Extensive Intestinal First-Pass Elimination and Predominant Hepatic Distribution of Berberine Explain Its Low Plasma Levels in Rats

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ABSTRACT:
Berberine, one of the most commonly used natural products, exhibits a poor plasma concentration-effect relationship whose underlying mechanisms remain largely unclear. This study was designed to test the hypothesis that extensive first-pass elimination and abundant tissue distribution of berberine may be its specific pharmacokinetic properties. For that, four different dosing routes, intragastric, intraduodenal, intraportal, and intravenous, were used to investigate the gastric, intestinal, and hepatic first-pass elimination of berberine. After intragastric administration, approximately half of berberine ran intact through the gastrointestinal tract and another half was disposed of by the small intestine, leading to an extremely low extent of absolute oral bioavailability in rats (0.36%). Moreover, the major berberine metabolites were identified and quantified in rat enterocyte S9 fractions, portal vein plasma, and intestinal perfusates; plasma concentrations and tissue distribution of berberine and its major metabolites were determined as well. Data indicated that M1, M2 glucuronide, and M3 were the major metabolites generated from the small intestine that there was a 70-fold increase in the ratio of the area under the concentration-time curve value for berberine (liver versus plasma). We conclude that intestinal first-pass elimination of berberine is the major barrier of its oral bioavailability and that its high extraction and distribution in the liver could be other important factors that lead to its low plasma levels in rats.

Introduction
Accumulating evidence has well documented that some (if not all) herbal products are characterized by very low absolute oral bioavailability and exposure but that they can exert their pharmacological effects well (Hao et al., 2007), suggesting that the distribution of their active ingredients in certain tissues or organs, particularly inside the active sites, may be contributory. Berberine, one of the frequently used herbal medications in Eastern Asians, has been found to have various pharmacological activities for treating certain diseases with antibacterial (Amin et al., 1969), antitumor (Ho et al., 2009), antioxidant (Hwang et al., 2002), cholesterol-lowering (Kong et al., 2004), and abundant tissue distribution of berberine. After intragastric administration, approximately half of berberine ran intact through the gastrointestinal tract and another half was disposed of by the small intestine, leading to an extremely low extent of absolute oral bioavailability in rats (0.36%). Moreover, the major berberine metabolites were identified and quantified in rat enterocyte S9 fractions, portal vein plasma, and intestinal perfusates; plasma concentrations and tissue distribution of berberine and its major metabolites were determined as well. Data indicated that M1, M2 glucuronide, and M3 were the major metabolites generated from the small intestine that there was a 70-fold increase in the ratio of the area under the concentration-time curve value for berberine (liver versus plasma). We conclude that intestinal first-pass elimination of berberine is the major barrier of its oral bioavailability and that its high extraction and distribution in the liver could be other important factors that lead to its low plasma levels in rats.

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ABBREVIATIONS: G6P, glucose 6-phosphate; G6PD, glucose-6-phosphate dehydrogenase; UDPGA, UDP-glucuronic acid; LC, liquid chromatography; MS/MS, tandem mass spectrometry; PK, pharmacokinetic; GI, gastrointestinal; OCT, organic cation transporter; P-gp, P-glycoprotein.
intravenous administration (Liu et al., 2009). However, less is known about how the small intestine and liver make their relative contributions to the extensive first-pass elimination of berberine in the body. To do that, this study was designed to dissect the entire first-pass elimination of berberine and tissue distribution of both berberine and its major metabolites (which are chemically synthesized in advance) in rats after dosing via four different routes, intragastric, intraduodenal, intraportal, and intravenous, and to further investigate the intestinal metabolism of berberine by identification and quantification of its major metabolites being generated in rat enteroctye S9 fractions, intestinal perfusates (using a single-pass intestinal perfusion model), and portal vein blood after intraduodenal dosing of berberine, respectively.

### Materials and Methods

#### Chemicals and Reagents
Berberine, tetrahydroberineper, and jatrorrhizine (purity >99% each) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Glucose 6-phosphate (G6P) (98–100%), glucose 6-phosphate dehydrogenase (G6PD) (≥150 units/mg protein), NADP (approximately 98%), UDP-glucuronic acid (UDPGA) (98–100%), D-saccharic acid 1,4-lactone (trisaccharalactone) (≥98%), alamethicin (≥90%), and β-glucuronidase (1240 units/mg of solid) were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography-grade acetonitrile was obtained from J.T. Baker Chemical Co. (Montgomery, PA). Water was purified using a Milli-Q system (Millipore Corporation, Milford, MA). All other reagents were of the highest grades available from standard commercial sources.

