Breast Cancer Resistance Protein and Multidrug Resistance Protein
2 Determine the Disposition of Esculetin-7-O-Glucuronide
and 4-Methylesculetin-7-O-Glucuronide

Yuhuan Li,1 Wenjie Song,1 Xiaojun Ou, Guangkuo Luo, Yushan Xie, Rongjin Sun, Ying Wang, Xiaoxiao Qi, Ming Hu, Zhongqiu Liu, and Lijun Zhu

Joint Laboratory for Translational Cancer Research of Chinese Medicine of the Ministry of Education of the People’s Republic of
China, International Institute for Translational Chinese Medicine, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong, People’s Republic of China (Y.L., W.S., X.O., G.L., Y.X., R.S., Y.W., X.Q., Z.L., L.Z.); State Key Laboratory of
Quality Research in Chinese Medicine, Macau University of Science and Technology, Macau (Special Administration Region), People’s
Republic of China (Z.L.); and Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, Texas (M.H.)

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ABSTRACT

Esculetin (ET)-7-O-glucuronide (ET-G) and 4-methylesculetin (4-ME)-7-O-glucuronide (4-ME-G) are the main glucuronide of ET
and 4-ME, respectively. The disposition mediated by efflux transporters for glucuronide has significant influence on the pharma-
cookinetic profile and efficacy of bioactive compounds. In the current study, transporter gene knockout mice and Caco-2 cells were used
to explore the effects of breast cancer resistance protein (BCRP) and multidrug resistance–associated protein 2 (MRP2) on the
disposition of ET-G and 4-ME-G. After oral or i.v. administration of ET and 4-ME, the area under the plasma concentration–time
curve from time 0 to the last data point or infinity values of ET, 4-ME, and their glucuronides (ET-G and 4-ME-G) were remarkably and signif-
ically increased in most Bcrp1−/− and Mrp2−/− mice compared with those in wild-type FVB mice (P < 0.05). These results were
accompanied with a significant increase of maximum plasma concentration values (P < 0.05). In Caco-2 monolayers, the efflux
and clearance rates of ET-G and 4-ME-G were markedly reduced by the BCRP inhibitor Ko143 and MRP2 inhibitor MK571 on the
apical side (P < 0.05). In an intestinal perfusion study, the excretion of ET-G was significantly decreased in perfusate and increased in
plasma in Bcrp1−/− mice compared with those in wild-type FVB mice (P < 0.05). The 4-ME-G concentration was also decreased in
the bile in transporter gene knockout mice. ET and 4-ME showed good permeability in both Caco-2 monolayers [apparent perme-
ability (Papp) ≥ 0.59 × 10−5 cm/s] and duodenum (Papp ≥ 1.81). In conclusion, BCRP and MRP2 are involved in excreting ET-G and
4-ME-G. ET and 4-ME are most likely absorbed via passive diffusion in the intestines.

Introduction

Esculetin [6,7-dihydroxy-coumarin (ET)] is a well-known naturally
occurring coumarin derivate found in many herbs, such as Fraxinus
rhynchophylla, Rehmannia glutinosa, and Artemisia capillaries (Li
et al., 2011; Venugopala et al., 2013). ET provides a broad range of
pharmacological activities, including antioxidant, anti-inflammatory,
antitumor, antiviral, antifungal, and neuroprotective properties (Witaicenis
et al., 2010; Viani et al., 2012; Wang et al., 2012; Jeon et al., 2015). The
4-methylesculetin [6,7-dihydroxy-4-methylcoumarin (4-ME)], a synthetic
coumarin derivate that contains a methyl group at C-4 in ET molecules, has
also been extensively investigated because it exhibits various pharmaco-
logical functions similar to those of ET (Hajime et al., 2007; Henshkekar
et al., 2013; Maistro et al., 2015). Thus, ET and 4-ME, as potential
therapeutic agents, have attracted substantial attention from researchers.

The disposition characteristics of bioactive compounds influence their
in vivo pharmacokinetics and pharmacodynamics. Elucidation of the
disposition characteristics and underlying mechanisms of the disposition
of these compounds would provide information to identify potential
drug candidates and drug–drug interactions. We have previously
demonstrated that the conjugation reactions of ET and 4-ME at the
7-C position generate ET-7-O-glucuronide (ET-G) and 4-ME-7-O-
glucuronide (4-ME-G), respectively. This reaction is mediated by
UDP-glucuronosyltransferase 1A9 and UGT1A6 in human liver and intestinal microsomes (Zhu et al., 2015). The elimination of glucuronides from cells requires the aid of efflux transporters [e.g., breast cancer resistance protein (BCRP)] because these metabolites are too hydrophilic to diffuse across the cellular membrane (Jeong et al., 2005b; Wang et al., 2006). The clinical evidence of impaired BCRP activity produced increased systemic exposure of gefitinib, rosuvastatin, sunitinib, and active metabolite of leflunomide, sulfasalazine, and diflomotecan (Lee et al., 2015). The importance of efflux transporters modulating the glucuronides excretion of bioactive compounds has been demonstrated in wild-type and transporter knockout mice (Ge et al., 2015; Qin et al., 2018). Thus, further investigation into the role of efflux transporters in the excretion of ET-G and 4-ME-G is important to accurately predict their disposition and efficacy in vivo.

BCRP and multidrug resistance–associated protein (MRP2) are ATP-binding cassette transporters that are widely located on the apical (AP) portions of the liver and intestine (Yang et al., 2017). BCRP and MRP2 are likely to excrete compounds with high hydrophilicity such as most conjugation metabolites (glucuronides and sulfates) (An and Morris, 2011; Zheng et al., 2016). The inhibition or deficiency of BCRP and MRP2 decreases the plasma exposure of parent drugs and their metabolites, which could result in reduced efficacy, although these drugs have good absorption characteristics (Pan et al., 2015; Kong et al., 2016). Transporter (e.g., P-glycoprotein, BCRP, and MRP2) gene knockout models, in which glucuronidation activities remain unaltered, are commonly used in understanding transporter-limited or transporter-mediated drug absorption, distribution, and excretion (Klaassen and Lu, 2008; Zamek-Gliszczynski et al., 2012, 2013). Mouse Bcrp1 protein and human BCRP protein are structurally and functionally similar. They share 87% sequence homology and efflux identical substrates (Natarajan et al., 2011). Murine in vivo models harboring Bcrp1 gene knockouts in FVB or C57BL/6J mouse strains are commonly used to predict the BCRP regulation of human small intestinal drug disposition (Zhou et al., 2002; Agarwal et al., 2012; Jiang et al., 2017). The amino acid sequence identity of human MRP2 with its mouse ortholog is approximately 78%, and MRP2 knockout mice are frequently used to investigate the impact of MRP2 on the pharmacokinetics of clinical drugs (Zimmermann et al., 2008).

