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Transport mechanisms of flavanone aglycones across Caco-2 cell monolayers and artificial PAMPA membranes

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Caco-2; flavanone; PAMPA; pH-partition hypothesis

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Abstract

Objectives We recently reported that flavanone aglycones (hesperetin, naringenin and eriodictyol) are efficiently absorbed via proton-coupled active transport, in addition to transcellular passive diffusion, in Caco-2 cells. Here, we aimed to evaluate in detail the absorption mechanisms of these flavanones, as well as homoeriodictyol and sakuranetin.

Methods We evaluated the absorption mechanisms of the above compounds by means of in vitro studies in Caco-2 cells in parallel with an artificial membrane permeation assay (PAMPA) under pH-gradient and iso-pH conditions.

Key findings Comparison of the permeability characteristics of flavanones in Caco-2 cells and in PAMPA under these conditions, as well as a consideration of the physicochemical properties, indicated that hesperetin, naringenin, eriodictyol and homoeriodictyol were efficiently transported by passive diffusion according to the pH-partition hypothesis, except in the case of sakuranetin. However, transport of all flavanones were remarkably temperature-dependent, and was significantly reduced when Caco-2 cells were treated with amino acid-modifying reagents.

Conclusions Our data confirm that both passive diffusion and an active transport mechanism contribute to flavanone absorption through human intestinal epithelium.

Introduction

Flavonoids are a subclass of polyphenol compounds found ubiquitously in edible plants. Epidemiological studies have indicated that flavonoid intake is associated with reduced incidence of diseases such as cancer, cardiovascular disease and other age-related diseases.[1,2]

Flavonoids are commonly present as glycosides in intact plants. Several in vivo studies have shown that orally administered flavanone glycosides are hydrolysed by intestinal microflora and subsequently absorbed as their aglycones;[3,4] the active species are almost always aglycones.[3,5] The aglycone form is more readily absorbed than complex glycosides.[6] On the other hand, enzymatic glucosyl conjugation, which is expected to increase water solubility, has been frequently applied to flavonoids to enhance their bioavailability.[7] Although flavonoids may have limited bioavailability due to their glycoside moiety,[5] a considerable number of publications have characterized transport activity mainly by using glycosylated forms.[5,6] Therefore, an understanding of the factors determining the bioavailability of flavonoid aglycones, and in particular their absorption mechanisms, is still needed for setting efficacious and safe levels of flavonoid intake.

For a long time, flavonoid aglycones were considered to be absorbed via passive diffusion.[8] However, we recently showed that, in Caco-2 cells (a human colon adenocarcinoma cell line), flavanone aglycones (hesperetin, naringenin and eriodictyol) are efficiently absorbed via proton-coupled active transport in addition to transcellular passive diffusion.[9,10] Apically loaded sodium azide, an ATPase inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone, a proton ionophore, or benzoic acid, an monocarboxylic acid transpor substrate, decreased the permeability of flavanone aglycones in the presence of a proton gradient.[9,10] Chabane *et al.* have indicated that naringenin seemed to be transported by the

multidrug resistance associated protein 1.[8] However, passive transport of weakly acidic compounds is also inhibited by addition of organic acids, including benzoic acid,[11] so the evidence for a specific transporter remains insufficient. Srirangam and Majumdar have reported pH-dependent passive transport of hesperetin across isolated rabbit cornea.[12] Neuhoff *et al*. reported that the pH-dependent acidic drug transport ratio is influenced by both passive and active transport.[13] Furthermore, they pointed out that pH-dependent transport in Caco-2 cell monolayers can be misinterpreted as active drug transport. Under in vivo conditions, the sodium/proton antiporter at the brush-border membrane can generate a proton gradient sufficient to facilitate the transport of some organic acids,[14] and it has been suggested that weakly ionic drugs would be absorbed at the intestine by passive diffusion according to the pH-partition theory, because rapid permeation would be facilitated by the gradient of permeable protonated forms between the extracellular and intracellular compartments.[11] It is well known that physicochemical properties, such as lipophilicity, hydrogen bonding, p*K*a and molecular weight, influence passive permeation. However, flavanones have not been investigated in this regard. Such proton gradient-dependent facilitated transport is difficult to distinguish from active transport and simple diffusion. Therefore, we considered that comparison of data from a Caco-2 cell-based assay and a passive diffusion permeability assay would provide further insight into the mechanisms of intestinal absorption of flavonoids.

