RESEARCH ARTICLE

Flavone potently stimulates an apical transporter for flavonoids in human intestinal Caco-2 cells

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Received: June 13, 2012
Revised: July 25, 2012
Accepted: August 6, 2012

Scope: Based on the studies suggesting that active transport mechanisms contribute to the absorption of flavonoids into human intestinal Caco-2 cells, we here used the structurally similar fluorescent rhodamine 123 to test a possible influence of flavonoids on its uptake.
Methods and results: Rhodamine absorption displayed saturation kinetics with a K_m of 1.1 μM and a pH-optimum of 8.5 and was stimulated by flavone four-fold in its V_{max}. Ring C of the other 16 flavonoids tested turned out to be of special importance in order to act as potent inhibitors for rhodamine transport, with a positive charge there, as present in the anthocyanidins, or a 2,3 double bond together with an aromatic ring fused to position 2, as present in flavones and flavonols, being essential structural requirements. Flavone-stimulated rhodamine uptake was unaffected by classical substrates of organic cation transporters or inhibitors of adenosine triphosphate (ATP)-dependent efflux pumps. Also, inhibitors of mitogen-activated protein kinases or tyrosine kinases did not influence the transport, whose stimulation, however, was essentially dependent on the simultaneous presence of flavone. The existence of a flavone-activated apical flavonoid transporter in Caco-2 cells was finally associated with the potently diminished transepithelial apical to basolateral fluxes of ^{14}C-kaempferol in the presence of competing unlabeled flavonoid substrates.
Conclusion: In conclusion, flavone activates an as yet unidentified transporter for flavonoids in the apical membrane of Caco-2 cells.

Keywords:
ABC transporter / Caco-2 cells / Flavonoids / Intestinal absorption / Rhodamine 123

1 Introduction

Flavonoids are a class of secondary plant compounds that may be beneficial in the prevention of disease such as cancer and cardiovascular diseases [1, 2]. They are generally ingested in the form of their glycosides [3], but in the gastrointestinal tract, they are cleaved by beta-glucosidases and may be also lactase phlorizin hydrolase to release their lipophilic aglycones [4, 5]. It is generally believed that the aglycones are absorbed mainly by simple diffusion in a range between 0 and 60% [6, 7], whereas for selected glucose-associated molecules transport via the sodium-dependent glucose transporter SGLT-1 has been suggested [8, 9]. In human intestinal Caco-2 cells, providing an in vitro cell culture model for the simulation of the absorption process in the small intestine [10], however, absorption of flavanones was remarkably temperature-dependent and significantly reduced when cells were treated with amino acid modifying reagents [11]. These results suggest an active transport mechanism exists through the human intestinal epithelium and also for the flavonoid aglycones [11].

Based on the structural common characteristics of flavonoids consisting of an O-heterocyclic ring fused to an aromatic ring and by a carbon–carbon bond to a second aromatic ring (Fig. 1), we believed rhodamine 123, a fluorescent compound providing these features also (Fig. 1), to be

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Abbreviations: MAP, mitogen-activated protein; **MDR**, multidrug resistance; **MRP-2**, multidrug resistance associated protein-2; **OCT**, organic cation transporter; **P-gp**, phosphoglycoprotein-170; **TEA**, tetraethylammonium

