Transport characteristics of isorhamnetin across intestinal Caco-2 cell monolayers and the effects of transporters on it

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ABSTRACT

Flavonoid isorhamnetin occurs in various plants and herbs, and demonstrates various biological effects in humans. This work will clarify the isorhamnetin absorption mechanism using the Caco-2 monolayer cell model. The isorhamnetin transport characteristics at different concentrations, pHs, temperatures, tight junctions and potential transporters were systematically investigated. Isorhamnetin was poorly absorbed by both passive diffusion and active transport mechanisms. Both trans- and paracellular pathways were involved during isorhamnetin transport. Active transport under an ATP-dependent transport mechanism was mediated by the organic anion transporting peptide (OATP); isorhamnetin's permeability from the apical to the basolateral side significantly decreased after estrone-3-sulfate was added (p < 0.01). Efflux transporters, P-glycoproteins (P-gp), breast cancer resistance proteins (BCRP) and multidrug resistance proteins (MRPs) participated in the isorhamnetin transport process. Among them, the MRPs (especially MRP2) were the main efflux transporters for isorhamnetin; transport from the apical to the basolateral side increased 10.8-fold after adding an MRP inhibitor (MK571). This study details isorhamnetin's cellular transport and elaborates isorhamnetin's absorption mechanisms to provide a foundation for further studies.

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1. Introduction

Flavonoids are a large class of polyphenolic compounds that are ubiquitous in green plants, particularly in integral and abundant dietary components, such as vegetables, fruits and plant-derived foods (Nait Chabane et al., 2009). Isorhamnetin (Fig. 1), or 3-O-methyl-quercetin, is a flavonoid found in various plant-based food products like apple and grapes (Wang et al., 2005), and herbal medicines such as seabuckthorn, Ginkgo biloba (Chi et al., 1997; Kumar et al., 2011). Recently, isorhamnetin has gained increased attention due to its chemopreventive effects against cancer (Kim et al., 2011), cardiovascular disease (Zhang et al., 2011), obesity (Lee et al., 2009) and Alzheimer’s disease (Muller et al., 2012); these effects are attributed to its excellent antioxidant activity. Therefore, this compound has potential for drug development and requires further investigation.

Oral administration is a popular drug delivery route because it is usually convenient and painless for both doctors and patients. To achieve successful therapeutic efficacy, drugs must be absorbed adequately and consistently after oral administration; this behavior depends heavily on the drug delivery system (Tian et al., 2009). Studying isorhamnetin’s absorption properties would help design its proper dose form. Moreover, studies have indicated that significant or even life-threatening interactions may occur between flavonoid-containing products and conventional drugs (Bailey et al., 1993; Rajnarayana et al., 2004). Therefore, elucidating isorhamnetin’s transport characteristics during intestinal membrane permeation would help mitigate any risky flavonoid-mediated pharmacokinetic interactions with conventional medications. Currently, flavonoid transport mechanisms have gained increasing research attention. For example, quercetin and baicalein are two typical flavonoids that utilize the passive diffusion mechanism (Nait Chabane et al., 2009; Zhang et al., 2007); some transporters, including organic anion transporting peptide (OATP), P-glycoproteins (P-gp) and multidrug resistance proteins (MRPs), have been proven to participate in the absorption of these two flavonoids (Akao et al., 2007; Li et al., 2012a; Walgren et al., 2000; Wong...
During our previous research (Li et al., 2012b; Zhao et al., 2013), intestinal efflux was a critical participant in the presystemic elimination of isorhamnetin after orally administering the total flavonoids of *Hippophae rhamnoides*. L. Wang et al. (2005) suggested that P-gp participated in isorhamnetin's transport while studying *G. biloba* extract with the Caco-2 cell model; Lan et al. (2008) implied that isorhamnetin interacted with P-gp after comparing the different transport properties observed in Caco-2 and Mardin-Darby canine kidney (MDCK) cell models. Additionally, Sesink et al. (2005) reported that isorhamnetin absorption was limited by breast cancer resistance proteins (BCRP) in rats. However, these data are insufficient for elucidating isorhamnetin's absorption mechanism for three main reasons: (1) isorhamnetin's transport pathways remain unclear; (2) it is unknown whether transporters other than P-gp and BCRP participated in the transport process; (3) isorhamnetin's absorption mechanism cannot be determined using herb extracts because its absorption properties might be altered by other components in the extract, as Lan et al. (2008) reported. Consequently, isorhamnetin's transport characteristics and mechanisms must be revealed systematically.

