Metabolism and transport of the citrus flavonoid hesperetin in Caco-2 cell monolayers

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ABC, ATP binding cassette; BCRP, breast cancer resistance protein; CsA, cyclosporin A; DAD, diode-array detector; DMEM, Dulbecco’s modified eagle medium; DMSO, dimethyl sulfoxide; EDTA, ethylene dinitrilotetraacetic acid; GF120918, \(N\)-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; HPLC, high performance liquid chromatography; Ko143, 3-(6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydropyrazino[1’,2’:1,6]pyrido[3,4-b]indol-3-yl)-propionic acid tert-butyl ester; MEM, minimal essential medium; MK571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-2(dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid; MRP, multidrug resistance protein; MS-MS, Tandem Mass Spectrometry; PBS, phosphate buffered saline; PCR, polymerase chain reaction; Pgp, P-glycoprotein; PSC-833, Valspodar; RT-qPCR, reverse transcription quantitative PCR; SD, standard deviation, SIR, selective ion recording; TEER, trans-epithelial electrical resistance; t\(_R\), retention time; uPLC, ultra performance liquid chromatography.
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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 (Pgp/MDR1/ABCB1), Multidrug Resistance Protein 2 (MRP2/ABCC2) and Breast Cancer Resistance Protein (BCRP/ABCG2) in the efflux of hesperetin and its metabolites was studied by co-administration of compounds known to inhibit several classes of ABC transporters, including cyclosporin A, GF120918, Ko143, MK571, and PSC-833. Apically-applied hesperetin (10 µM) was metabolized into hesperetin 7-O-glucuronide and hesperetin 7-O-sulfate, identified using HPLC-DAD, uPLC-DAD-MS-MS and authentic standards, which were transported predominantly to the apical side of the Caco-2 cell monolayer (1.12 cm²), at average (SD) rates of 14.3 (3.7) pmol/min/monolayer and 2.1 (0.8) pmol/min/monolayer, respectively. Hesperetin aglycone also permeated to the basolateral side, and this process was unaffected by the inhibitors used, possibly implying a passive diffusion process. Inhibition studies, however, showed that efflux of hesperetin conjugates to the apical side involved active transport, which from the pattern of inhibition, appeared to involve mainly BCRP. Upon inhibition by the BCRP inhibitor Ko143 (5 µM), the apical efflux of hesperetin conjugates was 1.9-fold reduced (P≤0.01) and transport to the basolateral side was 3.1-fold increased (P≤0.001). These findings elucidate a novel pathway of hesperetin metabolism and transport, and show that BCRP-mediated transport could be a limiting step for hesperetin bioavailability.
Introduction

Flavonoids consist of a large group of polyphenols which 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 anti-microbial effects (Garg et al., 2001).

Flavanones occur in the diet almost exclusively in citrus fruits or citrus fruit derived products, and 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 15.1 mg/day (after hydrolysis of glycosides) (Knekt et al., 2002). According to data on urinary and plasma concentrations, however, bioavailability of hesperetin 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, as well as 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/MDRI/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 co-exposure to compounds which 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 as well as 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 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 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 co-elution with authentic synthesized standards, by confirmation with uPLC-DAD-MS-MS and by specific enzymatic de-conjugation. By co-administering 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 (Hertfordshire, UK), Ko143 from Dr. Alfred H. Schinkel from the Netherlands Cancer Institute (Amsterdam, the Netherlands) and PSC-833 from Novartis Pharma AG (Basel, Switzerland). MK571 was purchased from Biomol (Plymouth Meeting, PA) and cyclosporin A 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 from J.T. Baker (Philipsburg, NJ), isopropanol (for molecular biology) from Acros (Geel, Belgium) and TRIzol reagent from Invitrogen (Paisley, UK). Di-potassium hydrogen phosphate, dimethyl sulfoxide (DMSO), ethanol (for molecular biology), ethylene dinitrilotetraacetic acid (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) from Roche (Mannheim, Germany). All cell culture reagents were purchased from Gibco (Paisley, UK). Authentic standards of hesperetin 7-O-glucuronide (purity 92.8%) and hesperetin 7-O-sulfate (purity <50%) were obtained from the Nestlé Research Center (Lausanne, Switzerland).