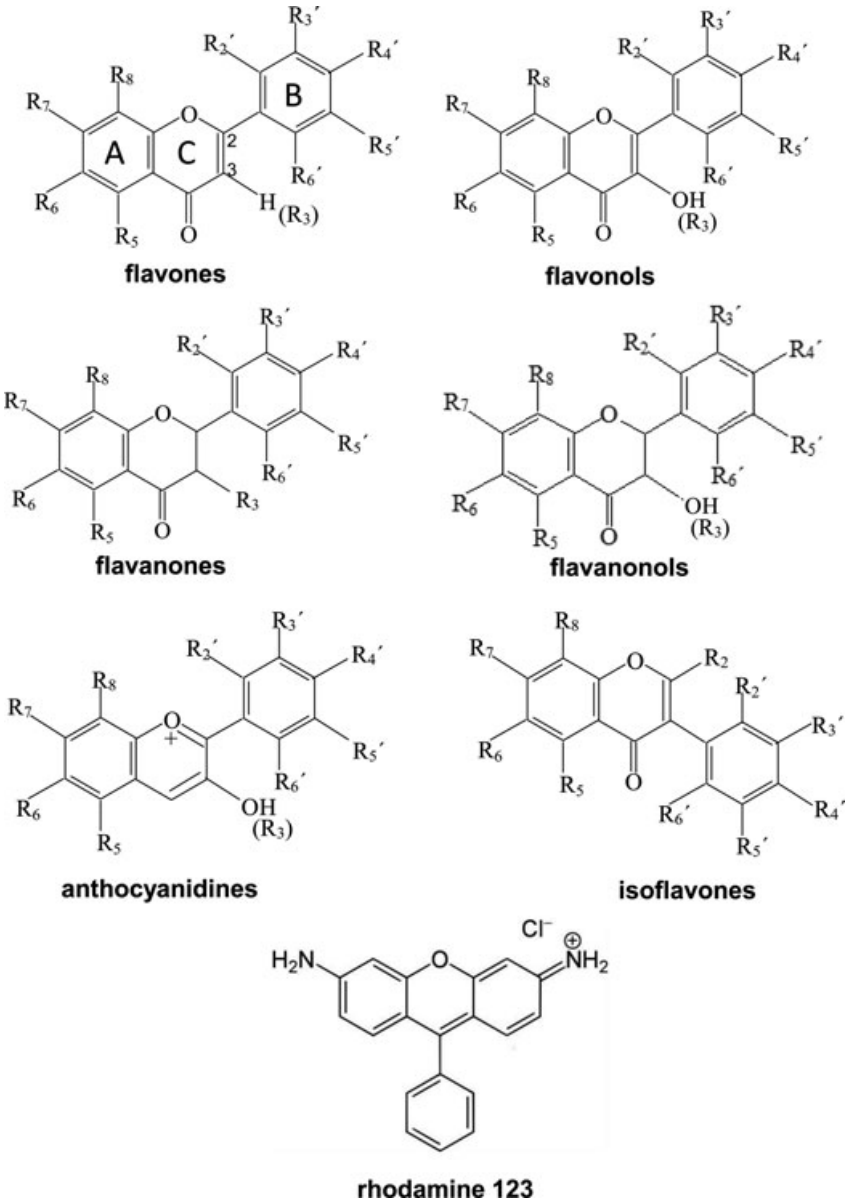

Figure 1. The basic structures of six main subclasses of flavonoids and of rhodamine 123. All flavonoid subclasses consist of an O-heterocyclic ring (C) fused to an aromatic ring (A) and by a carbon–carbon bond to a second aromatic ring (B). Classification of flavonoids is based on variations on the heterocyclic C-ring.

a potential competing substrate for the suggested flavonoid transporter. Although the transepithelial transport of rhodamine 123 in Caco-2 cells has been described to characterize phosphoglycoprotein-170 (P-gp) transport activities in many cases [12], we considered P-gp not a confounding factor for studying apical influx of rhodamine 123, because only apically directed rhodamine 123 transport was shown to be abolished by a P-gp inhibitor [13]. In contrast, P-gp-mediated efflux was not a barrier to rhodamine 123 absorptive transport across Caco-2 cell monolayers [13]. However, we observed in a former study an increased accumulation of rhodamine 123 in the presence of a P-gp inhibitor after extended incubation periods. Therefore the need of P-gp to reach adequate intracellular rhodamine concentrations could also determine its visibility [14]. Accordingly, we used short incubation times of only 30 min to characterize the apical influx of rhodamine 123. Thereafter, seventeen flavonoids were tested for their ability to interfere with the apical influx of rhodamine. Because rhodamine, according to its pK_a of 6.1 [13], at physiological pH values is partially positively charged, we considered organic cation transporters (OCTs) to contribute to absorption and tested classical substrates for interference. Finally, transgenic yeast, expressing recombinant, and partially purified key enzymes of flavonoid biosynthetic pathways, was used for the synthesis of ^{14}C-kaempferol from carbon-14 radiolabeled precursor substrates. The radiolabeled flavonoid enabled the characterization of transepithelial flavonoid transport as influenced by competing substrates and to evaluate the contribution of the identified transcellular transport route for flavonoids versus their paracellular fluxes.

2 Materials and methods

2.1 Materials

Flavonoids, verapamil and MK-571, tyrphostin 25, and PD98059 were purchased from Sigma (Deisenhofen, Germany). Fumitremorgin C was from VWR (Darmstadt, Germany). Cell culture plates, including ThinCert Transwell plates, and flasks were obtained from Greiner Bio-One (Frickenhausen, Germany). All other materials needed for cell culture and rhodamine 123 were purchased from Invitrogen (Karlsruhe, Germany). (2-^{14}C)Malonyl-CoA (53 mCi/mmol) was from Moravek Biochemicals (Brea, CA, USA) and *p*-Coumaroyl-CoA from TransMIT PlantMetaChem (Giessen, Germany).

2.2 Cell culture

Caco-2 cells (DSMZ, Braunschweig, Germany) were cultured and passaged in DMEM supplemented with 10% FCS, 2 mM glutamine, 1% MEM nonessential amino acids, and 70 μg/mL gentamycin in a humidified incubator at 37°C under an atmosphere of 5% CO_2. Cells between passages 15 and 45 were seeded at a density of 1×10^5 cells/well either onto 24 wells or ThinCert inserts and were used 7 days after reaching confluency. Fresh medium was given every second day and on the day before uptake measurements. For cells seeded onto ThinCerts transepithelial electrical resistance was measured with an epithelial voltmeter (EVOM, WPI, Berlin, Germany) and resistances of $\geq$300 Ω/cm^2 indicated the presence of an intact monolayer [15, 16].

2.3 Uptake of rhodamine 123 into Caco-2 cells from the apical compartment

Uptake studies in Caco-2 cells were performed in a modified Krebs buffer containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 0.3 mM KH_2PO_4, 10 mM glucose, and 10 mM HEPES-Tris for incubation buffer pH $\geq$ 7.4 or 10 mM MES-Tris for incubation buffer less than pH 7.4, respectively.