#### Synthesis of Two Major Phase I Metabolites: Berberrubine and Demethylberberine
To prepare berberrubine (M1), berberine chloride (0.7 g) was heated at 190°C in a dry oven under vacuum (20–30 mm Hg) for 20 min. The crude product was eluted with CHCl3-MeOH (10:1, v/v) through a silica gel column to separate M1 (0.35 g, yield 52.2%; m.p. 255–259°C).

For the preparation of demethylberberine (M2), berberine chloride (1.0 g, 2.7 mM), and phenol (0.76 g, 8.1 mM) were mixed with 60% H2SO4 (40 ml) and stirred at 100°C in an oil bath for 8 h. The mixture was cooled, filtered, and subjected to anion exchange into chloride form in HCl-acetone (1:1). The crude product was concentrated to prepare M2 (0.31 g, yield 31.3%). After cooling, the mixture was heated at 190°C in a dry oven under vacuum (20–30 mm Hg) for 20 min. The crude product was eluted through the column with a gradient of water-formic acid (100:0.05, 90:0.05, 80:0.05, 70:0.05, and 60:0.05, respectively). The separation and determination were performed using a Shim-pack VP-ODS analytical column (150 mm × 2.0 mm i.d.; Shimadzu, Kyoto, Japan). Samples were eluted through the column with a gradient of water-formic acid (100:0.05, v/v) and acetonitrile (0 min, 85:15; 5 min, 25:75; 6 min, 85:15; and 10 min, 85:15) at a flow rate of 0.2 ml/min at 40°C in the column oven. Berberine, tetrahydroberineper, M1 (berberrubine), M2 (demethylberberine), and M3 jatrorrhizine standards were used to validate the LC-MS/MS methods.

#### Relative Contribution of the Small Intestine and Liver to the First-Pass Elimination of Berberine
To differentiate the hepatic and gastrointestinal first-pass elimination of berberine, four different dosing routes, intragastric, intraduodenal, intraportal, and intravenous, were adopted (Kim and Lee, 2002). In brief, the carotid artery, jugular vein, and portal vein of each rat were cannulated under urethane anesthesia. Berberine was administered after 24 h of recovery. Rats were infused with berberine, intravenously or intraportally, for 15 min at a dose of 4 mg/kg, whereas the dose was set at 100 mg/kg for the intragastric or intraduodenal administration. Blood (approximately 0.15 ml) was collected from the carotid artery into heparinized tubes at pre-dose and at 0.083, 0.25 (the time for infusion), 0.5, 1, 2, 4, 6, 8, 12, 24, and 36 h postdose. The rats were euthanized by cervical dislocation at the end of the experiment, and the total gastrointestinal tract and its contents were collected to determine berberine concentrations. The quantification of plasma berberine concentrations was performed using an LC-MS/MS system with tetrahydroberineper as an internal standard.

#### Preparation of Enteroctye S9 Fractions
The small intestines from five male rats were rinsed with 10 mM phosphate buffer, pH 7.4, containing 0.5% NaCl (phosphate-buffered saline) and then were cut longitudinally; the mucous membrane was scraped gently with a slide. The mucosal scrapings were mixed with 5 volumes (w/v) of 10 mM phosphate buffer containing 5 mM EDTA, pH 7.4, and homogenized to prepare 20% homogenates. The homogenates were subjected to centrifugation to isolate cellular S9 fractions as described elsewhere (van de Kerkhof et al., 2007). The protein concentrations of the cellular fractions S9 were determined using a commercially available kit (BCA protein assay; Pierce Chemical, Rockford, IL) following the manufacturer’s instructions. The cellular fractions S9 were stored at −80°C before use.