The parallel artificial membrane permeability assay (PAMPA) was developed by Kansy *et al*. in 1998.[15] PAMPA consists of hydrophobic filters coated with lecithin in an organic solvent solution. It is a quick and simple test to measure passive permeability and has been used for the prediction of oral absorption and blood-brain barrier penetration.[16]

The aim of this study was to clearly establish the nature of the absorption mechanisms of flavanones hesperetin, naringenin and eriodictyol, together with homoeriodictyol and sakuranetin, by means of evaluation of passive and active transport mechanisms using PAMPA and Caco-2 cells under pH-gradient and iso-pH conditions. We show that the relationship between LogD and permeability in PAMPA or Caco-2 is useful to define the absorption mechanisms of flavanones.

Materials and Methods

Chemicals

The structures of flavanones and test drugs are presented in Figure 1. Paracetamol (acetaminophen), antipyrine and

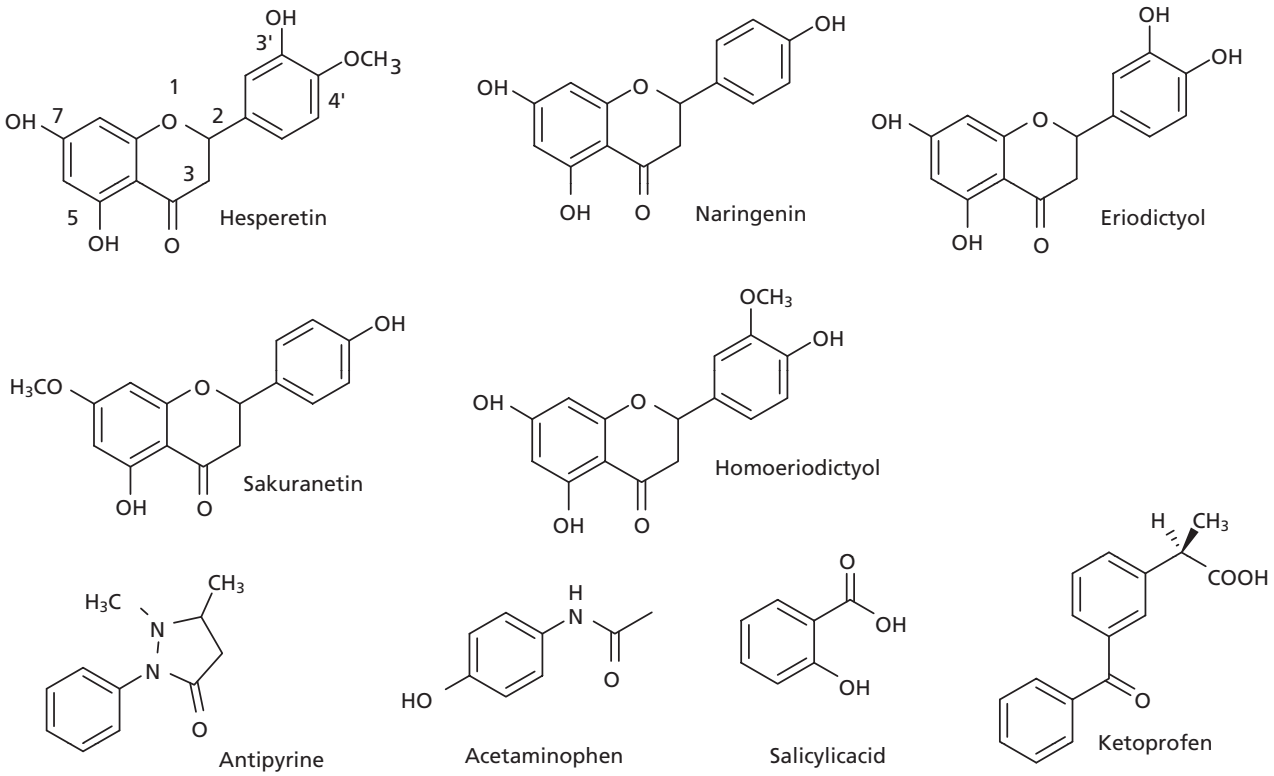

Figure 1 Chemical structures of flavanones and test drugs.

