL, 1.07 (0.99-1.14); $P = 0.158$] (Figure 3B and Table 2). In addition, the plasma concentration-time profile of arginine was not influenced by the administration of BMS-986371 in combination with MTX/SSZ EC as compared with MTX/SSZ EC alone treatment [$C_{\text{max}}$: $19886 \pm 3295$ versus $19026 \pm 3358$ ng/mL, 0.96 (0.90-1.01); $P = 0.178$; and $AUC(0-24\text{h})$: $379421 \pm 67669$ versus $366609 \pm 61756$ ng$\cdot$h/mL, 0.97 (0.92-1.01); $P = 0.228$] (Figure 3C and Table 2).
DISCUSSION

There are particular challenges in discovering endogenous biomarkers of efflux transporters P-gp and BCRP as the intestinal P-gp and BCRP are unlikely involved in the disposition of endogenous substrates in the body. As the intestinal P-gp/BCRP inhibition is the primary mechanism causing most clinically significant drug interactions with orally administered exogenous drugs (Taskar et al., 2022), P-gp- and BCRP-mediated DDIs may not always be reflected by the systemic exposure changes to those endogenous substrates. Unlike endogenous compounds, however, riboflavin (also known as vitamin B2) must be obtained from the diet since humans cannot synthesize it. One of the major findings of this study is that BCRP activity may be monitored by plasma riboflavin level. Statistically significant increases in riboflavin plasma concentrations were observed when combined with BMS-986371 as GMR with 90% CI and P-values of the riboflavin AUC(0-4h), AUC(0-24h), and C\text{max} of MTX/SSZ EC with and without BMS-986371 (MTX/SSZ EC + BMS-986371 versus MTX/SSZ EC) were 1.25 (1.11-1.35) \( P = 0.003 \), 1.14 (1.05-1.21) \( P = 0.009 \), and 1.11 (1.03-1.18) \( P = 0.025 \), respectively (Table 2). Cutoff value of DDI was previously defined as 1.25-fold (bioequivalence) by the FDA. As BMS-98637 increased the AUC(0-4h) of riboflavin by 25% and the increases of all pharmacokinetic parameters were statistically significant, the presence of a biomarker-drug interaction was likely. BMS-986371 did not substantially alter the plasma riboflavin concentrations (Figures 3 and 4) and this is not unexpected, considering that the magnitude of clinical P-gp and BCRP inhibitions are generally smaller than 2-fold (Zhou et al., 2019, Costales et al., 2021). Although SSZ is an inhibitor of BCRP and OAT1/3 (Elsby et al., 2011, Elsby et al., 2016), the riboflavin levels in the MTX/SSZ EC group served as a control for the assessment of transporter activity in the presence of BMS-986371 to avoid confounding factors (Figure 1). Moreover, these results are consistent with the small magnitudes of BCRP-mediated DDIs caused by BMS-986371 as the exposures of rosuvastatin and SSZ, known BCRP drug probes, were increased by 38% and 51%, respectively, after coadministration with BMS-986371 relative to the probes alone in the first clinical DDI study (NCT04268394) (Table 1). Additionally, the preliminary result of this DDI
study (i.e., the second clinical study NCT05445440) shows that BMS-986371 increases the AUC od SSZ by 79% [AUCR of 1.79 (1.21, 2.64)] (unpublished data). We verified the previous findings in mice and monkeys that BCRP inhibitors elacridar and ML753286, respectively, significantly increased riboflavin $C_{\text{max}}$ and AUC(0-24h) (Zhang et al., 2023), indicating the underlying mechanism of riboflavin-BMS-986371 interaction is BCRP inhibition. Digoxin AUC from 0 to 4 hours [AUC(0–4h)] has been used as an indicator of intestinal P-gp activity (Larsen et al., 2007, Kirby et al., 2012). Similarly, riboflavin AUC(0–4h) was selected to indicate the intestinal BCRP activity in this study as the riboflavin $T_{\text{max}}$ values were 4.1 ± 3.1 and 5.6 ± 2.1 h in the MTX/SSZ EC + BMS-986371 and MTX/SSZ EC alone groups, respectively (Table 2). Consistently, riboflavin AUC(0-4h) showed higher GMR compared with the AUC(0-24h) and $C_{\text{max}}$ (1.25 versus 1.14 and 1.11) (Table 2). Taken together, these results suggest that a change of riboflavin from baseline may be used to assess BCRP inhibition in lieu of a dedicated DDI study of healthy volunteers.