Caco-2 cells, which express various phase I and phase II enzymes as well as ATP-binding cassette transport proteins such as P-glycoprotein, BCRP, and MRPs (e.g., MRP1, MRP2, MRP3, and MRP4), are frequently used to study human intestinal absorption, metabolism, and transport (Ikeda et al., 2008; Meintl et al., 2008; Ming and Thakker, 2010). Inhibition assays in Caco-2 monolayers are also commonly used to identify transporter substrates. Ko143 (at concentrations from 0.5 to 20 μM) and MK571 (at concentrations from 10 to 100 μM) are frequently used to confirm the role of BCRP and MRPs [including MRP2 expressed on the AP membrane and MRP3 and MRP4 expressed on the basolateral (BL) membrane] in the disposition of xenobiotics in previous studies (Sheng et al., 2015; Shi et al., 2016; Ma et al., 2017; Zang et al., 2018).

In the current study, the pharmacokinetics of ET and 4-ME in Bcrp1 and Mnp2 knockout mice, as well as that in wild-type FVB mice, was studied. Inhibition assays in Caco-2 monolayers were conducted to verify the role of BCRP and MRP2 in the disposition of ET-G and 4-ME-G in vitro. The absorption characteristics of ET and 4-ME were also evaluated in Caco-2 cells. The perfused transporter knockout mice intestinal model was employed to confirm the role of BCRP and MRP2 in the disposition of ET and 4-ME. The concentrations of ET, 4-ME, and their glucuronides in different samples, such as plasma, cell culture medium, perfusate, and bile, were determined by ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS).

### Materials and Methods

#### Chemicals and Reagents

ET, 4-ME, ET-G, and 4-ME-G were obtained, as described previously (Zhu et al., 2015). Testosterone [used as internal standard (IS); purity >98%], Ko143, MK571, and Hanks’ balanced salt solution (HBSS; powder form) were purchased from Sigma-Aldrich (St. Louis, MO). Six-well polycarbonate cell culture inserts (with an absorption surface area of approximately 4.2 cm² and a pore size of 3 μm) were obtained from Corning (Corning, NY). Cloned Caco-2 cells (TC7) were provided by M.H. (Department of Pharmaceutical Sciences, College of Pharmacy, University of Houston, TX). All other chemicals and solvents were of analytical grade or better.

#### Animals

Male wild-type FVB mice (9–11 weeks) were purchased from Vital River Laboratory Animal Technology (Beijing, China). Male Bcrp1−/− and Mrp2−/− mice (9–11 weeks old) of a >99% FVB genetic background were purchased from Biomed Model Organism Science & Technology Development (Shanghai, China). Mice were kept in an environmentally controlled room (temperature of 25 ± 2°C, relative humidity of 50% ± 5%, and 12-hour dark/light cycle) for at least 1 week before the experiment. The animal experiments used in this study were approved by the Guangzhou University of Chinese Medicine’s Ethics Committee.

#### Cell Culture

Caco-2 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, and 1% antibiotics (penicillin and streptomycin) at 37°C with 5% CO₂. For the transport experiments, 2.5 × 10⁵ cells/well were seeded onto the 4.2-cm² inserts and grown for 19–22 days. Transepithelial electrical resistance values less than 460 Ω/cm² were discarded.

#### UHPLC-MS/MS Conditions

ET, 4-ME, and their glucuronides (ET-G and 4-ME-G) were separated and quantified using an Agilent UHPLC 1290 Infinity-Triple Quad MS 6460 (UHPLC-MS/MS) equipped with an electrospray ionization source. The chromatographic separation was achieved on a Zorbax C₁₈ column (100 × 3.0 mm², 1.8 μm; Agilent Technologies, Santa Clara, CA) using a mixture of 0.1% formic acid aqueous solution (A) and methanol (B) as the mobile phase with the following gradient elution: 20% (v/v) B from 0 to 1 minute; 90% B from 3 to 6 minutes; 20% B at 7 minutes; and the post-time was 0.5 minutes. The injection volume was 5 μL, the flow rate was 0.3 ml/min, and the column temperature was maintained at 35°C. The mass spectrometer parameters were as follows: capillary voltage, 4.0 kV; nozzle voltage, 500 V; nebulizer, 45 psi; gas temperature, 300°C with a gas flow of 5 l/min; and sheath temperature, 350°C with a sheath gas flow of 11 l/min.

The optimized fragmentation voltages of ET, ET-G, and testosterone (IS) were 90, 100, and 120 V, respectively. Quantification was performed in positive ion and multiple reaction monitoring mode using the following transitions: m/z 179.0→122.9 for ET with a collision energy of 22 eV; m/z 355.0→179.0 for ET-G with a collision energy of 15 eV; and m/z 289.0→97.1 for testosterone with a collision energy of 22 eV. The δ electron multiplier voltage was 250 V, and the cell acceleration voltage of all was 4 V. The method was to waste before 1 minute, then to mass spectrometer.

The optimized fragmentation voltages of 4-ME, 4-ME-G, and testosterone (IS) were 105, 135, and 120 V, respectively. Quantification was performed in positive ion and multiple reaction monitoring mode using the following transitions: m/z 193.0→147.0 for 4-ME with a collision energy of 20 eV; m/z 369.0→193.0 for 4-ME-G with a collision energy of 20 eV; and m/z 289.0→97.1 for testosterone with a collision energy of 22 eV. The δ electron multiplier voltage was 400 V, and the cell acceleration voltage of all was 4 V. The method was to waste before 2 minutes, then to mass spectrometer.

