RESEARCH ARTICLE

The effect of co-administered flavonoids on the metabolism of hesperetin and the disposition of its metabolites in Caco-2 cell monolayers

Walter Brand[1,2], Beatriz Padilla[1], Peter J. van Bladeren[1,2], Gary Williamson[2,3] and Ivonne M. C. M. Rietjens[1]

[1] Division of Toxicology, Wageningen University, Wageningen, The Netherlands
[2] Nestlé Research Center, Nestec Ltd., Lausanne, Switzerland
[3] School of Food Science and Nutrition, University of Leeds, Leeds, UK

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Metabolism by phase II enzymes and transport from intestinal cells back into the lumen by ATP binding cassette (ABC) transporters limits the bioavailability of the flavanone hesperetin, the aglycone of hesperidin. This study investigates to what extent other flavonoids modulate the metabolism and transport of hesperetin by characterizing the effect of co-administrating a series of flavonoids using Caco-2 cell monolayers in a two-compartment transwell system. Flavonoids may interfere with hesperetin metabolism and can also inhibit the apically located ABC transporter breast cancer resistance protein (*ABCG2*) which was previously shown to be responsible for the apical transport of hesperetin metabolites. Co-exposure of Caco-2 cell monolayers to hesperetin with specific flavonoids reduced the ratio of apical efflux to basolateral transport of hesperetin metabolites, and in some cases, also reduced the amount of hesperetin metabolites detected extracellularly. As intracellular accumulation of hesperetin metabolites did not account for this decrease, inhibition of metabolism of hesperetin is likely the underlying mechanism for the reduced metabolite formation and excretion. In spite of the reduction in metabolism the amount of hesperetin metabolites transported to the basolateral side significantly increased upon co-exposure with specific flavonoids and therefore co-administration of specific flavonoids could be a strategy to improve the bioavailability of hesperetin.

Keywords:
Breast cancer resistance protein / Hesperetin / *In vitro* bioavailability / Metabolism / Quercetin

1 Introduction

Flavonoids are polyphenols and can be divided into different classes including flavones, flavonols (3-hydroxyflavones), isoflavones, flavanones, flavanols, chalcones and anthocyanins (Fig. 1). They are present in fruits, vegetables and plant-derived products, often occurring as β-glycosides [1]. Flavonoids and flavonoid-rich products have been implicated as beneficial agents to reduce the risk of chronic diseases [2]. Despite their relatively high dietary intake, the bioavailability of many flavonoids and/or their metabolites is relatively poor [3]. Dependent on the type of flavonoid, a significant proportion can be attributed to efficient intestinal metabolism and/or efflux mediated by ATP binding cassette (ABC) transport proteins located in the apical (AP) membrane of enterocytes, including P-glycoprotein (Pgp/*MDR1*/*ABCB1*), multidrug resistance proteins (MRPs/*ABCCs*) and breast cancer resistance protein (BCRP/*ABCG2*) [4–7].

Correspondence: Dr. Walter Brand, Division of Toxicology, Wageningen University, Tuinlaan 5, 6703 HE Wageningen, The Netherlands
E-mail: Walter.Brand@WUR.nl
Fax: +31-317-484931

Abbreviations: ABC, ATP binding cassette; **AP,** apical; **BL,** basolateral; **BCRP,** breast cancer resistance protein; **EGCG,** (−)-epigallocatechin gallate; **MRP,** multidrug resistance protein; **TEER,** trans-epithelial electrical resistance

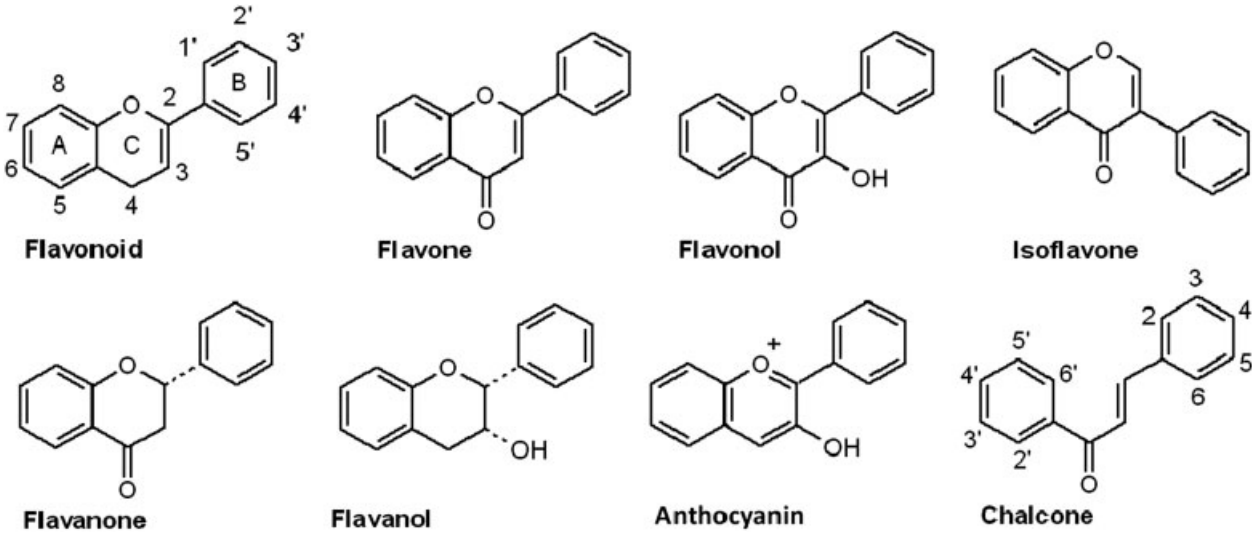

Figure 1. Basic chemical flavonoid structure and basic chemical structures of different flavonoid subclasses.