Cell culture. Caco-2 cells were obtained from ATCC (Rockville, MD) and cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in Dulbecco’s Modified Eagle Medium (DMEM) containing 25 mM HEPES buffer, 4500 mg/l glucose, L-glutamine and phenol red, and was supplemented with 10% (v/v) heat inactivated (30 min at 56 °C) fetal bovine serum, 1% (v/v) MEM non-essential amino
acids and 0.2% (v/v) 50 mg/ml gentamycin. When the cell culture reached 70 to 90% confluency, it was rinsed with phosphate buffered saline (PBS) containing 22 mg/l EDTA and split using trypsin. The cell density of the suspension was determined using a Bürker-Türk counting chamber from Labor Optik (Friedrichsdorf, Germany). For transport experiments, 1×10^5 cells per cm^2 (0.5 ml of 2.24×10^5 cells/ml) were seeded in Costar 12-well transwell plate inserts from Corning (Corning, NY) with an insert membrane pore size of 0.4 μm and a growth area of 1.12 cm^2. The passage number of the cells used in the experiments was between 37 and 47. The medium was changed 3 times a week and the transport experiments were performed 18 or 19 days post seeding.

**RNA isolation.** Medium was removed from a culture flask containing a Caco-2 cell monolayer (passage number 46) and the cells were lysed in TRIzol reagent (100 μl/cm^2) and stored at -80°C. After defreezing, 200 μl chloroform was added to 1 ml aliquots, which were shaken (1 min), incubated at room temperature (3 min) and centrifuged at 15500 g and 4°C (15 min). The aqueous phases were transferred to a sterile tube, followed by addition of an equal volume isopropanol to precipitate the RNA. The samples were mixed and left to incubate at room temperature (10 min), after which they were centrifuged at 15500 g and 4°C (10 min). After removing the supernatant the pellets were washed with 70% (v/v) ethanol. After centrifugation at 12000 g and 4°C (5 min), the supernatant was removed and the pellets were air dried and resuspended in 100 μl RNase free water. The samples were pooled, cleaned and concentrated using a RNeasy Mini kit from Qiagen (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 (Paisley, UK), 2 µl dNTPs (10 mM) from Fermentas (Vilnius, Lithuania) and RNase free water, together incubated 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 DTT (100 mM), 0.5 µl RNase OUT (40 units/µl), 1 µl M-MLV (200 units/µl) from Invitrogen (Paisley, UK) 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, 160 times) of the cDNA using the iCycler and iQ SYBR Green from Bio-Rad (Hercules, CA). Each 25 µl PCR reaction contained 5 µl cDNA, 12.5 µl Mastermix SYBR Green, RNase free water and 1 µl of both the specific forward and reverse primers (10 mM) which were synthesized by Biolegio (Nijmegen, the Netherlands). The sequences of the primers used were described by Taipalensuu et al. (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 pre-incubation at 95 ºC, 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 (C_T), was used for semi-quantification of the PCR product and the relative ABC transporter mRNA expression levels, normalized to villin, are given by ∆C_T=CT_ABC transporter-CT_villin converted to 2^-∆CT.

