Metabolism and Transport of the Citrus Flavonoid Hesperetin in Caco-2 Cell Monolayers

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ABSTRACT:

Metabolism and transport from intestinal cells back into the lumen by ATP-binding cassette (ABC) transporters is believed to limit the bioavailability of flavonoids. We studied metabolism and transport of the citrus flavonoid hesperetin, the aglycone of hesperidin, using a two-compartment transwell Caco-2 cell monolayer system, simulating the intestinal barrier. The role of apically located ABC transporters P-glycoprotein (MDR1/ABCB1), multidrug resistance protein 2 (ABCC2), and breast cancer resistance protein (BCRP/ABCG2) in the efflux of hesperetin and its metabolites was studied by coadministration of compounds known to inhibit several classes of ABC transporters, including cyclosporin A, GF120918, MK571, 3-[(3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl)-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid; PSC-833, Valspodar; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; HPLC, high-performance liquid chromatography; MS-MS, tandem mass spectrometry; DAD, diode array detector; uPLC, ultra-performance liquid chromatography; DMSO, dimethyl sulfoxide; DEME, Dulbecco’s modified Eagle’s medium; CsA, cyclosporine A; C7, threshold cycle; TEER, trans-epithelial electrical resistance; t½, retention time.

Flavonoids consist of a large group of polyphenols that can be divided into different classes and are present in fruits, vegetables, and other plant-derived products. In foods, flavonoids often occur as β-glycosides of aglycones, which become deglycosylated upon ingestion. The flavanone hesperetin (Fig. 1) is the aglycone of hesperidin (hesperetin-7-O-rutinoside), which is the major flavonoid present in sweet oranges (Citrus sinensis) and orange juice (Tomás-Barberán and Clifford, 2000). Both hesperidin and hesperetin exhibit some anti-inflammatory and antimicrobial effects (Garg et al., 2001).

Flavanones occur in the diet almost exclusively in citrus fruits or citrus fruit derived products; as a result, the daily intake of hesperidin is largely dependent on dietary habits. In general, the amount of hesperidin can form an important part of the total flavonoid intake and has been estimated to be 15.1 mg/day (after hydrolysis of glycosides) (Knekt et al., 2002). According to data on urinary and plasma concentrations, however, bioavailability of hesperidin is limited (Ameer et al., 1996; Manach et al., 2003; Nielsen et al., 2006; Gardana et al., 2007).

It is believed that hesperidin (Fig. 1), which has a disaccharide rutinoside at position C7, has to be hydrolyzed by colonic microflora prior to its absorption whereas hesperetin aglycone, as well as the
monosaccharide hesperetin 7-O-glucoside, can already be absorbed in the small intestine (Nielsen et al., 2006). Passive diffusion, absorption of hesperetin 7-O-glucoside, and hydrolysis of hesperidin by colonic microflora result in occurrence of intestinal intracellular hesperetin aglycone (Nielsen et al., 2006), which is subsequently conjugated into glucuronidated and sulfated metabolites, which were detected in human blood and urine (Ameer et al., 1996; Manach et al., 2003; Gardana et al., 2007).

Flavonoids and/or their metabolites are well known substrates of ATP-binding cassette (ABC) transporters (Morris and Zhang, 2006), which are present in epithelial cells throughout the intestinal tract (Taipalensuu et al., 2001; Englund et al., 2006). In general, ABC transporters are specifically located in the apical (lumen side) or basolateral (blood/plasma side) membrane of enterocytes and facilitate excretion back into the intestinal lumen or uptake into the blood, respectively. Intestinal ABC transporters that have been related to flavonoid transport include P-glycoprotein (Pgp/MDR1/ABCB1), multidrug resistance proteins (MRPs/ABCCs), and breast cancer resistance protein (BCRP/ABCG2), of which Pgp, MRP2, and BCRP are localized in the apical membrane (Morris and Zhang, 2006).

A common way to investigate the role of ABC transporters is coexposure to compounds that more or less specifically inhibit individual ABC transport proteins. In the present study, we used cyclosporin A, GF120918, Ko143, MK571, and PSC-833 (Table 1). Cyclosporin A is a broad-spectrum inhibitor reported to inhibit Pgp, MRPs, and BCRP (Qadir et al., 2005; Pawarode et al., 2007), GF120918 is a dual inhibitor of both Pgp and BCRP (de Bruin et al., 1999), PSC-833 is a more specific inhibitor of Pgp (Qadir et al., 2005), Ko143 is a highly specific inhibitor of BCRP (Allen et al., 2002; Xia et al., 2005), and MK571 is an MRP inhibitor (Leier et al., 1994; Gekeler et al., 1995).