#### In Vitro Incubation
The phase I reactions were conducted in a medium containing 100 mM potassium phosphate buffer (pH 7.4), 10 mM MgCl2, 0.5 mM NADP, 10 mM G6P, 1 unit/ml G6PD, 25 μM berberine (based on the Km values determined in rat liver microsomes) (Liu et al., 2009), and 2 mg/ml protein of intestinal S9 in a total volume of 200 μl. After preincubation for 5 min, the reaction was initiated by addition of the NADPH-generating system. After incubation at 37°C for 120 min (the long incubation time was designed to generate sufficient metabolites), the reaction was terminated by addition of 2 volumes of ice-cold acetonitrile. Blank incubations were performed without NADP or with boiled microsomes.

To determine the phase II glucuronide-conjugated metabolites, the intestinal S9 preparations were pretreated with alamethicin (a helical pore-forming peptide) at 25 μg of alamethicin/mg subcellular protein on ice for 15 min (Hao et al., 2007). The reaction mixture consisted of 25 μM berberine, 2 mg of subcellular protein, 5 mM UDPGA, 10 mM MgCl2, 1 mM saccharic acid-1,4-lactone, and the NADPH-generating system (containing 0.5 mM NADP, 10 mM G6P, 1 unit/ml G6PD, and 100 mM potassium phosphate buffer, pH 7.4) in a final volume of 200 μl. After preincubation for 5 min at 37°C, the reaction was started by addition of UDPGA. Blank incubations were performed without UDPGA or the NADPH-generating system or with boiled subcellular proteins. All reactions were incubated at 37°C for 120 min and terminated by adding 2 volumes of ice-cold acetonitrile. Blank incubations were performed without UDPGA or with boiled microsomes.

#### Identification of the Major Metabolites in Portal Vein Plasma
Rats were anesthetized with urethane, and a cannula was inserted into the portal vein to collect blood samples. An aliquot of blood was collected from the portal vein 24 h after surgery. Then berberine (100 mg/kg) was instilled into the duodenum and portal blood was collected at 5, 15, and 30 min after dosing to identify the intestinal metabolites of berberine. The blood was centrifuged immediately at 14,000g for 5 min at room temperature to obtain plasma. Plasma samples were extracted with 5 volumes of n-butanol.

#### Single-Pass Intestinal Perfusion
Single-pass intestinal perfusion studies were performed using an established method as described elsewhere (Cook and Shenoy, 2003). In brief, Sprague-Dawley rats (male, 180–220 g) were anesthetized using an intraperitoneal injection of ethyl urethane and placed on a heated pad to keep body temperature normal. The small intestine was surgically exposed, and 20-cm segments of duodenum and jejunum were ligated for perfusion and cannulated with plastic tubing (0.04 inch i.d. and 0.085 inch o.d.). The cannulated segment was rinsed with saline (37°C) and attached to a syringe pump. The biliary duct was ligated to prevent enterohepatic circulation. Blank perfusion buffer was infused for 20 min followed by the perfusion of berberine (20 μg/ml) at a flow rate of 0.2 ml/min for 120 min. Phenol red (20 μg/ml) was added as a nonabsorbable marker for water flux. The perfusate was collected every 20 min through microtubes. The length and perimeter of the segments were measured after the last collection, and finally the animal was sacrificed with a cardiac injection of a saturated solution of KCl. Samples were frozen immediately and stored at −20°C until analysis. After thawing at 37°C, β-glucuronidase (100 units) was mixed with 100 μl of the perfusate and incubated for 16 h at 37°C (Zuo et al., 2006). Samples pretreated with or
without β-glucuronidase were extracted by 2 volumes of acetonitrile for later LC-MS/MS analysis.

**Pharmacokinetic Study of Berberine and Its Major Metabolites after Oral and Intravenous Administrations.** Rats were given berberine, either 100 mg/kg via the intragastric route or 4 mg/kg via the intravenous route. Blood was collected predose and at 0.083, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, and 48 h postdose from five rats and kept in heparinized Eppendorf tubes. Plasma (100 μl) was separated by centrifugation at 5000g for 10 min and stored at −20°C before analysis. β-Glucuronidase (50 units) was mixed with 1/2 volume of thawed plasma (50 μl) and incubated for 16 h at 37°C (Zuo et al., 2006). Plasma samples pretreated with or without β-glucuronidase were extracted by 5 volumes of n-butanol.