The flavanone hesperetin (3′,5,7-trihydroxy-4′-methoxy-flavanone) is the aglycone of hesperidin (hesperetin 7-*O*-rutinoside) which contains a disaccharide moiety (Fig. 2), which is present in high amounts in sweet oranges (*Citrus sinensis*) and orange juice, but also in other citrus fruits including lemon, lime and mandarin [8]. For example, orange juice may contain more than 500 mg/L hesperidin [8]. Both hesperidin and hesperetin have been reported to provide beneficial health effects, as has been reviewed by Garg *et al.* [9]. The reported beneficial health effects of hesperetin include a reduced risk of osteoporosis, which has been demonstrated by an increased bone mineral density in ovariectomized or sham-operated rats or mice given hesperidin [10, 11]. Although the exact molecular mechanism for these effects has not yet been elucidated, some explanation arises from the anti-oxidant and anti-inflammatory properties of hesperetin affecting nuclear factor-κB and related signal transduction pathways [12].

Hesperidin must be hydrolyzed by colonic microflora before it can be absorbed, whereas the hesperetin aglycone, as well as the monosaccharide hesperetin 7-*O*-glucoside, is already taken up earlier in the digestive tract [13, 14]. The latter could be hydrolyzed by lactase phlorizin hydrolase whereafter the hesperetin aglycone can migrate into the intestinal cells and/or the hesperetin glucoside could be transported into the intestinal cells *via* the sodium-dependent glucose transporter (SGLT1) after which it is deglucosylated by β-glucosidase activity within the intestinal cell [13, 15, 16]. The resulting intracellularly located hesperetin aglycone is conjugated by UDP-glucuronosyltransferase and sulfotransferase enzymes into glucuronidated and sulfated metabolites, respectively, which have been detected in human and rat plasma [17–20]. An increased bioavailability of hesperetin by exposure to hesperetin 7-*O*-glucoside, which is already taken up in the small intestine, rather than to hesperidin [13], has been demonstrated to more efficiently prevent bone loss in ovariectomized rats [21].

Recently, we studied the intestinal metabolism and extracellular transport of hesperetin *in vitro* using Caco-2 cell monolayers in a two-compartment transwell system, simulating the intestinal transport barrier, and demonstrated that hesperetin was metabolized into hesperetin 7-*O*-glucuronide and hesperetin 7-*O*-sulfate metabolites which were preferentially transported to the AP compartment, simulating the intestinal lumen side [7]. Inhibition of the apically expressed BCRP by standard inhibitors reversed the predominant side of hesperetin metabolite efflux to the basolateral (BL) compartment, simulating the blood/plasma side [7]. Since intestinal BCRP-mediated AP

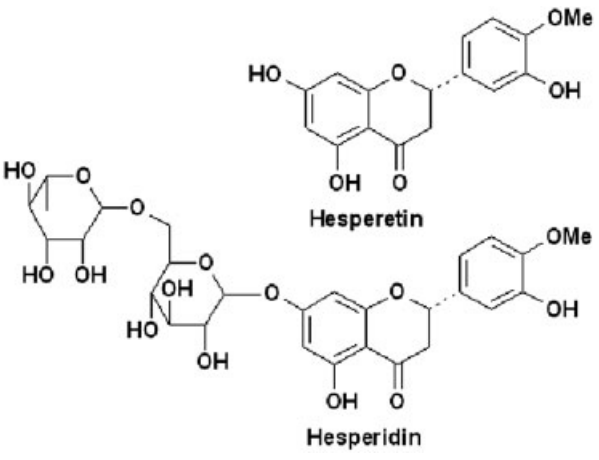

Figure 2. Chemical structures of the rutinoside hesperidin and its aglycone hesperetin ((+/−)-4′-methoxy-3′,5,7-trihydroxyflavone).

efflux of hesperetin metabolites could be a limiting factor in the bioavailability of hesperetin, co-administration of hesperetin with dietary compounds inhibiting BCRP mediated efflux could be a strategy to increase hesperetin bioavailability. Some dietary flavonoids are potent BCRP inhibitors [22, 23]. Furthermore, these flavonoids may also modulate the enzymes catalyzing phase II biotransformation thereby influencing the bioavailability of hesperetin and/or its metabolites by a second mechanism. The aim of this study was to investigate to what extent other flavonoids modulate the metabolism and intestinal transport of hesperetin. To this end we tested the ability of a selection of different flavonoids (Table 1) to modulate the metabolism and transport of hesperetin in Caco-2 cell monolayers as an intestinal *in vitro* model system. The results obtained provide insight in the flavonoid-mediated modulation of the two processes influencing hesperetin bioavailability: its metabolism by phase II enzymes and the extracellular transport of its conjugates. They also provide a possible strategy to improve the bioavailability of hesperetin.