Efficient intestinal metabolism and efflux, the latter mediated by ABC transporters located in the apical membrane, are believed to be the main reasons for poor bioavailability of flavonoids and their metabolites (Liu and Hu, 2007). Metabolites of the flavonoid quercetin, for instance, have been demonstrated to interact with MRP2 (Williamson et al., 2007) and BCRP (Sesink et al., 2005). In the present study, we focused on intestinal metabolism and transport of hesperetin in vitro, using Caco-2 cell monolayers grown on a permeable filter separating a two-compartment cell culture system, simulating the intestinal membrane barrier. Differentiated Caco-2 cells are known to display morphological and biochemical properties of intestinal enterocytes, including expression of Pgp, MRP2, and BCRP (Hirohashi et al., 2000; Taipalensuu et al., 2001; Englund et al., 2006; Seithel et al., 2006), of which we studied the mRNA expression levels in Caco-2 cell monolayers with RT-qPCR. By exposing Caco-2 cell monolayers to hesperetin (10 μM), in the absence or presence of inhibitors, we studied formation and transport of hesperetin conjugates, which were identified by HPLC and uPLC retention times and diode array detector (DAD) spectra, by coelution with authentic synthesized standards, by confirmation with uPLC-DAD-MS-MS, and by specific enzymatic deconjugation. By coadministering hesperetin with a range of different inhibitors, we investigated the role of Pgp, MRP2, and BCRP in the transport of hesperetin and its metabolites in Caco-2 cell monolayers.

**Materials and Methods**

**Materials.** Chloroform, hesperetin (purity ≥95%), L-ascorbic acid, and sulfatase (from *Helix pomatia*) were purchased from Sigma (St. Louis, MO). GF120918 was a generous gift from GlaxoSmithKline (Uxbridge, Middlesex, UK). Ko143 was from Dr. Alfred H. Schinkel from The Netherlands Cancer Institute (Amsterdam, The Netherlands), and PSC-833 was from Novartis (Basel, Switzerland). MK571 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA), and cyclosporin A was from Fluka (Buchs, Switzerland). Acetonitrile for the HPLC system and methanol were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile for the uPLC system and trifluoroacetic acid were from J.T. Baker (Philipsburg, NJ). Isopropanol (for molecular biology) was from Acros (Geel, Belgium), and TRIzol reagent was from Invitrogen (Paisley, UK). Dipotassium hydrogen phosphate, di-
methyl sulfoxide (DMSO), ethanol (for molecular biology), EDTA disodium salt dihydrate, ethyl acetate, formic acid, glacial acetic acid, hydrochloric acid, potassium dihydrogen phosphate, and sodium acetate were purchased from Merck (Darmstadt, Germany), and β-glucuronidase (from Escherichia coli) was from Roche Diagnostics (Mannheim, Germany). All cell culture reagents were purchased from Invitrogen. Authentic standards of hesperetin 7-O-glucuronide (purity 92.8%) and hesperetin 7-O-sulfate (purity 92.8%) were purchased from Invitrogen. Authentic standards of hesperetin 7-O-glucuronide (purity 92.8%) and hesperetin 7-O-sulfate (purity 92.8%) were purchased from Invitrogen.

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA), cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM HEPES buffer, 4500 mg/l glucose, L-glutamine, and phenol red, and supplemented with 10% (v/v) heat-inactivated (30 min at 56°C) fetal bovine serum, 1% (v/v) minimal essential medium nonessential amino acids, and 0.2% (v/v) 50 mg/ml gentamicin. When the cell culture reached 70 to 90% confluence, it was rinsed with phosphate-buffered saline containing 22 mg/l EDTA and split using 0.25% (w/v) trypsin. The cell density of the suspension was determined using a Bürker-Türk counting chamber from Labor Optik (Friedrichsdorf, Germany). For transport experiments, 1 × 10⁶ cells/cm² (0.5 ml of 2.24 × 10⁵ cells/ml) were seeded in Costar 12-well transwell plate inserts from Corning Inc. (Corning, NY) with an insert membrane pore size of 0.4 μm and a growth area of 1.12 cm². The passage number of the cells used in the experiments was between 37 and 47. The medium was changed three times a week, and the transport experiments were performed 18 to 19 days postseeding.