Noticeably, the extent of intra-individual variability of plasma riboflavin concentrations before drug administration over a 15-day period was low ($P = 0.336$) although high inter-individual variability was observed in the basal plasma riboflavin level ($P = 0.0001$) (Figure 5), in agreement with a previous study investigating dietary effects on plasma riboflavin concentrations in humans (Zhang et al., 2023). Low intra-individual and inter-meal variability of plasma riboflavin concentrations were noted in 64 healthy volunteers who consumed a standardized identical diet for 2 days compared with unregulated diets. Low intra-individual variability of plasma riboflavin concentrations is consistent with the fact that the absorption and elimination of riboflavin is tightly regulated (1998, Thakur et al., 2017). Primary absorption of riboflavin occurs in the proximal small intestine with a small amount of riboflavin absorbed in the large intestine. Very little riboflavin is absorbed from single doses beyond 25 mg when riboflavin intake meets minimal daily requirement (Jusko and Levy, 1967a, Roughead and McCormick, 1991, Zemplen et al., 1996). When excess amounts are consumed, they are excreted, primarily in the urine, and little is stored in the body tissues. Taken together, the pre-dose levels of riboflavin or those in the absence of an inhibitor for
each subject can serve as his or her own control for the assessment of BCRP activity in a clinical study. This conclusion is confirmed by the observations of high inter-individual variability (CV%) of riboflavin $AUC(0-4h)$ in the MTX/SSZ EC alone and MTX/SSZ EC + BMS-986371 groups (132% and 110%, respectively) whereas there was low inter-individual variability of the ratio of riboflavin $AUC(0-4h)$ (19.5%) in this study.

We reported that riboflavin was a selective substrate of human BCRP over P-gp previously (Zhang et al., 2023). To further examine the selectivity of riboflavin as a biomarker for BCRP, we evaluated whether it is a substrate for major hepatic and renal drug transporters including OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1, and MATE2-K. Based on the in vitro transporter phenotyping experiments using established transfected cell systems, the renal basolateral transporters OAT1 and OAT3 as well as apical transporter MATE2-K are involved in the transport of riboflavin (Figures 2B and 2C). But OAT1, OAT3, and MATE2-K are less sensitive to the inhibition by BMS-986371 compared with BCRP ($IC_{50}$ of 4.90, > 20, and 6.03 µM, respectively, versus 0.40 µM) (Table 1; Supplementary Tables 2, 5, 6, and 10). In addition, the risk for OAT1, OAT3, and MATE2-K inhibition by 150 mg BMS-986371 in humans can be excluded since the analyses using mechanistic static basic model methods indicate that the ratios of unbound maximum plasma BMS-986371 concentration divided by $IC_{50}$ are less than the cut-off values (0.004 and < 0.001 versus 0.1 and 0.003 versus 0.02, respectively) (Table 1). In agreement with our findings, clinical DDI studies have reported that BMS-986371 increased the $AUC$ of MTX (OAT1 and OAT3 substrate) 1.20-fold and decreased the $AUC$ of metformin (MATE2-K substrate) 0.95-fold, respectively (Table 1). BMS-986371 is also an inhibitor of OATP1B1, OATP1B3, OCT2, MATE1, and R-pg with $IC_{50}$ ranged from 1.45 to 2.58 µM (Table 1; Supplementary Tables 1, 3, 4, 8, and 9) (Zhang et al., 2023), which are highly expressed in the liver, kidney and intestine and are determinants of drug disposition. Oral administration of 150 mg BMS-986371, however, had minimal effect on the plasma concentrations of metformin (OCT2 and MATE1 substrate) and digoxin (P-gp substrate) in humans (0.95- and 1.22-fold AUCR, respectively). Although BMS-986371 increased the $AUC$ of rosuvastatin (OATP1B1 and OATP1B3 substrate) 1.38-fold, riboflavin
is not a substrate for human OATP1B1 and OATP1B3 (as well as OCT2, MATE1 and P-gp) (Figures 2A and 2C).