2 Materials and methods

2.1 Materials

The flavonoids acacetin (purity ≥ 85%), chrysin (purity ≥ 99%) and genistein (purity ≥ 98%) were obtained from ICN Biomedicals (Aurora, OH, USA), biochanin A (purity ≥ 97%), (+)-catechin hydrate (purity ≥ 98%), (−)-epigallocatechin gallate (EGCG) (purity ≥ 97%), (−)-epicatechin (purity ≥ 90%), hesperetin (purity ≥ 95%), hesperidin (purity ~ 90%), phloretin (purity ≥ 98%) and quercetin dihydrate (purity ≥ 95%) from Sigma (St. Louis, MO, USA), daidzein (purity ≥ 98%) from Indofine Chemical Company (Belle Mead, NJ, USA), isorhamnetin (purity ≥ 99%) and kaempferide (purity ≥ 99%) from Extrasynthèse (Genay, France), galangin (purity ≥ 95%) from Aldrich (Milwaukee, WI, USA) and rutin (purity ≥ 97%) from Acros (Morris Plains, NJ, USA). 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). L-Ascorbic acid was purchased from Sigma, ACN and methanol from Sigma-Aldrich (Steinheim, Germany), trifluoroacetic acid from J.T. Baker (Philipsburg, NJ, USA), DMSO and EDTA disodium salt dihydrate from Merck (Darmstadt, Germany) and all cell culture reagents from Gibco (Paisley, UK).

2.2 Cell culture

Caco-2 cells from ATCC (Manassas, VA, USA) were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C in DMEM supplemented with 10% heat inactivated fetal bovine serum, MEM non-essential amino acids and 0.1 mg/mL gentamycin, as described previously [7]. For transport experiments, 1×10^5 cells *per* cm^2 were seeded in Corning Costar 12-well transwell plate inserts with an insert membrane pore size of 0.4 μm and a growth area of 1.12 cm^2. The medium was changed three times a week and the experiments were performed 18 or 19 days post seeding. The passage number of the cells used in the experiments was between 39 and 50.

2.3 Caco-2 cell monolayer experiments

Before exposure, 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, USA). Only monolayers demonstrating a TEER value between 500 and 1000 Ω cm^2 were used. Transport experiments were carried out with 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 of the flavonoids, which was filtered through a cellulose acetate sterile syringe filter (0.2 μm) from VWR (West Chester, PA, USA). To study the effect of co-administration of flavonoids, Caco-2 cell monolayers were exposed at the AP side to 10 μM hesperetin, in the absence or presence of 10 μM of a specific flavonoid added to the AP side. The flavonoids tested are listed in Table 1, and include flavonoids reported to inhibit BCRP as well as flavonoids reported not to inhibit BCRP. References reporting these flavonoid characteristics with respect to BCRP are also listed in Table 1. The concentration of 10 μM reflects a physiologically relevant dose and corresponds with our earlier study [7]. All flavonoids were added to the exposure medium from 400 times concentrated stock solutions in DMSO. The concentration of DMSO at the AP side was kept at 0.5% in each transport experiment. After 120 min exposure, 150 μL samples were taken from both the BL and the AP compartment and the TEER value was rechecked to confirm the quality of the monolayer after the experiment. In an additional experiment, Caco-2 cell monolayers were exposed to hesperetin in the presence or absence of quercetin and in addition to the hesperetin metabolites in the BL and AP media also the intracellular levels of hesperetin metabolites and hesperetin were determined. To determine the intracellular hesperetin and hesperetin metabolite levels, the filters of two transwell plates covered with Caco-2 cell monolayers exposed for 120 min to 10 μM hesperetin with or without 10 μM quercetin added at the AP side were washed with phosphate-buffered saline containing 22 mg/L EDTA, cut out of the insert, suspended in 200 μL of 65% v/v methanol and sonicated for 15 min using a Bandalin Sonorex RK100 (Berlin, Germany). All samples were stored at −80°C until further analysis by HPLC-DAD.

Table 1. Flavonoids used in this study which have been reported to inhibit (+), or not to inhibit (−), BCRP in different *in vitro* studies [27, 28, 34–36]