RNA Isolation. Medium was removed from a culture flask containing a Caco-2 cell monolayer (passage no. 46), and the cells were lysed in TRIZol reagent (100 μl/cm²) and stored at −80°C. After defrosting, 200 μl of chloroform was added to 1-ml aliquots, which were shaken (1 min), incubated at room temperature (3 min), and centrifuged at 15,500g and 4°C (15 min). The aqueous phases were transferred to a sterile tube, followed by addition of an equal volume of isopropanol to precipitate the RNA. The samples were mixed and left to incubate at room temperature (10 min), after which they were centrifuged at 15,500g and 4°C (10 min). After removing the supernatant, the pellets were washed with 70% (v/v) ethanol. After centrifugation at 12,000g and 4°C (5 min), the supernatant was removed, and the pellets were air dried and resuspended in 100 μl of RNAse free water. The samples were pooled, cleaned, and concentrated using a RNeasy Mini kit from QIAGEN GmbH (Hilden, Germany) following the instructions of the manufacturer. The RNA concentration was spectrophotometrically determined using a Nanodrop ND-1000 from Nanodrop Technologies (Wilmington, DE).

Real-Time RT-qPCR. A 5 μl mix containing 2 μg total RNA was reverse transcribed using 0.25 μg random primers from Invitrogen, 2 μl of dNTPs (10 mM) from MBI Fermentas (Hanover, MD), and RNAse-free water, incubated together at 65°C for 5 min in an iCycler from Bio-Rad (Hercules, CA). The product was added to a 9-μl enzyme mix containing RT buffer, 2 μl of dithiothreitol (100 mM), 0.5 μl of RNAse OUT (40 units/μl), 1 μl of murine myeloleukemia virus reverse transcriptase (200 units/μl) from Invitrogen, and RNAse-free water and incubated for 10 min at 25°C, 50 min at 37°C, 15 min at 70°C, and cooled to 4°C, in the iCycler. To quantify the amount of mRNA of Pgp, MRP2, and BCRP, real-time qPCR was performed in duplicate with a dilution series (5, 10, 20, 40, 80, and 160 times) of the cDNA using the iCycler and iQ SYBR Green from Bio-Rad. Each 25-μl polymerase chain reaction contained 5 μl of cDNA, 12.5 μl of Mastermix SYBR Green, RNAse-free water, and 1 μl of both the specific forward and reverse primers (10 mM), which were synthesized by Biologeo (Nijmegen, The Netherlands). The sequences of the primers used were described by Taipalensuu et al. (2001) and are given in Table 2. Villin, an actin cross-linking structural protein, was used to normalize the mRNA expression levels (Lown et al., 1997). The amplification program consisted of a 15-min preincubation at 95°C, followed by 45 amplification cycles with denaturation at 95°C for 30 s, annealing at 60°C (MDR1, MRP2, and BCRP) or 56°C (villin) for 30 s, and extension at 72°C for 45 s. The cycle number at the threshold (Ct) was used for semiquantification of the polymerase chain reaction product, and the relative ABC transporter mRNA expression levels, normalized to villin, are given by ∆Ct = Ct ABC transporter − Ct villin converted to 2^-ΔCt.

Transport Experiments. Before experiments were started, Caco-2 cell monolayers were washed with DMEM (without phenol red). The integrity of the monolayers was checked by measuring trans-epithelial electrical resistance (TEER) values with a Millicell ERS voltohmeter from Millipore (Bedford, MA). Only monolayers that demonstrated a TEER value between 500 and 1000 Ω/cm² were used. Transport experiments were carried out with transport medium consisting of DMEM (without phenol red) supplemented with 1% (v/v) minimal essential medium nonessential amino acids and 1 mM ascorbic acid to prevent auto-oxidation, which was filtered through a sterile 0.2-μm filter unit from Whatman Schleicher and Schuell (Dassel, Germany).

To study the transport of hesperetin and the formation of metabolites with time, transport studies were performed in which the monolayers were exposed at the apical side to 10 μM hesperetin for 0, 20, 40, 60, 80, 100, or 120 min, whereupon samples of medium were taken from both the apical and basolateral compartment.

