Role of P-glycoprotein in the Intestinal Absorption of Glabridin, an Active Flavonoid from the Root of Glycyrrhiza glabra

Jie Cao, Xiao Chen, Jun Liang, Xue-Qing Yu, An-Long Xu, Eli Chan, Wei Duan, Min Huang, Jing-Yuan Wen, Xi-Yong Yu, Xiao-Tian Li, Fwu-Shan Sheu, and Shu-Feng Zhou

Department of General Surgery, the First Municipal Hospital of Guangzhou, Guangzhou, China (J.C.); Departments of Pharmacy (X.C.) and Nephrology (X.-Q.Y.), the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; Department of Pharmacology and Toxicology, Australian Institute of Chinese Medicine, Sydney, New South Wales, Australia (J.L., S.-F.Z.); Department of Biochemistry, School of Life Sciences (A.-L.X.), and Institute of Clinical Pharmacology, School of Pharmaceutical Sciences (M.H.), Sun Yat-sen University, Guangzhou, China; Departments of Pharmacy (E.C.) and Biological Sciences (F.-S.S.), Faculty of Science, National University of Singapore, Singapore; School of Pharmacy, Faculty of Medical and Health Sciences, the University of Auckland, Auckland, New Zealand (J.-Y.W.); School of Medicine, Deakin University, Waurn Ponds, Victoria, Australia (W.D.); Department of Molecular & Clinical Pharmacology, Guangdong Provincial Cardiovascular Institute, Guangzhou, China (X.-Y.Y.); and Department of Maternal Medicine, Obstetrics and Gynecology Hospital, Fudan University, Shanghai, China (X.-T.L.)

Received April 24, 2006; accepted January 3, 2007

ABSTRACT:

Glabridin is a major constituent of the root of Glycyrrhiza glabra, which is commonly used in the treatment of cardiovascular and central nervous system diseases. This study aimed to investigate the role of P-glycoprotein (PgP/MDR1) in the intestinal absorption of glabridin. The systemic bioavailability of glabridin was approximately 7.5% in rats, but increased when combined with verapamil. In single-pass perfused rat ileum with mesenteric vein cannulation, the permeability coefficient of glabridin based on drug disappearance in luminal perfusates ($P_{\text{lumen}}$) was approximately 7-fold higher than that based on drug appearance in the blood ($P_{\text{blood}}$). Glabridin was mainly metabolized by glucuronidation, and the metabolic capacity of intestine microsomes was 1/15 to 1/20 of that in liver microsomes. Polarized transport of glabridin was found in Caco-2 and MDCKII monolayers. Addition of verapamil in both apical (AP) and basolateral (BL) sides abolished the polarized transport of glabridin across Caco-2 cells. Incubation of verapamil significantly altered the intracellular accumulation and efflux of glabridin in Caco-2 cells. The transport of glabridin in the BL-AP direction was significantly higher in MDCKII cells overexpressing PgP/MDR1 than in the control cells. Glabridin inhibited PgP-mediated transport of digoxin with an IC50 value of 2.56 μM, but stimulated PgP/MDR1 ATPase activity with a $K_m$ of 25.1 μM. The plasma AUC0→24h of glabridin in mdrl−/− mice was 3.8-fold higher than that in wild-type mice. These findings indicate that glabridin is a substrate for PgP and that both PgP/MDR1-mediated efflux and first-pass metabolism contribute to the low oral bioavailability of glabridin.

There is an increasing consumption of herbal medicines in recent years in Asian and Western countries. Their incorporation into the medical care system has been encouraged by the World Health Organization despite the lack of evidence for the efficacy of most herbal drugs. Herbal medicines are usually orally administered with long-term regimens. However, the nature of intestinal absorption of the major ingredients of most herbal medicines is unknown, probably because of a lack of sensitive analytical methods, difficulties in the choice of marker components, and difficulties in the establishment and validation of efficient study models. The widely used traditional Chinese medicine, the root of Glycyrrhiza glabra (licorice), is one of the most commonly used herbal medicines in the world because of its exceptional pharmacological properties recognized by traditional Chinese medicine (Zhu, 1998). Licorice has been used as antioxidants...
demulcants, expectorants, antioxidants, and remedies for inflammation, as well as flavoring and sweetening agents in Asia and Europe (Zhu, 1998). Licorice contains glycyrrhizin, oleane triterpenoids, glucose, and flavonoids. Glabridin (Fig. 1) is a major polyphenolic flavonoid and a main constituent in the hydrophobic fractions of licorice extract. It has antioxidant, antimicrobial, antiatherosclerotic, hypoplipidemic, anti-inflammatory, estrogen-like, hypoglycemic, cardiovascular protective, antihepatic, and radical scavenging activities (Belinky et al., 1998; Yokota et al., 1998; Rosenblat et al., 1999; Tamir et al., 2000). The hydroxyl groups on the glabridin B ring were found to be most important for its antioxidative activity (Belinky et al., 1998). Because of the wide spectrum of pharmacological activity, licorice is widely used as a single preparation or, more often, in combination with other herbs to treat diseases in respiratory, cardiovascular, endocrine, and digestive systems. The Pharmacopoeia of the People’s Republic of China recommends a dosage of 8 to 25 g daily for licorice in decoction form, or up to 100 g in treatment of severe diseases (Zhu, 1998). Since the typical content of glabridin in licorice is approximately 0.08 to 0.35% of dry weight (Hayashi et al., 2003), approximately 20 to 87.5 mg of glabridin is administered daily if 25 g of crude licorice is dosed (or 80–350 mg when 100 g of crude extract was dosed in some cases). However, in some licorice extract products, the contents of glabridin are up to 1.2 to 11.6% (Vaya et al., 1997), and 300 to 2900 mg of glabridin is taken when 25 g of crude licorice extract is used.

P-glycoprotein (PgP/MDR1) was initially found to be expressed at high levels in many tumor cell lines and conferred multidrug resistance (MDR) to a variety of anticancer drugs, including vinca alkaloids, epipodophyllotoxins, taxanes, and anthracyclines. It is a 170- to 300 to 2900 mg of glabridin is taken when 25 g of crude licorice extract is used. Glabridin [(R)-(3,4-dihydro-8,8-dimethyl-2H,8H-benzo[1,2-b;3,4-b']dipryran-3-yl)-1,3-benzenediolo] extracted and purified from the root of Salvia miltiorrhiza was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). This compound has a purity >99.0%, as determined by high performance liquid chromatography (HPLC). Dulbecco’s modified Eagle’s medium, fetal bovine serum, 0.05% trypsin-EDTA, penicillin-streptomycin, nonessential amino acids, and sterilized Hanks’ balanced salt solution (HBSS; pH 7.0) containing 25 mM HEPES and 25 mM glucose were obtained from Invitrogen (Carlsbad, CA). Krebs-Ringer buffer (Kreb-Ringer buffer) was used as buffer for rat intestinal perfusion study. Phenol red, trypan blue, propranolol, dimethyl sulfoxide (DMSO), α-saccharic acid 1,4-lactone, verapamil hydrochloride, Brij 58 (polyoxyethylene 20 cetyl ether), nifedipine, probenecid, quinidine sulfate dihydrate, indomethacin, diclofenac, mefenamic acid, mannitol, and antipyrine were all purchased from Sigma-Aldrich (St. Louis, MO). α-Nicotinamide adenine dinucleotide phosphate in reduced form (NADPH) and uridine diphosphoglucose (UDP-GA) were purchased from Roche Diagnostics Ltd. (Sydney, Australia). The leukotriene D4 receptor antagonist, 3-[[3-[2-(7-chloro-2-quinoxalinyl) (E)-ethenyl]phenyl][1-(3-dimethylamino)-3-oxopropyl]thio]methyl[thio]propionic acid (MK-571 or L-660,711), was a gift from Dr. Ford Hutchinson (Merck Frosst Canada, Inc., Kirkland, QC, Canada). Tissue culture plastics and 0.4-μm pore size 12-mm i.d. Transwell polycarbonate inserts were obtained from Corning Co. (Corning, NY). The control MDCKII cells with empty vector and their human MDR1 recombinantly transfected derivative, MDR1-MDCKII, were obtained as a kind gift from Professor Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The water used was purified by a Milli-Q purification system (Millipore, Billerica, MA). All other chemicals and reagents were of analytical or HPLC grade as appropriate.

Animals. Male healthy Sprague-Dawley rats (200–260 g) were kept in a room under controlled temperature (22 ± 1°C) and automatic day-night rhythm (12-h cycle) and housed in wire-bottom cages with paper underneath. The ethical approval of this study was obtained from the Ethical Committee of the Australian Institute of Chinese Medicine, Sydney, Australia. Animals were treated humanely, and the animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health of the United States of America (National Institutes of Health publication 85-23, 1985).

Cell Culture. Caco-2 cells were obtained from The American Type Culture Collection (Manassas, VA). Caco-2, control MDCKII, and MDR1-MDCKII cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 100 U/ml penicillin and gentamicin in an atmosphere of 5% CO2 and 90% relative humidity at 37°C. The expression levels of MDR1 in Caco-2 cells, control MDCKII cells, and the MDR1-transfected MDCKII cells were monitored every two to four passages by Western blotting analysis.