Flavonoid class	Flavonoid	Systematic name	Inhibition of BCRP	Effect reported in literature
Rutinosides	Hesperidin	Hesperetin-7-*O*-rutinoside		
	Rutin	Quercetin-3-*O*-rutinoside	−	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells [27, 35]
Flavonols	Quercetin	3,3′,4′,5,7-Pentahydroxyflavone	+	Mitoxantrone and bodipy-FL-prazosin accumulation in drug selected BCRP overexpressing MCF-7 cells and BCRP transfected K562 cells [34] and mitoxantrone accumulation in drug selected BCRP overexpressing MCF-7 and NCI-H460 cells [36]
			−	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells[27, 35]
	Isorhamnetin	3,4′,5,7-Tetrahydroxy-3′-methoxyflavone		
	Galangin	3,5,7-Trihydroxyflavone	+	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells [27, 35] and mitoxantrone accumulation in BCRP-transfected HEK-293 cells [28]
	Kaempferide	3,5,7-Trihydroxy-4′-methoxyflavone	+	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells [27, 35]
Flavones	Chrysin	5,7-Dihydroxyflavone	+	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells [27, 35], mitoxantrone accumulation in BCRP-transfected HEK-293 cells [28] and mitoxantrone accumulation in drug selected BCRP overexpressing MCF-7 and NCI-H460 cells [36]
	Acacetin	5,7-Dihydroxy-4′-methoxyflavone	+	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells[27, 35]
Isoflavones	Genistein	4′,5,7-Trihydroxyisoflavone	+	SN-38 and mitoxantrone accumulation in BCRP transfected K562 cells [27, 35], mitoxantrone accumulation in BCRP-transfected HEK-293 cells [28] and mitoxantrone accumulation in drug selected BCRP overexpressing MCF-7 and NCI-H460 cells [36]
	Daidzein	4′,7-Dihydroxyisoflavone	+	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells [27, 35], mitoxantrone and bodipy-FL-prazosin accumulation in drug selected BCRP overexpressing MCF-7 cells and BCRP-transfected K562 cells [34] and mitoxantrone accumulation in drug selected BCRP overexpressing MCF-7 and NCI-H460 cells [36]
	Biochanin A	5,7-Dihydroxy-4′-methoxyisoflavone	+	Mitoxantrone accumulation in drug selected BCRP overexpressing MCF-7 and NCI-H460 cells [36]
Flavanols	(+)-Catechin	(+)-3,3′,4′,5,7-Pentahydroxyflavane	−	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells [27, 35]
	(−)-EGCG	(−)-3,3′,4′,5,5′,7-Hexahydroxyflavan-3-gallate	−	Mitoxantrone accumulation in drug selected BCRP overexpressing MCF-7 and NCI-H460 cells [36]
	(−)-Epicatechin	(−)-3,3′,4′,5,7-Pentahydroxyflavane		
Chalcones	Phloretin	2′,4,4′,6′-Tetrahydroxydihydrochalcone	−	SN-38 and mitoxantrone accumulation in BCRP-transfected K562 cells [27, 35] and mitoxantrone accumulation in drug selected BCRP overexpressing MCF-7 and NCI-H460 cells [36]

Table 2. Effect of different flavonoids (10 μM) on the apical and basolateral amounts of hesperetin metabolites as percentage of the control detected after 120 min incubation of Caco-2 cell monolayers with 10 μM hesperetin with or without 10 μM of the respective flavonoids added to the apical side expressed as mean (±SD) percent of control (only 10 μM hesperetin)

	Apical			Basolateral		
	M1 (%)	M2 (%)	Sum (%)	M1 (%)	M2 (%)	Sum (%)
Acacetin	59.1±14.4[a)]	80.2±19.5[b)]	61.0±13.7[a)]	165±39[a)]	154±38[b)]	163±36[a)]
Biochanin A	64.0±11.7[a)]	143±34[a)]	75.5±9.4[a)]	160±51[c)]	171±30[a)]	159±38[a)]
(+)-Catechin	112±37	95.3±10.3	104±25	124±67	103±30	115±51
Chrysin	61.4±11.6[a)]	84.5±17.6[b)]	64.6±11.5[a)]	201±53[c)]	136±45	179±49[a)]
Daidzein	70.4±19.5[c)]	130±22[a)]	79.4±17.7[c)]	129±48	114±29	122±36
(−)-EGCG	115±39	94.1±11.5	107±27	105±53	90.5±28	99.3±42
(−)-Epicatechin	114±36	94.1±16.4	106±26	122±55	111±37	117±45
Galangin	47.6±6.8[a)]	111±33	56.2±6.2[a)]	140±37[c)]	190±46[c)]	151±55[c)]
Genistein	59.9±14.5[a)]	119±23[b)]	71.1±14.4[a)]	167±46[a)]	144±39[b)]	157±38[a)]
Hesperidin	94.4±11.7	101±13	95.5±11.7	95.2±22.6	88.3±24.6	93.4±21.7
Isorhamnetin	49.3±16.6[c)]	79.2±3.0[a)]	38.6±1.3[a)]	176±42[a)]	336±46[a)]	178±23[c)]
Kaempferide	51.0±15.8[a)]	94.0±20.8	55.7±12.6[a)]	164±51[c)]	227±61[a)]	175±43[a)]
Phloretin	91.0±21.5[b)]	78.1±13.8[c)]	86.7±16.0[c)]	120±39	120±51	120±43
Quercetin	38.0±11.8[a)]	80.6±52.8	43.2±15.3[a)]	202±77[a)]	235±92[a)]	202±76[a)]
Rutin	103±23	98.6±14.3	99.1±17.4	87.0±14.1[b)]	97.2±20.4	91.3±10.9

$n = 9$–11. M1 = hesperetin 7-*O*-glucuronide, M2 = hesperetin 7-*O*-sulfate.
a) $p < 0.001$ significantly different from the corresponding controls.
b) $p < 0.05$.
c) $p < 0.01$.

2.4 HPLC-DAD analysis

The HPLC system consisted of a Waters (Milford, MA, USA) Alliance 2695 separation module with autosampler connected to a Waters 2996 photodiode array detector and was equipped with an Alltech (Breda, the Netherlands) Alltima C18 5 μm 150 × 4.6 mm reverse phase column with 7.5 × 4.6 mm guard column. Before injection, samples were centrifuged at 16 000 × *g* for 4 min and 50 μL of the supernatant was injected and eluted at a flow rate of 1 mL/min. The gradient of the method to analyze the medium samples from the Caco-2 monolayer transport and metabolism experiments was reported previously [7]. The HPLC chromatograms of the medium from Caco-2 cell monolayers co-exposed with other flavonoids demonstrated no peak overlap of hesperetin or hesperetin metabolite peaks with other peaks resulting from the simultaneously added flavonoids, which was confirmed by analysis of medium from Caco-2 monolayers exposed only to the flavonoids used for co-administration (data not shown). All DAD spectra were recorded between 200 and 420 nm, and HPLC chromatograms acquired at 280 nm were used for quantification.