To study the role of metabolism and different ABC transporters, Caco-2 cell monolayers were exposed at the apical side to 10 μM hesperetin, in the absence or presence of an inhibitor, all added from 400 times concentrated stock solutions in DMSO. Cyclosporin A, GF120918, Ko143, MK571, and PSC-833 were used in concentrations, which are often used in inhibition studies and have demonstrated to potently inhibit specific ABC transporters (Table 1). The concentration of DMSO at the apical side was kept at 0.5% in each transport experiment.

After 120-min exposure, in which the apical efflux of hesperetin metabolites was linear with time, 150-μl samples were taken from both basolateral and apical compartment. Finally, the TEER value was rechecked to confirm the quality of the monolayer after the experiment. On some occasions, the filters covered with Caco-2 cell monolayers were washed with phosphate-buffered saline, cut out of the insert, suspended in 250 or 500 μl of 65% (v/v) methanol, and sonificated for 15 min in a Bandelin Sonorex RK100 (Berlin, Germany) to...
collect the intracellular contents. All samples were stored at −80°C until further analysis.

**HPLC-DAD Analysis.** The HPLC system consisted of a Waters (Milford, MA) Alliance 2695 separation module connected to a Waters 2996 photodiode array detector, equipped with an Alttech (Breda, The Netherlands) Altima C18 5-μm 150-× 4.6-mm reverse phase column with a 7.5-× 4.6-mm guard column. Before injection, samples were centrifuged at 16,000g for 4 min, and 50 μl was injected and eluted at a flow rate of 1 ml/min starting at 0% acetonitrile in nanopure water containing 0.1% trifluoroacetic acid, increasing to 10% acetonitrile in 5 min, to 15% in the following 16 min, and to 50% in the next 16 min. Thereafter, the percentage acetonitrile was increased to 80% in 1 min. This condition was kept for 1 min, followed by a decrease to 0% acetonitrile in 1 min, keeping this condition for 10 min, allowing the column to re-equilibrate at the initial conditions (total run time, 50 min). DAD spectra were detected between 200 and 420 nm, and HPLC chromatograms acquired at 280 nm were used for quantification and presentation.

**uPLC-DAD Analysis.** The uPLC system consisted of a Waters Acquity binary solvent manager, sample manager, and photodiode array detector, equipped with a Waters BEH C18 1.7-μm 50-× 2.1-mm column. After centrifugation at 16,000g for 4 min, 10-μl samples were injected and eluted at a flow rate of 0.440 ml/min, starting at 95% Millipore water and 5% acetonitrile, both containing 0.1% formic acid, increasing to 21% acetonitrile in 2 min, keeping this condition for 1.5 min, followed by an increase to 25% acetonitrile in 0.25 min; this condition was kept for 1.75 min and followed by an increase to 80% acetonitrile in 0.5 min. This condition was kept for 0.5 min, after which the percentage acetonitrile was decreased to 5% in 0.5 min, keeping this condition for 1 min, allowing the column to re-equilibrate at the initial conditions (total run time, 8 min). DAD spectra were detected between 230 and 420 nm, and uPLC chromatograms acquired at 280 nm were used for quantification.

**uPLC-DAD-MS-MS Analysis.** Before injecting, samples were pretreated by a solvent extraction in which 300 μl of sample was mixed with an equal volume of 200 mM HCl/methanol and extracted three times with 900 μl of ethyl acetate. The collected organic fractions were pooled, dried under nitrogen, and dissolved in 60 μl Millipore water containing 5% acetonitrile and 0.1% formic acid. A sample injection of 1.5 μl was eluted according to the uPLC-DAD method described above; however, a 1-mm inner diameter column at a flow rate of 0.1 ml/min was used on a similar Waters Acquity UPLC system connected to a Micromass (Manchester, UK) Quattro Micro Triple Quadrupole equipped with an electrospray ionization probe. The instrument was operated on negative ion scan mode. The following parameters were used for the ion source for the mass spectrometry scans: 3-kV capillary needle temperature, 400°C; cone gas (nitrogen) flow, 50 l/h; and desolvation gas flow rate of 80°C until analysis by HPLC-DAD.