**Tissue Distribution of Berberine and Its Major Metabolites after Oral Administration.** After rats were given a single oral dose of 100 mg/kg berberine, their heart, liver, spleen, lung, kidney, and brain were collected at 0.5, 2, 8, and 24 h postdose, respectively. There were five rats for each time point. These tissue samples were homogenized to make 20% homogenates and stored at −20°C before analysis. β-Glucuronidase (100 units) was mixed with 100 μl of the homogenates and incubated for 16 h at 37°C (Zuo et al., 2006). Tissue samples pretreated with or without β-glucuronidase were extracted by 5 volumes of n-butanol.

**Data Handling and Biostatistical Analysis.** The area under the drug concentration-time curve (AUC(t)) was estimated by trapezoidal rule (non-compartmental analysis). If we assume that berberine is chemically stable in the gastrointestinal tract and metabolized in the small intestine and liver in rats and that PK profiling of berberine is linear in the range of berberine doses used, total absolute oral bioavailability (F), hepatic bioavailability (Fh), and gastrointestinal bioavailability (Fg) were calculated using eqs. 1 to 4:

\[F_1 = F_g 	imes F_h \times F_6 \quad (1)\]
\[F_1 = \frac{AUC_{i.d.}}{AUC_{i.p.v.}} \times \frac{D_{i.d.}}{D_{i.p.v.}} \quad (2)\]
\[F_1 = \frac{AUC_{i.p.v.}}{AUC_{i.d.}} \times \frac{D_{i.p.v.}}{D_{i.d.}} \quad (3)\]
\[F_g = 1/F_1 \times F_1/F_6 = 1/F_2 \times AUC_{i.d.}/AUC_{i.p.v.} \times \frac{D_{i.p.v.}}{D_{i.d.}} \quad (4)\]

where D denotes the dose of berberine and Fn is the fraction of absorption, which equals total dosage minus the sum of both the fraction not being absorbed and the fraction excreted by the efflux transporters into the intestinal lumen and bile after berberine was absorbed; i.e., indicates the gastrointestinal route, i.v. indicates the intravenous route, i.d. indicates the intraduodenal route, and i.p.v. indicates the intraperitoneal route.

The effective permeability coefficient of berberine (Papp) was calculated using eq. 5 (Zakeri-Milani et al., 2007) according to the parallel model:

\[P_{app} = -Q \ln [C_{int}/C_{int}]/2 \pi r \quad (5)\]

where Cint denotes the concentration in the intestinal lumen and Cint denotes the outlet concentration of berberine, both of which are normalized by volume change in the segment using phenol red concentration in inlet and outlet tubing. Q denotes the flow rate (0.2 ml/min), r is the intestinal radius of each rat, and l is the length of the segment. In addition, eq. 6 was used to make a correction:

\[C_{corrected} = C_{measured} \times [\text{phenol red}]_{\text{in}}/[\text{phenol red}]_{\text{out}} \quad (6)\]

To estimate the velocity of the major phase I metabolites of berberine present in the perfusate, eq. 7 was used:

\[Velocity = C_{metabolite} \times \text{Volume}_{\text{perfu}}/2 \pi r \quad (7)\]

where Cmetabolite denotes the concentration of phase I metabolite in the perfusate, t is the time of perfusion (20 min for each point), r is the intestinal radius of each rat, and l is the length of the segment.

**Results**

**Hepatic First-Pass Elimination of Berberine.** As shown in Table 1, after a 15-min infusion of berberine at 4 mg/kg intraportally or intravenously, intraportal administration (ruling out the role played by the small intestine in the overall first-pass elimination of berberine) led to a significant reduction in AUC(t) of berberine in plasma compared with intravenous dosing (466.0 ± 109.9 versus 649.2 ± 99.5 ng · h/ml; n = 5; P < 0.05). According to eq. 3, the Fh was estimated at 71.8% of the fraction of berberine that entered the portal vein. In other words, first-pass elimination of berberine in the liver was estimated at 28.2% of the fraction of berberine that entered the portal vein.

**Gastrointestinal First-Pass Elimination of Berberine.** The fraction of berberine not being absorbed during the period of 36 h after intragastric administration was at least 56% of the total dose given, of which 32% was recovered from rat stools and 24% was recovered from the entire gastrointestinal (GI) tract. Thus, the Fg value was estimated at 44%.