ketoprofen were purchased from Wako Pure Chemicals Inc., Ltd (Osaka, Japan). Hesperetin, naringenin and salicylic acid were from Alfa Aesar (Ward Hill, USA), Sigma (St Louis, USA) and Kanto Chemical Co., Inc. (Tokyo, Japan), respectively. All other chemicals used in this study were of analytical grade.

Calculation of physicochemical properties

The Marvin Sketch program (http://www.chemaxon.com/products/marvin) was used to evaluate the ionization profiles, p*Ka* values, LogPs, LogDs, ionization curves and ionisable OH groups present in each molecule; the results are summarized in Table 1.

Theoretical partition ratio according to the pH-partition hypothesis

The concentration ratio of an acidic compound at the gut lumen/plasma, C_1/C_2, is given by the following relation for a weak acid:

$$C_2/C_1 = \left(1+10^{pH_2\text{-}pKa}\right)/\left(1+10^{pH_1\text{-}pKa}\right) \quad (1)$$

Permeability studies through artificial membranes

Permeability studies through artificial membranes (PAMPA) studies were done according to the Millipore Protocol Note (Lit. no: PC040EN00). This model consists of a 96-well microtitre plate, which serves as the donor chamber, and a 96-well filter plate (MultiScreen-IP, Millipore), which serves as the acceptor compartment. The acceptor plate, placed directly on the donor plate, is fitted with a hydrophobic microfilter disc (Immobilion-P membrane, 0.45 μm), impregnated with a phospholipid solution. In these tests, 5 μl of a 1% (w/v) solution of soy lecithin in dodecane was added to each well as a phospholipidic membrane. Buffers of pH 6.0 or 7.4 were prepared from pH 7.0 phosphate buffer (28 mM KH_2PO_4 and 41 mM Na_2HPO_4), which was adjusted to the required pH value with NaOH (pH 7.4) or H_3PO_4 (pH 6.0). Assays for each of the samples were performed under two conditions: with a pH gradient between the acceptor and the donor reservoirs, and with the same pH in both compartments. In both assays, the concentration of the samples was 500 μM; the solutions were prepared by dilution of stock solutions, and all contained 5% dimethyl sulfoxide (DMSO). After 16 h the two plates were separated, and the acceptor solutions were analysed with an HPLC-electrochemical detector (ECD) using an ESA coulometric detection system (ESA Inc., Boston, USA) or HPLC-UV system (JASCO, Tokyo, Japan). The permeability coefficient through the artificial membrane, $\mathrm{Log}P_{\text{app-PAMPA}}$, was calculated using Equation 2.

$$\mathrm{Log}P_{\text{app-PAMPA}} = \mathrm{Log}\left\{C\cdot\text{-}\ln\left(1\text{-}[\mathrm{drug}]_{\text{acceptor}}/[\mathrm{drug}]_{\text{equilibrium}}\right)\right\},$$
$$\text{where } C = \left(V_D\cdot V_A/(V_D+V_A)\,\mathrm{Area}\cdot\mathrm{time}\right) \quad (2)$$

In this equation, V_D (cm^3) is the donor volume (0.15 cm^3), V_A (cm^3) is the volume of the acceptor compartment (0.30 cm^3), Area (cm^2) is the accessible filter area (0.24 cm^2), time (s) is the incubation time, $[\mathrm{drug}]_{\text{acceptor}}$ is the concentration of compound in the acceptor compartment at the completion of the assay and $[\mathrm{drug}]_{\text{equilibrium}}$ is the concentration of compound at theoretical equilibrium. Paracetamol, antipyrine, salicylic acid, and ketoprofen were used as reference compounds.