It is worth noting that the effects of oral doses of PROB, a known inhibitor of OAT1 and OAT3, on the urinary excretion of riboflavin in humans were evaluated (Jusko and Levy, 1967b, Jusko et al., 1970). PROB inhibited the active renal tubular secretion of riboflavin in male participants. Therefore, further studies are required to confirm the interaction and understand the contribution of BCRP- and OAT1/3-mediated transport to riboflavin disposition. Little information is available regarding the diurnal variation in human blood riboflavin concentration. Zempleni et al. reported that concentration of riboflavin in plasma was slightly lower during afternoon with less than 25% decrease in 9 healthy subjects (Zempleni et al., 1996). Finally, it is worth noting that there may be a gender difference in dietary riboflavin intake (Mataix et al., 2003, Wan et al., 2023). Further study is needed to evaluate the effect of a BCRP inhibitor on riboflavin levels in both male and female subjects.

BMS-986371 administration did not significantly alter the plasma concentrations of isobutyryl carnitine and arginine (0.96- to 1.07-fold) (Figures 3B and 3C; Table 2), suggesting that isobutyryl carnitine and arginine are not appropriate endogenous biomarkers of BCRP in humans. Isobutyryl carnitine has also been recently suggested as a potential OCT1 biomarker (Luo et al., 2020). OCT1 inhibitors ritilectinib and trimethoprim decreased plasma isobutyryl carnitine concentration as OCT1 mediates the net efflux of isobutyryl carnitine out of hepatocytes (Vora et al., 2023, Wang et al., 2023), in agreement with the reduced plasma isobutyryl carnitine level in subjects carrying the OCT1 mutation (Jensen et al., 2021). Inhibition on both BCRP-mediated efflux and OCT1-mediated uptake in the intestine and liver, respectively, may explain the lack of effect of BMS-986371 on plasma isobutyryl carnitine. However, a second reason for the lack of change of plasma isobutyryl carnitine concentration by BMS-986371 is that both BCRP and OCT1 have a limited role. Therefore, further investigation on isobutyryl carnitine level with selective BCRP or OCT1 inhibitor is needed.
In conclusion, two clinical DDI studies for BCRP-inhibitor BMS-986371 were utilized for the measurement of riboflavin as a biomarker of BCRP to evaluate its utility. Riboflavin exposures [\(AUC(0-4h)\), \(AUC(0-24h)\), and \(C_{\text{max}}\)] were significantly increased by BMS-986371 exposure, indicating possible inhibitory effect of BMS-986371 on BCRP in humans. To the best of our knowledge, this is the first report exploring the utility of riboflavin as a novel BCRP biomarker in a clinical study. The utility of riboflavin as a BCRP biomarker warrants further validation since a minor degree of changes in \(AUC\) and \(C_{\text{max}}\) were observed in this study, and a relatively small sample size was used in this study.
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DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Shen, Tong, W Chen, Paris, Mensah, M Chen, Xue, Li, and Sinz.

Conducted experiments: Huo, Zhang, and Wang.

Contributed to new reagents or analytic tools: Shen, Huo, Zhang, and Xue.


Wrote or contributed to the writing of the manuscript: Shen, Huo, Zhang, Tong, Mensah, and Sinz.