Subsequent to the incubation with rhodamine 123, cells were washed twice with ice-cold Krebs buffer and fluorescence of rhodamine 123 was measured at emission of 538 nm after excitation at 485 nm, using a microtiter plate reader (Fluoroskan Ascent, Thermo Electron, Dreieich, Germany).

2.4 Transepithelial transport of ^{14}C-kaempferol

Using the same buffer as described under Section 2.3 for the estimation of transepithelial fluxes of ^{14}C-kaempferol cell monolayers grown on ThinCert transwells were washed free of serum-containing medium and incubated from the apical side of the monolayer with 2.5 μM radiolabeled ^{14}C-kaempferol in the presence of 250 μM of unlabeled flavone and in presence or absence (control) of 250 μM of unlabeled kaempferol, morin, or pelargonidin for 30 min at 37°C. The basolateral side of the monolayers contained the same buffer of pH 7.4 without substrates. After the incubation period the cells were washed three times with ice-cold incubation buffer and the cells were subsequently digested with a tissue solubilizer. Cellular accumulation of ^{14}C-kaempferol was measured subsequent to the addition of scintillation cocktail by liquid scintillation spectroscopy. Fluorescein isothiocyante (FITC)-dextran was used as an extracellular marker to account for paracellular transport of kaempferol.

2.5 Synthesis of ^{14}C-kaempferol

Synthesis of ^{14}C-kaempferol was done using recombinant and partially purified proteins of chalcone synthase, chalcone isomerase, flavanone 3-beta-hydroxylase, and flavonol synthase as described [17,18]. The three-step synthesis procedure was initiated from *p*-coumaroyl-CoA and (2-^{14}C)-malonyl-CoA that were condensed in a preparative scale (50× scale-up) to yield (2*S*) ^{14}C-naringenin by the action of chalcone synthase and chalcone isomerase [17]. After 1-h incubation at 37°C, flavanone 3-hydroxylase and flavonol synthase were stepwise added to the synthesis together with the respective cofactors [18] following another incubation time for 1 h at 30°C for each, respectively. The products were extracted twice with 5 mL ethyl acetate. Extracts were concentrated, analyzed and purified on TLC. The final yield of ^{14}C-kaempferol is approximately 14 μCi ^{14}C-kaempferol (64% yields) with a specific activity of 99 μCi/μmol.

2.6 Calculations and statistics

All calculations were performed by using Prism 5.01 (Graph PAD, Los Angeles, CA, USA). For each variable, four to eight independent experiments were carried out. Data are given as the mean ± SD Variance analysis between groups was performed by one-way ANOVA and significance of differences between control and treated cells was determined using Tukey´s test.

3 Results

3.1 Apical rhodamine 123 influx into Caco-2 cells is saturable and pH dependent

Uptake of fluorescent rhodamine 123 into Caco-2 cells occurred linearly over the first 30 min (Fig. 2A). When measured over that time period, transport rates increased with increasing pH, reaching an optimum at pH 8.5 (Fig. 2B). Concentration-dependent kinetics revealed the inclusion of