Cytotoxicity Assay. The cytotoxic effect of glabridin on various cells examined was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were exposed to the drug for 48 h, and the absorbance of formazan, a metabolite of MTT, was measured at a wavelength of 595 nm using a microplate reader (Tecan Instrument Inc., Research Triangle Park, NC). The MTT assays were the means of at least six
independent experiments, each performed in replicates of eight for each drug concentration.

**Systemic Bioavailability of Glabridin in Rats.** The animals were fasted overnight with free access to water before drug administration. Glabridin was freshly prepared by dissolving in DMSO and then diluted with distilled water, resulting in a final DMSO concentration of 0.2% (v/v). Rats were randomized to three groups (n = 6) to receive 5 or 20 mg/kg glabridin by gavage, or 5 mg/kg by i.v. bolus injection through the tail vein. Blood samples were collected through jugular vein cannulation into heparinized tubes at predetermined times over 24 h after drug administration. Plasma was obtained by centrifugation at 5000g for 6 min at 4°C and the plasma was then transferred to clean 1.5-ml tubes. All samples were stored at −20°C until analysis.

**Preparation of Rat Intestinal and Hepatic Microsomes.** Hepatic and intestinal mucosal tissues were collected from healthy male Sprague-Dawley rats (190–250 g) and stored at −80°C. Hepatic and intestinal microsomes were prepared by differential centrifugation as described previously (Zhou et al., 2000). The rat liver homogenates were centrifuged at 9000g for 20 min at 4°C. The supernatant was then centrifuged at 105,000g for 1 h at 4°C using an ultracentrifuge with a Type 70 Ti rotor (Beckman Coulter, Inc., Fullerton, CA). Microsomal protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985). Tissue microsomes were stored at −80°C until use.

**In Vitro Metabolism and Metabolic Inhibition Study in Rat Hepatic and Intestinal Microsomes.** Initial incubations containing either 1.0 mg/ml rat hepatic microsomes or 5.0 mg/ml rat intestinal microsomes in the presence of NADPH or UDPGA were performed to investigate whether phase I and/or glucuronidation reaction were involved in the metabolism of glabridin. The depletion of substrate was determined to monitor glabridin metabolism. A single ion monitoring determined the possible formation of new metabolites of glabridin in intestinal and hepatic microsomes using liquid chromatography-mass spectrometry (LC-MS). Once any metabolite from phase I or phase II reaction was detected, the incubation conditions in hepatic and intestinal microsomes were examined with respect to microsomal protein concentration and incubation time. Typical incubations (200 µl) for glabridin glucuronidation contained hepatic (0.1 mg/ml) or intestinal (1.0 mg/ml) microsomal protein, 10 mM UDPGA, 5 mM MgCl2, 0.1 mg/ml β-saccharic acid 1,4-lactone, Brij 58 (0.1–0.2:1, ratio of Brij 58 over microsome, w/w), and verapamil, indomethacin, and diclofenac (all at 100 µM) to monitor the hepatic glucuronidation of glabridin in vitro by various compounds including B-phenolsulfonphthalein was used as a nonabsorbable marker for measuring water flux and to correct for changes in the water flux across the incised ileal segment (Zhang et al., 2006). Additional control experiments were conducted to examine the disappearance (in ileal lumen) and appearance (in mesenteric vein blood) rates of the passive transcellular (antipyrine, 100 µM) and paracellular (mannitol, 1.0 mM) markers to validate our single-pass rat ileum perfusion system (n = 6 per group). The gut does not metabolize mannitol and antipyrine and absorbs these two compounds in an unchanged form. The effects of verapamil at 100 µM, probenecid at 200 µM, MK-571 at 100 µM, and celecoxib at 100 µM on the intestinal transport of both probe compounds, antipyrine and mannitol, were also investigated.

**Uptake and Efflux Study of Glabridin by Cells.** The uptake and efflux of glabridin by Caco-2, MDCKII, and MDR1-MDCKII cells were examined in confluent cell cultures grown on 60-mm plastic culture dishes (Corning) as described previously (El Hafny et al., 1997; Zhou et al., 2005). For the uptake assay, exponentially growing cells were exposed to 0 to 50 µM glabridin over 120 min at 37°C. For the concentration-dependence study, the incubation time was 30 min. The medium was aspirated off at indicated times, and the dishes were rapidly rinsed five times with 50 ml of ice-cold phosphate-buffered saline (PBS). HPLC analysis ensured that the final wash contained no residual glabridin. The cells were harvested and each cell pellet was suspended in 200 µl of extraction solution (acetonitrile/methanol 1:1, v/v, with 0.01 N HCl) with the addition of 10 µl of 1.0 mg/ml mefenamic acid, which was used as IS. For the efflux assay, glabridin (0.1–50 µM) was added to confluent cell cultures grown on 60-mm plastic culture dishes (Corning) before three washes with 20 ml of warm PBS and incubated for 120 min. After five washes of the cells with 4°C PBS to eliminate the extracellular drug, cells were incubated in culture medium for 20 min at 37°C. After centrifugation of the cells in culture medium at 5000g for 10 min, the supernatant was dried using a rotary concentrator and the residues were reconstituted with the mobile phase; 10 to 20 µl was then injected into the LC-MS system for glabridin concentration determination. The cellular uptake and efflux of glabridin was expressed as ng/min/mg cellular proteins and corrected by subtraction of the mean extracellular adsorption value of [14C]sucrose by cells tested.

The effects of various ATP inhibitors (sodium azide at 10 mM and 2,4-dinitrophenol at 5 mM), and PgP and MRP inhibitors, including verapamil, nifedipine (both PgP inhibitors, 100 µM), MK-571 (a MRP1/2 inhibitor, 100 µM), MK-717 (a PgP/MRP inhibitor, 100 µM), and dinitrophenol at 5 mM, and PgP and MRP inhibitors, including verapamil, nifedipine (bothPgP inhibitors, 100 µM), MK-571 (a MRP1/2 inhibitor, 100 µM), MK-717 (a PgP/MRP inhibitor, 100 µM), on glabridin cellular uptake and efflux were investigated in Caco-2 cells. All inhibitors were freshly prepared by dissolving in DMSO and then diluted by PBS. The final concentration of DMSO was 0.2% (v/v). These inhibitors at indicated concentrations showed little cytotoxicity (<8.0%) to the cells tested using the MTT assay when incubated for 2 h. For uptake inhibition assay, all inhibitors were preincubated with cells for 2 h and then incubated for 30 min after addition of glabridin. For efflux inhibition assay, glabridin at 0.1 or 1.0 µM was added to the cells and incubated for 120 min to achieve maximum drug uptake. After five washes with ice-cold PBS, the cells were incubated in the presence of an inhibitor for 30 min at 37°C. Thereafter, cells were washed with cold PBS buffer five times. The cell were then harvested, lysed by sonication, and extracted using ice-cold acetonitrile/methanol mixture (1:1 v/v, with 0.01 N HCl) as described above. All uptake and efflux assays in the absence and presence of inhibitor were studied in at least three independent experiments. DMSO at a final concentration of 0.2% (v/v) in the culture buffer, used to dissolve all the inhibitors, did not change the accumulation of glabridin in Caco-2 and MDCKII cells.

The uptake of a known PgP substrate, daunomycin, was performed with or without 100 µM verapamil as described above. Preliminary experiments showed that daunomycin uptake was at equilibrium after 60 to 90 min of incubation in Caco-2 cells. Cells were then incubated for 30 min with 1.0 µM
daunomycin (0.3 μCi/well [3H]daunomycin; PerkinElmer Life and Analytical Sciences, Boston, MA) and unlabeled daunomycin with or without 100 μM verapamil. Cells were then washed with HBSS and further processed as described above. In addition, control uptake assays were performed using the extracellular marker [14C]sucrose (565 mCi/mmole) and [3H]propranolol (both from GE Healthcare, Buckinghamshire, UK). For the efflux assay, [3H]vinblastine (GE Healthcare) was used as a model substrate, and the radioactivity was determined by an LC-6000 liquid scintillation counter (Beckman Coulter, Inc.).

Transport Study of Glabridin in Caco-2, Control MDCKII, and MDR1-MDCKII Monolayers. For the transport studies of glabridin, Caco-2 cells, control MDCKII cells, or MDR1-MDCKII cells were seeded at a density of 5 to 10 × 10^5 cells/well onto polycarbonate membrane Transwell inserts (Corning) in 12-well plates. The effective transepithelial electrical resistance (TEER) of the monolayers (equal to total TEER value – value in empty filter membranes) was examined routinely before and after the experiment using a Millicell ERS apparatus (Millipore). Caco-2 cells were used for transport experiments 21 days after seeding, when the effective TEER values typically exceeded 260 to 350 Ω·cm². The transport experiments in Caco-2 cells were conducted on cells between passages 30 and 35. For MDCKII and MDR1-MDCKII monolayers, only cells at passages 5 to 9 were used for transport studies after receipt from The Netherlands Cancer Institute. Cells were used in transport experiments at days 5 to 7 after cell seeding, when the effective TEER values for MDCKII monolayers were typically 40 to 60 Ω·cm² and 120 to 150 Ω·cm² for MDR1-MDCKII monolayers. [3H]Mannitol (GE Healthcare) was used as a probe for paracellular transport, and a value of 0.5% per hour indicated acceptable integrality for the monolayers examined. The transport of glabridin by Caco-2, control MDCKII, and MDR1-MDCKII monolayers was investigated on an orbital shaker as described previously (Zhang et al., 2006). In brief, the monolayers were washed twice with warm HBSS containing 25 mM HEPES (pH 7.0) before the transport experiments. A pH of 7.0 was chosen because it was close to the ileal pH value, and this pH resulted in maximum apical (AP) to basolateral (BL) and BL to AP transport of glabridin.