According to eqs. 2 and 4, the F1 and Fg values were estimated at 0.37 and 1.16%, respectively. Moreover, the GI bioavailability (Fgi) after drug intake by the intragastric or intraduodenal routes was estimated by eqs. 8 and 9, respectively:

\[F_{gi} = \frac{AUC_{i.g.} \times D_{i.p.v.}}{AUC_{i.p.v.} \times D_{i.g.}} \quad (8)\]
\[F_{gi} = \frac{AUC_{i.d.} \times D_{i.p.v.}}{AUC_{i.p.v.} \times D_{i.d.}} \quad (9)\]

According to eqs. 8 and 9, Fgi was 0.50 and 0.51%, respectively. If the hepatic first-pass effect was ignored, the first-pass elimination in the GI tract was 99.5%, of which the gastric first-pass effect was 0.01% and the intestinal first-pass effect was 99.49%. When the fraction of berberine not being absorbed (56%) was taken into account, gastric and intestinal first-pass effects were adjusted to be 0.004 and 43.8%, respectively. Therefore, the fraction of berberine that entered the portal vein was estimated at 0.2% of the total oral dose, and hepatic first-pass effect was 0.056% after oral intake of berberine in rats.

As shown in Table 1, the AUC(t) values of berberine after intraduodenal dosing were not significantly different from that after intraduodenal dosing, indicating that the gastric first-pass elimination was almost negligible (if any) in rats. However, the AUC(t) values of berberine after intraduodenal dosing were significantly less than those after intraportal administration (that is, mean AUCi.d. was nearly 0.5% of that of the AUCi.p.v. when normalized by the dose given), suggesting that first-pass elimination of berberine occurs predominantly in rat small intestine but not in the liver and stomach.

**Identification of Major Metabolites of Berberine In Vivo and In Vitro.** The major metabolites of berberine were identified in several important matrices, including systemic plasma, portal plasma, enterocyte S9 fractions, and intestinal perfusates. LC-MS/MS spectrum analysis together with the synthetic standards of berberine metabolites indicated that berberine phase I metabolites were M1 via demethylation, M2 via demethylation, and M3 (jatrorrhazine), whereas its phase II metabolites were the corresponding glucuronide conjugates of M1, M2, and M3, respectively (Fig. 1).
These results were consistent with previous findings as described elsewhere (Tsai and Tsai, 2004; Zuo et al., 2006). All six metabolites appeared in systemic plasma and portal vein plasma after intragastric and intraduodenal dosing. M1, M2, M3, M1 glucuronide, and M2 glucuronide were identified in enterocyte fractions S9 and intestinal perfusates.

**Single-Pass Intestinal Perfusion.** An effective permeability coefficient ($P_{eff}$) was estimated on the basis of the berberine steady-state concentrations (occurring at approximately 60 min after perfusion, as measured by the desired phenol red concentrations at steady state) in collected perfusates. Consequently, $P_{eff}$ of berberine was estimated at $1.78 \pm 0.90 \text{ cm/s} \ (10^{-5})$, according to eq. 5.

For the major metabolites of berberine being identified in the perfusates, their formation velocity was calculated and is summarized in Table 2. M1, M2 glucuronide, and M3 are the major metabolites generated from the small intestine.

**Metabolism of Berberine and the Formation of Its Major Metabolites Vary by Its Dosing Route.** The AUC$_{0-t}$ values of berberine and its phase I metabolites (M1, M2, and M3) after intragastric and intravenous dosing are summarized in Table 3. Oxidative demethylation (M1) and its subsequent glucuronidation were predominantly metabolic pathways after oral intake, in contrast with our more recent study (Liu et al., 2009), in which oxidative demethylation (M2) of berberine and subsequent glucuronidation of M2 were major metabolic pathways after intravenous dosing in rats. After digestion with $\beta$-glucuronidase, the AUC$_{0-t}$ ratio of M1 to berberine was 6.8-fold, whereas the AUC$_{0-t}$ ratio of M2 to berberine was 2.7-fold after intragastric dosing. In contrast, the AUC$_{0-t}$ ratio of M1 to berberine was 37%, whereas the AUC$_{0-t}$ ratio of M2 to berberine was 1.6-fold after intravenous dosing. These data suggest that intestinal first-pass elimination of berberine is the major barrier of its absolute oral bioavailability and that its metabolic pathway is dependent on its dosing route.