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 volt/ohmmeter from Millipore (Bedford, MA). Only monolayers that demonstrated a TEER value between 500 and 1000 \( \Omega \cdot \text{cm}^2 \) were used. Transport experiments were carried out with transport medium consisting of DMEM (without phenol red) supplemented with 1% (v/v) MEM non-essential amino acids and 1 mM ascorbic acid to prevent auto-oxidation, which was filtered through a sterile 0.2 \( \mu \text{m} \) filter unit from Schleicher & 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 \( \mu \text{M} \) 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 \( \mu \text{M} \) 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 \( \mu \text{l} \) samples were taken from both basolateral and apical compartment. Finally, the TEER value was re-checked to confirm the quality of the monolayer after the experiment. On some occasions the filters covered with Caco-2 cell monolayers were washed with PBS, cut out of the insert, dissolved in 250 or 500 \( \mu \text{l} \) 65% (v/v) methanol and sonificated for 15 min in a Bandelin Sonorex RK100 (Berlin, Germany) in order to collect the intracellular contents. All samples were stored at -80 \( ^\circ \text{C} \) 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 Alltech (Breda, the Netherlands) Alltima C18 5 µm 150 x 4.6 mm reverse phase column with 7.5 x 4.6 mm guard column. Before injection, samples were centrifuged at 16000 g 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, which condition was kept for 1 min, followed by a decrease to 0% acetonitrile in 1 min, keeping this condition for 10 minutes 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 x 2.1 mm column. After centrifugation at 16000 g for 4 min samples of 10 µl 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, which condition was kept for 1.75 min, and followed to 80% acetonitrile in 0.5 min. This percentage 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 400 nm and UPLC chromatograms acquired at 280 nm were used for quantification.
**uPLC-DAD-MS-MS analysis.** Before injecting, samples were pre-treated by a solvent extraction in which 300 µl of sample was mixed with an equal volume of 200 mM HCl/methanol and 3 times extracted with 900 µl ethyl acetate. The collected organic fractions were pooled, dried under nitrogen and dissolved in 60 µl in 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, with a 1 mm inner diameter column at a flow rate of 0.1 ml/min, on a similar Waters Acquity UPLC system connected to a Micromass (Manchester, UK) Quattro Micro Triple Quadrupole equipped with an electrospray ionization (ESI) probe. The instrument was operated on negative ion scan mode. The following parameters were used for the ion source for the MS Scans: 3 kV capillary needle voltage, 38 V cone voltage, source block temperature 100 °C, desolvation temperature 400 °C, cone gas (nitrogen) flow 50 L/h, and desolvation gas (nitrogen) flow 500 L/h. The scanned mass range was between 200-700 m/z and the scan time 0.4 s with interscan delay of 0.05 s. For the selective ion recording mode (SIR) the following masses of metabolites were monitored: 217, 301, 381, 395, 463, 477, 491, and 609 m/z. For the SIR mode, the cone voltage change was between 30 to 40 V depending on the analyte. Dwell time was 0.02 s and the other parameters as above.

**Enzymatic deconjugation.** To confirm the presence of glucuronide or sulfate conjugates, samples were treated with β-glucuronidase or sulfatase/β-glucuronidase. For β-glucuronidase incubations 50 µl sample was added to 50 µl 400 mM potassium phosphate buffer (pH 6.2) and after addition of 4 µl β-glucuronidase solution (0.8 units), the mixture was incubated for 60 min at 37 °C. To hydrolyze both glucuronide and sulfate conjugates, samples were treated with sulfatase (containing β-glucuronidase activity). A sample volume of 50 µl was added to 50 µl 1 M sodium
acetate buffer (pH 4.5) and upon addition of 4 µl sulfatase/β-glucuronidase solution (0.8 units) the sample was incubated for 18 h at 37 ºC. Control samples were given the same treatment but with 4 µl water instead of enzyme solution. After incubation the mixtures were stored at -80 ºC until further analysis by HPLC-DAD.

**Quantification.** Hesperetin was quantified by peak area measurement using HPLC-DAD analysis, based on detection at 280 nm, using a ten-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 since 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 hours) from wells on a Corning Costar 12-well plate (Corning, NY) which was stored in the incubator used for cell culture and contained in each well 2 ml 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.syrres.com/esc/est_kowdemo.htm) from Syracuse 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 ($R^2=0.98$).