Nine independent incubations were performed in triplicate for all experiments. The effects of pH, Na⁺, temperature, and ATP on glabridin transport across Caco-2 monolayers were investigated. The effect of apical or basolateral pH (5.5–7.4) on the AP to BL and BL to AP flux of glabridin at 0.1 and 1.0 μM was examined at pH 7.4 for the receiving side. The pH was altered by substituting appropriate amounts of HEPES in the incubation medium by equimolar (25 mM) 2-[N-morpholino]ethanesulfonic acid. In experiments to investigate the effect of Na⁺ on the flux of glabridin at 0.1 and 1.0 μM across the Caco-2 monolayers, sodium chloride in the HBSS was replaced by equimolar amounts (140 mM) of potassium chloride. In addition, the permeability of glabridin from AP to BL and BL to AP was measured after incubation for 30 min at 4°C or 37°C. To determine whether there was an energy dependence of glabridin flux in Caco-2 cells, the transport medium depleted in glucose was used in both sides of the monolayers. Like all other ABC transporters, PgP acts as an efflux pump by exporting its substrate from the membrane or cell cytosol to the exterior of the cells, with ATP hydrolysis as the driving force. Sodium azide (10 mM) or 2,4-dinitrophenol (5.0 mM) (both ATPase inhibitors) was added to the monolayers (equal to total TEER value in empty filter membrane fractions). Verapamil (0.25–100 μM) was used as the positive control in these experiments. To estimate the reaction kinetics with glabridin and verapamil, the ATP hydrolysis rate was fitted to several nonlinear kinetic models using the Prism 3.0 program (GraphPad Software Inc., San Diego, CA).

Effects of Coadministered Verapamil on the Plasma Pharmacokinetics of Glabridin in Rats. In separate kinetic experiments, we examined the effects of coadministered verapamil at 25 or 100 mg/kg on the plasma pharmacokinetics of glabridin in healthy male Sprague-Dawley rats. The dose of verapamil selected was approximately the maximum tolerated dose, as assessed in pilot experiments in male Sprague-Dawley rats. Rats were randomized to receive the following different treatments (n = 6 per group): glabridin at 5 mg/kg by gavage plus water (0.3 ml control vehicle), and glabridin at 5 mg/kg by gavage in combination with verapamil at 50 or 100 mg/kg by oral gavage dissolved in water. The inhibitor was administered 2 h before glabridin dosing. Blood was collected as described above. The concentrations of glabridin in plasma were determined by LC-MS.

Pharmacokinetic Study of Glabridin in mdrla(−/−) Gene-Deficient and Wild-Type Mice. FVB/NJ (20–35 g) and mdrla gene-deficient mice (25–30 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) and Taconic Farms, Inc. (Germantown, NY), respectively. The mice were treated with oral glabridin at 5 mg/kg by gavage (n = 4 per time point). At predetermined time points, the mice were sacrificed by neck dislocation. Blood was immediately collected and plasma was obtained by centrifugation at 5000 g for 10 min at 4°C, and all tissues were processed as described above. The concentration of glabridin in plasma and tissues was determined by LC-MS analysis.

Liquid Chromatography-Mass Spectrometry and High Performance Liquid Chromatography Analysis of Glabridin. The concentrations of glabridin in rat and mouse plasma, perfusates, and transport medium in cellular monolayers and cellular lysates were determined by an LC-MS system equipped with an Agilent 1100 LC (Agilent Technologies, Palo Alto, CA) connected to an Applied Biosystems Q-Trap 4000 mass spectrometer (Applied Biosystems, Foster City, CA) through an electrospray ionization source. Chromatographic separation was achieved using a C18 HyperClone ODS column (200 mm × 4.6 mm i.d.) (Phenomenex, Torrance, CA) preceded by a Phenomenex C18 guard cartridge at room temperature (22°C). Mobile phase composed of methanol and 0.1% (v/v) formic acid (85/15, v/v) had a flow rate of 0.2 ml/min. Injection volume was 20 μl of a sample kept in an autosampler set at 10°C. Air set at 600°C and a pressure of 70 psi was used for heating, and the nebulizing gas was set at 40 psi. The capillary temperature was 450°C and the spray voltage was 4000 V. The product ions were recorded using a negative ion detection mode. The monitored ions and collision energy were m/z 323.1→201.3 and 30 eV for glabridin, and m/z 240.1→196.1 and 25 eV for the
IS. The lower limit of quantitation of glabridin was 0.025 to 0.05 ng/ml in rat and mouse plasma, perfusates, and other matrices tested in this study. Glabridin was recovered by >95%, and was stable when kept at 10°C for 36 h, at -20°C for 3 months, and after five to eight freeze-thaw cycles.

The antipyrene and mannitol in perfusates were determined by a validated HPLC method as described previously (Miki et al., 1996; Hung et al., 2001). The lower limits of quantitation for antipyrene and mannitol were 30.0 and 195.0 ng/ml, respectively.

Pharmacokinetic Calculation. The plasma concentration-time curves of glabridin in rats were obtained by plotting the mean plasma concentrations of glabridin versus time on a semilogarithmic scale. Pharmacokinetics parameters were calculated by standard model-independent pharmacokinetic formulas using the WinNonlin program (Pharsight Inc., Mountain View, CA). The elimination half-life (t1/2β) value was calculated as 0.693/β, where β is the elimination rate constant calculated from the terminal linear portion of the log plasma concentration-time curve. The total area under the plasma concentration-time curve from time 0 to the last quantifiable time point (AUC0-t) and elimination rate constant calculated from the terminal linear portion of the log plasma concentration-time curve from time 0 to the last quantifiable time point (AUC0-t) were calculated using the log trapezoidal rule. The AUC0-t, was calculated by the following equation:

\[ \text{AUC}_{0\rightarrow t} = \text{AUC}_{0\rightarrow t-1} + C/\beta \]  

where \( C \) is the last measurable plasma concentration. The maximum plasma concentration (\( C_{\text{max}} \)) for glabridin was obtained by visual inspection of the plasma concentration-time curve, whereas the initial drug concentration (the extrapolated concentration at zero time) of the drug after i.v injection was calculated by back-extrapolation of the plasma concentration-time curve to the y-axis. The plasma clearance (CL) was estimated by dividing the total administered dose by the AUC0-\( t \). The volume of distribution (\( V_d \)) was calculated by dividing CL by \( \beta \).

The systemic bioavailability (\( F \% \)) of glabridin after oral administration was determined as follows:

\[ F(\%) = \left( \frac{\text{AUC}^{\text{p.o.}}/\text{Dose}^{\text{p.o.}}}{\text{AUC}^{\text{i.v.}}/\text{Dose}^{\text{i.v.}}} \right) \times 100 \]  

where \( \text{AUC}^{\text{p.o.}} \) and \( \text{AUC}^{\text{i.v.}} \) are the areas under the plasma concentration curves calculated after oral and i.v. administration, respectively.

Data Analysis. Data are presented as mean ± S.D. The initial statistical analysis to evaluate the differences in the mean kinetic parameters among the different groups was carried out by a one-way analysis of variance followed by a post hoc test (Dunnett’s multiple comparison test). Student’s \( t \) test was conducted for the between-group comparisons with a significance level of \( P < 0.05 \).

The permeability values of glabridin across rat ileum were calculated based on the disappearance of the drug from the lumen (\( P_{\text{lumen}} \)) as well as the appearance of drug in the blood (\( P_{\text{blood}} \)) using the following equations (Zhang et al., 2006):

\[ P_{\text{lumen}} = \frac{Q}{2\pi l} \ln \frac{C_{\text{lumen}}}{C_0} \]  

\[ P_{\text{blood}} = \frac{dX/dt}{A \times C_0} \]  

where \( r \) is the radius of the intestinal lumen (0.18 cm), \( l \) is the length of the segment (cm), \( Q \) is the flow rate of drug through the intestinal segment, \( C_0 \) is the concentration of glabridin at the start of perfusion (in the syringe), \( C_{\text{lumen}} \) is the steady-state concentration of glabridin exiting the lumen, \( dX/dt \) is the rate of glabridin appearance in mesenteric venous blood (\( \mu g/s \)), and \( A \) is the surface area of the ileal segment (= 2\( \pi rl \), cm²).