2.5 Quantification

Hesperetin and hesperetin 7-*O*-glucuronide were quantified by peak area measurement using calibration curves ($R^2 > 0.99$) of relevant concentration series of available reference compounds. The limit of detection was 0.02 μM, and the lower limit of quantification was 0.06 μM (injection volume 50 μL). Hesperetin 7-*O*-sulfate was quantified on the basis of the hesperetin 7-*O*-glucuronide calibration curve, since it demonstrated a comparable molar extinction coefficient and maximum absorption wavelength [7].

2.6 Statistics

The Student's two-tailed paired *t*-test was used to evaluate statistical differences between the hesperetin metabolite transport in Caco-2 monolayers exposed to hesperetin in the presence of another flavonoid and the control (only exposed to hesperetin) from the same transwell plate. Statistical differences in the experiment analyzing the intracellular content were evaluated using Student's two-tailed unpaired *t*-test. Differences were considered significant when *p*-values were less than 0.05. Values are expressed as mean and variances as SD.

3 Results

When hesperetin (10 μM) is incubated for 120 min with Caco-2 cell monolayers, it is efficiently metabolized into hesperetin 7-*O*-glucuronide and hesperetin 7-*O*-sulfate, of which the efflux is linear with time, and 28% of the initial amount of hesperetin aglycone can be recovered from the AP, BL and intracellular compartment [7]. On average (±SD) 1.73 (±0.48) nmol and 0.47 (±0.18) nmol of hesperetin 7-*O*-glucuronide were transported to the AP and BL side, respectively, and 0.34 (±0.24) nmol and 0.19

(±0.36) nmol of hesperetin 7-*O*-sulfate were transported to the AP and BL side, respectively (average values of all controls, $n = 18$).

On co-incubation with 10 μM of several of the tested flavonoids the efflux of hesperetin metabolites to the AP side of the Caco-2 cell monolayer decreased, whereas the transport to the BL side increased (Table 2 and Fig. 3). Co-incubation with hesperetin and especially flavonols, including galangin, isorhamnetin, kaempferide and quercetin, as well as with the flavones acacetin and chrysin, had the most pronounced effect. Significant effects were also demonstrated upon co-incubation of Caco-2 cell monolayers with hesperetin and the isoflavones biochanin A, daidzein and genistein. Interestingly, the AP efflux of specifically hesperetin 7-*O*-sulfate was increased instead of decreased by the co-administration of the isoflavones (Table 2). Co-incubation with hesperetin and the chalcone phloretin significantly decreased the AP efflux of hesperetin metabolites only by 13.3%, and co-incubation with flavanols including (+)-catechin, EGCG and (−)-epicatechin, as well as with the rutinosides hesperidin and rutin, did not affect the disposition of hesperetin metabolites under the applied experimental conditions (Table 2 and Fig. 3).

In the controls, the average BL/AP ratio of hesperetin metabolite transport was 0.36 (Fig. 4). Co-incubation of the Caco-2 cell monolayers with hesperetin and chrysin, isorhamnetin or quercetin increased this ratio above 1, which effectively reverses the predominant side of hesperetin metabolite efflux (Fig. 4), with quercetin being the most potent modulator (BL/AP ratio 1.7). However, co-administration of the flavonoids affecting the disposition of hesperetin metabolites also negatively affected the sum of amounts of hesperetin metabolite transported to the AP and BL sides (Fig. 5). The possible mechanism underlying this effect was investigated further for the experiments with co-administration with quercetin, which significantly ($p < 0.01$) decreased the total amount of hesperetin metabolites detected in the AP and BL side by 23% (Fig. 5). An additional experiment was performed in which the intracellular contents of the Caco-2 cell monolayers exposed to hesperetin or the combination of hesperetin and quercetin after 120 min was analyzed. Only small amounts (~3%) of the total amount of hesperetin metabolites detected were present inside the Caco-2 cells (Fig. 6). Although co-administration of quercetin showed a significant ($p < 0.05$) increase of 16% in the amount of hesperetin metabolites inside the cell, this amount accounts for only 5% of the total decrease of hesperetin metabolites detected extracellularly, demonstrating that the major part of the decrease (95%) in the total amount of hesperetin metabolites (0.69 nmol) is likely caused by inhibition of the phase II conjugation by co-exposure to quercetin. This is also reflected by a 70% higher total amount of hesperetin aglycone (0.68 nmol) (Fig. 6). The amount of 3.5 nmol of hesperetin and hesperetin metabolites recovered 120 min upon exposure in the AP, BL and intracellular compartments account for only 70% of the initial dose (5 nmol). At least part of the explanation for the residual loss of hesperetin 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 [7].