Flavonoids display relatively poor oral absorption; for instance, oral bioavailability of quercetin and kaempferol in rats were reported as 16.2% (Khaled et al., 2003) and 1.9% (Barve et al., 2009) respectively. Moreover, Gao et al. (2009) reported that quercetin absorption's main rate-limiting barrier was the intestinal epithelial cell layer. Because it is structurally similar to quercetin and kaempferol, isorhamnetin may exhibit similarly low absorption properties. Therefore, its membrane permeability and oral bioavailability must be enhanced, while elucidating its transport mechanisms would provide a theoretical basis for this objective.

Different models, such as animal everted sacs, parallel artificial membrane permeability assays (PAMPA) and MDCK cells, etc. were developed to investigate and predict intestinal drug absorption mechanisms. Of these in vitro models, the Caco-2 cell line is frequently chosen because it is derived from human colonic adenocarcinoma and shares many morphological and functional characteristics with mature enterocytes. Moreover, Caco-2 cells exhibit a well-differentiated brush border on its apical surfaces and at its tight junctions (Meunier et al., 1995), in addition to expressing many typical transporters found in the small intestine (Maubon et al., 2007); the transporter expression is important for predicting drug transport pathways (e.g., passive versus carrier mediated).

Therefore, Caco-2 cells were used as an absorption model to clarify isorhamnetin's transport mechanism, allowing us to discuss the in vivo pharmacokinetics assay results and provide some information useful for designing effective isorhamnetin delivery systems. Specifically, the effects of drug concentration, pH, temperature and ethylene diamine tetraacetic acid (EDTA) on the transport of isorhamnetin and the related transporters were investigated systemically.

2. Experimental

2.1. Materials

Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Thermo-Fisher Scientific Product (Beijing) Co. Ltd. Heat-inactivated fetal bovine serum (FBS), a non-essential amino acid solution, EDTA, antibiotic solutions (100,000 U/l penicillin and 100,000 mg/l streptomycin) and Hank’s balanced salt solution (HBSS) were obtained from Gibco Laboratory (Invitrogen Co, Grand Island, NY, USA). Isorhamnetin, baicalin, apigenin and verapamil were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Sodium vanadate, cimetidine and indomethacin were purchased from Aladdin Reagent (Shanghai, China). CTK (3-45,5-dimethyl-2-thiazoyl)-2,5-di-phenyl-1H-tetrazolium), MCE571 and benzobromarone were purchased from Sigma Chemical Co. (St Louis, MO, USA). Estrene-3-sulfate (ES) was purchased from Toronto Research Chemical Inc (Toronto, Canada) and Koi143 was purchased from Tocris Bioscience (Missouri, USA). Acetominitole and methanol (HPLC grade) were purchased from SK Chemicals (Ulsan, Korea). All other chemicals were of analytical grade.

2.2. Cell culture

Caco-2 cells were obtained from the Shanghai Institutes for Biological Sciences (SIBS, Shanghai, China). The cells were grown in culture flasks (Corning® Costar, Cambridge, MA, USA) using DMEM supplemented with 10% (v/v) FBS, 1% (v/v) non-essential amino acid solution and 100 000 U/l antibiotic–antimycotic solution. The cells were grown in 5% CO2 and 95% O2 with 90% relative humidity at 37 °C. The medium was replaced every 2–3 days during incubation. The cells were passaged every 5 days between 70% and 80% confluence at a 1:5 split ratio using 0.25% trypsin and 0.02% EDTA. For the transport experiments, the cells from passages between 30 and 45 were seeded at 1 × 104 cells/cm2 onto permeable polycarbonate inserts (0.45 μm pore size, seeding surface of 0.6 cm2, Millipore, MA, USA) in 24-well plastic plates. The media in the culture plates were changed every two days for the first week after seeding and were replaced daily afterward. The integrity of the cell monolayer was examined by measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS-electrode (Millipore Corp, Billerica, MA, USA); Caco-2 cells were used for the transport experiments 21–28 days after seeding: only monolayers with a TEER value above 420 ± 2 cm2 were used during the studies.