#### Pharmacokinetic Studies of ET and 4-ME in Wild-Type, Bcrp1−/−, and Mrp2−/− FVB Mice

Mice were fasted for 10 to 12 hours with free access to water before the pharmacokinetics experiments. For oral administration, ET and 4-ME suspensions (1 mg/ml) were prepared with 20% (v/v) hydroxypropyl β-cyclodextrin aqueous solution. Wild-type and transporter knockout FVB mice were orally administered with 10 mg/kg ET (56.14 μmol/kg) or 4-ME (52.03 μmol/kg). For i.v. injection, ET and 4-ME solutions (0.3 mg/ml) were prepared with 5% (v/v) hydroxypropyl β-cyclodextrin aqueous solution. Wild-type and transporter knockout FVB mice were i.v. injected with 2 mg/kg ET (11.23 μmol/kg) and 4-ME (10.41 μmol/kg). Blood samples (approximately 25 μl) were collected from the tail vein and then placed in dried heparinized tubes at 0, 3, 5, 10, 15, 30, 45, 60, 120, 240, 360, 480, 720, and 1440 minutes. The first blood sample was collected by cutting the tail; the following blood samples were collected by scraping the cut. The blood samples were centrifuged at 11,040g for...
8 minutes. The plasma supernatant was removed to a new tube and stored at −80°C until analysis. Plasma samples were prepared by mixing 10 μl plasma sample and 200 μl methanol containing 200 nM IS, followed by vortexing for 3 minutes. The mixture was centrifuged at 19,357 g for 30 minutes. Supernatant (160 μl) was transferred to a new tube and evaporated to dryness in a vacuum drying oven. The residue was dissolved with 80 μl 50% methanol aqueous solution. After centrifugation at 19,357 g for 30 minutes, 10 μl supernatant was injected into the UHPLC-MS/MS for analysis.

The pharmacokinetic parameters, including area under the plasma concentration–time curve (AUC) from time 0 to the last data point or infinity (AUC0–∞, and AUC0–t), half-life, maximum plasma concentration (Cmax), mean residence time, t1/2 and clearance (CL), were analyzed using the noncompartmental model in WinNonlin 3.3 (Pharsight, Mountain View, CA). The AUC0–t or AUC0–∞ was calculated by the trapezoidal rule–extrapolation method. The average absolute bioavailability (F) values of ET and 4-ME were determined by using the following formula:

\[
F = \frac{AUC_{0-\infty,\text{ET or 4-ME}} \times D_{p,o}}{AUC_{0-\infty,\text{IS}} \times D_{p,o}} \times 100\% 
\]

(1)

where \(AUC_{0-\infty,\text{ET or 4-ME}}\) and \(AUC_{0-\infty,\text{IS}}\) represent the average area under the concentration–time curve from zero to infinity after ET (or 4-ME) oral and injection administration, respectively. \(D_{p,o}\) and \(D_{p,i}\) represent the dose of ET (or 4-ME) oral and injection administration, respectively.

Bidirectional Transport Study of ET and 4-ME in Caco-2 Monolayers

The transport experiments were conducted, as described previously (Ye et al., 2013). Before the experiment, Caco-2 monolayers were washed thrice with warm HBSS at 37°C. Testing compound (2 ml; 10 μM ET or 4-ME) was loaded on the AP side or BL side of the cell monolayers, and 2 ml blank HBSS was loaded on the other side. In the inhibition experiment, BCRP inhibitor (Ko143, 5 μM) and MRP2 inhibitor (MK571, 10 μM) were added to the AP side to determine whether AP efflux transporters (BCRP and MRP2) participate in the efflux of ET, 4-ME, and their glucuronides. Samples (0.5 ml) were collected from both sides of each transwell at designated times (0, 0.5, 1, 1.5, and 2 hours), and the same volume of the testing compound or blank HBSS was immediately replenished. Then 250 μl methanol containing 200 nM IS was added to 500 μl 50% methanol aqueous solution for 30 minutes, followed by vortexing for 3 minutes. The mixture was centrifuged at 19,357 g for 30 minutes, and the supernatant was separated into two aliquots, as follows: one of them was diluted with methanol containing 200 nM IS and used for the qualitative analysis of ET, 4-ME, and their metabolites, and the other side was used for the quantitative analysis. The mean concentration of compounds on the donor side; and 

The apparent permeability (Papp) coefficient of ET and 4-ME across a cellular membrane was estimated using the following equation:

\[
P_{\text{app}} = \frac{dQ/dt}{A_{\text{C0}}} 
\]

(2)

where \(dQ/dt\) is the rate of ET and 4-ME transported on the receiver side; \(A_{\text{C0}}\) is the initial concentration of compounds on the donor side; and \(A\) is the monolayer growth surface area of 4.2 cm².

The fraction of the metabolized dose (Fmet) was calculated in this study in response to the extent of metabolism in Caco-2 cells by the following equation:

\[
F_{\text{met}} = \frac{\sum \text{metabolite}}{\sum \text{metabolite} + \sum \text{parent compound}} 
\]

(3)

The efflux rate (J) of glucuronide was obtained from the rate of change in the concentration of the substrate (or its metabolite), and this parameter was expressed as a function of time and volume of the sampling chamber (V), as follows:

\[
J = \frac{dC}{dt} \times V 
\]

(4)

The CL of efflux transporter was calculated in this study because the extracellular concentration of glucuronides differed from their intracellular concentration. CL was determined using the excretion rate of glucuronides (\(C_{\text{in}}\)) divided by the intracellular concentration of glucuronides (\(C_{\text{in}}\), as follows:

\[
CL = \frac{J}{C_{\text{in}}} = \frac{J_{\text{max}}}{K_m + C_{\text{in}}} 
\]

(5)

where \(J_{\text{max}}\) is the maximal excretion rate of glucuronides, and \(K_m\) is the Michaelis constant of glucuronide efflux. To determine \(C_{\text{in}}\), we assumed that the average cytosolic water volume of the cells was 4 μl/mg protein. The intracellular metabolite concentration was calculated from the total amount of intracellular glucuronides divided by the total volume of intracellular protein.

Perfused Mice Intestinal Model for Glucuronidation Disposition of ET and 4-ME

Two segments (upper small intestine and colon) of the mouse intestine were perfused simultaneously with perfusate containing 70 μM ET (or 4-ME) using an infusion pump (model PHD2000; Harvard Apparatus, Cambridge, MA) at a flow rate of 0.167 ml/min. Before the surgery, each mouse was anesthetized with 10 mg/ml urethane (0.03 ml/10 g, i.p.). The mouse intestinal surgical procedures were modified from the rat model previously described (Chen et al., 2003; Jeong et al., 2005a). Briefly, after the mouse was anesthetized, it was put over a heating blanket and under a heating lamp to keep its normal body temperature. Then the mouse abdominal cavity was opened and the duodenum was located as the intestinal segment immediately adjacent to the stomach. First, two cannulae at approximately 10 cm apart were inserted into two ends of the duodenum and secured with suture. Second, the colon inlet cannula was inserted into the colon at approximately 2 cm below the junction, and the outlet cannula was inserted through the anus. After a 30-minute washout period, which is considered to achieve steady-state absorption, perfusate were collected from the outlet cannula every 15 minutes during the 1-hour perfusion period. Perfusion that did not cross the intestinal segments was also collected every 30 minutes as a control. The blood samples were withdrawn from tail vein at the end of the perfusion. The gallbladder was excised, and the bile was collected. The length of the intestine was measured, as described previously (Hu et al., 1998). The effective intestinal permeability (\(P_{\text{eff}}\)) coefficient and absorption amounts of ET and 4-ME and the percentage of glucuronides (ET-G and 4-ME-G) excreted into the perfusate in the mice were measured and calculated, as described previously (Chen et al., 2003; Dai et al., 2015). The exposure of ET-G and 4-ME-G was determined by using the peak area in the perfusion experiment because the commercial source of ET-G and 4-ME-G was unavailable.