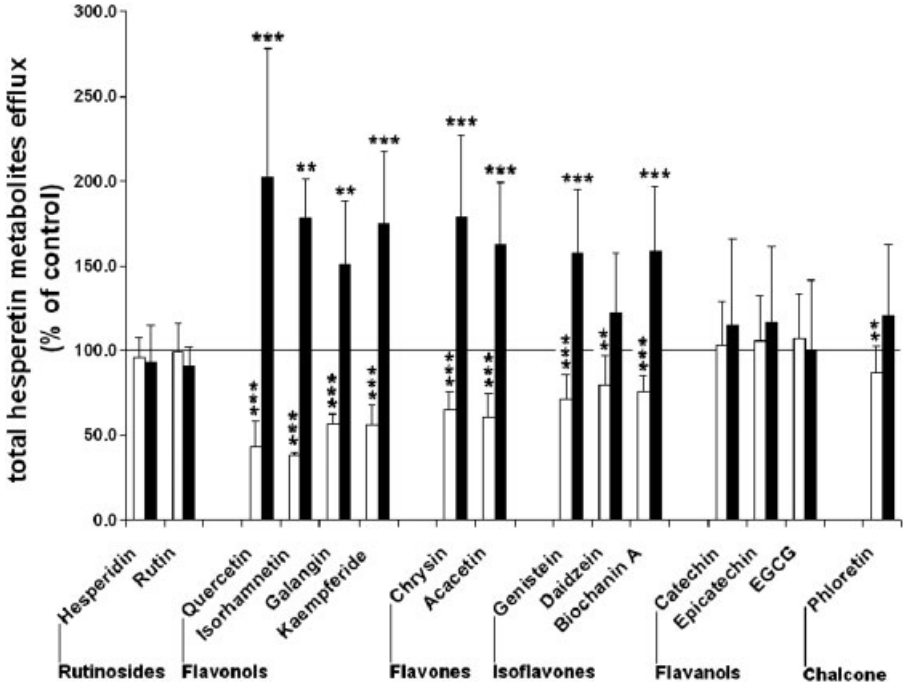

Figure 3. Effect of different flavonoids, grouped *per* class, on the AP (□) and BL (■) efflux of hesperetin metabolites (hesperetin 7-*O*-glucuronide plus hesperetin 7-*O*-sulfate) as percentage of the control, detected after 120 min incubation of Caco-2 cell monolayers with 10 μM hesperetin with or without 10 μM of the respective flavonoids added to the AP side. Mean ± SD values shown ($n = 9$–11). $^{**}p < 0.01$; $^{***}p < 0.001$ significantly different compared with the paired controls.

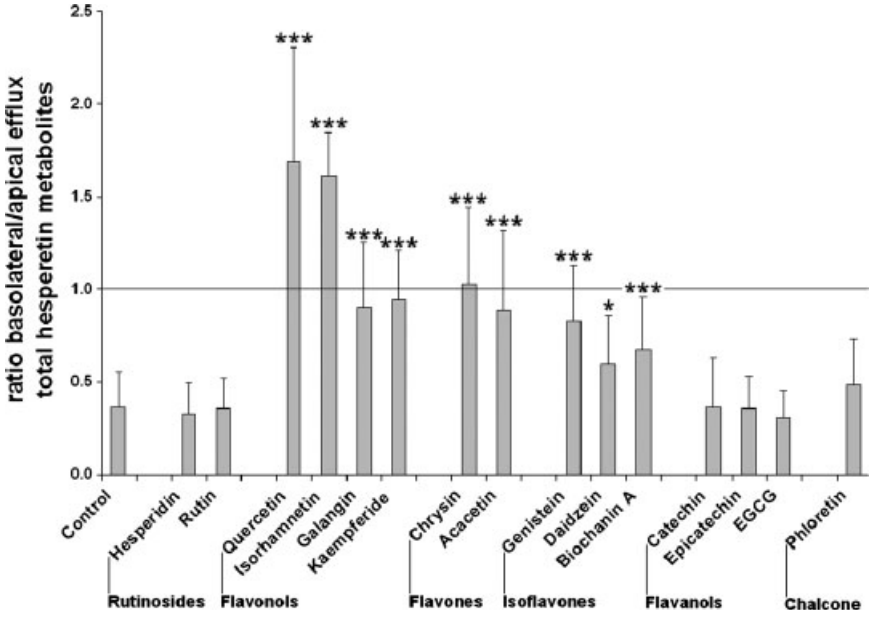

Figure 4. Effect of co-administration of different flavonoids, grouped *per* class, on the ratio BL/AP efflux of hesperetin metabolites (hesperetin 7-*O*-glucuronide plus hesperetin 7-*O*-sulfate), detected after 120 min incubation of Caco-2 cell monolayers with 10 μM hesperetin with or without 10 μM of the respective flavonoids added to the AP side. Mean ± SD values shown ($n = 9$–11, control $n = 18$). $^{*}p < 0.05$; $^{***}p < 0.001$ significantly different compared with the paired controls.