**Statistical analysis.** 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 ± standard deviation (SD).
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) compartment 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 (t_R 36.7 min), as well as 2 major metabolites, M1 (t_R 30.9 min) and M2 (t_R 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, sholder 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 co-elution 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) hydrolysed 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 hours at 95% of its initial concentration. Hesperetin 7-O-sulfate from the authentic standard also was very stable and present after both 120 min and 24 hours 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 hours at only 67% of the initial concentration.

Hesperetin was extensively metabolized 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. 3), at average rates of 14.3 ± 3.7 pmol/min/monolayer 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 compartment of the transwell system, 120 min upon exposure to 10 µM hesperetin (5 nmol/0.5 ml). Upon 120 min incubation, the residual amount of hesperetin at the donor side amounts to only 0.78 nmol (15.5% of the initial amount added). The amount of hesperetin appearing in the basolateral compartment and the amount of both hesperetin metabolites transported to the apical and basolateral compartment together make up for only 59.3% of the initial amount hesperetin added. The residual 25.2% may be accounted for by the amount of
hesperetin and/or hesperetin metabolites accumulating in the cellular compartment of the Caco-2 monolayer transwell system. Indeed, we detected intracellular amounts of hesperetin, hesperetin 7-\textit{O}\-glucuronide and hesperetin 7-\textit{O}\-sulfate. The total 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, and so the real time qPCR data from the 20-fold diluted cDNA was 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 respectively 12- and 41-fold higher compared to the mRNA expression level of BCRP.

**Effect of ABC transporter inhibitors on hesperetin metabolism and efflux by Caco-2 cell monolayers.** Since flavonoids and/or their metabolites are known to be substrates of ABC transporters, the effect of co-administering 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 co-administrated 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 weight (302 Da), would imply the latter.
The efflux of hesperetin metabolites (Fig. 7 and 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 7-0-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 co-administration of the highly specific BCRP inhibitor Ko143, which caused an even greater decrease in the apical efflux, for both hesperetin 7-0-glucuronide (Fig. 7A) and hesperetin 7-0-sulfate (Fig. 7B), and a concomitant increase in basolateral efflux of both hesperetin metabolites. Co-administration 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 7-0-glucuronide and hesperetin 7-0-sulfate efflux was reversed from the apical to the basolateral side. Although not significant, co-administration of MK571 (24 μM) decreased the apical efflux of hesperetin 7-0-glucuronide by 19% (Fig. 7A and Table 3), implying a minor role for apically localized MRP transporters (i.e. MRP2) in the transport of hesperetin 7-0-glucuronide as well.
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 as 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, while no conjugates were detected which were only sulfated (Manach et al., 2003). Systemic plasma analysis does not reveal the organs in which conjugation has taken place, but likely 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.

Co-administration 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 consequently increased efflux of hesperetin metabolites to the basolateral side, while co-administration 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). Co-administration
of 10 µM cyclosporin A, which is generally regarded as a non-specific 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 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, since 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), while co-administration of up to 50 µM hesperetin did not interact with Pgp-mediated transport in Caco-2 cell monolayers (Mertens-Talcott et al., 2007) and interactions of hesperetin with MRP2, on the basis of structural similarity to other flavonoids, seem 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 co-administration 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, since co-administration of 24 µM MK571 did seem to negatively affect the apical efflux of hesperetin 7-O-glucuronide, although not 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 shown to inhibit glucuronidation at concentrations higher than 25 µM (Hu et al., 2003).

Since 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 expression of BCRP in the intestine (Taipalensuu et al., 2001; Englund et al., 2006; Seithel et al., 2006), which could both result in an underestimation of its relevance in *in vitro* experiments. The real time RT-qPCR analyses in the present study demonstrated a 12- and 41-times lower mRNA expression of BCRP compared to 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.* (Xia *et al.*, 2005), making the Caco-2 cells used in the present study represent 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.* non-transporter 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.*, in which hesperetin (15 µM) was perfused *in situ* in rats, more than 95% of the hesperetin found in the mesenteric vein was conjugated (Silberberg *et al.*, 2006). In the study of Manach *et al.*, in which humans were given hesperidin, no unconjugated hesperetin was detected in plasma (Manach *et al.*, 2003) and in the
study of Gardana et al., in which juice from blood oranges was given to humans, also more than 95% of hesperetin in plasma was conjugated (Gardana et al., 2007). 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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References


Footnote

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Legends to the figures

FIG. 1. Chemical structures of the rutinoside hesperidin and its aglycone hesperetin (4’-methoxy-3’,5,7-trihydroxyflavanone) and an illustration of possible pathways for their intestinal uptake.