Statistical Analysis

SPSS 17.0 was used to evaluate significant differences, and unpaired Student’s t test was used to analyze the data. Data were presented as the mean ± S.D. Differences were considered significant at \(P < 0.05\).

Results

Pharmacokinetic Profile of ET and 4-ME in Wild-type, Bcrp1<sup>−/−</sup>, and MRP2<sup>−/−</sup> FVB Mice

The mean plasma concentration–time curves after the oral administration of 10 mg/kg (56.14 μmol/kg) ET in wild-type, Bcrp1<sup>−/−</sup>, and Mrp2<sup>−/−</sup> FVB mice are shown in Fig. 1, A and B, and the pharmacokinetic parameters are shown in Table 1. After oral administration, ET was quickly absorbed and metabolized into ET-G, and lower ET was detected in plasma samples. The AUC<sub>0–2</sub> values of ET and ET-G in wild-type FVB mice were 2.96 ± 0.98 and 306.68 ± 113.93 minutes • μmol/l, respectively, and those in Bcrp1<sup>−/−</sup> mice were significantly increased to 32.68 ± 14.49 and 1535.30 ± 666.51 minutes • μmol/l, respectively (\(P < 0.05\)). The AUC<sub>0–2</sub> value of ET was significantly increased from 2.96 ± 0.98 minutes • μmol/l in wild-type FVB mice to 23.48 ± 9.96 minutes • μmol/l in Bcrp1<sup>−/−</sup> mice (\(P < 0.05\)). The Cmax<sub>0–2</sub> values of ET and ET-G were also increased from 0.20 ± 0.11 μmol/l in wild-type FVB mice to 8.21 ± 5.78 μmol/l in Bcrp1<sup>−/−</sup> mice for ET and 2.46 ± 0.36 μmol/l in wild-type FVB mice to 7.58 ± 1.65 μmol/l for ET-G in Bcrp1<sup>−/−</sup> mice (\(P < 0.05\)). The Cmax<sub>0–2</sub> value of ET in Mrp2<sup>−/−</sup> mice was more than threefold higher than that in wild-type FVB mice (\(P < 0.05\)). The AUC<sub>0–2</sub> ratios of ET-G to ET (Metabolite/Parent AUC ratios, M/P AUC ratios) in wild-type FVB mice, Bcrp1<sup>−/−</sup> mice, and Mrp2<sup>−/−</sup> mice were 103.61, 41.50, and 15.98, respectively. The pharmacokinetic profiles after i.v. injection (i.v.) of 2 mg/kg (11.23 μmol/kg) ET are shown in Fig. 1, C and D, and the pharmacokinetic parameters are shown in Table 2. The AUC<sub>0–2</sub> value of ET was significantly increased from 5.88 ± 4.93 minutes • μmol/l in
wild-type FVB mice to 32.04 ± 8.47 minutes • μmol/l in Mrp2−/− mice (P < 0.05). The AUC0–∞ value of ET-G was also significantly increased from 155.76 ± 50.41 minutes • μmol/l in wild-type FVB mice to 259.85 ± 69.20 minutes • μmol/l in Bcrp1−/− mice (P < 0.05). The average absolute F% of ET in wild-type FVB mice, Bcrp1−/− mice, and Mrp2−/− mice were 10.07%, 17.10%, and 14.66%, respectively. Additionally, the AUC0–∞/AUC∞ values of ET-G to ET (M/P AUC ratios) in wild-type FVB mice, Bcrp1−/− mice, and Mrp2−/− mice were 26.50, 6.80, and 4.52, respectively.

The mean plasma concentration–time curves after oral administration of 10 mg/kg (52.03 μmol/kg) 4-ME in wild-type, Bcrp1−/−, and Mrp2−/− FVB mice are shown in Fig. 2, A and B, and the pharmacokinetic parameters are shown in Table 3. The AUC0–∞ value of 4-ME in Bcrp1−/− mice (193.56 ± 42.58 minutes • μmol/l) was significantly higher than that in wild-type FVB mice (64.23 ± 18.52 minutes • μmol/l) (P < 0.05). The AUC0–∞ values of 4-ME-G in Bcrp1−/− mice (733.70 ± 202.51 minutes • μmol/l) and Mrp2−/− mice (535.13 ± 81.20 minutes • μmol/l) were also significantly higher than that in wild-type FVB mice (375.21 ± 114.50 minutes • μmol/l) (P < 0.05). The AUC0–∞ of 4-ME-G to 4-ME (M/P AUC ratios) in wild-type FVB mice, Bcrp1−/− mice, and Mrp2−/− mice were 5.84, 3.79, and 5.18, respectively. The pharmacokinetic profiles after the i.v. injection (i.v.) of 2 mg/kg (10.41 μmol/kg) 4-ME are shown in Fig. 2, C and D, and the pharmacokinetic parameters are shown in Table 4. The AUC0–∞ values of 4-ME-G in Bcrp1−/− (162.83 ± 33.01 minutes • μmol/l) and Mrp2−/− mice (175.29 ± 33.66 minutes • μmol/l) were significantly higher than that in wild-type FVB mice (55.08 ± 13.80 minutes • μmol/l) (P < 0.05). The F% of 4-ME in wild-type FVB mice, Bcrp1−/− mice, and Mrp2−/− mice were 22.28%, 45.70%, and 22.97%, respectively. The AUC0–∞ values of 4-ME-G to 4-ME (M/P AUC ratios) in wild-type FVB mice, Bcrp1−/− mice, and Mrp2−/− mice were 0.96, 1.92, and 1.95, respectively.

**Absorption and Metabolic Characteristics of ET and 4-ME in Caco-2 Monolayers.** Figure 3 shows the absorption and metabolic characteristics of ET and 4-ME in Caco-2 cells. The mean Papp values of ET were significantly higher than those of 4-ME regardless of whether the transportation direction was from the AP side to basolateral side (AP-BL) or from the BL side to AP side (BL-AP) (Fig. 3A) (P < 0.05).