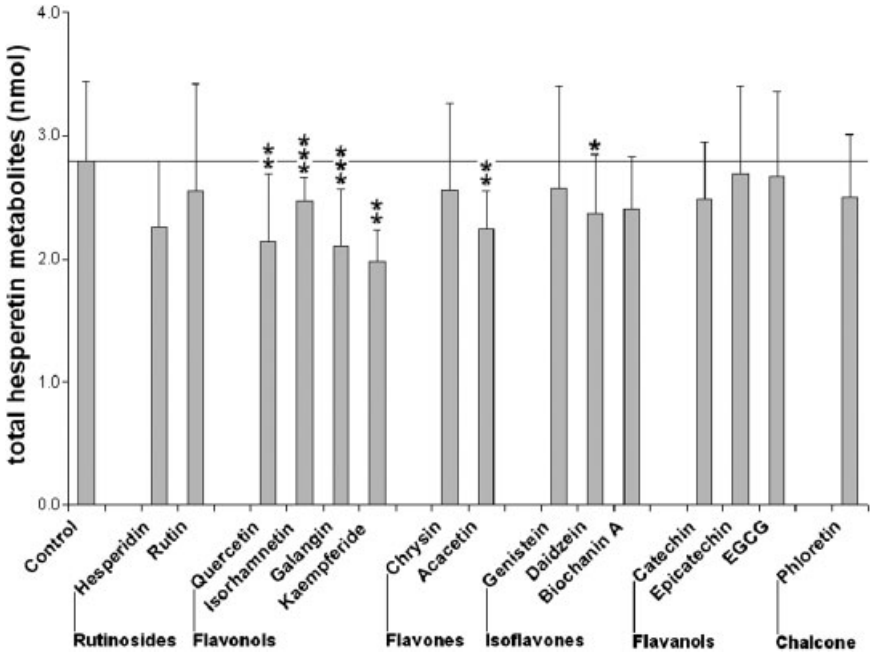

Figure 5. Effect of co-administration of different flavonoids, grouped *per* class, on the sum of hesperetin metabolites at the BL and AP side of Caco-2 cell monolayers, detected after 120 min incubation of Caco-2 cell monolayers with 10 μM hesperetin with or without 10 μM of the respective flavonoids added to the AP side. Mean ± SD values shown ($n = 9$–11, control $n = 18$). $^{*}p < 0.05$; $^{**}P < 0.01$; $^{***}p < 0.001$ significantly different compared with the paired controls.

4 Discussion

Metabolism by phase II enzymes and transport from intestinal cells back into the lumen by ABC transporters limit the bioavailability of flavonoids [4–7]. Recently, we demonstrated an important role for the ABC transporter BCRP in the efflux of hesperetin metabolites to the AP side of Caco-2 cell monolayers, an *in vitro* model of the intestinal barrier, by co-administration of standard BCRP inhibitors such as GF120918 and Ko143 [7]. The objective of this study was to identify whether this effect could also be achieved by co-administration of hesperetin with other flavonoids, since certain flavonoids were demonstrated to be potent inhibitors of BCRP (Table 1 and references therein), and also to define to what extent flavonoids may affect the phase II metabolism of hesperetin, representing the second mechanism that may influence the bioavailability of hesperetin [24].

Hesperetin is metabolized by Caco-2 cell monolayers into hesperetin 7-*O*-glucuronide and hesperetin 7-*O*-sulfate which are mainly transported to the AP side [7], and hesperetin partly migrates through the monolayer passively

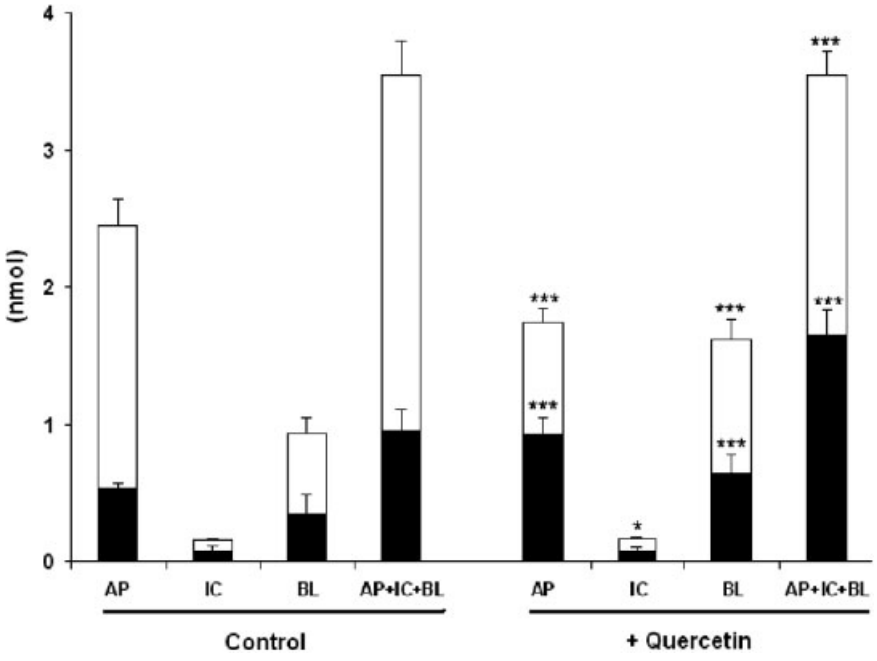

Figure 6. Effect of co-administration of quercetin (10 μM) on the amount of hesperetin metabolites (hesperetin 7-*O*-glucuronide plus hesperetin 7-*O*-sulfate) (□) and hesperetin aglycone (■) detected in the AP, intracellular (IC) and BL compartment of the Caco-2 cell monolayers, and the sum of amounts in these compartments (AP+IC+BL), detected after 120 min, compared with the Caco-2 cell monolayers only exposed to hesperetin (control). Mean+SD values shown ($n = 10$). $^{*}p < 0.05$; $^{***}p < 0.001$ significantly different compared with the control.