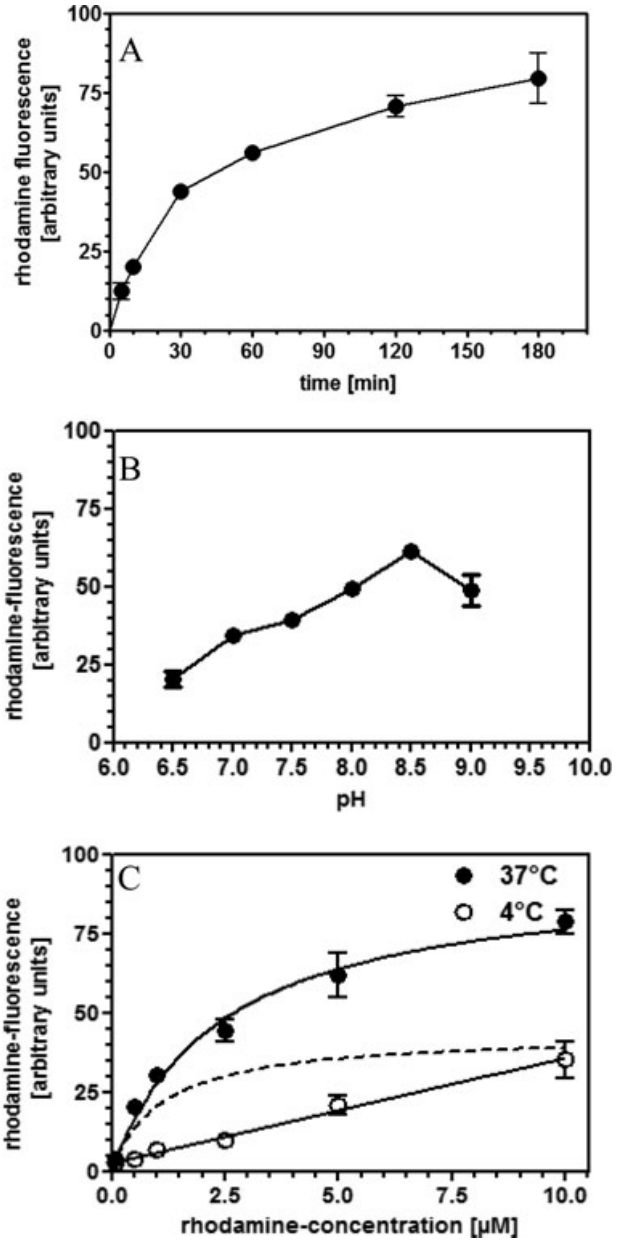

Figure 2. Uptake of rhodamine 123 into Caco-2 cells from the apical side is saturable and pH-dependent. Transport of rhodamine 123 into Caco-2 cells was quantified by measuring the fluorescence of cells using a microtiter plate reader. (A) Time-dependent kinetics was recorded over 3 h using 2.5 μM rhodamine at pH 7.4. Dependence of transport, as measured over 30 min at 2.5 μM rhodamine, on pH is given in (B). Absorption as affected by increasing rhodamine concentrations at pH 7.4 is shown in (C). The stippled line denotes the resultant kinetics after subtracting the diffusion (4°C) from the overall transport (37°C).

saturable and diffusional processes for overall transport (Fig. 2C). Extrapolating the transporter-mediated kinetics by subtracting the diffusion rates from overall transport revealed a K_m of 1.1 ± 0.8 μM (Fig. 2C).

3.2 Flavone-stimulated rhodamine transport is inhibited by other flavonoids

Based on the structural similarities between rhodamine 123 and flavonoids, we tested 17 flavonoids from the flavone (flavone, chrysin, apigenin, and luteolin), flavonol (kaempferol, morin, fisetin, quercetin, and myricetin), flavanone (naringenin), flavanonol (taxifolin and 2,3-dihydrokaempferol), isoflavone (biochanin A, daidzein, and genistein), and anthocyanidin (delphinidin and pelargonidin) subgroups regarding their effects on rhodamine uptake. Except for the basic structure flavone, all tested flavones, flavonols, and anthocyanidins inhibited the influx of rhodamine significantly, whereas taxifolin, 2,3-dihydrokaempferol, naringenin, and the isoflavones displayed no influence (Fig. 3A). Flavone-stimulated uptake of rhodamine is more than three-fold (Fig. 3A). In the simultaneous presence of flavone, an almost identical inhibition pattern was achieved for rhodamine transport by the other flavonoids (Fig. 3B). The stimulation of rhodamine accumulation was dose-dependently increased by flavone, starting with a significant increase of rhodamine accumulation at 100 μM flavone ($p < 0.05$) (Fig. 3C). Flavone affected the V_{max} of transport without a significant influence on the affinity of the transporter for rhodamine (Fig. 3D).

3.3 Flavone-stimulated rhodamine transport into Caco-2 cells is not dependent on Na^+ or Cl^- ions or transport activities of MDR transporters or OCT

In order to investigate the ion requirements for flavone-stimulated rhodamine transport, potentially co-transported ions were exchanged in the medium. Na^+ and Cl^- ions revealed to be completely dispensable for rhodamine transport in the presence of flavone (Table 1). Because rhodamine has been described as a P-gp substrate and moreover, ATP-binding cassette transporters relevant for multidrug resistance (MDR) could be blocked by flavone, we tested their contribution to the observed increased rhodamine accumulation. Inhibition of breast cancer related protein by fumitremorgin C displayed no influence at all on rhodamine transport in the presence of flavone (Table 1) or its absence (data not shown). Inhibition of P-gp by verapamil or multidrug resistance associated protein-2 by MK-571 increased rhodamine accumulation in the absence and also in the presence of flavone (Table 1), demonstrating that flavone-induced rhodamine accumulation is not caused under the conditions described by the blockade of these three ATP-binding cassette transporters. Because rhodamine 123 is an organic cation, we finally tested the contribution of OCT to the cellular rhodamine increase caused by flavone. Typical competitive inhibitors of OCT were without significant effects on flavone-stimulated rhodamine transport (Table 1).