**Table 1**

Pharmacokinetic parameters of ET and ET-G in wild-type, Bcrp1−/−, and Mrp2−/− FVB mice after oral administration of 10 mg/kg (56.14 μmol/kg) ET

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ET</th>
<th>ET-G</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FVB</td>
<td>Bcrp1−/−</td>
</tr>
<tr>
<td>Cmax (μmol/l)</td>
<td>0.20 ± 0.11</td>
<td>0.64 ± 0.43*</td>
</tr>
<tr>
<td>Tmax (min)</td>
<td>5.83 ± 2.24</td>
<td>5.00 ± 0.00</td>
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<tr>
<td>AUC0–∞ (min • μmol/l)</td>
<td>2.85 ± 0.94</td>
<td>16.98 ± 6.10*</td>
</tr>
<tr>
<td>AUC0–∞ (min • μmol/l)</td>
<td>2.96 ± 0.98</td>
<td>32.68 ± 14.49*</td>
</tr>
<tr>
<td>T1/2 (min)</td>
<td>32.54 ± 21.32</td>
<td>278.39 ± 201.85*</td>
</tr>
<tr>
<td>MRT (min)</td>
<td>33.20 ± 25.88</td>
<td>330.15 ± 254.85*</td>
</tr>
<tr>
<td>Vd (l/kg)</td>
<td>125.77 ± 67.89</td>
<td>114.56 ± 32.14</td>
</tr>
<tr>
<td>CL/F (l/min per kilogram)</td>
<td>0.43 ± 0.22</td>
<td>0.28 ± 0.05*</td>
</tr>
<tr>
<td>M/P AUC ratios</td>
<td>103.61</td>
<td>41.50</td>
</tr>
</tbody>
</table>

MP AUC ratios, the AUC0–∞ ratio of ET-G to ET; T1/2, half-life; Tmax, time to reach maximum plasma concentration.

*P < 0.05 indicates a statistically significant difference between wild-type FVB mice and Bcrp1−/− mice (or Mrp2−/− mice).
metabolized fractions (3A). In the transport experiment, 4-ME showed significantly larger
P \text{ET-G} on the BL and AP sides, respectively (Fig. 3E) (experiment, the
were 6.64- and 3.98-fold higher than those of ET-G on the AP and BL
BL-AP transport experiment, the
0.0055 mM for 4-ME-G) (Fig. 3D) (ET-G vs. 0.0068 mM for 4-ME-G) and BL-AP (0.022 mM for ET-G vs.
4-ME glucuronides in the transport experiment, 4-ME-G also produced
in both AP-BL (0.039 mM for ET vs. 0.0048 mM for 4-ME) and BL-AP
(0.10 for ET vs. 0.27 for 4-ME) (Fig. 3B)

Unlike that of ET-G, Ko143 reduced the
excretion of 4-ME-G in the presence of 5

Effects of Inhibitors on the Glucuronidation Disposition of ET
and 4-ME in Caco-2 Monolayers. The effects of Ko143 (BCRP
inhibitor) and MK571 (MRPs inhibitor) on the glucuronidation
disposition of ET and 4-ME are presented in Fig. 4. The excretion amounts
of ET-G and 4-ME-G on the BL sides were 5- to 8-fold more, respectively,
than those of ET-G and 4-ME-G on the AP side regardless of the side
where the substrates were loaded. The excretion amounts of ET-G on
both the AP and BL sides were significantly decreased in the presence of
5 \mu M Ko143 and 10 \mu M MK571 (Fig. 4, A–D). Similar results were
obtained in the excretion of 4-ME-G in the presence of 5 \mu M Ko143 and
10 \mu M MK571 (Fig. 4, E–H). The effects of Ko143 and MK571 on the J
and CL of glucuronides (ET-G and 4-ME-G) are shown in Fig. 5. Ko143
and MK571 significantly reduced the J and CL values of ET-G in the
bidirectional transport experiment (Fig. 5, A, B, E, and F) (P < 0.05).
Unlike that of ET-G, Ko143 reduced the J of 4-ME-G on both the AP
and BL sides when 4-ME was loaded on the AP side (Fig. 5C) (P < 0.05), but
The mean \text{P}_{\text{app}} values of ET and 4-ME in AP-BL were \(2.36 \times 10^{-5}\) and
0.59 \times 10^{-5} \text{ cm/s}, respectively. The mean \text{P}_{\text{app}} values of ET and 4-ME
in BL-AP were \(2.55 \times 10^{-5}\) and 0.69 \times 10^{-5} \text{ cm/s}, respectively (Fig.
3A). In the transport experiment, 4-ME showed significantly larger
metabolized fractions (F_{\text{met}}) than those of ET in AP-BL (0.11 for ET vs.
0.25 for 4-ME) and BL-AP (0.10 for ET vs. 0.27 for 4-ME) (Fig. 3B)
(P < 0.05), and 4-ME yielded lower intracellular concentrations than ET
in both AP-BL (0.039 mM for ET vs. 0.0048 mM for 4-ME) and BL-AP
(0.042 mM for ET vs. 0.007 mM for 4-ME) (Fig. 3C). For the ET and
4-ME glucuronides in the transport experiment, 4-ME-G also produced
lower intracellular concentrations than ET-G in both AP-BL (0.016 mM for
ET-G vs. 0.0068 mM for 4-ME-G) and BL-AP (0.022 mM for ET-G vs.
0.0055 mM for 4-ME-G) (Fig. 3D) (P < 0.05). For the AP-BL transport
experiment, the J of 4-ME-G were 2.65- and 1.58-fold higher than those
of ET-G on the BL and AP sides, respectively (Fig. 3E) (P < 0.05). For the
BL-AP transport experiment, the J of 4-ME-G were 2.58- and 1.82-fold
higher than those of ET-G on the AP and BL sides, respectively (Fig. 3F)
(P < 0.05). For the AP-BL transport experiment, the CL rates of 4-ME-G
were 6.64- and 3.98-fold higher than those of ET-G on the AP and BL
sides, respectively (Fig. 3G) (P < 0.05). For the BL-AP transport
experiment, the CL rates of 4-ME-G were 11.12- and 8.16-fold higher than
those of ET-G on the AP and BL sides, respectively (Fig. 3H) (P < 0.05).