The apparent permeability coefficient (\( P_{\text{app}} \)) in cellular monolayers is expressed in centimeters per second and calculated as the following equation:

\[ P_{\text{app}} = \frac{\Delta Q/\Delta t}{A \times C_0} \]  

where \( \Delta Q/\Delta t \) is the permeability rate (\( \mu g/s \)), \( A \) is the surface area of the membrane (cm²), and \( C_0 \) is the initial drug concentration in the donor chamber (\( \mu g/ml \)). Samples from the 30-min point were used for \( P_{\text{app}} \) calculations since, at this time, steady state has been achieved. The net BL to AP efflux of glabridin (\( R_{\text{net}} \)) was determined by calculating the ratio of \( P_{\text{app}} \) in the BL to AP direction versus \( P_{\text{app}} \) in the AP to BL direction (\( P_{\text{app(BL-AP)}}/P_{\text{app(AP-BL)}} \)) as eq. 6 (Zhou et al., 2005).

\[ R_{\text{net}} = \frac{P_{\text{app(BL-AP)}}}{P_{\text{app(AP-BL)}}} \]  

The passive diffusion flux rate (excluding the influence of efflux transporter) of glabridin in Caco-2 monolayers was estimated by conducting the transport experiment in the presence of verapamil (100 \( \mu g \)), assuming that other transporters play a minimal role in glabridin transport. The active transport flux rates were then estimated by subtracting the passive diffusion rates from total flux rates. Several models to describe the kinetics of the calculated active transport and ATPase stimulation activity of glabridin (single- and two-binding sites with and without a nonsaturable component, substrate inhibition, and the sigmoid models) were fitted with the following models and using the Prism 3.0 program (GraphPad Software Inc., San Diego, CA).

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]  

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} + K_d \]  

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} + \frac{V_{\text{max}2}[S]}{K_m2 + [S]} \]  

\[ v = \frac{V_{\text{max}}[S]}{K + [S] + [S]/K_m} \]  

\[ v = \frac{V_{\text{max}}[S]}{K + [S] + [S]/K_m} \]  

where \( v \) is the rate of glabridin transport or ATP hydrolysis; \( V_{\text{max}} \) is the maximum velocity; \( K_{\text{m}} \) is the Michaelis-Menten constant; \( [S] \) is the substrate concentration; \( K_d \) is the nonsaturable component; \( K_{\text{h}} \) is the substrate inhibition constant; \( h \); the Hill coefficient for cooperative substrate binding; and subscripts 1 and 2 represent the first and the second type of enzyme binding sites. The choice of model was confirmed by \( F \) test and comparison of Akaike’s information criterion values (Yamaoka et al., 1978).

Apparent inhibition constant (\( K_i \)) was estimated using eqs. 13 to 15 as previously described (Zhou et al., 2005).

\[ K_i = \frac{P_i}{P_0} - 1 \]  

\[ P_0 = P_{\text{app}} - P_{\text{app}1} \]  

\[ P_i = P_{\text{app}} - P_{\text{app}1} \]  

where \( P_i \) and \( P_0 \) are the \( P_{\text{app}} \) values of glabridin in the direction of BL to AP in the presence and absence of the inhibitor, respectively; and \( P_i/P_0 \) is a reflection of the inhibitory effect of the test compound on the active BL to AP transport of glabridin across the Caco-2 monolayers. \( [I] \) is the concentration of inhibitor in the donor and the receiver side. \( P_{\text{app}1} \) is the total transport in the absence of any inhibitory compound, \( P_{\text{app}2} \) is the total transport in the presence of a potential inhibitor, and \( P_{\text{app}3} \) is the passive diffusion component.

Results

Oral Bioavailability of Glabridin in Rats. The representative plasma concentration-time profiles of glabridin after oral (5 and 20 mg/kg) and i.v. (5 mg/kg) administration in rats are shown in Fig. 2 and the pharmacokinetic parameters of glabridin are listed in Table 1. After oral administration of glabridin at 5 or 20 mg, the \( C_{\text{max}} \) and \( T_{\text{max}} \) of glabridin were 15.10 ± 4.72 ng/ml and 60.41 ± 18.87 ng/ml; 4.33 ± 1.86 h and 4.50 ± 2.17 h; and 96.49 ± 32.34
ng/ml · h and 387.98 ± 137.28 ng/ml · h, respectively. These results indicated that the oral pharmacokinetics of glabridin is linear (dose-independent), as indicated by the proportional increase of \( C_{\text{max}} \) and AUC of glabridin when its oral dose was increased from 5 mg/kg to 20 mg/kg. The \( t_{1/2A} \) of glabridin was 2.38 to 2.41 h after administration at 5 to 20 mg/kg.

In addition, after i.v. bolus injection of glabridin at 5 mg/kg, the \( C_{\text{max}} \), AUC\( _{0-24h} \), and CL, and \( V_d \) were 1.92 h, 1301.48 ± 375.79 ng/ml · h, 1.92 h, 59.01 ml/min/kg and 2.72 l/kg, respectively. Thus, the \( F \) values of glabridin in rats were 7.45% and 7.44%, respectively, after oral dosing of glabridin at 5 and 20 mg/kg. These findings indicate that the oral absorption and oral bioavailability of glabridin are low and dose-independent.

**In Vitro Metabolism of Glabridin in Rat Intestinal and Hepatic Microsomes.** In rat intestinal and hepatic microsomes, no oxidative metabolites of glabridin were observed when glabridin was incubated in the presence of NADPH using LC-MS and HPLC methods. Marked formation of glabridin glucuronide was found when glabridin was incubated with rat hepatic microsomes in the presence of UDPGA. The formation of glabridin glucuronide, when monitored by relative metabolite ion density using LC-MS, was linear up to 60 min incubation time and 50 \( \mu M \) substrate concentration. A minimal peak of glabridin glucuronide using HPLC and a weak signal of glabridin glucuronide ion using LC-MS were observed when the substrate was incubated in rat intestinal microsomes. The formation rate of glabridin glucuronide in rat intestinal microsomes was approximately 1/15 to 1/20 of that when incubated with rat hepatic microsomes. In addition, diclofenac and indomethacin (both at 100 \( \mu M \)) inhibited the formation of glabridin glucuronide in rat hepatic and intestinal microsomes by 68.5 ± 15.2% and 72.3 ± 14.3%, respectively. However, verapamil at 100 \( \mu M \) did not inhibit glabridin glucuronidation in both intestinal and hepatic microsomes.

**Transport of Glabridin in Single-Pass Perfusion Study of Rat Ileum.** There was insignificant loss of glabridin (<6.0%) when the drug was perfused through the perfusion apparatus used in this study, indicating that there was no significant adsorption of glabridin to the tubing wall of the system. The compound was stable in the perfusion buffer as well as intestinal perfusate at 37°C for at least 12 h. There were no oxidative metabolites formed in the perfusates or mesenteric vein blood after glabridin was loaded, as determined by both HPLC and LC-MS analysis. No detectable peak of glabridin glucuronide in the perfusates or mesenteric vein blood was found using HPLC analysis, but a weak signal of glabridin glucuronide ion using LC-MS was observed. This indicated that the glucuronidation of glabridin by rat perfused ileum segment was minimal or just detectable, and gut metabolism had a minor impact on the determination of permeability coefficients of glabridin in this model.

For intestinal perfusions with glabridin, samples were collected from the outlet of the ileal segment and mesenteric vein at 5-min intervals over 60 min. The permeability values of glabridin at 0.1, 0.5, and 2.0 \( \mu M \) are shown in Table 2. The permeability of glabridin based

---

**TABLE 1**

The pharmacokinetics parameters \((n = 6)\) of glabridin after p.o. administration at 5 and 20 mg/kg, and i.v. bolus injection at 5 mg/kg

<table>
<thead>
<tr>
<th>Parameter</th>
<th>p.o. (5 mg/kg)</th>
<th>p.o. (20 mg/kg)</th>
<th>i.v. (5 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{\text{max}} ) (h)</td>
<td>4.33 ± 1.86</td>
<td>4.50 ± 2.17</td>
<td>N.C.</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (ng/ml)</td>
<td>15.10 ± 4.72</td>
<td>60.41 ± 18.87</td>
<td>422.96 ± 123.92</td>
</tr>
<tr>
<td>( t_{1/2A} ) (h)</td>
<td>2.38 ± 0.70</td>
<td>2.41 ± 0.74</td>
<td>1.92 ± 0.60</td>
</tr>
<tr>
<td>AUC(_{0-24h}) (ng/ml · h)</td>
<td>96.49 ± 32.34</td>
<td>387.98 ± 137.28</td>
<td>1301.48 ± 375.79</td>
</tr>
<tr>
<td>AUC(_{0-\infty}) (ng/ml · h)</td>
<td>103.54 ± 34.03</td>
<td>413.30 ± 127.72</td>
<td>1368.49 ± 400.22</td>
</tr>
<tr>
<td>CL (ml/min/kg)</td>
<td>N.C.</td>
<td>N.C.</td>
<td>59.01</td>
</tr>
<tr>
<td>( V_d ) (l/kg)</td>
<td>7.45%</td>
<td>7.44%</td>
<td>N.C.</td>
</tr>
</tbody>
</table>

N.C., not calculated.