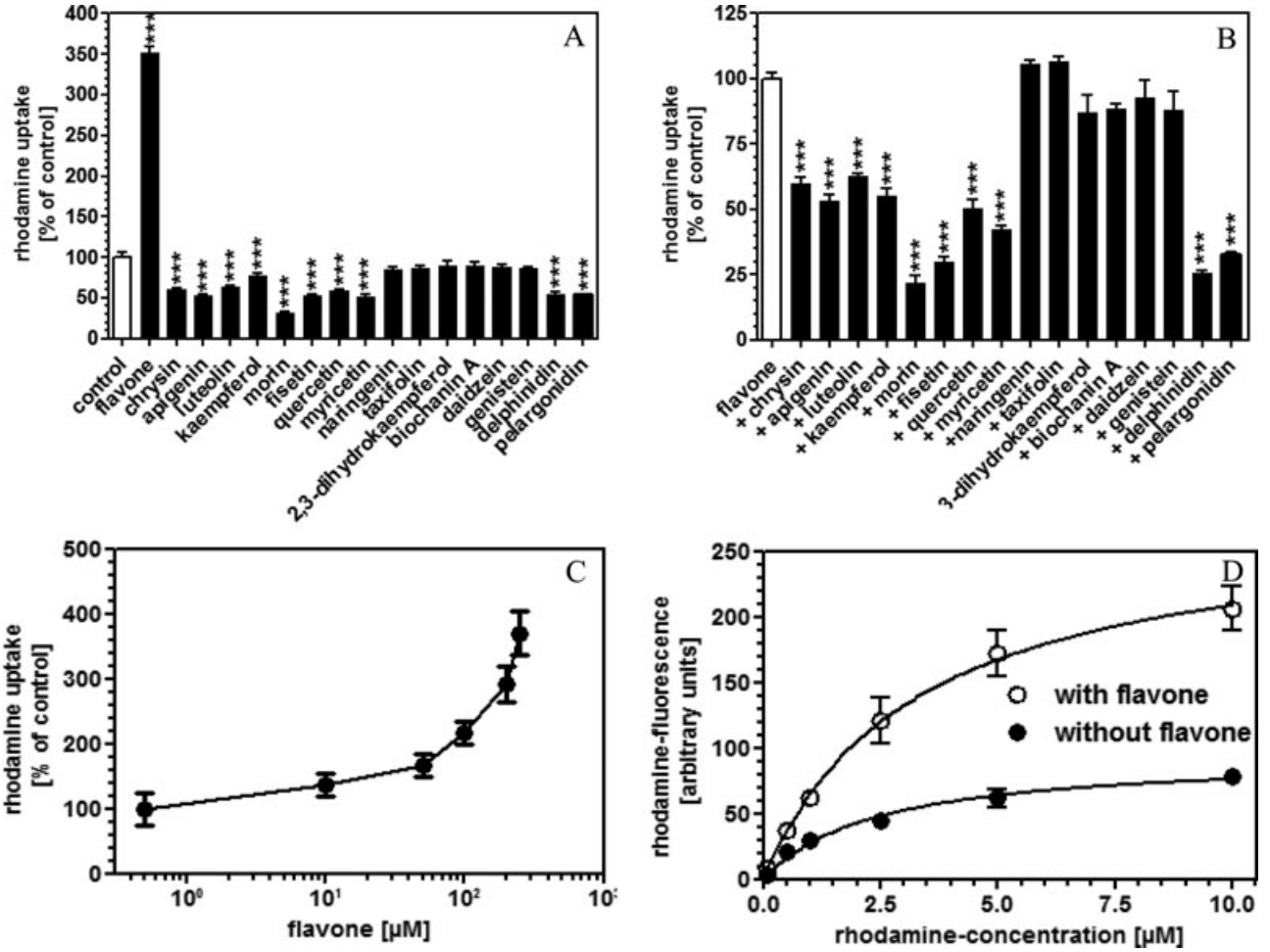

Figure 3. Flavone-stimulated uptake of rhodamine is inhibited by flavones, flavonols, and anthocyanidins. (A) Uptake of 2.5 μM rhodamine 123 was measured for 30 min at pH 7.4 in the presence of 250 μM of flavonoids. Rhodamine uptake in the control was set to 100%. The inhibition of flavone-stimulated rhodamine uptake by the same flavonoids is given in (B). Transport rates of rhodamine are dependent upon flavone concentrations and are shown in (C). Determination of concentration-dependent rhodamine transport in the presence and absence of flavone revealed an increase in V_{max} whereas K_m was not changed by the flavonoid (2.3 ± 0.4 μM in the absence and 3.2 ± 0.7 μM in the presence of flavone; data are uncorrected for diffusion) (D). ***$p < 0.001$ versus the control in (A) or flavone-stimulated transport in (B).

3.4 Flavone does not affect the expression of rhodamine transporters in the cell membrane nor does it act through MAP kinase or tyrosine kinase pathways

To find out whether flavone increases the number of transporters, cells were preincubated for 3 h with 250 μM flavone before they were washed and uptake of rhodamine was measured (in the absence of flavone). Under these circumstances flavone was no longer able to increase the transport of rhodamine (Table 2). Also did the inhibition of vesicular trafficking by colchicine, an inhibitor of the cytoskeleton, display any effect on flavone-stimulated rhodamine transport (Table 2). The inhibition of mitogen-activated protein (MAP) kinase pathways or tyrosine kinase pathways also had no impact on the increased rhodamine influx under flavone (Table 2).