Effects of Inhibitors on the Glucuronidation Disposition of ET
and 4-ME in Caco-2 Monolayers. The effects of Ko143 (BCRP
inhibitor) and MK571 (MRPs inhibitor) on the glucuronidation
disposition of ET and 4-ME are presented in Fig. 4. The excretion amounts
of ET-G and 4-ME-G on the BL sides were 5- to 8-fold more, respectively,
than those of ET-G and 4-ME-G on the AP side regardless of the side
where the substrates were loaded. The excretion amounts of ET-G on
both the AP and BL sides were significantly decreased in the presence of
5 \mu M Ko143 and 10 \mu M MK571 (Fig. 4, A–D). Similar results were
obtained in the excretion of 4-ME-G in the presence of 5 \mu M Ko143 and
10 \mu M MK571 (Fig. 4, E–H). The effects of Ko143 and MK571 on the J
and CL of glucuronides (ET-G and 4-ME-G) are shown in Fig. 5. Ko143
and MK571 significantly reduced the J and CL values of ET-G in the
bidirectional transport experiment (Fig. 5, A, B, E, and F) (P < 0.05).
Unlike that of ET-G, Ko143 reduced the J of 4-ME-G on both the AP
and BL sides when 4-ME was loaded on the AP side (Fig. 5C) (P < 0.05), but

![Fig. 2. Mean plasma concentration-time curves of 4-ME (A and C) and 4-ME-G (B and D)
following oral 10 mg/kg (52.03 \mu mol/kg) and i.v. 2 mg/kg (10.41 \mu mol/kg) administration
of 4-ME in wild-type, Bcrp1^{-/-}, and Mrp2^{-/-} FVB mice. Blood samples were collected from
the tail vein of mice and were analyzed using UHPLC-MS/MS. The pharmacokinetic parameters
of oral administration and i.v. injection are shown in Tables 3 and 4, respectively. The data
points are presented as the average plasma concentrations of 4-ME or 4-ME-G, and the error bars
represent the S.D. of the mean (n = 5).](image-url)
their $J$ and $CL$ values were not significantly inhibited by MK571 (Fig. 5, D, G, and H). The intracellular concentrations of ET-G were significantly decreased by Ko143 in the BL-AP transport experiment ($P < 0.05$), whereas the intracellular concentrations of 4-ME-G were not changed on both sides (Fig. 6, A and B). MK571 did not alter the intracellular concentrations of ET-G and 4-ME-G regardless of the side on which the ET (or 4-ME) was loaded. MK571 significantly decreased the $F_{\text{net}}$ of ET-G (Fig. 6C). Ko143 and MK571 did not influence the $F_{\text{net}}$ of 4-ME-G in the transport experiment (Fig. 6D).

Glucuronidation Disposition of ET and 4-ME in Wild-Type, Bcrp1$^{-/-}$, and Mrp2$^{-/-}$ FVB Mice. The perfused mouse model was used to further confirm the role of Bcrp1 and Mrp2 in the disposition of ET (Fig. 7) and 4-ME (Fig. 8). The $P^{*}_{\text{eff}}$ of ET (or 4-ME) and amount of ET (or 4-ME) absorbed and percentage of excreted ET-G (or 4-ME-G) in a 15-minute interval were determined and normalized over a 10-cm intestinal length. The exposure levels of ET-G (or 4-ME-G) in bile and plasma at the end of the perfusion were determined by the ratio value ($A_{\text{ET-G}}/A_{\text{IS}}$) of the peak area of ET-G to the peak area of IS (testosterone) (or $A_{\text{ET-G}/A_{\text{IS}}}$ or $A_{\text{ET-G}/A_{\text{IS}}}$). The $P^{*}_{\text{eff}}$ values of ET in the upper small intestine (duodenum) ranged from 1.84 ± 0.45 to 2.18 ± 0.37 without a significant difference among the three mouse genotypes (Fig. 7A). The $P^{*}_{\text{eff}}$ values of ET in colon are lower than those in duodenum and ranged from 0.58 ± 0.59 to 1.10 ± 0.23 and without significant difference among the three mouse genotypes (Fig. 7A). The absorption features are also reflected by the absorbed amount of ET. The amount of ET absorbed ranged from 41.47 ± 9.99 to 50.05 ± 5.44 nmol/15 minutes without a significant difference among the three mouse genotypes (Fig. 7B). Compared with the duodenum, the amount of ET absorbed was lower and the values ranged from 13.00 ± 13.31 to 18.43 ± 5.57 nmol/15 minutes and without a significant difference among the three mouse genotypes (Fig. 7B). The percentage of ET-G excreted in the duodenum was significantly decreased from 34.71% ± 9.84% in wild-type FVB mice to 7.52% ± 1.91% in Bcrp1$^{-/-}$ mice (Fig. 7C) ($P < 0.05$), whereas that in Mrp2$^{-/-}$ mice is 37.27% ± 9.91%. Meanwhile, the peak area ratio of ET-G was significantly increased from 2.17 ± 0.99 in wild-type FVB mice to 9.64 ± 1.75 in Bcrp1$^{-/-}$ mice (Fig. 7E) ($P < 0.05$), whereas the ratio value of ET-G ($A_{\text{ET-G}/A_{\text{IS}}}$) in Mrp2$^{-/-}$ mice was 2.68 ± 0.25. The exposure of ET-G in bile showed no significant alteration in both duodenum and colon among the three mouse genotypes. However, the ratio of ET-G ($A_{\text{ET-G}/A_{\text{IS}}}$) in the bile had decreased from 5.99 ± 6.55 in wild-type FVB mice to 1.58 ± 0.75 and 1.42 ± 0.88 in Bcrp1$^{-/-}$ and Mrp2$^{-/-}$ mice, respectively (Fig. 7D).

The $P^{*}_{\text{eff}}$ values of 4-ME in the upper small intestine (duodenum) ranged from 1.81 ± 0.39 to 2.54 ± 0.52 (Fig. 7A). A slight but significant increase was noted in the $P^{*}_{\text{eff}}$ values of Mrp2$^{-/-}$ mice compared with that in wild-type FVB mice (Fig. 8A) ($P < 0.05$). The duodenum and colon showed comparatively $P^{*}_{\text{eff}}$ values and absorbed amounts for 4-ME (Fig. 8, A and B). The duodenum excreted more 4-ME-G than the colon in perfusate (Fig. 8C). The 4-ME-G showed higher concentrations in both bile and plasma than 4-ME (Fig. 8, D and E). The concentrations of 4-ME-G (13.67 ± 10.10) were increased in the bile of Bcrp1$^{-/-}$ (7.48 ± 5.34) and Mrp2$^{-/-}$ (2.25 ± 1.72) mice compared with that in wild-type FVB mice, but the increase was not statistically significant.
Discussion

In our previous study, we had demonstrated that both ET and 4-ME were metabolized to 7-O-glucuronides (ET-G and 4-ME-G, respectively) in human liver and intestinal microsomes (Zhu et al., 2015). The metabolism studies in rats and mice also showed that the 7-C position glucuronidation reaction is the unique metabolic pathway for ET and 4-ME (unpublished data). The transportation of glucuronides depends mostly on the excretion of transporters that are commonly located on the AP and BL membranes of many cells. Thus, in the current study, transporter knockout mice and Caco-2 cells were employed to explore the role of BCRP and MRP2 in the disposition of ET-G and 4-ME-G in vitro and in vivo.