**Tissue Distribution of Berberine and Its Major Metabolites after Oral Intake.** The AUC$_{0-t}$ values of berberine and its phase I metabolites (M1, M2, and M3) in different tissues as well as their ratios (tissue/plasma) after oral intake are summarized in Table 4. In addition, the concentrations of berberine and its phase I metabolites at different time points in the liver are also summarized in Fig. 2. After intragastric dosing, berberine was widely distributed into various tissues, including liver, heart, kidney, spleen, lung, and even brain, with the liver being the most predominant organ, in which the mean level of berberine was approximately 70-fold greater than that in plasma (Table 4). As for the major metabolites of berberine, the unconjugated metabolites were the major forms present in tissues, including liver, heart, and kidney; however, glucuronides of phase I metabolites of berberine were the major forms in plasma after oral intake. The liver is the key organ for the distribution of berberine and its unconjugated metabolites (Fig. 2), and the ranking order of their amount was M2 > berberine > M1 >> M3, consistent with our most recent studies on the metabolism of berberine in the liver (Liu et al., 2009).

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
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<tr>
<td></td>
<td>$\text{ng/(min \cdot cm}^2\text{)}$</td>
<td></td>
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<tr>
<td>Enzyme-digested</td>
<td>$0.181 \pm 0.057$</td>
<td>$0.148 \pm 0.09^*$</td>
<td>$0.42 \pm 0.11$</td>
</tr>
<tr>
<td>Undigested</td>
<td>$0.163 \pm 0.042$</td>
<td>$0.022 \pm 0.012$</td>
<td>$0.44 \pm 0.122$</td>
</tr>
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* $P < 0.05$ vs. undigested.
In this study, we dissected the role the GI tract and liver could play in the overall first-pass elimination of berberine in rats and found that the first-pass elimination of berberine occurs predominantly in the small intestine rather than in the liver and stomach, that the liver dominates over the other organs in its tissue distribution, and that oxidative demethylation of berberine (generating M1) and subsequent glucuronidation are the major mechanisms responsible for the intestinal metabolism of berberine after oral intake. Using four different dosing routes (intragastric, intraduodenal, intraportal, and intravenous) to estimate various bioavailabilities of berberine, we observed very low $F_t$ (0.36%) and $F_{GI}$ (0.5% each by the intragastric or intraduodenal route) values, confirming a very low exposure of berberine after oral intake in rats and further providing some underlying mechanisms to explain this observation.

In theory, an extremely low plasma concentration of berberine is associated mainly with the following PK causes: 1) extensive metabolism in the gut and/or liver (Tsai and Tsai, 2004; Zuo et al., 2006; Hao et al., 2007; Qiu et al., 2008); 2) marked excretion to intestinal lumen, bile (Tsai and Tsai, 2004), and urine (Qiu et al., 2008) (as a substrate of certain efflux transporters) (Xie, 2010), as well as enterohepatic circulation (Zuo et al., 2006; Xie, 2010); 3) poor absorption (due to some unique physicochemical properties); and 4) predominant tissue distribution.

Previous studies in vivo (Tsai and Tsai, 2004; Zuo et al., 2006; Qiu et al., 2008), including our more recent study in vitro (Liu et al., 2009), have well demonstrated that berberine is extensively metabolized in the body to generate M1 and M1 glucuronide via oxidative demethylation and subsequent glucuronidation and to form M2 and M2 glucuronide via oxidative demethylation and subsequent glucuronidation. Therefore, UDP-glucuronosyltransferases have been identified as the major drug-metabolizing enzymes responsible for the formation of the phase II metabolites of berberine as shown by our more recent study in vitro (Liu et al., 2009) and this study.

Marked efflux transporter-mediated excretion of berberine to intestinal lumen, bile, and urine may be a second PK cause leading to the very low absorption and exposure of berberine after oral intake.

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Previous observations have shown that, in addition to being a substrate of certain influx transporters (OCT1 and OCT2) (Nies et al.,...


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