3.5 Anthocyanidins are efficient inhibitors of the rhodamine transporter

For three selected flavonoids displaying inhibitory effects on rhodamine transport, i.e. kaempferol, morin, and pelargonidin, we performed Dixon plots to reveal whether the interactions are competitive and what the affinities of the inhibitors are. Dixon plots showed that kaempferol and pelargonidin competitively interacted with rhodamine transport with K_i-values of 104.7 μM and 99.4 μM, respectively (Fig. 4). Morin, on the other hand, displayed a noncompetitive mode of

Table 1. Flavone-stimulated rhodamine uptake into Caco-2 cells remains unaffected by Na^+- or Cl^--ions, and also by inhibitors of MDR transporters or OCT

Modification	% of flavone-stimulated rhodamine uptake	Significance
Exchange of ions		
KCl (for Na^+ salts)	95.3 ± 6.4	
LiCl (for Na^+ salts)	105.2 ± 6.5	
NaSCN (for Cl^- salts)	110.7 ± 14.3	
Inhibitors of MDR transporters		
Verapamil (40 μM)	145.3 ± 8.7	$p < 0.001$
MK-571 (50 μM)	490.3 ± 22.0	$p < 0.001$
Fumitremorgin C (10 μM)	103.9 ± 4.2	
Inhibitors of OCT		
Guanidine (250 μM)	96.3 ± 5.1	
1-Methyl-4-phenylpyridinium (250 μM)	104.5 ± 4.6	
TEA (250 μM)	90.7 ± 12.3	

Rhodamine was applied at 2.5 μM and flavone at 250 μM. In the absence of flavone only verapamil ($p < 0.05$) and MK-571 ($p < 0.05$) stimulated rhodamine accumulation significantly (not shown).

Table 2. Flavone-stimulated rhodamine uptake is not mediated by the expression of rhodamine transporters or activation through MAP or tyrosine kinase pathways

Modification	% of flavone-stimulated rhodamine uptake	Significance
Preincubation (3 h)		
Flavone (250 μM)	35.1 ± 5.5	$p < 0.001$
Inhibitor of vesicular trafficking		
Colchicine (100 μM)	79.6 ± 13.7	
Inhibitor of MAP kinases		
PD 98059 (40 μM)	102.2 ± 3.5	
Inhibitor of tyrosine kinases		
TEA (250 μM)	90.0 ± 9.4	

None of the interventions affected the uptake of 2.5 μM rhodamine in the absence of flavone.

interaction as indicated by the convergence of the lines on the x-axis of a Dixon plot (Fig. 4).

3.6 Flavonoids inhibit potently the transcellular and transepithelial transport of ^{14}C-kaempferol

Kaempferol, morin, and pelargonidin at concentrations of 250 μM, all three potently inhibited transcellular fluxes when 5 μM ^{14}C-kaempferol was applied in the presence of 250 μM flavone from the apical compartment (Fig. 5). Taking into account that the apical to basolateral paracellular diffusion, as estimated by FITC-dextran appearing in the basolateral compartment, was 104.4 ± 6.5 pmol/cm^2 h, this result means that fluxes of ^{14}C-kaempferol occur predominantly via the transcellular route and were almost completely blocked by an excess of unlabeled flavonoids under conditions where the apical rhodamine transporter is activated by flavone.

4 Discussion

Flavonoids are a class of secondary plant compounds that are suggested to have beneficial health effects such as the prevention of atherosclerosis or certain types of cancer [1,2]. Accordingly, their bioavailability is of importance in order to reach adequate concentrations at their site of action [19]. Although there are considerable differences among the different types of dietary flavonoids, the site of main absorption appears to be the small intestine [6]. Nevertheless, substantial amounts of applied flavonoids or their metabolites appear in ileal fluid of ileostoma patients [20], indicating that these compounds also pass to the large intestine where they are subject to microbial metabolism. In the small intestine flavonoids, being enzymatically released from their glycosides, appear to be absorbed predominantly by simple diffusion [7,21]. On the other hand, various flavonoids have been described in human intestinal Caco-2 cells to interfere with facilitated glucose transport by GLUT-2 [22,23] or monocarboxylate transport by MCT-1 [24], although it must be questioned whether those transporters contribute substantially to the absorption of flavonoids, if at all [25]. However, active transport of flavonoids cannot be neglected per se, since absorption of some flavonoids was demonstrated to be temperature-dependent, clearly indicating the contribution of proteins to the transport process [11].

In the present study, we searched for a flavonoid transport system in human intestinal Caco-2 cells, by using the fluorescent substrate rhodamine 123, which displays a structure similar to flavonoids, and by assessing the interference of unlabeled flavonoids with rhodamine uptake. Transport of rhodamine via the apical membrane of Caco-2 cells is controversial in as far as it was described not to be affected by P-gp transport activity [13], whereas others, including ourselves, showed that inhibition of P-gp increases the accumulation of rhodamine [14,26]. In the study presented here, we show that initial uptake of rhodamine 123 during the first 30 min of apical exposure is increased 3.5-fold by flavone and occurs in a saturable, pH- and temperature-dependent manner with Michaelis–Menten kinetics. Although these results could be explained by the inhibition of P-gp as caused by flavone, we believe the characterized rhodamine uptake to be largely independent of P-gp, because accumulation as affected by P-gp requires a longer time of incubation in order to become visible [27] and the P-gp inhibitor verapamil does not further increase rhodamine accumulation after 3 h [27], whereas it does at 30 min. Moreover, chrysin was shown formerly to inhibit ATP-dependent drug-efflux pumps such as P-gp [14], whereas it reduced the accumulation of rhodamine when measured at 30 min of incubation.