**TABLE 2**

Permeability values of glabridin across isolated perfused rat ileum with mesenteric vein cannulation

<table>
<thead>
<tr>
<th>Concentration of Glabridin in Perfusion Solution</th>
<th>( \frac{P_{\text{lumen}}}{P_{\text{blood}}} \times 10^{-5} )</th>
<th>( \frac{P_{\text{blood}}}{P_{\text{lumen}}} \times 10^{-5} )</th>
<th>Ratio of ( P_{\text{lumen}}/P_{\text{blood}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ( \mu M )</td>
<td>6.51 ± 0.72</td>
<td>8.63 ± 0.97*</td>
<td>7.54</td>
</tr>
<tr>
<td>+ Verapamil (100 ( \mu M ))</td>
<td>6.64 ± 0.63</td>
<td>21.32 ± 3.12*</td>
<td>3.11</td>
</tr>
<tr>
<td>+ Probenecid (200 ( \mu M ))</td>
<td>6.57 ± 0.59</td>
<td>9.23 ± 1.14*</td>
<td>7.12</td>
</tr>
<tr>
<td>+ MK-571 (100 ( \mu M ))</td>
<td>6.72 ± 0.43</td>
<td>9.55 ± 1.03*</td>
<td>7.12</td>
</tr>
<tr>
<td>+ Celecoxib (100 ( \mu M ))</td>
<td>6.48 ± 0.59</td>
<td>8.65 ± 1.12*</td>
<td>7.49</td>
</tr>
<tr>
<td>0.5 ( \mu M )</td>
<td>8.22 ± 0.91*</td>
<td>10.23 ± 1.17*</td>
<td>8.04</td>
</tr>
<tr>
<td>+ Verapamil (100 ( \mu M ))</td>
<td>8.42 ± 0.63</td>
<td>28.31 ± 3.05*</td>
<td>2.97</td>
</tr>
<tr>
<td>+ Probenecid (200 ( \mu M ))</td>
<td>8.28 ± 0.51</td>
<td>11.13 ± 1.27*</td>
<td>7.44</td>
</tr>
<tr>
<td>+ MK-571 (100 ( \mu M ))</td>
<td>8.40 ± 1.01</td>
<td>10.83 ± 1.37*</td>
<td>7.76</td>
</tr>
<tr>
<td>+ Celecoxib (100 ( \mu M ))</td>
<td>8.24 ± 0.61</td>
<td>11.01 ± 1.06*</td>
<td>7.48</td>
</tr>
<tr>
<td>2.0 ( \mu M )</td>
<td>11.54 ± 1.31*</td>
<td>14.42 ± 1.55*</td>
<td>8.00</td>
</tr>
<tr>
<td>+ Verapamil (100 ( \mu M ))</td>
<td>12.24 ± 1.41</td>
<td>36.79 ± 4.07*</td>
<td>2.97</td>
</tr>
<tr>
<td>+ Probenecid (200 ( \mu M ))</td>
<td>11.89 ± 1.41</td>
<td>13.93 ± 1.63*</td>
<td>8.54</td>
</tr>
<tr>
<td>+ MK-571 (100 ( \mu M ))</td>
<td>12.22 ± 1.42</td>
<td>16.67 ± 1.82*</td>
<td>7.33</td>
</tr>
<tr>
<td>+ Celecoxib (100 ( \mu M ))</td>
<td>12.04 ± 1.61</td>
<td>15.48 ± 1.95*</td>
<td>7.78</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \), \( P_{\text{blood}} \) vs \( P_{\text{lumen}} \).

* \( P < 0.05 \), presence of a transporter inhibitor vs \( P_{\text{blood}} \) in the absence of a transporter inhibitor.

* \( P < 0.05 \), \( P_{\text{blood}} \) at 0.5 or 2.0 \( \mu M \) vs \( P_{\text{blood}} \) at 0.1 \( \mu M \).

---

Fig. 2. The representative plasma concentration-time profiles of glabridin after oral administration at 5 and 20 mg/kg and intravenous bolus injection at 5 mg/kg (n = 6 per group).
on the luminal disappearance of the compound was estimated at steady state (i.e., samples obtained from 30 to 60 min). The \( P_{\text{lumen}} \) values of glabridin were \( 6.51 \pm 0.72 \times 10^{-4}, 8.22 \pm 0.91 \times 10^{-4}, \) and \( 11.54 \pm 1.31 \times 10^{-4} \) cm/s, respectively, when the concentrations of glabridin in perfusates were 0.1, 0.5 and 2.0 \( \mu \)M. With the increase of glabridin concentration in perfusates, the \( P_{\text{lumen}} \) values significantly increased \((P < 0.05)\). The appearance of glabridin in mesenteric vein blood increased when the drug concentration was 0.1, 0.5, and 2.0 \( \mu \)M in perfusates. The concentration-dependent increase in \( P_{\text{lumen}} \) of glabridin may reflect the relatively low to moderate intrinsic permeability of glabridin and possible involvement of a saturable active mechanism for its intestinal transport. As for the permeability based on appearance of glabridin in the mesenteric blood \( (P_{\text{blood}}) \), concentration-dependent increases in permeability were evident, whereas the \( P_{\text{blood}} \) values at 0.1, 0.5, and 2.0 \( \mu \)M were 6.5- to 7.0-fold lower than \( P_{\text{lumen}} \) \((P < 0.05)\).

Verapamil, probenecid, MK-571, and celecoxib all did not significantly alter the \( P_{\text{lumen}} \) values of glabridin at 0.1, 0.5, or 2.0 \( \mu \)M \((P > 0.05)\). However, the \( P_{\text{blood}} \) values of glabridin at 0.1, 0.5, and 2.0 \( \mu \)M increased significantly in the presence of 100 \( \mu \)M verapamil, with the ratio of \( P_{\text{lumen}} / P_{\text{blood}} \) decreased from 7.5 to 8.0 to 3.0 to 3.3 (Table 2). However, probenecid, MK-571, and celecoxib insignificantly affected the \( P_{\text{blood}} \) values at all substrate concentrations tested. These data suggest that PgP-mediated efflux effectively limited the absorption of glabridin across the intestinal wall, which was at least partially reversed by verapamil, a known PgP inhibitor, but not by MRP inhibitors including probenecid (MRP1), MK-571 (MRP1/2), and celecoxib (MRP4).

We also examined the transport of two probe compounds, antipyrine (a well absorbed passive transcellular transport marker) and mannitol (a well absorbed paracellular transport marker), using the single-pass intestinal perfusion model. The results showed that the \( P_{\text{lumen}} \) and \( P_{\text{blood}} \) for antipyrine were \( 5.96 \pm 1.33 \times 10^{-5} \) cm/s and \( 6.12 \pm 2.21 \times 10^{-5} \) cm/s \((n = 6 \text{ per group})\), respectively; and 7.63 \pm 1.25 \times 10^{-6} \text{ and } 7.74 \pm 1.68 \times 10^{-6} \text{ cm/s for mannitol, respectively.} \) The addition of verapamil at 100 \( \mu \)M or other inhibitor did not significantly affect the \( P_{\text{lumen}} \) and \( P_{\text{blood}} \) of both antipyrine and mannitol (data not shown). Notably, the \( P_{\text{lumen}} \) values of glabridin were significantly higher than those for antipyrine and mannitol. These results indicate that there was no significant difference between

![Fig. 3. Effects of incubation time (A and C) and substrate concentration (B and D) on the uptake and efflux of glabridin in Caco-2 cells cultured in HBSS. The curves in plots B and D represent the best fit of the one-binding-site model. E, the kinetic profiles of glabridin efflux from Caco-2 cells when preloaded at 1.0 \( \mu \)M and the intracellular concentrations of glabridin were monitored by LC-MS over 120 min of incubation. The data are the mean \pm S.D. of at least six to nine determinations.]
Cytotoxicity and Metabolism of Glabridin in Caco-2, Control MDCKII, and MDR1-MDCKII Cells. Glabridin at 0.1 to 100 μM did not show significant cytotoxicity (<10%) to Caco-2, control MDCKII, and MDR1-MDCKII cells when incubated for up to 48 h as determined by the MTT assay.

No detectable oxidative metabolites were observed when glabridin at 0.1 to 100 μM was incubated with Caco-2, control MDCKII, and MDR1-MDCKII cells for 2 to 48 h as determined by HPLC and LC-MS analysis. A minor signal was detected for glabridin glucuronide ion when the substrate was incubated with Caco-2 for 2 to 48 h, as determined by LC-MS analysis. The minimal formation of glabridin glucuronides in Caco-2 cells indicated that Caco-2 cells had a weak metabolic ability for glabridin, possibly due to low levels or activity of uridine diphosphate glucuronosyltransferases that metabolize this compound, and metabolism had a minor impact on the assessment of transport of glabridin in this model.

Uptake and Efflux of Glabridin in Caco-2 Cells. The intracellular accumulation and efflux of glabridin in Caco-2 cells was examined with regard to incubation time and substrate concentration (Fig. 3). The uptake of glabridin into Caco-2 cells was linear up to 60 min, whereas efflux of glabridin out of Caco-2 cells was linear up to 20 min. Its accumulation and efflux in Caco-2 cells also increased depending on the substrate concentration and followed Michaelis-Menten kinetics with a one-binding site model being the best fit. The estimated $K_m$ and $V_{max}$ for glabridin uptake by Caco-2 cells and efflux from Caco-2 cells were $12.38 \pm 1.41$ and $4.68 \pm 1.03$ μM, and $17.44 \pm 0.76$ ng/min/mg cellular protein and $0.67 \pm 0.04$ pg/min/mg cellular protein, respectively. We also monitored the intracellular concentration of glabridin at 1.0 μM upon incubation over 120 min (Fig. 3). It appeared that the efflux of glabridin from Caco-2 cells was characterized by a monoeponential kinetics with a half-life of 9.84 min, indicating a rapid exit of the drug from Caco-2 cells during the first 10 min, followed by a slow exit requiring several hours.