Transepithelial transport experiments across Caco-2 monolayers

Caco-2 cells (passage 30–60) were cultivated as previously described (i.e. the source, culture medium and seeding density were as reported).[9,10] Studies investigating the transport of samples across Caco-2 cell monolayers were performed as previously described.[9,10] Briefly, apical-to-basolateral transport rates for the test compounds

Table 1 Physicochemical properties of test compounds. Molecular weights, estimated p*Ka*, logP and logD and presence of non-ionized forms

						% of non-ionized forms	
Flavanone	MW	p*Ka*[a]	log*P*[a]	$\log D_{6.0}$[a]	$\log D_{7.4}$[a]	pH 6.0[a]	pH 7.4[a]
Hesperetin	302	7.27	2.38	2.36	2.02	94.94	42.68
Naringenin	272	7.27	2.64	2.61	2.27	94.93	42.49
Eriodictyol	288	7.27	2.35	2.33	1.98	94.90	42.20
Homoeriodictyol	302	7.27	2.38	2.36	2.20	94.95	42.70
Sakuranetin	286	9.36	2.67	2.67	2.66	99.96	98.27
Paracetamol	151	9.46	1.09	1.09	1.08	99.97	99.15
Antipyrine	188	–0.63	1.22	1.22	1.22	100.00	100.00
Ketoprofen	254	3.88	3.46	1.35	0.17	0.76	0.03
Salicylic acid	138	2.79	1.93	–1.16	–1.70	0.06	0.00

[a]Marvin Sketch (http://www.chemaxon.com/products/marvin).

(50 μmol/l) were measured across Caco-2 monolayer cultures grown for 2–3 weeks in Transwell inserts (Costar, Cambridge, USA) coated with type-I collagen. The integrity of the cell layer was evaluated by measurement of transepithelial electrical resistance (TER) with Millicell-ERS equipment (Costar, Cambridge, USA). Only monolayers with a TER of more than 300 $\Omega\,cm^2$, measured before and after each transport experiment, were used. Samples were prepared by dilution of stock solutions, and all solutions contained 1% DMSO, which was confirmed not to affect transport.[17] The amount of sample transported was determined in the same manner as for PAMPA. The permeability coefficient through Caco-2 cells, $\mathrm{Log}P_{\text{app-Caco-2}}$, was calculated using Equation 3.

$$\mathrm{Log}P_{\text{app-Caco-2}} = \mathrm{Log}\left\{C\cdot\left([\text{drug}]_{\text{acceptor}}/[\text{drug}]_{\text{initial,donor}}\right)\right\},\quad \text{where } C = (V_A/\text{Area}\cdot\text{time}) \tag{3}$$

In this equation, V_A (cm^3) is the volume of the acceptor compartment (0.30 cm^3), Area (cm^2) is the accessible filter area (1 cm^2), time (s) is the incubation time, $[\text{drug}]_{\text{acceptor}}$ is the concentration of compound in the acceptor (basolateral) compartment at the completion of the assay and $[\text{drug}]_{\text{initial, donor}}$ is the concentration of compound in the donor (apical) compartment.

The slope of the initial linear portion of the curve of the "permeated amount (nmol/mg protein)" versus "time (min)", calculated by linear regression analysis, was defined as the permeation rate (nmol/min/mg protein).

If a membrane protein is treated with an amino acid-modifying reagent, the targeted amino acid(s) of the membrane proteins will be modified, and as a result, the membrane permeability will be changed. The procedures for treatments with diethylpyrocarbonate (DEPC) and *N*-ethylmaleimide (NEM) were essentially the same as those described by Miyamoto *et al.*[18] Treatment with dithiothreitol (DTT) was performed according to Tuner *et al.*[19] Each reagent was added to the apical side of the Caco-2 cell monolayer and incubation was carried out at 37°C for 30 min. The pre-incubation solution was removed, and cells were washed three times with Hanks' balanced salt solution (HBSS) before the transport study.