2.3. Cell viability assay with isorhamnetin

Cell viability was assessed using an MTT assay. Caco-2 cells were cultured in 96-well plates at 1 × 104 cells/well in 200 μl medium. The cells were grown to 70% confluence over approximately 48 h. Subsequently, the culture medium was replaced with HBSS containing different concentrations of isorhamnetin (5, 15, 30 and 50 μM); the final concentration of dimethyl sulfoxide (DMSO) in HBSS remained at < 1%. The negative control was HBSS containing 1% DMSO. The cells were exposed to the drug for 2 h, and subsequently the HBSS containing isorhamnetin was replaced with 200 μl 0.5 mg/ml MTT solution in HBSS. After 4 h, the MTT medium was discharged, and the formazan crystals were dissolved using 150 μl DMSO per well. The plates were gently shaken for 15 min, and the formazan concentration was measured spectrophotometrically at 490 nm using a Multiskan Spectrum microplate reader (Thermo Labsystems, MA, USA). All MTT assays were carried out using five separate trials. The viability of the non-treated control cells was arbitrarily defined as 100%. The relative cell viability was calculated according to Eq. (1) (Kowapradit et al., 2010)

Relative cell viability (%) = \[ \frac{OD_{90\text{ min}} - OD_{0\text{ min}}}{OD_{90\text{ min}} - OD_{0\text{ min}}} \] \times 100%

(1)

2.4. Transport experiments

Before the experiments, the Caco-2 cell monolayers were washed twice with warm HBSS solution and subsequently preincubated (37 °C, 15 min). Afterward, the HBSS solution on both sides of the cell monolayers was removed using aspiration. To measure the apical (AP) to basolateral (BL) transport, 400 μl HBSS containing isorhamnetin was added to the AP side, while 600 μl blank HBSS was added to the BL side. To measure the BL to AP transport, 600 μl HBSS containing isorhamnetin was added to the BL side and 400 μl blank HBSS was added to the AP side. The isorhamnetin solution was freshly prepared in DMSO. The final DMSO concentration in the HBSS/dye mixture was below 1%. The monolayers were incubated at 37 °C, and 100 μl samples were taken at 20, 40, 60 and 80 min from the acceptor
compartment followed and immediately replaced with the same volume of pre-warmed fresh blank HBSS. TEER measurements for assessing the membrane integrity took place before and after the experiments.

Isorhamnetin transport was assessed in both directions at different concentrations (5, 15, 30, and 50 μM). The pH's effect on isorhamnetin transport (30 μM) in the AP to BL direction was studied using the following pH combinations for the HBSS in the acceptor/donor compartments: 6.5; 6.5/6.5 and 7.4/7.4. The isorhamnetin transport at 4°C and 37°C was evaluated in the AP to BL direction to investigate the temperature effects. To reveal the paracellular isorhamnetin transport, the cell monolayer was incubated with 5 mM EDTA in HBSS without Ca²⁺ and Mg²⁺ for 5 min. Subsequently, the isorhamnetin transport solution (30 μM) was prepared in a medium containing 50% HBSS without Ca²⁺ and Mg²⁺ and 50% HBSS and subsequently added to the AP compartment. Concurrently, EDTA's efficiency for modulating the cell junctions was monitored by testing the TEER before and after EDTA treatment.

The experiments were performed alongside several transporter inhibitors. Inhibiting the influx during isorhamnetin transport across the Caco-2 cell monolayers was investigated by adding 50 μM sodium vanadate and cimetidine to evaluate the selectivity of Na/K-ATPase (Nait Chabane et al., 2009) and organic anion transporter (OAT) (Ahn et al., 2009; Han et al., 2010). Fifty μM and 100 μM estrone-3-sulfate (ES) was used to investigate OATP (Yang et al., 2010) at the same condition. Inhibiting the efflux of P-gp during isorhamnetin transport was undertaken by adding 100 μM verapamil and nifedipine (Cao et al., 2007). One hundred μM MK571, 50 μM benzbramarone and 200 μM indomethacin were used to inhibit the efflux of MRPs probes substrates (Zhang et al., 2007; Prime-Chapman et al., 2004; Ursic et al., 2009), respectively. Ten μM Ko143 and 25 μM apigenin were used as BCRP inhibitors (Brand et al., 2008; Zhang et al., 2004).

Before the transport experiment, the inhibitors were added on both sides of the Caco-2 cell monolayers and preincubated (37°C, 30 min). The transporter inhibitors were present on the AP (BL) side during the experiments. The category, concentration and modulatory effect of the inhibitors are summarized in Table 1.

The apparent permeability coefficient (P_{app}) was calculated as Buyukozturk et al. (2010) reported:

\[ P_{app} = \frac{dQ}{dt} \over A \cdot C_0 \]  

where \( dQ/dt \) is the steady-state flux, \( A \) is the membrane surface area, and \( C_0 \) is the initial drug concentration in the donor compartment. The value of \( dQ/dt \) was calculated as the change in cumulative amount of isorhamnetin in the receiver solution over 20, 40, 60, 80 min. P_{app} values were calculated using the slope of the steady-state rate constant \( dQ/dt \) over an 80 min incubation period.