**FIG. 4.** Effects of sodium azide, 2,4-dinitrophenol, verapamil, nifedipine, probenecid, MK-571, and celecoxib on the uptake in Caco-2 cells (A) and efflux (B) of glabridin at 1.0 μM from Caco-2 cells. The inhibitor was preincubated with the cells for 2 h and incubated further for 30 min after addition of glabridin for uptake inhibition assay. For efflux inhibition assay, the cells were washed five times with ice-cold PBS after incubation with glabridin at 1.0 μM for 120 min and incubated further in the presence of an inhibitor for 30 min at 37°C. The data are the mean ± S.D. of at least six to nine determinations. *, $P < 0.05$; **, $P < 0.01$.

**FIG. 5.** Transport of glabridin across Caco-2 monolayers. A, effect of concentration of glabridin (0.1–100 μM) on the permeability ($P_{app}$) of glabridin from the apical (AP) to basolateral (BL) and BL to AP side; and B, effect of incubation time (0–60 min) on the permeability ($P_{app}$) of glabridin at 1.0 μM in the AP to BL and BL-AP directions. Glabridin was loaded on either the AP or BL side and incubated at 37°C. Samples from the receiving side were collected at indicated time points and glabridin was determined by LC-MS. Data are the mean ± S.D. from at least six to nine determinations. **, $P < 0.01$. 

$P_{lumen}$ and $P_{blood}$ for antipyrine and mannitol, and our single-pass intestinal perfusion model was a valid system for study of drug transport.
The effects of potential inhibitors including verapamil, nifedipine, probenecid, MK-571, and celecoxib on the intracellular accumulation and efflux of glabridin in Caco-2 cells are shown in Fig. 4. When verapamil or nifedipine (both at 100 μM) was preincubated for 2 h with the cells, glabridin accumulation was significantly \( P < 0.05 \) increased by 83.3 ± 13.3% and 116.9 ± 15.2%, respectively. Addition of MK-571 (100 μM), celecoxib (100 μM), or probenecid (200 μM) insignificantly increased glabridin intracellular accumulation by 2.4 to 13.5% \( P > 0.05 \). On the other hand, when verapamil or nifedipine (both at 100 μM) was incubated in Caco-2 cells for 30 min, glabridin efflux was significantly \( P < 0.05 \) decreased by 27.8 ± 3.3% and 32.5 ± 4.2%, respectively. Addition of MK-571 (100 μM), celecoxib (100 μM), or probenecid (200 μM) insignificantly increased glabridin efflux \( P > 0.05 \). These findings demonstrated that incubation with the PgP inhibitors verapamil and nifedipine, instead of MRP inhibitors including MK-571, probenecid, and celecoxib, significantly altered glabridin accumulation in Caco-2 cells and efflux from Caco-2 cells, suggesting that glabridin is probably a substrate for PgP/MDR1, but not for MRP1–4. A significantly altered glabridin accumulation in Caco-2 cells and efflux from these cells by addition of verapamil or nifedipine, but not MK-571, probenecid, and celecoxib, suggested that glabridin is probably a substrate for PgP/MDR1, but not for MRP1–4.

Uptake of the probe markers sucrose and propranolol into Caco-2 cells was determined upon incubation up to 120 min. Propranolol penetrated into Caco-2 cells to a low extent \( 0.25 ± 0.03 \) to \( 0.44 ± 0.52 \) ng/min/mg cellular protein), and diffusion of sucrose into the cells was minimal \( 0.04 ± 0.00 \) to \( 0.07 ± 0.01 \) ng/min/mg cellular protein). The uptake of sucrose and propranolol did not increase with incubation time and substrate concentration in Caco-2 cells. In addition, when daunomycin was used as a model PgP/MDR1 substrate, its uptake by Caco-2 cells was significantly increased by 75.2 ± 9.4% when coincubated with 100 μM verapamil. As well, the efflux of the model substrate vinblastine was also dependent on the substrate concentration and incubation time, but its cellular efflux was characterized by a biexponential kinetics with half-lives of 9.8 min and 2.7 h, respectively.

Transport of Glabridin in Caco-2 Monolayers. After incubation of glabridin at 0.1 to 100 μM loaded at either the AP or BL side, the sample was collected from the receiving side for LC-MS analysis. No detectable metabolites were observed when glabridin was loaded on the AP or BL side at all concentrations over 60 min. The time course and concentration effect of glabridin flux from AP to BL or BL to AP were determined by LC-MS methods. Data are the mean ± S.D. from at least six to nine determinations. * \( P < 0.05 \); ** \( P < 0.01 \).

Results indicated that theflux rate (ng/min/cm²) of glabridin from AP to BL or BL to AP transport of glabridin at 0.1 μM (A) or 1.0 μM (B) in Caco-2 monolayers upon incubation for 30 min. Glabridin (0.1 or 1.0 μM) was loaded on either the apical or basolateral side and incubated for 30 min at 37°C. Transport studies were undertaken in the absence or presence of the potential inhibitor added to both the apical and basolateral compartments. All inhibitors were preincubated for 2 h with Caco-2 cells. Samples from the receiving side were collected and glabridin concentrations were determined by LC-MS methods. Data are the mean ± S.D. from at least six to nine independent determinations. The curve represents the best fit of one saturable transport model.

Fig. 6. Model fitting for the passive and active transport of glabridin from apical (AP) to basolateral (BL) and BL to AP sides in Caco-2 monolayers. The passive and active BL-AP and AP-BL transport was calculated only based on verapamil inhibition of PgP in Caco-2 cells assuming that the role of other transporters is minimal and ignored. Glabridin (0.1–100 μM) was loaded on either the AP or BL side and incubated at 37°C over 30 min. Samples from the receiving side were collected and glabridin was determined by LC-MS analysis. Data are the mean ± S.D. from six to nine independent determinations. The curve represents the best fit of one saturable transport model.
transport rate of glabridin across Caco-2 monolayers from the BL to AP side was significantly \( P < 0.05 \) higher than that from the AP to BL side. The \( P_{\text{app}} \) of glabridin from the BL to AP side \( (8.12 - 25.63 \times 10^{-5} \text{ cm/s}) \) was approximately 3.3- to 8.4-fold higher than those from the AP to BL side \( (0.87 - 5.76 \times 10^{-5} \text{ cm/s}) \) with a marked decrease in \( P_{\text{app}} \) values for both directional transport at increasing glabridin concentrations (Fig. 5). The \( K_{\text{max}} \) values ranged from 4.3 to 9.4. These results demonstrated a polarization in the Caco-2 permeability toward glabridin and a predominantly secretory rather than absorptive transport. The BL to AP efflux rate of glabridin increased with increasing glabridin concentrations over 0.1 to 100 \( \mu M \) but appeared saturable when glabridin concentration was \( \geq 10 \mu M \), as indicated by a non-proportional increase in the efflux (data not shown). Consistently, there was a significant decrease in \( P_{\text{app}} \) values for BL to AP flux at glabridin concentrations \( \geq 10 \mu M \) \( (P < 0.001) \).

The passive and active AP-BL and BL-AP transport was calculated only based on verapamil inhibition of PgP in Caco-2 cells assuming that the role of other transporters for glabridin transport is minimal and negligible. Model fitting indicates that the one-binding-site model was the best fit for calculated active BL to AP efflux, with a \( K_m \) of 6.57 \( \pm 1.16 \mu M \), and \( V_{\text{max}} \) of 0.65 \( \pm 0.03 \text{ mmol/min/cm}^2 \) as shown in Fig. 6. The low \( K_m \) value suggested that glabridin was a substrate for PgP with high affinity.

Reducing the apical pH to 5.5 to 6.5 caused a significant \( P < 0.05 \) increase in glabridin flux by 24.5 to 56.8\% at 0.1 and 1.0 \( \mu M \) from the AP to BL or BL to AP side compared with the values at pH 7.4. A maximum \( P_{\text{app}} \) was observed at pH 7.0 for both AP-BL and BL-AP transport at 0.1 and 1.0 \( \mu M \) substrate concentration. Lower pH may reduce the ionization of glabridin and thus increase its intestinal transport. The substitution of potassium salts in the transport medium did not significantly affect the AP to BL flux of glabridin at either 0.1 or 1.0 \( \mu M \). In contrast, depletion of glucose significantly \( P < 0.05 \) decreased the BL to AP flux of glabridin at 0.1 and 1.0 \( \mu M \) by 50.5 to 65.2\%. These results indicated that the transport of glabridin across Caco-2 monolayers was pH-, energy-, and temperature-dependent, but not sodium-dependent.

The effects of ATP inhibitors and various ABC transporter inhibitors on the transport of glabridin \( (0.1 \) and \( 1.0 \mu M \) in Caco-2 monolayers were also investigated. Addition of the transport buffer at both sides with sodium azide \( (10 \text{ mM}, 2,4\text{-dinitrophenol (1 mM)}, \) or verapamil \( (100 \mu M) \) significantly \( P < 0.05 \) increased the AB to BL flux of glabridin at 0.1 \( \mu M \) by 52.8\%, 38.6\%, and 82.4\%, respectively \( (P < 0.05) \) (Fig. 7). In contrast, these compounds caused a significant \( P < 0.05 \) decrease in the BL to AP flux of glabridin at 0.1 \( \mu M \) by 48.4\%, 43.3\%, and 53.1\%, respectively. Similar results were observed when the concentration of glabridin was increased to 1.0 \( \mu M \) in the presence of the above inhibitors. The estimated \( K_i \) values based on eqs. 13 to 15 for sodium azide, 2,4-dinitrophenol, and verapamil were 8.8 \( \text{ mM}, 1.5 \text{ mM}, \) and 6.8 \( \mu M \), respectively. However, probenecid, MK-571, and celecoxib slightly altered the AB-AP \( P_{\text{app}} \) values \( (P > 0.05) \), suggesting that MRPs play a minor or negligible role in the intestinal transport of glabridin.