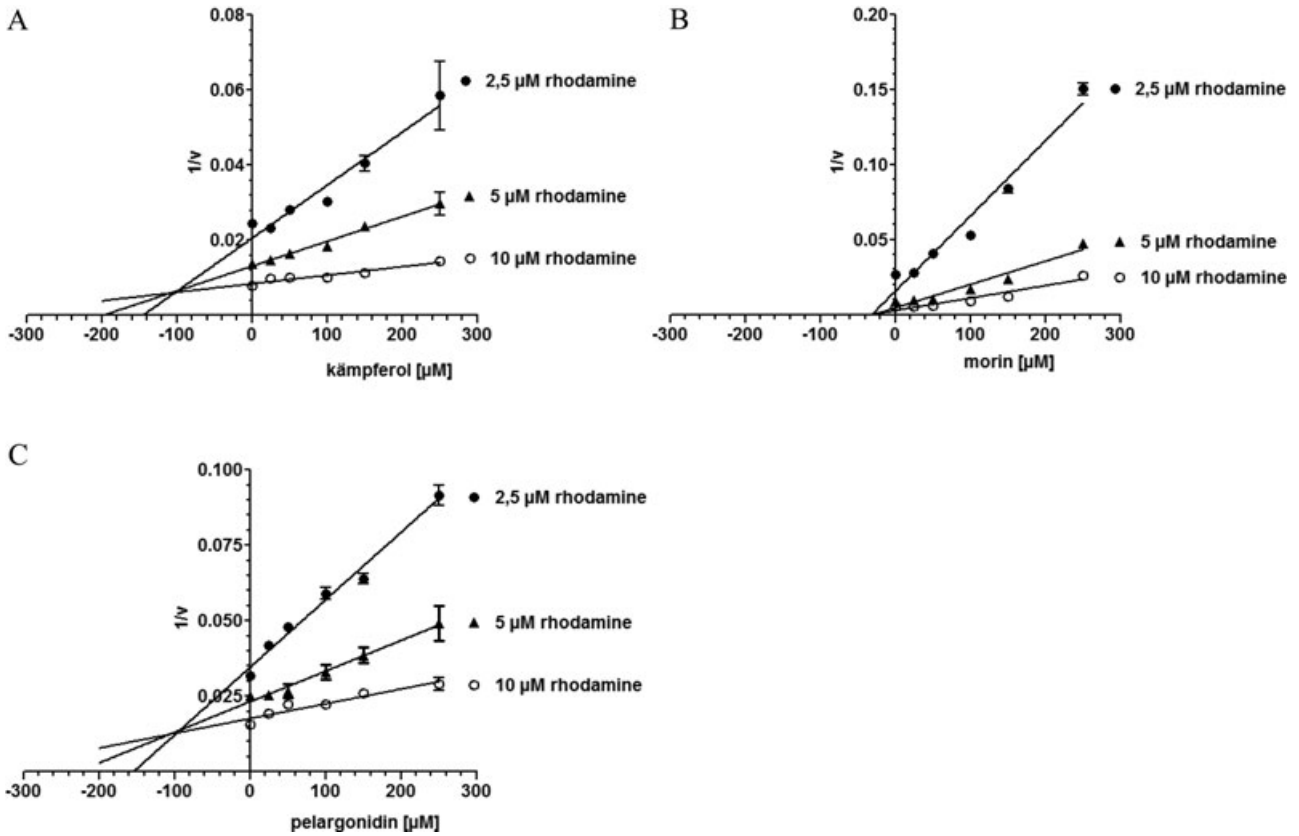

Figure 4. Dixon plots for inhibition of rhodamine uptake by flavonoids. Rhodamine 123 uptake into Caco-2 cells was measured at three different concentrations in the presence of increasing concentrations of either kaempferol (A), morin (B), or pelargonidin (C). Reciprocal values for uptake are depicted over the concentrations of flavonoid inhibitors. Crossing of the kinetics in the left quadrants of the graphs point to a competitive mode of interaction.

Of the 17 flavonoids tested, only flavone stimulated the apical uptake of rhodamine into the Caco-2 cells. Representatives of the flavanone, flavanonol, and isoflavone subgroups were all without effect on rhodamine uptake in the presence or absence of flavone. Those of the flavone (except flavone itself), flavonol, and anthocyanidin subgroups, all significantly inhibited rhodamine absorption. The most potent inhibition was achieved by the flavonol morin, although the type of inhibition appears to be noncompetitive according to the results of the Dixon plot [28]. The potent competitive inhibition by kaempferol clearly indicates that a positive charge, a characteristic of rhodamine 123 and anthocyanidins such as pelargonidin, is not an essential requirement to interact with the rhodamine transporter. However, if the positive charge is missing, a double bond between positions 2 and 3 of ring C, as present in flavones and flavonols, becomes absolutely essential for an affinity toward the rhodamine transporter. This is at best exemplified by the potent inhibition achieved by kaempferol, whereas 2,3-dihydrokaempferol does no longer interfere with rhodamine transport. If the 2,3 double bond is present a carbon–carbon bond at position 2 of ring C becomes an additional requirement for interference of flavonoids with rhodamine transport, as evidenced by the lack of inhibition in case of isoflavones.