**Quantification.** Hesperetin was quantified by peak area measurement using HPLC-DAD analysis, based on detection at 280 nm, using a 10-point linear (R² > 0.99) calibration line of a concentration range of 0.02 to 20 μM hesperetin in transport medium containing a final concentration of 0.5% DMSO. Similarly, the amount of hesperetin 7-O-glucuronide was quantified based on an eight-point linear (R² > 0.99) calibration curve of a concentration range of 0.02 to 2.5 μM with the authentic synthesized standard of hesperetin 7-O-glucuronide. The limit of detection of both compounds in transport medium was 0.02 μM (injection volume, 50 μl). The amount of hesperetin 7-O-sulfate was quantified using the calibration curve of hesperetin 7-O-glucuronide because the authentic hesperetin 7-O-sulfate sample was not pure enough to allow definition of a calibration curve and the enzymatic deconjugation of hesperetin metabolites with sulfatase/β-glucuronidase, compared with the deconjugation with only β-glucuronidase, demonstrated a comparable molar extinction coefficient for hesperetin 7-O-sulfate and hesperetin 7-O-glucuronide metabolites.

**Stability.** The stability of hesperetin, hesperetin 7-O-glucuronide, and 7-O-sulfate standards under experimental conditions was tested separately by taking samples with time (at 0, 1, 2, 3, 6, and 24 h) from wells on a Corning Costar 12-well plate, which was stored in the incubator used for cell culture and contained in each well with 2 ml of transport medium supplemented with known concentrations of hesperetin aglycone, hesperetin 7-O-glucuronide, or hesperetin 7-O-sulfate standards (final concentration: 0.5% DMSO). The samples were stored at −80°C until analysis by HPLC-DAD.

**Partition Coefficient Determination.** The log P value of hesperetin was calculated using the online LogKow (KowWin) program (available at http://www.syrrx.com/es/est_kowdemo.htm) from Syr Arc Research Corporation (Syracuse, NY). This program uses fragmental analysis of the chemical structure for the prediction, and obtained log P values demonstrate high correlation with quoted experimental log p values (R² = 0.98).

**Statistical Analysis.** The Student’s two-tailed unpaired t test was used to evaluate statistical differences. Differences were considered significant when p values were less than 0.05. Values are expressed as mean ± S.D.

**Results**

**Metabolism and Transport of Hesperetin by Caco-2 Cell Monolayers.** Using the Caco-2 cell monolayer two-compartment transwell system, we studied transport and metabolism of hesperetin. Figure 2 shows representative sections of HPLC chromatograms of samples taken from the 0.5-ml apical (A) and 1.5-ml basolateral (B) compartments of a Caco-2 cell monolayer upon 120-min exposure to 10 μM hesperetin added to the apical compartment. Hesperetin was detected at the basolateral side (tq, 36.7 min), as well as two major metabolites, M1 (tq, 30.9 min) and M2 (tq, 31.4 min), which were detected at about 3.9- and 3.1-fold higher amounts in the apical compartment compared with the basolateral compartment, respectively.

Upon further analysis by uPLC-DAD, the major metabolite M1 (amounting to 86% of the total amount of metabolites determined by peak area) demonstrated the same retention time (3.50 min) and DAD spectrum (UV_{max} 284.1 nm) as the authentic hesperetin 7-O-glucuronide standard, and metabolite M2 demonstrated the same retention time (4.30 min) and UV spectrum (UV_{max} 280.5 nm; shoulder at 335 nm) as the authentic hesperetin 7-O-sulfate standard. Equal retention times and DAD spectra for M1 and the hesperetin...
7-O-glucuronide standard, as well as for M2 and the hesperetin 7-O-sulfate standard, were demonstrated by coelution on the HPLC-DAD system as well.

Additional identification of M1 as hesperetin 7-O-glucuronide was achieved by confirming the correct molecular mass by uPLC-DAD-MS-MS. Both M1 from the transport medium and the corresponding peak from the metabolite standard showed an [M-H]-ion of m/z 477, consistent with the molecular mass of hesperetin (302 Da) containing a deprotonated additional glucuronic acid moiety (175 Da). The mass of M2, however, could not be determined on the uPLC-DAD-MS-MS system, due to instability of the sulfate moiety during the required sample preparation. However, enzymatic deconjugation with sulfatase (which contains also β-glucuronidase activity) hydrolyzed both M1 and M2, as well as the corresponding standards, into hesperetin aglycone, whereas treatment with β-glucuronidase deconjugated only M1. In control incubations, without enzyme activity added, M1 and M2 were unaffected.