[7, 25, 26]. Co-administration of hesperetin with flavonoids, previously reported to inhibit BCRP *in vitro*, decreased the AP efflux of hesperetin metabolites and increased the transport to the BL side, while flavonoids from classes reported not to inhibit BCRP (flavanols, rutinosides) did not affect the disposition (Tables 1 and 2, Figs. 3 and 4). In general, the order of potency was flavonols > flavones > isoflavones. This order is in line with structure–activity relationships which have been proposed for flavonoid-mediated BCRP inhibition: the 2,3-double bond in ring C as well as ring B attached at position 2, are structural requirements for effective BCRP inhibition, although the lack of hydroxylation at position 3, as in flavones, not flavonols, has been defined as an important structural requirement as well [27–29]. The rutinoside hesperidin is probably not taken up by Caco-2 cells [25], although there is one report where rutin has been proposed to occur intracellularly in Caco-2 cells [26]. Moreover, rutin has been described to decrease the AP to BL transport of genistein through Caco-2 cell monolayers, albeit at relatively high concentrations of 50 and 150 μM rutin [30]. This effect might be due to an inhibitory effect of rutin on a BL transporter, which is in line with the slight decrease in the BL amount of hesperetin 7-*O*-glucuronide detected in our studies upon co-exposure to rutin (Table 2).

Under the applied experimental conditions in this study, that include the use of only a single dose, quercetin, isorhamnetin and chrysin were demonstrated to be the most potent modulator of hesperetin metabolite disposition, reversing the predominant side of efflux from the AP to the BL side (Fig. 4). Co-exposure to flavonoids that significantly modulated the BL/AP ratio also resulted in a decreased amount of hesperetin metabolites excreted from the cells (Fig. 5), but intracellular accumulation of hesperetin metabolites did not account for this decrease (Fig. 6). In fact, only a small amount of the total amount of hesperetin metabolites was detected in the cellular compartment, which indicates a high affinity of ABC transporters toward conjugates of hesperetin, a conclusion advanced previously to explain the negligible amounts of metabolites of the flavone baicalein in Caco-2 cells [31]. Since quercetin is also known to be metabolized by Caco-2 cells into glucuronidated and sulfonated metabolites [32, 33], a (competitive) inhibitory effect of quercetin on the phase II metabolism of hesperetin might be the reason for the 27% decrease in the total amount of hesperetin metabolites formed by the Caco-2 cells upon co-exposure with quercetin.

Specific flavonoids have been described as potent modulators of BCRP-mediated activity [22, 23], including hesperetin itself [27, 34–37], and specific combinations of flavonoids have demonstrated an additive effect on the inhibition of BCRP *in vitro* [37], although the exact mechanism of interaction is not precisely defined. Flavonoids and/or their metabolites could interact directly with BCRP-associated ATP-ase activity [34], however, as high-affinity substrates they probably also (competitively) inhibit BCRP-mediated transport. Since different flavonoids are substrates and/or modulators, interaction between different flavonoids may affect their respective bioavailability.

Inhibition of murine BCRP (Bcrp1) has been demonstrated to increase the bioavailability of total plasma quercetin while limiting the intestinal efflux of quercetin glucuronide metabolites [5], indicating a role for BCRP *in vivo* in the efflux of quercetin glucuronides to the intestinal lumen and bioavailability of total plasma quercetin. In a study on the bioavailability of biochanin A, a single oral

co-administration of biochanin A together with the combination of quercetin and EGCG did increase the bioavailability of biochanin A in Sprague–Dawley rats [38]. This could be explained by the fact that BCRP plays an important role in limiting the bioavailability of both quercetin [5] and biochanin A [39], and that both have been demonstrated to interact with BCRP [34, 36, 37]. In another study, a 3-wk period of co-administration of quercetin and (+)-catechin, the latter was reported not to interact with BCRP [27, 35], did not result in an increased bioavailability of both compounds in Wistar rats fed a diet containing (+)-catechin, quercetin or both [40]. Flavanols including (+)-catechin, (−)-epicatechin and EGCG and/or their metabolites, preferably interact with other classes of ABC transporters such as MRPs [41–43]. Modulation of the intracellular amount or bioavailability of EGCG has been successfully demonstrated in HT-29 cells and CF-1 mice, respectively, in combination with genistein [44], which could be explained by the fact that both compounds have been reported to be substrates of MRPs [41, 45].

In conclusion, the amount of hesperetin metabolites excreted to the BL side of the Caco-2 cell monolayer, representing the blood/plasma side of the intestinal barrier, was doubled upon co-exposure with micromolar concentrations of quercetin, in spite of the 27% reduction in phase II metabolite formation also resulting from the co-exposure with quercetin. Since the effect of quercetin on transport dominates over the effect on metabolism, co-administration of quercetin, or other specific flavonoids, could be a strategy to improve the limited bioavailability of hesperetin, which in turn could lead to an improved bioefficacy. Altogether, whether co-administration of other flavonoids could indeed increase the bioavailability of hesperetin *in vivo* remains to be elucidated and is currently under investigation in our laboratory.

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