2.5.1. Extraction procedure

One hundred μL samples were transferred into polypropylene tubes and 10 μL baiacalein solution (7.3 μg/mL, 1.5). 20 μL 10% (w/v) ascorbic acid solution and 200 μL ethyl acetate were added and vortexed vigorously for 2 min, followed by centrifugation at 13,000 rpm for 5 min. The supernatant (organic layer) was then collected and evaporated to dryness under a stream of nitrogen at 37°C and reconstituted using 100 μL of the mobile phase before UPLC–MS analysis.

2.5.2. UPLC–MS analysis

The UPLC system was an Agilent 1290 series instrument with a G4220A binary pump, a G4226A autosampler, a G1330B thermostat for ALS/FC/Spotter and a G1316 thermostatted column compartment. Chromatographic separation was performed using a Waters UPLC column (Acquity UPLC BEH C18 1.7 μm, 2.1 x 50 mm, Waters, Ireland). The mobile phase consisted of (A) acetonitrile and (B) 0.1% aqueous formic acid. The column was eluted using a simple gradient: 30% A (0–1.2 min), 30–90% A (3.2–4 min), 90–30% A (4.4–5 min) and 30% A (4.5–5.5 min). The flow rate was 0.3 mL/min at 40°C.

Mass spectrometric analysis was performed using an Agilent 6410B triple-quadrupole mass spectrometer (Agilent Technologies, CA, USA) with an electrospray ionization (ESI) interface operating in the negative ionization mode. The mass spectra were obtained in selected-ion monitoring (SIM) mode using the mass spectrometer settings that follow: nebulizer temperature, 300°C; nebulizer pressure (N2), 5 psi; drying gas flow (N2), 11 L/min; fragmentor voltage, 150 and 160 V for isorhamnetin and baiacalein, respectively; capillary voltage, 7 kV for isorhamnetin and 4 kV for baiacalein, respectively. The analytes were assayed by quantifying the [M − H]⁺ ions at m/z 315.2 for isorhamnetin and m/z 269.2 for baiacalein. The Agilent MassHunter (B.05.00) software package was used to acquire and analyze the data (Agilent Technologies, CA, USA).

2.6. Statistical analysis

The data were expressed as the mean ± SD. To compare the two groups, a two-tailed unpaired Student's t-test was employed. p < 0.05, p < 0.01 and p < 0.001 were considered statistically significant.

3. Results

3.1. Cytotoxicity of isorhamnetin

Over 90% of the cells were viable when up to 50 μM isorhamnetin was used during the experiments (data not shown), demonstrating that isorhamnetin was not significantly cytotoxic.

3.2. Isorhamnetin transport across Caco-2 cell monolayers over time

The transcellular isorhamnetin transport across Caco-2 cell monolayers was investigated over time. The Caco-2 cells were incubated at 37°C with 30 μM isorhamnetin on either the AP or BL side (Fig. 2a). The transcellular isorhamnetin transport from the AP to the BL side and from the BL to the AP side increased linearly over 80 min. Moreover, in the AP to BL direction, the isorhamnetin permeability was below \( P_{appAB} = (1.56 ± 0.11) \times 10^{-6} \text{cm/s} \), that of propranolol \( P_{appAB} = (82.4 ± 5.10) \times 10^{-6} \text{cm/s} \) (Hovgaard et al., 1995), which is a transcellular flux marker, suggesting that isorhamnetin was poorly absorbed. The AP to BL isorhamnetin transport was 6.8–9.3-fold higher than transport in the opposite direction. The efflux ratio \( P_{ratio} = P_{appBA}/P_{appAB} \) was 0.1; this value is below 0.5, suggesting that the uptake mechanisms are all involved (Hubatsch et al., 2007).