The pharmacokinetic study and perfusion experiment in knockout mice and inhibition assays in Caco-2 monolayers provided considerable evidence supporting the important role of BCRP and MRP2 in the excretion of ET-G and 4-ME-G. The pharmacokinetic data showed that...
ET and 4-ME are mainly present as ET-G and 4-ME-G in plasma (Figs. 1 and 2). This result was consistent with our previous study showing that ET and 4-ME easily undergo 7-O glucuronidation metabolism by human hepatic and intestinal microsomes (Zhu et al., 2015). The pharmacokinetic parameters of ET-G and 4-ME-G in knockout mice exhibited significant differences from those in wild-type FVB mice (Tables 1–4).

Fig. 4. Effects of Ko143 (inhibitor of BCRP) and MK571 (inhibitor of MRP2) on the excretion amounts of ET-G (A–D) and 4-ME-G (E–H) from the cellular membrane to the AP and BL sides. The experiment was set up as follows: ET or 4-ME (10 μM) was loaded on the AP or BL side; Ko143 (5 μM) or MK571 (10 μM) was loaded on the AP side; or no inhibitor was added as the control group. The amounts of ET-G or 4-ME-G on the AP or BL side were determined at 0.5, 1, 1.5, and 2 hours after incubation. The data points represent the average values of the triplicate samples, and the error bars represent the S.D. of the mean. Unpaired Student’s t test was used to analyze the data. The asterisk (*) indicates a statistically significant difference between the control and inhibited groups at P < 0.05.
The AUC\textsubscript{0-\infty} values of ET and 4-ME, and their glucuronides (ET-G and 4-ME-G), were significantly increased in most of Bcrp1\textsuperscript{+/−} and Mrp2\textsuperscript{+/−} mice compared with those in wild-type FVB mice after oral or i.v. administration of ET or 4-ME. The average absolute $F\%$ of ET and 4-ME were increased in Bcrp1\textsuperscript{+/−} mice (17.10% for ET and 45.70% for 4-ME) compared with those in wild-type FVB mice (10.07% for ET.

Fig. 5. Effect of Ko143 and MK571 on the $J$ of ET-G (A and B) and 4-ME-G (C and D) as well as the CL rates of ET-G (E and F) and 4-ME-G (G and H) at both the AP side to BL side (AP-BL) and BL side to AP side (BL-AP) directions in Caco-2 monolayers. The experiment was set up as follows: 10 $\mu$M ET or 4-ME was loaded on the AP or BL side; Ko143 (5 $\mu$M) or MK571 (10 $\mu$M) was loaded on the AP side; or no inhibitor was added as the control group. The amounts of ET-G or 4-ME-G on the AP and BL sides as well as their intracellular concentrations were determined at 0.5, 1, 1.5, and 2 hours after incubation. The data points represent the average values of the triplicate samples, and the error bars represent the S.D. of the mean. Unpaired Student’s $t$ test was used to analyze the data. The asterisk (*) indicates a statistically significant difference between the control and inhibited groups at $P < 0.05$. 

A. Efflux rate of ET-G (AP-BL) 
B. Efflux rate of ET-G (BL-AP) 
C. Efflux rate of 4-ME-G (AP-BL) 
D. Efflux rate of 4-ME-G (BL-AP) 
E. Clearance of ET-G (AP-BL) 
F. Clearance of ET-G (AP-BL) 
G. Clearance of 4-ME-G (AP-BL) 
H. Clearance of 4-ME-G (AP-BL)
22.28% for 4-ME) (Tables 2–4). These results suggested that Bcrp1 and Mrp2 might be involved in the disposition of ET-G and 4-ME-G. The inhibition assays in Caco-2 cells were conducted to verify the role of BCRP and MRP2 on the disposition of ET-G and 4-ME-G in vitro. In the inhibition assays in Caco-2 monolayers, the excretion of ET-G and 4-ME-G was significantly inhibited in the presence of the Ko143 (BCRP inhibitor) or MK571 (MRP2 inhibitor) on the AP side of Caco-2 monolayers (Fig. 4). The $J$ and $CL$ of ET-G and 4-ME-G were also significantly decreased by Ko143 and MK571 (Fig. 5). These results also suggested that BCRP and MRP2 are probably involved in excreting ET-G and 4-ME-G. Additionally, the perfusion experiment in transporter knockout mice showed that Bcrp1 deficiency significantly

![Graphs A and B: Intracellular of ET-G and 4-ME-G](image1)

![Graphs C and D: $F_{met}$ of ET and 4-ME](image2)

**Fig. 6.** Effect of Ko143 and MK57 on the intracellular concentrations of ET-G (A) and 4-ME-G (B), and metabolism fraction ($F_{met}$) of ET (C) and 4-ME (D) on both the AP side to BL side (AP-BL) and BL side to AP side (BL-AP) in Caco-2 monolayers. The experiment was set up as follows: 10 μM ET or 4-ME was loaded on the AP or BL side; Ko143 (5 μM) or MK571 (10 μM) was loaded on the AP side; or no inhibitor was added as the control group. The amounts of ET-G and 4-ME-G on the AP and BL sides as well as their intracellular concentrations were determined at 0.5, 1, 1.5, and 2 hours after incubation. $F_{met}$ was determined by comparing the total amounts of glucuronides and the parent compound. The data points represent the average values of the triplicate samples, and the error bars represent the S.D. of the mean. Unpaired Student’s $t$ test was used to analyze the data. The asterisk (*) indicates a statistically significant difference between the control and inhibited groups at $P < 0.05$.

**Fig. 7.** Effective intestinal permeability as well as the absorption and metabolism of ET in the mouse intestinal perfusion model. Two segments (upper small intestine and colon) were perfused simultaneously at a flow rate of 0.167 ml/min using concentration of 70 μM ET. The $P^{eff}_*$ of ET (A), amount of ET absorbed (B), and percentage of excreted ET-G (C) in a 15-minute interval were determined and normalized over a 10-cm intestinal length. The exposure levels of ET-G in bile (D) and plasma (E) at the end of the perfusion were determined by the ratio of the peak area of ET-G to that of the IS. Each column represents the average of the determinations, and the error bar represents the S.D. of the mean (the number of mice in each group ranged from 3 to 5). Unpaired Student’s $t$ test was used to analyze the data. The asterisk (*) indicates a statistically significant difference between wild-type FVB mice and knockout mice at $P < 0.05$. 