FIG. 2. Representative sections of the HPLC chromatograms of medium samples from the apical (A) and basolateral (B) side of a Caco-2 monolayer 120 min upon exposure to 10 μM hesperetin added to the apical medium. The volume of the apical and basolateral compartment is 0.5 and 1.5 ml, respectively. M1 = hesperetin 7-O-glucuronide, M2 = hesperetin 7-O-sulfate.

FIG. 3. Amounts of hesperetin 7-O-glucuronide (M1), hesperetin 7-O-sulfate (M2) and hesperetin aglycon in the apical compartment (A) and basolateral compartment (B) with time upon exposure to 5 nmol (=10 μM / 0.5 ml) apically-applied hesperetin. Data are the average of 2 determinations.

FIG. 4. Mean amounts of hesperetin 7-O-glucuronide (M1), hesperetin 7-O-sulfate (M2) and hesperetin aglycone in the apical compartment and basolateral compartment 120 min upon exposure to 5 nmol (=10 μM / 0.5 ml) apically-applied hesperetin (n=7), *** = p<0.001 significantly different.

FIG. 5. Relative ABC transporter mRNA expression levels in Caco-2 cells normalized to the expression of villin. Mean ± SD values of two determinations are shown.
FIG. 6. Effect of different ABC transport inhibitors on basolateral amounts of hesperetin detected 120 min upon apical addition of 10 µM hesperetin compared with the control (10 µM hesperetin without inhibitors). Mean ± SD values are shown (n=4, control n=7). CsA = cyclosporin A.

FIG. 7. Effect of different ABC transport inhibitors on amounts of hesperetin 7-O-glucuronide (A), hesperetin 7-O-sulfate (B) and on the sum of both hesperetin metabolites (C) in the apical and basolateral medium 120 min after addition of 10 µM hesperetin to the apical side of Caco-2 monolayers compared to the control (10 µM hesperetin without inhibitors). Mean ± SD values shown (n=4, control n=7), * = p<0.05, ** = p<0.01 and *** = p<0.001 significantly different compared to the control. CsA = cyclosporin A.
### Table 1.

*Overview from selected literature on the ability of the inhibitors used in this study to inhibit different ABC transporters in different specific model systems*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration(s) used in present study</th>
<th>Affected ABC transporter(s)</th>
<th>Effect reported in literature</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin A</td>
<td>10 µM</td>
<td>Pgp, MRP1 and BCRP</td>
<td>2.5 µM inhibited mitoxantrone efflux in Pgp, MRPl and BCRP overexpressing cell lines.</td>
<td>(Qadir et al., 2005; Pawarode et al., 2007)</td>
</tr>
<tr>
<td>GF120918</td>
<td>1, 5 and 10 µM</td>
<td>BCRP and Pgp</td>
<td>25-250 nM inhibited rhodamine efflux in Pgp overexpressing cells and 1-10 µM inhibited mitoxantrone efflux in BCRP overexpressing cells.</td>
<td>(De Bruin et al., 1999)</td>
</tr>
<tr>
<td>Ko143</td>
<td>1, 2 and 5 µM</td>
<td>BCRP</td>
<td>EC$_{90}$ of 23 and 26 nM for inhibiting mitoxantrone and topotecan efflux, respectively, in Bcrp1 overexpressing cells.</td>
<td>(Allen et al., 2002)</td>
</tr>
<tr>
<td>MK571</td>
<td>2.4 and 24 µM</td>
<td>MRP1/MRP2</td>
<td>Inhibited LTC$_4$ transport in MRP overexpressing cells with a K$_i$ of 0.6 µM and 30 µM completely inhibited vincristine efflux in MRP overexpressing cells.</td>
<td>(Leier et al., 1994; Gekeler et al., 1995)</td>
</tr>
<tr>
<td>PSC-833</td>
<td>5 µM</td>
<td>Pgp, MRPs</td>
<td>2.5 µM inhibited mitoxantrone efflux in Pgp overexpressing cells.</td>
<td>(Qadir et al., 2005; Pawarode et al., 2007)</td>
</tr>
</tbody>
</table>
Table 2.