| Table 1 | Inhibitory effects on bidirectional isorhamnetin transport in Caco-2 cell monolayers. |
|---------|---------------------------------|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Transporter | Inhibitors | Concentration (μM) | \( P_{appBA} \) (10⁻⁶ cm/s) | \( P_{appAB} \) (10⁻⁶ cm/s) | \( P_{ratio} \) | Modulatory effect |
| Batch 1 | Control | / | 1.56 ± 0.12 | 0.14 ± 0.01 | 0.09 | / |
| Na⁺/K⁺ pump | Sodium vanadate | 50 | 0.36 ± 0.02 | 0.12 ± 0.01 | 0.33 | + |
| OATs, OATPs | Cimetidine | 50 | 0.56 ± 0.14 | 0.14 ± 0.02 | 0.09 | - |
| Estrone-3-sulfate | 50 | 1.13 ± 0.06 | 0.16 ± 0.03 | 0.14 | + |
| Estrone-3-sulfate | 100 | 1.09 ± 0.01 | 0.15 ± 0.03 | 0.14 | + |
| BCRP | Ko143 | 10 | 1.07 ± 0.08 | 0.15 ± 0.03 | 0.08 | - |
| Apigenin | 25 | 2.69 ± 0.08 | 0.15 ± 0.02 | 0.06 | + |
| MRP1 | MK571 | 100 | 16.85 ± 0.73 | 0.17 ± 0.01 | 0.01 | - |
| MRP2 | Indomethacin | 200 | 2.62 ± 0.24 | 0.15 ± 0.01 | 0.06 | + |
| MRP3, MRP4 | Benzbromarone | 50 | 1.86 ± 0.04 | 0.13 ± 0.02 | 0.07 | - |
| Batch 2 | Control | / | 1.42 ± 0.12 | 0.13 ± 0.01 | 0.09 | - |
| P-gp | Verapamil | 100 | 1.83 ± 0.05 | 0.14 ± 0.03 | 0.06 | + |
| P-gp | Nifedipine | 100 | 2.29 ± 0.02 | 0.14 ± 0.03 | 0.06 | + |

* Indicates that the inhibitor has significant effect on the isorhamnetin transport.
- Indicates that the inhibitor has no significant effect on the isorhamnetin transport.

** Mean ± SD for n = 3 monolayers. \( P_{app} \) was monitored by sampling media from receiver compartments at 20 min as an interval over an 80 min incubation period.

* Denote results significantly different from those of the control experiments (p < 0.01).
** Denote results significantly different from those of the control experiments (p < 0.001).
3.3. Concentration effects on transcellular isorhamnetin transport across Caco-2 cell monolayers

Isorhamnetin transport was evaluated in both directions from 5 μM to 50 μM (Fig. 2b). The apparent permeability coefficient decreased with increasing concentrations over the experimental range, and the behavior in both directions was consistent with a saturable mechanism (Spyridopoulos et al., 2009): from (3.86 ± 0.43) × 10^{-6} cm/s at 5 μM to (1.82 ± 0.20) × 10^{-6} cm/s at 50 μM (AP to BL) and from (1.64 ± 0.12) × 10^{-6} cm/s at 5 μM to (0.10 ± 0.01) × 10^{-6} cm/s at 50 μM (BL to AP). Interestingly, as Fig. 2b indicated, isorhamnetin’s $P_{app}$ value changed from 0.4 to 0.05 from 5 μM to 50 μM, indicating that the uptake mechanisms might be important, especially at higher concentrations. Isorhamnetin’s $P_{app}$ value at 30 μM was the smallest ($P_{app} = (1.56 ± 0.11) \times 10^{-6} \text{ cm/s}$) measured from the four concentrations, but it was still higher than that of mannitol that is a marker for paracellular transport ($P_{app} = (0.38 ± 0.12) \times 10^{-6} \text{ cm/s}$) (Liang et al., 2000). Therefore, the isorhamnetin transport followed the transcellular route. In the following experiments, 30 μM of isorhamnetin was utilized because the $P_{app}$ values were relatively consistent from 30 μM to 50 μM.

3.4. pH effects on transcellular isorhamnetin transport across Caco-2 cell monolayers