We also tested the stability of the hesperetin derivates dissolved in transport medium and incubated under applied experimental conditions. The concentration of hesperetin 7-O-glucuronide from the authentic standard was stable and present after 120 min at 97% and after 24 h at 95% of its initial concentration. Hesperetin 7-O-sulfate from the authentic standard also was stable and present after both 120 min and 24 h at >98% of its initial concentration. Hesperetin aglycone, however, seemed less stable (or soluble) and after 120 min was present at 88% and after 24 h at only 67% of the initial concentration.

Hesperetin was extensively metabolized in the presence of Caco-2 cells into hesperetin 7-O-glucuronide and hesperetin 7-O-sulfate, which were predominantly excreted to the apical side, linear with time up to 120 min (Fig. 3A), at average rates of 14.3 ± 3.7 and 2.1 ± 0.8 pmol/min/monolayer, respectively, in the transport experiments (Fig. 4). Figure 4 shows the amounts of hesperetin, hesperetin 7-O-glucuronide, and hesperetin 7-O-sulfate in the apical and basolateral compartments of the transwell system, 120 min after exposure to 10 μM hesperetin added to the apical medium. The volumes of the apical and basolateral compartments are 0.5 and 1.5 ml, respectively. M1, hesperetin 7-O-glucuronide; M2, hesperetin 7-O-sulfate.

amount, however, accounted for only 6% of the initial dose. At least part of the explanation for the residual 19.2% loss of hesperetin that remained unaccounted for could be the apparent instability or insolubility of hesperetin aglycone under experimental conditions or during storage, leading to losses in the overall amount of hesperetin plus metabolites.

**Real-Time RT-qPCR.** From the cDNA dilution series, the C_T values were for all genes most stable between 10- and 40-fold dilution; therefore, the real-time qPCR data from the 20-fold diluted cDNA were used for the calculations. Figure 5 depicts the relative mRNA expression levels of Pgp, MRP2, and BCRP normalized to villin. All three ABC transporter genes are expressed, with the relative levels of Pgp and MRP2 mRNA being 12- and 41-fold higher, respectively, compared with the mRNA expression level of BCRP.

**Effect of ABC Transporter Inhibitors on Hesperetin Metabolism and Efflux by Caco-2 Cell Monolayers.** Because flavonoids and/or their metabolites are known to be substrates of ABC transporters, the effect of coadministering different ABC transporter inhibitors to the apical compartment on the transport of hesperetin aglycone and the hesperetin metabolites was investigated. Figure 6 demonstrates no significant effect of the range of several coadministered inhibitors on the appearance of hesperetin aglycone at the basolateral side, which could indicate that hesperetin moves through the Caco-2 monolayer by passive paracellular or transcellular diffusion. The relatively high lipophilicity of hesperetin, represented by the calculated log P value of 2.44 and the molecular mass (302 Da), would imply the latter.

The efflux of hesperetin metabolites (Fig. 7; Table 3), however, was affected by the dual Pgp/BCRP inhibitor GF120918, which caused a concentration-dependent decrease in the apical efflux, accompanied by a concentration-dependent increase in basolateral efflux of hesperetin 7-O-glucuronide. Relevant doses of other Pgp-inhibiting compounds did not alter the efflux of hesperetin metabolites significantly, which suggested BCRP to be the major ABC transport protein involved in the apical efflux of hesperetin metabolites. This was corroborated by the effect of coadministration of the highly specific BCRP inhibitor Ko143, which caused an even greater decrease in the apical efflux, for both hesperetin 7-O-glucuronide (Fig. 7A) and hesperetin 7-O-sulfate (Fig. 7B), and a concomitant increase in basolateral efflux of both hesperetin metabolites. Coadministration of 5 μM Ko143 resulted in a 1.9-fold decrease (p ≤ 0.01) in the total amount of hesperetin metabolites transported to the apical side of the Caco-2 cell monolayer and in a 3.1-fold increase (p ≤ 0.001) in the total amount of hesperetin metabolites transported to the basolateral compartment (Fig. 7C). As a result, the predominant side of both hesperetin 7-O-glucuronide and hesperetin 7-O-sulfate efflux was...
reversed from the apical to the basolateral side. Although not statistically significant, coadministration of MK571 (24 μM) decreased the apical efflux of hesperetin 7-O-glucuronide by 19% (Fig. 7A; Table 3), implying a minor role for apically localized MRP transporters (i.e., MRP2) in the transport of hesperetin 7-O-glucuronide.