Gene specific PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1(Pgp)</td>
<td>5’-CAGACAGCAGGAATGAAATGTTGGA-3’</td>
<td>5’-TGAAGACATTTCCAAGGCATCA-3’</td>
<td>(Taipalensuu et al., 2001)</td>
</tr>
<tr>
<td>MRP2</td>
<td>5’-TGCAGCCCTCCATAACCATGAG-3’</td>
<td>5’-GATGCCTGCCATGGACCTA-3’</td>
<td>(Taipalensuu et al., 2001)</td>
</tr>
<tr>
<td>BCRP</td>
<td>5’-CAGGTCTGTTGGTCAATCTCACA-3’</td>
<td>5’-TCCATATCGTGAATGCTGAAG-3’</td>
<td>(Taipalensuu et al., 2001)</td>
</tr>
<tr>
<td>Villin</td>
<td>5’-CATGAGCCATGCCCTGAAC-3’</td>
<td>5’-TCATTCTGCACCTCCACCTGT-3’</td>
<td>(Taipalensuu et al., 2001)</td>
</tr>
</tbody>
</table>
Table 3.

Effect of different ABC transport inhibitors on amounts of hesperetin 7-O-glucuronide and hesperetin 7-O-sulfate in the apical and basolateral medium 120 min after addition of 10 µM hesperetin at the apical side of Caco-2 monolayers compared to the control (10 µM hesperetin without inhibitors). Mean ± SD values (n=4, control n=7), * = p<0.05, ** = p<0.01 *** = p<0.001 significant different compared to the control. CsA = cyclosporine A.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>(µM)</th>
<th>hesperetin 7-O-glucuronide (nmol)</th>
<th>hesperetin 7-O-sulfate (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Apical</td>
<td>Basolateral</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.71 ± 0.44</td>
<td>0.44 ± .12</td>
</tr>
<tr>
<td>MK571</td>
<td>2.4</td>
<td>1.68 ± 0.42</td>
<td>0.41 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.39 ± 0.44</td>
<td>0.46 ± 0.15</td>
</tr>
<tr>
<td>CsA</td>
<td>10</td>
<td>1.66 ± 0.43</td>
<td>0.53 ± 0.12</td>
</tr>
<tr>
<td>PSC-833</td>
<td>5</td>
<td>1.70 ± 0.31</td>
<td>0.48 ± 0.10</td>
</tr>
<tr>
<td>GF120918</td>
<td>1</td>
<td>1.39 ± 0.28</td>
<td>0.71 ± 0.17 *</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.11 ± 0.25 *</td>
<td>0.85 ± 0.30 *</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Ko143</td>
<td>1</td>
</tr>
<tr>
<td>---</td>
<td>--------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>1.09 ± 0.34 *</td>
<td>0.88 ± 0.17 ***</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>Ko143</td>
<td>1.50 ± 0.23</td>
<td>0.82 ± 0.12 ***</td>
<td>0.23 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.89 ± 0.31 **</td>
<td>1.14 ± 0.40 **</td>
<td>0.14 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>0.96 ± 0.13 **</td>
<td>1.34 ± 0.18 ***</td>
<td>0.10 ± 0.04 *</td>
</tr>
</tbody>
</table>
Figure 3
Figure 4
Figure 5: villin normalized mRNA expression level for MRP2, Pgp, and BCRP.
Figure 7