The assay studying the AP to BL transport in HBSS with various pH values is presented in Fig. 3; isorhamnetin’s $P_{app}$ values at pH 7.4 were significantly higher than those at pH 6 ($p < 0.01$) and pH 6.5 ($p < 0.01$). In the AP to BL direction, isorhamnetin transport across the cell monolayer at high pHs (7.4/7.4) was easier than at lower pHs (6.5/6.5, 6/6): $P_{app}$ 7.4 = (2.97 ± 0.21) × 10^{-6} cm/s > $P_{app}$ 6.5 = (1.56 ± 0.08) × 10^{-6} cm/s > $P_{app}$ 6 = (1.27 ± 0.09) × 10^{-6} cm/s. Of the three tested pH values, isorhamnetin’s $P_{app}$ at pH 6.5 was the smallest, and the $P_{app}$ values were relatively steady from pH 6 to 6.5. This phenomenon may occur because the $pK_a$ of isorhamnetin (predicted as 6.3 on Scifinder) is between pH 6 and pH 6.5. When the solution pH was similar to its $pK_a$, isorhamnetin existed mainly in its molecular form; the ionized form appears at pH levels above and below the $pK_a$. The different isorhamnetin forms present at different pH values might account for the changes in its directional transport through the cell membranes (Estudante et al., 2012). Furthermore, previous research indicated that both the influx (Troutman and Thakker, 2003) and efflux transport (Crowe and Diep, 2008; Kuwayama et al., 2008) were activated at lower pHs; the efflux transporters might be central at pH 6 but the influx transporters might be more critical at pH 7.4 during isorhamnetin transport. Consequently, the data indicated that isorhamnetin transport was pH-dependent.

3.5. Temperature effects on transcellular isorhamnetin transport across Caco-2 cell monolayers

As presented in Fig. 4a, the isorhamnetin transport across Caco-2 cell monolayers was markedly reduced because $P_{app}$ was changed from (1.42 ± 0.12) × 10^{-6} cm/s to (0.22 ± 0.02) × 10^{-6} cm/s when lowering the temperature from 37 °C to 4 °C ($p < 0.01$). Isorhamnetin’s decreased $P_{app}$ at 4 °C might indicate that the transport was energy-dependent because decreasing the temperature would slow down cellular metabolism (Rocha et al., 2013). However, further studies are necessary to confirm the energy dependence of Caco-2 cells for decreasing the temperature may also slow down passive diffusion.

3.6. The paracellular pathway’s role during the isorhamnetin transport by absorption

The cellular junctions were modified to study the paracellular transport by adding EDTA (5 mM) to remove Ca^{2+} ions from the...
values in the AP to BL direction across the Caco-2 monolayers increased from 0.09 to 0.14 (Table 1). There was decreased when P-gp inhibitors were modest but significantly increased. Therefore, decreased from (1.56 ± 0.12) ** to (2.41 ± 0.19) **, indicating that the paracellular pathway was involved in theisorhamnetin transport process, as Menez et al. (2007) reported previously.

3.7. Effects of transporters on isorhamnetin transport

Interestingly, the majority of $P_{\text{app}}$ values in the AP to BL direction showed a statistical difference compared to the control, but no significant difference was found in the BL to AP direction in the experiments with inhibitors. Thus, the results were focused on the effects of inhibitors on isorhamnetin transport in the AP to BL direction.

3.7.1. Effects of influx transporters on transcellular isorhamnetin transport

Fig. 5 and Table 1 presented the data from transport experiments using various selective influx transport inhibitors. Sodium vanadate is an ATPase Na$^+/K^+$-dependent inhibitor: during the experiments, it completely inhibited isorhamnetin’s absorption, and the permeability in the AP to BL direction was decreased (Fig. 5, Table 1, $P < 0.001$), while $P_{\text{ratio}}$ was increased. Therefore, isorhamnetin transport across the Caco-2 cells most likely occurred with apical sodium-dependent glucose transporters (SGLT, active, sodium coupled). Further investigations of the isorhamnetin influx mechanism utilized cimetidine and ES (OAT and OATP inhibitors, respectively) on both sides. As Fig. 5 and Table 1 indicated, no significantly decrease of isorhamnetin transport amount at each sampling points, as well as $P_{\text{app}}$ and $P_{\text{ratio}}$, occurred after cimetidine addition ($P > 0.05$) during the experiment, implying that OAT did not cause the isorhamnetin influx. However, the isorhamnetin absorption was significantly decreased when 50 μM (Fig. 5, $P < 0.01$) and 100 μM (Fig. 5, $P < 0.01$) ES were added on the AP side; the $P_{\text{app}}$ decreased from (1.56 ± 0.12) × 10^{-6} cm/s to (1.13 ± 0.06) × 10^{-6} cm/s and (1.09 ± 0.01) × 10^{-6} cm/s, respectively, while the $P_{\text{ratio}}$ increased from 0.09 to 0.14 (Table 1). Therefore, isorhamnetin was transported into the Caco-2 cells via OATP. Moreover, there was no significant difference between the two ES concentration groups, meaning that the inhibition of isorhamnetin absorption was independent of the ES concentrations. In summary, isorhamnetin might be an OATP substrate but not an OAT substrate.