**Uptake and Efflux of Glabridin in Control MDCKII and MDR1-MDCKII Cells.** As shown in Fig. 8, the intracellular accumulation and efflux amounts of glabridin in both control MDCKII and MDR1-MDCKII cells were linear up to 120 min of incubation time. The accumulation and efflux rate of glabridin in both control MDCKII and MDR1-MDCKII cells over 0.1 to 50 \( \mu M \) increased in a concentration-dependent manner, following the Michaelis-Menten kinetics with the one-binding-site model being the best fit (Fig. 8). The estimated \( K_m \) and \( V_{\text{max}} \) in both control MDCKII and MDR1-MDCKII...
cells for glabridin uptake were 12.75 ± 1.43 and 10.91 ± 2.23 μM, and 16.22 ± 0.70 and 4.47 ± 0.34 ng/min/mg cellular proteins, respectively (Fig. 8). The uptake of glabridin by control MDCKII cells was significantly (approximately 3-fold) higher than that in MDR1-MDCKII cells (P < 0.05). The estimated $K_m$ and $V_{max}$ in both control MDCKII and MDR1-MDCKII cells were 5.70 ± 1.75 and 5.60 ± 0.73 μM, and 8.44 ± 0.81 and 60.03 ± 2.44 pg/min/mg cellular proteins, respectively. The efflux rate of glabridin from MDR1-MDCKII cells was significantly (approximately 5- to 7-fold) higher than that in control MDCKII cells (P < 0.05 or < 0.01).

These results suggest that PgP/MDR1 diminished the uptake and increased the efflux of glabridin by MDCKII cells and glabridin is probably a substrate for PgP/MDR1. Nevertheless, the diminished accumulation and increased efflux in MDR1-MDCKII cells compared with the control MDCKII cells cannot be attributable to drug-induced plasma membrane damage, which in turn could cause cellular leakage and increase drug influx and efflux. The cells remained viable during drug accumulation studies over 120 min as measured using trypan blue exclusion.

Uptake of the probe markers sucrose and propranolol into both control MDCKII and MDR1-MDCKII cells was also determined upon incubation up to 120 min. Propranolol penetrated into both cell lines to a low degree with a value of 0.18 ± 0.02 to 0.37 ± 0.40 ng/min/mg cellular protein, and diffusion of sucrose into the cells was minimal (0.03 ± 0.00 to 0.06 ± 0.01 ng/min/mg cellular protein). The uptake of sucrose and propranolol did not increase with incubation time and substrate concentration in either cell line. These findings indicate that PgP/MDR1 did not significantly affect the uptake of both sucrose and propranolol.

In addition, the efflux of the probe drug, vinblastine, from MDR1-MDCKII cells was significantly (approximately 4- to 8-fold) higher than that in control MDCKII cells (data not shown). These results indicated that glabridin was a potent PgP inhibitor in vitro.

To further investigate the nature of the polarized transport of glabridin, transport studies were conducted in control MDCKII and MDR1-MDCKII cells which stably and functionally overexpress the human PgP/MDR1. Our Western blotting analysis demonstrated that control MDCKII cells expressed constitutive canine P-glycoprotein at a much lower level than that in the recombinant MDR1-MDCKII cells (data not shown).

The transport data across these two MDCKII cell lines for glabridin are shown in Fig. 9. Consistent with the data for glabridin in the Caco-2 monolayer studies, glabridin at 0.1 to 50 μM in the control MDCKII monolayers showed a significantly (P < 0.05 or 0.01) greater (approximately 2-fold) permeability in the BL-AP direction compared with that in the AP-BL direction. The transport of glabridin across MDR1-MDCKII monolayers was also examined and compared with the control cells. Most apparent is that the extent of polarized transport is now more profound, with $P_{app}$ values in both AP-BL and BL-AP directions.

At all substrate concentrations, the permeability of glabridin in the BL-AP direction in the MDR1-MDCKII cells was significantly (P < 0.01 or 0.001) greater than that in the BL-AP direction in control MDCKII cells. As for the Caco-2 data, increased glabridin concentration also resulted in lower $P_{app}$ values in both AP-BL and BL-AP directions for both MDCKII and MDR1-MDCKII monolayers.

**Inhibition of P-glycoprotein-Mediated Digoxin Transport by Glabridin.** We examined the effects of glabridin on PgP-mediated transport of the probe digoxin in Caco-2 monolayers. The results are shown in Fig. 10. Glabridin inhibited digoxin transport in a concentration-dependent manner with an IC$_{50}$ value of 2.56 ± 0.04 μM. In addition, verapamil exhibited potent inhibitory effects on PGp-mediated transport of digoxin, with an IC$_{50}$ value of 2.34 ± 0.03 μM. These results indicated that glabridin was a potent PgP inhibitor in vitro.

**Stimulation of P-glycoprotein ATPase Activity by Glabridin.** The affinity of glabridin to PgP/MDR1 was assessed by the ATPase activity.
The systemic bioavailability of glabridin after oral administration at 5 and 20 mg/kg was very low (approximately 7.5%). Moderate to high bioavailability and elimination in vivo.

**Discussion**

The systemic bioavailability of glabridin after oral administration at 5 and 20 mg/kg was very low (approximately 7.5%). Moderate to high plasma clearance (59.0 ml/min/kg) of glabridin was observed in rats, which was partially attributed to partitioning into red blood cells based on an approximate blood/plasma concentration ratio of 1.6 (M. Huang, X. Chen, and S. F. Zhou, unpublished data). Therefore, blood clearance of glabridin was 1.6-fold lower than plasma clearance.
was approximately 43% of hepatic blood flow in rats (approximately 85.0 ml/min/kg). If glabridin was well absorbed and cleared mainly in the liver, its oral bioavailability would be expected to be approximately 57% calculated from 100% minus 43%.

Microsomal study demonstrated that glabridin was extensively glucuronidated in the liver. To a much lesser extent, the intestine could also metabolize glabridin by glucuronidation. The low water solubility of glabridin, intestinal transporter-mediated efflux, and first-pass metabolism are considered the major factors for its low oral bioavailability.

The absorption of glabridin in the single-pass perfused rat ileum was monitored by determination of disappearance permeability (P_{lumen}) and appearance permeability (P_{blood}). The P_{lumen} for glabridin (6.51–11.54 × 10^{-6} cm/s at substrate concentrations of 0.1–2.0 μM) was higher than that of verapamil (3.07 × 10^{-5} cm/s) (Johnson et al., 2003), lidocaine (7.5 × 10^{-5} cm/s) (Berggren et al., 2004), and RU60079 (a novel angiotensin II antagonist; 1.4 × 10^{-5} cm/s) (Boisset et al., 2000), but lower than that of warfarin (7.7 × 10^{-5} cm/min) (Okudaira et al., 2000). The much lower P_{blood} (approximately 7-fold) than P_{lumen} of glabridin indicated extensive intestinal efflux and/or gut metabolism. Such marked difference between P_{blood} and P_{lumen} occurs because permeability calculations based only on drug disappearance from the lumen cannot distinguish drug losses due to absorption from those due to extensive gut metabolism (Dackson et al., 1992). These data also illustrated the potentially different conclusions that might be drawn as to the importance of PgP/MDR1 efflux on drug absorption when evaluating permeability based on drug disappearance as opposed to drug appearance values in the single-pass intestinal perfusion model.

There was only minimal metabolite formation detected using LC-MS analysis in the perfusates or mesenteric vein blood when glabridin was loaded, suggesting that extensive metabolism of glabridin in the gut did not occur. The lack of effect of verapamil on P_{lumen} but a great effect on P_{blood} instead was considered to be due to the PgP/MDR1 inhibition, rather than inhibition of intestinal first-pass metabolism. A lack of effect of verapamil on P_{lumen} was also found in previous studies with verapamil (Sandstrom et al., 1999; Johnson et al., 2003). PSC833 (a known potent PgP/MDR1 inhibitor, significantly increased the P_{blood} of verapamil but insignificantly affected its disappearance in perfused rat jejunum (Johnson et al., 2003). Similar results were observed with verapamil in the presence of ketoconazole (a known potent cytochrome P450 3A4 and PgP/MDR1 inhibitor) in a human intestinal perfusion study (Sandstrom, 1999).

These findings most likely reflected the small impact of PgP/MDR1-mediated efflux on the disappearance of glabridin in the lumen and, thus, verapamil had minor effect on the permeability of glabridin. Substantial tissue uptake and binding of both substrate and potential inhibitors could also compromise the effect of PgP inhibitors.