Other: None.
REFERENCES


FOOTNOTES

- This work received no external funding.

- The authors are employees of and may own shares/stock option in Bristol Myers Squibb.

- No author has an actual or perceived conflict of interest with the contents of this article.
LEGENDS FOR FIGURES

Figure 1. Outline of the second clinical DDI study (NCT05445440) to evaluate the utility of riboflavin as an biomarker for BCRP inhibition in healthy male adult participants (N = 14). In this open-label, fixed-sequence, and cross-over study, blue, red, and green highlights indicate clinical dosing scheme for oral administration of methotrexate (MTX) (7.5 mg) and enteric coated (EC) sulfasalazine (SSZ) (1,000 mg) alone (MTX/SSZ EC alone), MTX and SSZ EC concurrently with BMS-986371 (150 mg) (MTX/SSZ EC + BMS-986371), and MTX and immediate release (IR) SSZ (1,000 mg) concurrently with BMS-986371 (MTX/SSZ IR + BMS-986371) on Days 1, 10, and 17, respectively. BMS-986371 was given once daily from Day 7 through Day 21. Blood samples were collected to measure drug and biomarker levels on Days 1, 10, and 17.

Figure 2. Profiling of the transport of riboflavin by major drug transporters. Uptake in the HEK-293 cells stably transfected with the control vector (HEK-Mock), hepatic OATP1B (A), and renal organic anion transporters (B) and organic cation transporters (C) was measured after a 2-min incubation at 37°C with riboflavin (RFV) (0.5 and 2.5 μM). The experimental systems with the transporter activity was confirmed using radio-labelled probe substrates [1 μM [3H]E17βG (OATP1B1), 1 μM [3H]CCK-8 (OATP1B3), 1 μM [3H]PAH (OAT1), 1 μM [3H]E3S (OAT3), and 2 μM [14C]MFM (OCT2, MATE1, and MATE2-K) for hepatic OATP1B (D), renal organic anion transporters (E), and renal organic cation transporters (F). Incubations were carried out in the absence and presence of 100 μM RIF (OATP1B1/1B3), 1 mM PROB (OAT1/3), or 100 μM PYR (OCT2/MATE1/2-K) to evaluate the effects of these inhibitors on riboflavin uptake. Each value represents fold uptake of compound over mock cells treated without inhibitor. Each bar represents the mean ± SD (N = 3).
Figure 3. Mean plasma concentration versus time profiles for (A) riboflavin, (B) isobutyryl carnitine, and (C) arginine following oral administration of methotrexate (MTX) (7.5 mg) and enteric coated (EC) sulfasalazine (SSZ) (1,000 mg) alone (MTX/SSZ EC alone; closed squares) or in combination with BMS-986371 (150 mg) (MTX/SSZ EC + BMS-986371; open triangles) to 14 healthy male adult participants (N = 14). Data are expressed as the arithmetic mean ± SD.

Figure 4. Individual area under the plasma concentration-time curve from zero to 24 hours \([AUC(0-24h)]\), \(AUC(0-4h)\), and maximum plasma concentration \((C_{\text{max}})\) values for (A, B and C) riboflavin, (D and E) isobutyryl carnitine, and (F and G) arginine following oral administration of methotrexate (MTX) (7.5 mg) and enteric coated (EC) sulfasalazine (SSZ) (1,000 mg) alone (MTX/SSZ EC alone; closed squares) or in combination with BMS-986371 (150 mg) (MTX/SSZ EC + BMS-986371; open triangles) to 14 healthy male adult participants (N = 14).

Figure 5. Plasma concentrations of riboflavin just prior to a dose (i.e., \(T = 0\) h) on days 1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 14, and 15 in 14 healthy male adult participants (N = 14).
Table 1: Predicted and observed DDIs due to transporter inhibition by 150 mg BMS-986371 from first clinical DDI trial (NCT04268394).