Discussion

The present study showed that hesperetin was intensively metabolized by Caco-2 cells into 7-O-glucuronide and 7-O-sulfate metabolites. Other studies describing metabolism of flavonoids by Caco-2 cells also reported a relatively high rate of conjugation into glucuronidated, sulfated, and/or methylated metabolites, the relative formation of all these metabolites depending on the type of flavonoid (Galijatovic et al., 1999; Vaidyanathan and Walle, 2001; Liu and Hu, 2002; Zhang et al., 2004a).

About 86% of the total amount of hesperetin metabolites formed consisted of hesperetin 7-O-glucuronide, a percentage similar to that reported in a study in which the rutinoside hesperidin was given to humans, and 87% of hesperetin in plasma consisted of glucuronides (Manach et al., 2003). The remaining 13% in this human study consisted of sulfoglucuronides, whereas no conjugates were detected that were only sulfated (Manach et al., 2003). Systemic plasma analysis does not reveal the organs in which conjugation has taken place, but it is likely that at least part of the conjugation reactions of hesperetin take place in the intestinal epithelia. The results with the Caco-2 cells in the present study would support a role for intestinal cells in phase II metabolism of hesperetin and of hesperidin after its deglycosylation.

Coadministration of compounds known to potently inhibit BCRP-mediated transport, including the specific BCRP inhibitor Ko143 and the dual BCRP/Pgp inhibitor GF120918, decreased efflux of hesperetin metabolites to the apical compartment and increased efflux of hesperetin metabolites to the basolateral side, whereas coadministration of PSC-833, MK571, and cyclosporin A, known to inhibit several other classes of ABC transporters (Table 1), did not modify the efflux of hesperetin metabolites significantly (Fig. 7; Table 3). Coadministration of 10 μM cyclosporin A, which is generally regarded as a...
nonspecific inhibitor of Pgp but has been reported to inhibit BCRP as well (Qadir et al., 2005; Pawarode et al., 2007), did not demonstrate any effect on disposition of hesperetin metabolites in the present study. A reason for this could be that its BCRP-inhibiting properties were demonstrated in a cell line specifically overexpressing BCRP, whereas in Caco-2 cells, cyclosporin A might have a higher affinity for other ABC transporters present. In addition, Ejendal and Hrycyna (2005) did not demonstrate inhibition of BCRP by cyclosporin A in BCRP-overexpressing HeLa- and MCF-7-derived cells (Ejendal and Hrycyna, 2005). The addition of Ko143 seemed to have a more profound effect on inhibition of the apical efflux of hesperetin 7-O-sulfate than hesperetin 7-O-glucuronide, which further supports a role of BCRP in the transport of hesperetin metabolites, because the affinity of BCRP for sulfated metabolites has been reported to be greater than that for glucuronidated metabolites (Chen et al., 2003).

Altogether, our results suggest that efflux of hesperetin metabolites to the apical compartment in our Caco-2 cell transwell system mainly involves BCRP.

Specific flavonoids and/or their metabolites interact with apically located ABC transporters, especially with Pgp and BCRP (Morris and Zhang, 2006), representing high-affinity substrates. Hesperetin and/or its metabolites have been demonstrated to interact with BCRP in vitro (Cooray et al., 2004; Zhang et al., 2004b), whereas coadministration of up to 50 μM hesperetin did not interact with Pgp-mediated transport in Caco-2 cell monolayers (Mertens-Talcott et al., 2007), and interaction of hesperetin with MRP2, on the basis of structural similarity to other flavonoids, seems unlikely (van Zanden et al., 2005), together further supporting a role for BCRP in the efflux of hesperetin metabolites. The important role for BCRP in the intestinal efflux of sulfate and glucuronide conjugates has also been established for other compounds (Adachi et al., 2005; Ebert et al., 2005), including glucuronide metabolites of the flavonol quercetin (Sesink et al., 2005).