![Graphs A and B: F$_{met}$ of ET and 4-ME](image3)
BCRP & MRP2 Excrete Glucuronide of Esculetin and 4-Methylesculetin 213

Fig. 8. Effective intestinal permeability as well as absorption and metabolism of 4-ME in a mouse intestinal perfusion model. Two segments (upper small intestine and colon) were perfused simultaneously at a flow rate of 0.167 ml/min using concentration of 70 μM 4-ME. The P_{app} of 4-ME-G (A), amount of 4-ME absorbed (B), and percentage of excreted 4-ME (C) in a 15-minute interval were determined and normalized over a 10-cm intestinal length. The exposure levels of 4-ME-G in bile (D) and plasma (E) at the end of the perfusion were determined by the ratio of the peak area of 4-ME-G to that of the IS. Each column represents the average of determinations, and the error bar represents the S.D. of the mean (the number of mice in each group ranged from three to five). Unpaired Student’s t test was used to analyze the data. The asterisk (*) indicates a statistically significant difference between wild-type FVB mice and knockout mice at P < 0.05.

decreased the ET-G excretion into the intestine and increased the concentration of ET-G in plasma (Fig. 7, C and E). These results demonstrated that Bcrp1 plays a primary role in excreting ET-G into the intestinal lumen. The bile excretions of ET-G in the Bcrp1^{−/−} and Mrp2^{−/−} mice were markedly decreased compared with that in wild-type FVB mice, but the effect was not statistically significant (Fig. 7D). The lack of significance of these data can be explained by the large derivation. However, these results also suggested that Mrp2 is probably involved in the ET-G excretion. No significant decrease was observed for 4-ME-G intestinal and bile excretions in Bcrp1- or Mrp2-deficient mice, implying that Mrp2 compensated the functional Bcrp1 when there was a BCRP deficiency, and vice versa (Fig. 8, C and D). These results suggested that both Bcrp1 and Mrp2 had a comparable capacity in excreting 4-ME-G into the intestine and bile.

Other MRPs, including MRP3 and MRP4, that are expressed on the BL membrane of the Caco-2 cells also might be involved in the excretion of ET-G and 4-ME-G because MK571 is not a specific inhibitor of MRP2 (Gao et al., 2018). In the inhibition assays in Caco-2 monolayers, the excretion of ET-G and 4-ME-G was inhibited by MK571, which was loaded on the AP side (Fig. 4). Furthermore, MK571 showed a stronger inhibition effect on the excretion of ET-G and 4-ME-G than Ko143. These results were probably due to two reasons. First, no BCRP was expressed on the BL side in the Caco-2 cells. Second, MRP3 and MRP4, which are expressed on the BL side, were most likely inhibited by MK571. The excretion amounts of ET-G and 4-ME-G on the BL sides were higher than those of ET-G and 4-ME-G on the AP side, implying that MRPs on the BL side play a major role in excreting ET-G and 4-ME-G into the system circulation. These results could elucidate that the plasma concentrations of ET-G and 4-ME-G are higher than those of ET and 4-ME in the pharmacokinetic study. The role of MRPs on the BL membrane in ET-G and 4-ME-G excretion will be considered in a future study. In addition, MRPs were silenced by using specific small interfering RNA, or double-knockout mice could be used. Meanwhile, dosing the pure glucuronides via the i.v. route to thoroughly evaluate the disposition of the ET-G and 4-ME-G will also be conducted. Although the inhibition specificity of Ko143 and MK571 has been debated frequently, they are commonly used in screening BCRP and MRP2 substrates in vitro assays (Sheng et al., 2015; Shi et al., 2016). These results of the inhibition assay were consistent with previous findings in which most glucuronides are substrates of BCRP and MRP2 (An and Morris, 2011; Zheng et al., 2016). Efflux transporters have been considered one of the most important factors in governing the F% and efficacy, as well as the toxicity and drug–drug interaction susceptibility, of most market drugs and drug candidates (Li et al., 2012; Planas et al., 2012). Thus, the study of disposition characteristics of ET and 4-ME will provide valuable information for further research and development.

In the Caco-2 monolayers, the directional rates of transport of ET and 4-ME were similar (the ratio of BL-AP P_{app} to AP-BL P_{app} is close to 1), indicating that ET and 4-ME are most likely transported via passive diffusion in Caco-2 cells (Fig. 3A). Meanwhile, the P_{app} values of ET and 4-ME were close to 2 (or were greater than 2) in the duodenum in the perfusion experiment, also suggesting that ET and 4-ME possess a good permeability (Fig. 7A; Fig. 8A). However, the F% values of ET and 4-ME in wild-type FVB mice were 10.07% and 22.28%, respectively. These results suggested that the extensive glucuronidation of ET and 4-ME resulted in their poor intestinal absorption and metabolism. The low F% values indicated that the extensive glucuronidation of ET and 4-ME resulted in their poor 4-ME-G intestinal and bile excretions in Bcrp1- or Mrp2-deficient mice, suggesting one of the most important factors in governing the F% and efficacy, as well as the toxicity and drug–drug interaction susceptibility, of most market drugs and drug candidates (Li et al., 2012; Planas et al., 2012). Thus, the study of disposition characteristics of ET and 4-ME will provide valuable information for further research and development.
the intestinal lumen. ET and 4-ME are most likely absorbed via passive diffusion, and the extensive glucuronidation resulted in their poor FF%. 

**Authorship Contributions**

**Participated in research design:** Li, Song, Ou, Sun, Liu, Zhu.  
**Conducted experiments:** Song, Ou, Luo, Xie, Qi.  
**Contributed new reagents or analytic tools:** Wang, Qi, Hu, Liu.  
**Performed data analysis:** Song, Ou, Luo, Xie, Zhu.  
**Wrote or contributed to the writing of the manuscript:** Li, Ou, Zhu.

**References**


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**Address correspondence to:** Dr. Lijun Zhu, Joint Laboratory for Translational Cancer Research of Chinese Medicine of the Ministry of Education of the People’s Republic of China, International Institute for Translational Chinese Medicine, Guangzhou, Guangdong, 510006, PR China. E-mail: zhulijun@gzucm.edu.cn; or Dr. Zhongqiu Liu, Joint Laboratory for Translational Cancer Research of Chinese Medicine of the Ministry of Education of the People’s Republic of China, International Institute for Translational Chinese Medicine, Guangzhou, Guangdong, 510006, PR China. E-mail: liuzq@gzucm.edu.cn.