<table>
<thead>
<tr>
<th>Transporter</th>
<th>( IC_{50} ) (µM)</th>
<th>( I_{gut}, C_{\text{max,inlet,u}} ), and ( C_{\text{max,u}} ) at 150 mg (µM)</th>
<th>FDA and EMA Transporter Inhibition Equation and Cutoff</th>
<th>Estimated Ratio</th>
<th>Observed Probe AUCR (Probe + BMS-986371/Probe alone) (N = 16): GMR (90% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>2.58</td>
<td>1277 (I_{gut})</td>
<td>( I_{gut}/IC_{50} &gt; 10 )</td>
<td>495**</td>
<td>Digoxin AUCR: 1.22 (1.10, 1.36)</td>
</tr>
<tr>
<td>BCRP</td>
<td>0.40</td>
<td></td>
<td></td>
<td>3216**</td>
<td>Sulfasalazine AUCR: 1.51 (1.09, 2.10)</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>2.24</td>
<td></td>
<td></td>
<td>1.20**</td>
<td>Rosuvastatin AUCR: 1.38 (1.23, 1.56)</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>3.56</td>
<td>0.44 (C_{\text{max,inlet,u}})</td>
<td>( R )-value = (1 + C_{\text{max,inlet,u}}/IC_{50}) &gt; 1.1</td>
<td>1.12*</td>
<td></td>
</tr>
<tr>
<td>OCT1</td>
<td>0.13</td>
<td></td>
<td></td>
<td>4.40**</td>
<td></td>
</tr>
<tr>
<td>OAT1</td>
<td>4.90</td>
<td></td>
<td></td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>OAT3</td>
<td>&gt; 20</td>
<td>0.02 (C_{\text{max,u}})</td>
<td>( C_{\text{max,u}}/IC_{50} &gt; 0.1 )</td>
<td>&lt; 0.001</td>
<td>Methotrexate AUCR: 1.20 (0.93, 1.54)</td>
</tr>
<tr>
<td>OCT2</td>
<td>1.45</td>
<td></td>
<td></td>
<td>0.014</td>
<td>Metformin AUCR: 0.95 (0.89, 1.02)</td>
</tr>
<tr>
<td>MATE1</td>
<td>2.44</td>
<td>0.02 (C_{\text{max,u}})</td>
<td>( C_{\text{max,u}}/IC_{50} &gt; 0.02 )</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>MATE2-K</td>
<td>6.03</td>
<td></td>
<td></td>
<td>0.003</td>
<td>Metformin AUCR: 0.95 (0.89, 1.02)</td>
</tr>
</tbody>
</table>

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\(^{a}\text{IC}_{50}\) values were generated in transporter-transfected cells using radiolabelled probe substrates (Supplementary Tables 1 to 10).

A probe drug cocktail containing substrates of important drug transporters was performed for assessment of mutual transporter inhibitions by interactions in the first clinical DDI trial with BMS-986371. This is an open-label, fixed-sequence, and cross-over study (N = 14). The geometric mean ratios (\(GMRs\)) and 90% confidence intervals (\(CI\)) of \(AUCR\) are presented.** above cutoff, and * borderline above cutoff.