3.7.2. Effects of efflux transporters on transcellular isorhamnetin transport

Fig. 6 presented the results from transport experiments performed using various selective efflux transport inhibitors. Verapamil and nifedipine (both P-gp inhibitors) were chosen because they are more selective for P-gp than other efflux transporter inhibitors. The transport amount of isorhamnetin at 80 min was significantly increased (Fig. 6a, $P < 0.01$), its $P_{\text{app}}$ was modest but significantly increased and $P_{\text{ratio}}$ was decreased when P-gp inhibitors were added (Table 1, $P < 0.01$), implying that isorhamnetin was a P-gp substrate under our experimental conditions. These results agreed with the previous reports indicating that isorhamnetin was a P-gp substrate in animals (Li et al., 2012b; Wang et al., 2005); moreover, other flavonoids were effluxed by P-gp as Vaidyanathan and Walle (2003) and Tourniaire et al. (2005) described. Similar experiments with verapamil and nifedipine, the isorhamnetin efflux was
affected by BCRP inhibitor Ko143 and apigenin. As displayed in Fig. 6b and Table 1, isorhamnetin’s transport amount at 80 min and permeability were all significantly enhanced by Ko143 and apigenin (p < 0.01). However, P\textsubscript{ratio} values were modest weaken, suggesting that both P-gp and BCRP transporters were involved in isorhamnetin secretion.

However, isorhamnetin might have been preferentially effluxed by MRP2. When MK571 (a typical MRPs inhibitor) was added to the apical side, isorhamnetin transport amount at 80 min and \( P_{\text{appAB}} \) was increased 6.0-fold and 10.8-fold respectively, and \( P_{\text{ratio}} \) was decreased from 0.09 to 0.01 (Fig. 6c, Table 1, p < 0.001), implying that the MRPs transporters governed the isorhamnetin secretion. Earlier observations indicated that Caco-2 cells express more MRP2 and MRP3 and less MRP1 and MRP5; moreover, MRP2 and MRP3 are located separately at the AP and BL sides of the Caco-2 cells (Prime-Chapman et al., 2004). Therefore, the inhibitory effect of MK-571 implied that MRP2 was primarily responsible for the isorhamnetin efflux in the AP to BL direction. This result was validated by the values of \( P_{\text{appAB}} \) and \( P_{\text{ratio}} \) when the efflux of isorhamnetin was inhibited by benzbromarone and indomethacin. Indomethacin is a MRP2 inhibitor that markedly increased isorhamnetin transport from AP to BL direction (2.0-fold) and \( P_{\text{appAB}} \) value (1.7-fold), respectively (Fig. 6d, Table 1, p < 0.01). However, OAT may still mediate the isorhamnetin transport because the OAT expression in Caco-2 cells is low (Seithel et al., 2006). Additionally, this is the first report that an influx transporter, such as OATP, participated in isorhamnetin transport; previous reports revealed that flavonoids, such as

In Summary, the three selected ATP-binding cassette (ABC) transporter inhibitors (P-gp, BCRP, MRPs) were all involved during isorhamnetin secretion. The MRPs, especially MRP2, revealed a high affinity for isorhamnetin because MK571 enhanced isorhamnetin’s permeability in the AP to BL direction more than other inhibitors.

4. Discussion

We evaluated the mechanisms possibly involved during intestinal isorhamnetin transport with an \textit{in vitro} human intestinal epithelium model (Caco-2 cells). The apparent permeability coefficients under various conditions (concentration and temperature) were determined, revealing that transporters, particularly influx transporters, affected isorhamnetin transport. Firstly, isorhamnetin permeability in the AP to BL direction was higher than that from BL to AP (\( P_{\text{ratio}} < 0.5 \) at 30 \( \mu \)M, Fig. 2a). Secondly, isorhamnetin transport in the AP to BL direction revealed that the mechanism could be saturated from 5 \( \mu \)M to 50 \( \mu \)M (Fig. 2b). Thirdly, isorhamnetin’s \( P_{\text{appAB}} \) was markedly decreased at 4 °C (Fig. 4a). Moreover, isorhamnetin was mainly transported by apical SGLT (active, sodium coupled) mediated by OATP rather than OAT (the modulatory effects in Table 1). However, OAT may still mediate the isorhamnetin transport because the OAT expression in Caco-2 cells is low (Seithel et al., 2006). Additionally, this is the first report that an influx transporter, such as OATP, participated in isorhamnetin transport; previous reports revealed that flavonoids, such as
isorhamnetin, naringenin and eriodictyol, were absorbed via influx transporters in Caco-2 cells (Kobayashi et al., 2008; Seithel et al., 2006).