The fact that the anthocyanidins and rhodamine, both with a positive charge, compete for uptake into Caco-2 cells led us to suggest that members of the OCT family of transporters might be responsible for flavonoid absorption. Moreover, selected flavonoids without a positive charge have been shown to interact with OCT in Caco-2 cells [29]. Prototypical organic cations displayed similar inhibitory potency on N-[methyl-3H]4-phenylpyridinium uptake in Caco-2 cells as compared to CHO-hOCT3 cells, suggesting hOCT3 to be the predominant apical transporter for organic cations in Caco-2 cells [30]. However, the independence on sodium and chloride as well as the missing inhibitory effects of classical OCT-substrates, such as 1-methyl-4-phenylpyridinium, tetraethylammonium (TEA) or guanidine [29–31] exclude the contribution of OCT to rhodamine uptake under the applied conditions. Finally, MCT-substrates such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid or alpha-cyano-4-hydroxycinnamic acid [32] were tested based on the

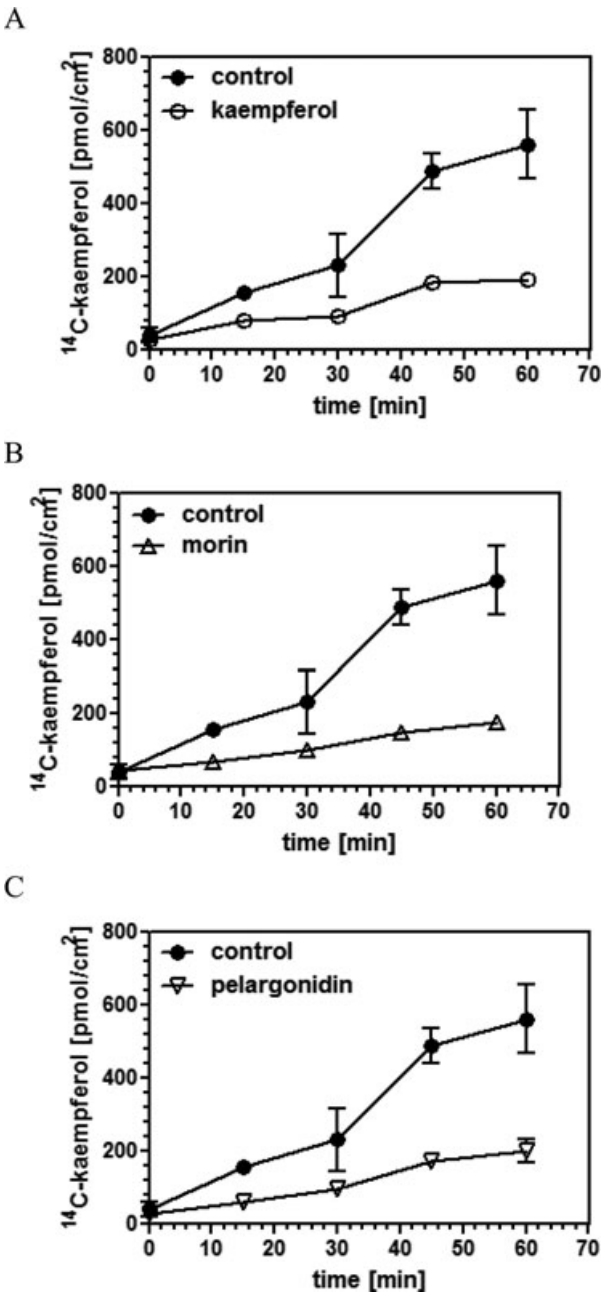

Figure 5. Transepithelial transport of ^{14}C-kaempferol is inhibited by flavonoids. The uptake of 2.5 μM ^{14}C-kaempferol was measured in the presence of 250 μM flavone from the apical to basolateral compartment using ThinCert transwells. Flux via the paracellular route was estimated using FITC-dextran as an extracellular marker. Together with the radiolabeled kaempferol 250 μM of unlabeled (A) kaempferol, (B) morin, or (C) pelargonidin were applied.

findings that showed interference of flavonoids with such transporters [24] but were also without effect at concentrations of 250 μM (data not shown).

Because neither preincubation with flavone increased rhodamine uptake nor inhibitors of vesicular trafficking affects the transport, it was plausible to conclude that flavone leads to interconversion of the rhodamine transporter and thereby increases its transport capacity. However, contribution of classical MAP kinase or tyrosine kinase triggered pathways can be excluded by the absence of effects through inhibitors. Irrespective of the underlying mechanism, the data suggest that the characterized rhodamine transporter is able to transport a number of flavonoids carrying the described structural requirements. This was finally proven by the use of ^{14}C-labeled substrate kaempferol, whose transport from the apical to the basolateral site was potently inhibited by excess of unlabeled flavonoids competing with ^{14}C-kaempferol for uptake via the apical membrane.

In conclusion, our studies provide the evidence for a flavonoid transporter in the apical membrane of human intestinal Caco-2 cells that is potently activated by flavone through an as-yet-unidentified mechanism of interconversion.

This work was financially supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG), Grant numbers: MA 2476/6-1 and 6-2 to S. Martens and WE 2684/7-1 and 7-2 to U. Wenzel.

The authors have declared no conflict of interest.

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