Abbreviations: \(AUCR\), area under the plasma concentration-time curve ratio; \(C_{\text{max, inlet, u}}\), predicted unbound maximum plasma liver inlet concentration; \(C_{\text{max, u}}\), unbound maximum plasma concentration; \(GMR\), geometric mean ratio; \(\text{IC}_{50}\), concentration required to inhibit transport by 50%; \(I_{\text{gut}}\), estimated intestinal luminal concentration.
Table 2: Pharmacokinetics of riboflavin, isobutryl carnitine, and arginine in the absence and presence of BMS-986371 in the second clinical DDI trial (NCT05445440) (N = 14).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MTX/SSZ EC Alone</th>
<th>MTX/SSZ EC + BMS-986371</th>
<th>MTX/SSZ EC + BMS-986371 Period to MTX/SSZ EC Alone Period Ratio (90% CI); P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Riboflavin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>4.93 ± 6.19</td>
<td>5.27 ± 5.88</td>
<td>1.11 (1.03-1.18); $P = 0.025$</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>5.6 ± 2.1</td>
<td>4.1 ± 3.1</td>
<td>NC</td>
</tr>
<tr>
<td>$AUC(0-24h)$ (ng•h/mL)</td>
<td>94.1 ± 118</td>
<td>99.2 ± 106</td>
<td>1.14 (1.05-1.21); $P = 0.009$</td>
</tr>
<tr>
<td>$AUC(0-4h)$ (ng•h/mL)</td>
<td>15.5 ± 20.5</td>
<td>17.4 ± 19.2</td>
<td>1.25 (1.11-1.35); $P = 0.003$</td>
</tr>
<tr>
<td><strong>Isobutryl Carnitine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>36.7 ± 13.4</td>
<td>38.1 ± 13.5</td>
<td>1.06 (0.97-1.13); $P = 0.323$</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>9.6 ± 6.1</td>
<td>10.4 ± 5.8</td>
<td>NC</td>
</tr>
<tr>
<td>$AUC(0-24h)$ (ng•h/mL)</td>
<td>692 ± 240</td>
<td>725 ± 231</td>
<td>1.07 (0.99-1.14); $P = 0.158$</td>
</tr>
<tr>
<td><strong>Arginine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>19886 ± 3295</td>
<td>19026 ± 3358</td>
<td>0.96 (0.90-1.01); $P = 0.178$</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>7.0 ± 2.9</td>
<td>7.5 ± 3.1</td>
<td>NC</td>
</tr>
<tr>
<td>$AUC(0-24h)$ (ng•h/mL)</td>
<td>379421 ± 67669</td>
<td>366609 ± 61756</td>
<td>0.97 (0.92-1.01); $P = 0.228$</td>
</tr>
</tbody>
</table>
This is an open-label, fixed-sequence, and cross-over study (N = 14). Pharmacokinetic parameters were determined as described in Materials and Methods. Data are shown as mean ± SD. The GMRs between treatments are given with 90% CI.

Abbreviations: $AUC_{0-4h}$, area under the plasma concentration-time curve from zero to 4 hours; $AUC_{0-24h}$, area under the plasma concentration-time curve from zero to 24 hours; $CI$, confidence interval; $C_{max}$, maximum plasma concentration; $GMR$, geometric mean ratio; NC, not calculated; $T_{max}$, time to reach maximum plasma concentration.
Plasma samples over a 48-hour period

**Figure 1**
A). Riboflavin Transport by OATP1B1/1B3

B). Riboflavin Transport by OAT1/3

C). Riboflavin Transport by OCT2/MATE1/2-K

D). Probe Transport by OATP1B1/1B3

E). Probe Transport by OAT1/3

F). Probe Transport by OCT2/MATE1/2-K

Figure 2
Figure 3

A) Plasma Riboflavin Concentration (ng/mL)

B) Plasma Isobutyl Carnitine Concentration (ng/mL)

C) Plasma Arginine Concentration (ng/mL)

- □ MTX/SSZ EC alone
- ▲ MTX/SSZ EC + BMS-986371
Figure 4

A) Riboflavin AUC(0-24h) (ng/mL)

B) Riboflavin Cmax (ng/mL)

C) Isobutryl Carnitine AUC(0-4h) (ng/mL)

D) Isobutryl Carnitine Cmax (ng/mL)

E) Arginine AUC(0-24h) (ng/mL)

F) Arginine Cmax (ng/mL)

G) Arginine AUC(0-24h) (ng/mL)

MTX/SSZ EC alone

MTX/SSZ EC + BMS-986371
Figure 5

Basal Plasma Riboflavin Concentration (ng/mL) vs. Hours