Although it was not the focus of this study, the liver uptake of isorhamnetin might be important during the isorhamnetin absorption in vivo because OATP and other influx transporters (OCT for example) are highly expressed by the liver (Hilgendorf et al., 2007). Consequently, enterohepatic recirculation was a pharmacokinetic property of isorhamnetin in rats, as described previously (Li et al., 2012b). Further experiments should verify whether other carriers were involved during isorhamnetin transport.

Isorhamnetin might also be a substrate for active efflux pumps. According to Table 1, MRP2, P-gp and BCRP were proposed to limit transcellular isorhamnetin transport. Previous reports indicated that isorhamnetin was effluxed across intestine cells via BCRP and P-gp (Lan et al., 2008; Wang et al., 2005; Sesink et al., 2005), but no studies reported the effects of MRP2 during isorhamnetin transport. Our results demonstrated that isorhamnetin might have a higher affinity for MRPs than other efflux transporters on the AP side of Caco-2 cells because it contains a methoxy group; previous studies revealed that MRP2 mediated methylated (Chen et al., 2005; Hong et al., 2003), glucuronidated and sulfated flavonoid transport (Brand et al., 2008).

Of the selected inhibitors, Ko143 and apigenin inhibited the apical isorhamnetin efflux less than MK571 in the Caco-2 cells, but BCRP’s role during isorhamnetin’s intestinal efflux in vivo still should be emphasized. The expression of P-gp and MRP2 in Caco-2 cells was comparable to that in normal human jejunum (van Dijk et al., 2000), but the BCRP expression in Caco-2 cells was 100-fold lower than that in the jejunum (Taipalensuu et al., 2001); therefore, the Caco-2 cell model underestimated BCRP’s effect on isorhamnetin transport. Totally speaking, the transporter’s contributions to a specific isorhamnetin absorption process will depend on the substrate dose available, the specific affinity and the specific transporter expression levels. Moreover, because P-gp, BCRP, MRP2 and other ABC transporters have overlapping substrates (Seithel et al., 2006) and because isorhamnetin might be a substrate for at least four transporter categories used during the study, the absorption, distribution, metabolism and elimination of isorhamnetin in vivo might be more complicated than that in vitro. Therefore, these results have only begun to provide enough useful information to elucidate isorhamnetin pharmacokinetics and drug-isorhamnetin interactions; in vivo evaluations must be undertaken further.

As demonstrated by the concentration and paracellular transport data, passive diffusion across the Caco-2 monolayer cells was a valid pathway for isorhamnetin. Both transcellular (P_{appAB} isorhamnetin > P_{appAB} mannitol) (Krishna et al., 2001) and paracellular pathways (Fig. 4b) were involved during isorhamnetin transport. Similar to other models, the Caco-2 cell model also has intrinsic limitations; for example, the tight junctions formed by this cell line are usually closer than in the small intestine, producing a decrease in the paracellular permeability (Rocha et al., 2013). Consequently, the isorhamnetin absorption parameters obtained using Caco-2 cells are most likely lower than those observed in vivo.

A drug’s oral bioavailability depends primarily upon its solubility and permeability. On one hand, isorhamnetin is weakly acidic and has low water solubility (sparingly soluble, 0.063 g/L at 25 °C in Scifinder). On the other hand, isorhamnetin was poorly absorbed (P_{appAB} isorhamnetin < P_{appAB} prropanol) (Krishna et al., 2001) through Caco-2 cells and mediated by efflux transporters. Isorhamnetin’s poor permeability was most likely observed because its four hydroxyl groups and planar configuration might hinder its cell membrane intercalation. Therefore, both low permeation and poor solubility caused isorhamnetin’s low oral absorption; improving these qualities would enhance its oral bioavailability.

5. Conclusions

In conclusion, isorhamnetin transport is a complicated process: (i) passive diffusion, as well as paracellular and transcellular pathways were involved; (ii) the polarized transport mechanism was mediated by influx transporters and efflux transporters. An ATP-dependent influx mechanism was critical for the isorhamnetin transport process. Influx transporters, particularly OATP instead of OAT, are involved in isorhamnetin transport. Efflux transporters, P-gp, BCRP and MRPs (especially MRP2) are vital for isorhamnetin transport in the intestines. The elucidated isorhamnetin absorption mechanism provides useful information for designing delivery systems and avoiding drug-isorhamnetin interactions.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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