The uptake and efflux assays of glabridin in Caco-2 cells were conducted under nonsink conditions. Under such nonsink conditions, the efflux of glabridin from Caco-2 cells followed a one-phase exponential kinetics, which reflected that the efflux process was entirely driven by passive diffusion. The intracellular accumulation of glabridin in Caco-2, control MDCKII, and MDR1-MDCKII cells was investigated in this study. The uptake of glabridin in these cells was different with different uptake rates. This may be due mainly to the different biological properties of these cell lines; for example, the differential nature of tight junctions, cellular membrane, and other factors, resulting in differential uptake rate. However, the K_{m} values for all these cell lines are similar, ranging from 10.91 to 12.75 μM, indicating a similar mechanism of passive diffusion for glabridin under nonsink condition in all these cells.

However, the observed difference in the estimated K_{m} for glabridin uptake and efflux (12.38 ± 1.41 versus 4.68 ± 1.03 μM) indicated the presence of an active transport mechanism because, for a compound entirely driven by passive diffusion, the affinities for uptake and efflux would not be expected to differ. Therefore, both active and passive transport were likely involved in the uptake and efflux of glabridin by the cells tested. A number of flavonoids can be readily transported across the cell membrane by a number of pathways.

Like glabridin, the P_{app} for BL-AP or AP-BL flux decreased with increasing substrate concentration for many compounds such as cryptotanshinone (a major active constituent of S. miltiorrhiza) (Zhang et al., 2006) and several aryloxy phosphoramidate derivatives (all PgP/MDR1 substrates) of the anti-HIV agent stavudine (Siccardi et al., 2003). This may reflect that P_{app} is affected by a number of factors associated with the intestine and drugs, for particular substrates with some intrinsic permeability and substantial tissue uptake and binding.

We found that the permeability of the Caco-2 monolayers to glabridin is nonlinear over the concentrations of 0.1 to 50 μM. The deviation from linearity suggests the presence of a polarized efflux pump and/or a saturable metabolic barrier to absorption. However, the intrinsic permeabilities of glabridin in Caco-2 and MDCKII monolayers compared with most lipophilic drugs and negligible metabolite (glucuronide) formation in Caco-2 cells indicated that the first-pass metabolism within the enterocytes played a minor role in limiting the
oral absorption of glabridin. The increased glabridin permeability in the apical to basolateral direction in Caco-2 monolayers by coinoculated verapamil was considered to be due to inhibition of PgP/MDR1 instead of glabridin metabolic inhibition. The permeability data from the single-pass intestinal perfusion model and Caco-2 monolayers (Table 2; Fig. 5) provided initial evidence that PgP-mediated efflux into the luminal side might play an important role in the low oral bioavailability of glabridin in rats and in humans, providing that there are no species differences with regards to the rate and extent of active transport and intestinal metabolism of glabridin.

The permeability coefficients for AP-BL transport of glabridin in Caco-2 monolayers (Fig. 5) were approximately 3.3- to 8.4-fold lower than those in the BL-AP direction. A significant increase in the AB-BL flux of glabridin in the presence of sodium azide at 10 mM or 2,4-dinitrophenol at 5 mM provided supportive evidence for the involvement of an ATP-dependent active mechanism for glabridin intestinal transport. Furthermore, the addition of the PgP/MDR1 inhibitor verapamil markedly reduced the transport of glabridin in the BL-AP direction. However, the MRPI inhibitors, probenecid, MK-571, and celecoxib, had insignificant effect on glabridin transport in Caco-2 monolayers, excluding glabridin as a substrate for MRPI-4.

Transport data in the MDCKII and MDR1-MDCKII model further provided evidence that glabridin was a substrate for PgP/MDR1. Glabridin was significantly more permeable in the BL-AP direction than in the AP-BL direction in both cell lines. The permeability coefficients in the BL-AP direction were significantly higher in the cell line overexpressing PgP/MDR1 (Fig. 9). In addition, the uptake and efflux of glabridin by control MDCKII cells were significantly different from those values observed in MDR1-MDCKII cells, and this difference could be significantly altered in the presence of PgP/MDR1 inhibitors. This provided additional evidence supporting our hypothesis.

Many PgP/MDR1 substrates are also PgP/MDR1 inhibitors or modulators. Thus, we examined the effect of glabridin on PgP/MDR1-mediated transport of digoxin using Caco-2 models. The observed IC50 value (2.34 μM) for glabridin indicated that glabridin was a potent inhibitor for PgP/MDR1 compared with many other reported potent PgP/MDR1 inhibitors such as ketoconazole (1.2 μM) and cyclosporine (1.3 μM) when using the translocation of digoxin across Caco-2 cells as the model (Choo et al., 2000). The observed IC50 value for verapamil (2.57 μM) in this study is similar to those reported by other groups (Choo et al., 2000). In addition, we found that glabridin-stimulated PgP/MDR1 ATPase activity with a Km of 25.1 μM, suggesting that glabridin as a modulator has moderate affinity to protein. This may provide some insight into the mechanism for the inhibition of PgP/MDR1-mediated transport of digoxin by glabridin. It would be interesting to investigate how glabridin interacts with the ATP binding sites of PgP/MDR1 and affects its hydrolysis.

Results from the present study suggested that PgP/MDR1 played a major role in the intestinal transport of glabridin, but the contribution of MDR3 cannot be excluded. Not all these proteins confer resistance to drugs; transfection experiments have shown that the expression of human PgP/MDR1 or rodent mdr1a or mdr1b is sufficient to confer resistance to anticancer agents, whereas MDR3 (ABCB4) and mdr2 are involved in the transport of other molecules, such as phospholipids in the bile (Borst and Ellerink, 2002). The role of MDR3 for intestinal transport of drugs is likely to be minor because MDR3 is expressed in the canicular membrane of hepatocytes, heart, muscle, and B cells but not in the intestine, whereas MDR3 transcripts were only detected at a very low level (Taipalensuu et al., 2001). MDR1-mediated drug-drug interactions have been reported, and drug interaction studies of glabridin with PgP/MDR1 substrates/inhibitors should be considered in future clinical trials.

To further address the role of PgP/MDR1 in the in vivo disposition of glabridin, we examined the effects of coadministered verapamil on the systemic bioavailability and plasma pharmacokinetics of glabridin. Verapamil significantly increased the oral bioavailability of glabridin in rats. Similar results in rats have been observed with colchicine or vinblastine as the substrate and verapamil as the inhibitor (Orion et al., 1996). In the same way, SDZ PSC 833 (valspodar), a potent PgP/MDR1 inhibitor, also significantly increased the systemic bioavailability of cyclosporine and vincristine in rats (Lemaire et al., 1996). Inhibition of intestinal PgP/MDR1 was considered one of the major reasons for increased oral bioavailability of glabridin by combined verapamil. A significantly increased half-life of glabridin by coadministered verapamil indicated that verapamil might inhibit PgP/MDR1/MRPI-mediated hepatobiliary and renal efflux of glabridin. Because glabridin has a low molecular mass (354 Da) and high lipophilicity, the most likely mechanism for its hepatobiliary and renal efflux would be a transporter-mediated process. Clearance of PgP/MDR1 substrates through biliary and/or renal secretion can be significantly decreased in the presence of a PgP/MDR1 inhibitor (Song et al., 1999). The localization of the protein also suggests that the function of PgP/MDR1 is related to a transport mechanism of glabridin in the liver and kidneys because there are high levels of PgP/MDR1 protein in the brush border of renal proximal tubules and the biliary surface of hepatocytes. However, it should be noted that verapamil might have other effects rather than PgP/MDR1 inhibition that lead to altered pharmacokinetics of PgP/MDR1 substrates.

The comparative study in mdr1a(−/−) and wild-type mice indicated that depletion of PgP/MDR1 in vivo significantly increased the oral bioavailability and elimination half-life of glabridin. This provided further evidence that PgP/MDR1 had an important impact on oral bioavailability and elimination in vivo.

Because our in vitro experiments indicated that glabridin only underwent minimal glucuronidation in rat intestinal microsomes and verapamil did not alter glabridin metabolism in the intestine, the significant inhibition of intestinal PgP/MDR1 by verapamil would not decrease intestinal glabridin first-pass metabolism in a synergistic manner. Such an effect would not have been seen in vivo using a multispecific inhibitor such as verapamil or cyclosporine. A combined use of specific inhibitors such as elacridar/zosquidar with a multispecific, i.e., PgP/MDR1 and cytochrome P450 3A4, inhibitor such as cyclosporine can provide an insight into the role of PgP/MDR1 on drug intestinal absorption and first-pass metabolism (Cummins et al., 2002).

Identification of glabridin as a PgP/MDR1 substrate, low intestinal first-pass metabolism that could not be inhibited by verapamil, and a marked shift in T1/2 after coadministration of verapamil as well as the data obtained from the perfusion experiments indicated that both PgP/MDR1-mediated efflux and first-pass metabolism contribute to the low oral bioavailability of glabridin in rats.

In conclusion, glabridin has a low oral bioavailability in rats. Glabridin is a substrate and an inhibitor for PgP/MDR1. PgP/MDR1-mediated active efflux across the intestine partly contributed to the low bioavailability of glabridin, as indicated by the verapamil-glabridin interactions in vitro and in vivo and the findings from the comparative study in mdr1a(−/−) and wild-type mice. First-pass metabolism is also considered to play an important role for a low oral bioavailability of glabridin. Further research addressing the role of PgP/MDR1 and other transporters in the disposition and in vivo distribution of glabridin is warranted.
References


Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au

Address correspondence to: Dr. Shu-Feng Zhou, Department of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, Brisbane, Queensland 4001 Australia. E-mail: S4.zhou@qut.edu.au