Previous studies on the transport and metabolism of flavonoids other than hesperetin by Caco-2 cell monolayers demonstrated a decreased apical efflux of flavonoids and their metabolites by coadministration of MK571, suggesting MRP2 to be responsible for the transport of flavonoid metabolites back to the intestinal lumen (Vaidyanathan and Walle, 2001; Hu et al., 2003; Zhang et al., 2004a). For instance, 10 μM MK571 inhibited both the apical and basolateral efflux of glucuronidated and sulfated metabolites of the flavone apigenin in Caco-2 monolayers (Hu et al., 2003). Our findings do not rule out a role for MRP2 in the transport of flavonoid hesperetin conjugates completely because coadministration of 24 μM MK571 did seem to negatively affect the apical efflux of hesperetin 7-O-
glucuronide, although not statistically significantly. Both BCRP and MRP2, as well as other ABC transporters, have an overlapping substrate specificity (Haimeur et al., 2004; Takano et al., 2006); thus, the class of transporter contributing most to specific transport will depend on the available dose of a substrate and the specific affinity, together with the specific levels of transporter expression. Earlier studies did not always focus on BCRP-mediated transport, and other studies sometimes used concentrations of 50 μM MK571 or higher, which could be problematic when studying the metabolism of the flavonoid together with transport of its metabolites because MK571 has been shown to inhibit glucuronidation at concentrations higher than 25 μM (Hu et al., 2003).

Because BCRP is a highly expressed ABC transporter throughout the intestinal tract (Taipalensuu et al., 2001; Gutmann et al., 2005; Englund et al., 2006; Seithel et al., 2006), an important role for BCRP in the intestinal efflux of hesperetin conjugates in vivo is very likely. Furthermore, the expression of BCRP in Caco-2 cells is often considered to be relatively low compared with the expression level of other classes of ABC transporters, including Pgp and MRP2, and with real-time RT-qPCR analyses. In the present study, the real-time RT-qPCR analyses in the present study demonstrated a 12 and 41 times lower mRNA expression of BCRP compared with the expression of Pgp and MRP2, respectively. This MRP > Pgp >> BCRP rank order of gene expression is in line with earlier studies on Caco-2 cells (Englund et al., 2006; Seithel et al., 2006). The expression of ultimate BCRP protein in Caco-2 cells has been demonstrated by Western blotting by Xia et al. (2005), making the Caco-2 cells used in the present study a good model to study intestinal BCRP-mediated transport, keeping in mind that effects observed may even be of greater importance in vivo.

Our experiments demonstrated that a portion of the apically applied hesperetin aglycone appeared at the basolateral side unconjugated. Administration of the different inhibitors did not affect this process, which could indicate that the small and relatively lipophilic hesperetin molecule moves through the Caco-2 monolayer by passive transcellular diffusion (i.e., nontransporter mediated). This suggests that passive diffusion of hesperetin could play a role in permeation across intestinal cells, not only in the Caco-2 monolayers, but possibly also in vivo. Lipophilicity is an important determinant of flavonoids for transfer across the intestinal barrier, as demonstrated in situ in rat models (Crespy et al., 2003); however, in a study by Silberberg et al. (2006), in which hesperetin (15 μM) was perfused in situ in rats, more than 95% of the hesperetin found in the mesenteric vein was conjugated. In the study of Manach et al. (2003), in which humans were given hesperidin, no unconjugated hesperetin was detected in plasma, and in the study of Gardana et al. (2007), in which juice from blood oranges was given to humans, also more than 95% of hesperetin in plasma was conjugated. Unfortunately, the only study in which the aglycone itself was given to humans did not study the chemical forms present in plasma (Kanaze et al., 2007); thus, the fate of hesperetin in vivo in humans remains to be elucidated.

In conclusion, hesperetin is extensively metabolized by Caco-2 cell monolayers into 7-O-glucuronide and 7-O-sulfate metabolites, which are predominantly transported to the apical side. Hesperetin aglycone, however, also permeates to the basolateral side of the Caco-2 cell monolayer unconjugated. The pattern of inhibition by different ABC transporter inhibitors suggests the apical efflux of hesperetin metabolites involves mainly BCRP. Moreover, inhibition of BCRP results not only in a decreased apical efflux but also in an increased transport of hesperetin metabolites to the basolateral side of Caco-2 cell monolayers. Altogether, these findings elucidate a novel pathway of hesperetin metabolism and transport and show that BCRP-mediated transport could be one of the main limiting steps for hesperetin bioavailability.

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