Analytical methods

The concentrations of flavanones, paracetamol and salicylic acid were determined by HPLC-ECD as follows. An HPLC-ECD instrument fitted with a coulometric detection system was used for analysis, as previously reported.[9,10] Chromatographic separation was performed on a C18 column (ODS150; MC Medical, Inc., Tokyo, Japan). Mobile phase A (solvent A) was 50 mM sodium acetate containing 5% methanol (pH 3.0), while mobile phase B (solvent B) was 50 mM sodium acetate containing 40% acetonitrile and 20% methanol (pH 3.5). The elution profile (0.6 ml/min) was as follows: 0–0.5 min, isocratic elution, 60% solvent A/40% solvent B; 0.5–28.5 min, linear gradient from 60% solvent A/40% solvent B to 0% solvent A/100% solvent B; 28.6–31 min, isocratic elution, 0% solvent A/100% solvent B; 31–34 min, isocratic elution, 60% solvent A/40% solvent B. Eight electrode detector potentials (0–700 mV in increments of 100 mV) were used to measure the amounts of flavanones, paracetamol and salicylic acid. In HPLC-ECD analysis, the detection limit for all samples on the column was <0.5 pmol.[20] The dominant oxidation potentials and retention times were as follows: hesperetin (400 mV, 17.5 min), naringenin (600 mV, 15.8 min), eriodictyol (200 mV, 11.9 min), homoeriodictyol (200 mV, 11.9 min), sakuranetin (700 mV, 24.30 min), paracetamol (400 mV, 5.81 min) and salicylic acid (700 mV, 10.62 min).

Analysis of ketoprofen and antipyrine was carried out with a JASCO GULLIVER PU-980 LC pump and JASCO UV-970 intelligent UV/VIS detector. Ketoprofen analysis was performed on a TST-GEL ODS-100V column (150 × 4.6 mm; TOSOH, Tokyo, Japan). Separation was performed with isocratic elution (50% methanol containing 0.1% trifluoroacetic acid aqueous) at a flow rate of 1.0 ml/min. The absorbance of the eluate was monitored at 258 nm. Antipyrine analysis was performed on an RSpak DE-613 column (150 × 6 mm; Shodex, Tokyo, Japan). Separation was performed with isocratic elution (10 mM potassium dihydrogenphosphate containing 20% aqueous acetonitrile) at a flow rate of 1.0 ml/min. The absorbance of the eluate was monitored at 254 nm. The retention times of antipyrine and ketoprofen were 9.08 min and 9.67 min, respectively.

Data analysis

Results are expressed as means ± SD. Two-group comparisons were made with the unpaired two-tailed Student's *t*-test (Figure 3a). The Kruskal–Wallis test was used for multiple comparisons of the data in Figure 3b.

Results

Determination of physicochemical properties

The physicochemical properties of the test compounds are summarized in Table 1. Sakuranetin, paracetamol and antipyrine exist almost entirely in non-ionized forms at pH 6.0 and 7.4, while hesperetin, naringenin, eriodictyol and homoeriodictyol were about 50% ionized at pH 7.4. Ketoprofen and salicylic acid were almost completely ionized at pH 6.0 and 7.4. For these flavanones, the p*K*a and ionization curves obtained from the Marvin Sketch program should reflect ionization of the 7-OH group resulting from deprotonation at pH 7.4, except in the case of sakuranetin.

Theoretical partition ratio according to the pH-partition hypothesis

The p*K*a values of hesperetin, naringenin, eriodictyol and homoeriodictyol are the same (7.27). In the presence of a proton gradient, C_{bl}/C_{ap} was calculated as 2.35/1.05 according to Equation 1. C_{bl}/C_{ap} of sakuranetin was 1.01/1.00 in the presence of a proton gradient.

Transport of flavanones through permeability studies through artificial membranes

The permeability coefficients through PAMPA are listed in Table 2. $P_{app\text{-}PAMPA}$ values of hesperetin, naringenin and homoeriodictyol in the presence of the proton gradient were higher than in its absence. However, $P_{app\text{-}PAMPA}$ values of sakuranetin were almost the same in the presence and absence of a proton gradient. It was difficult to obtain reliable values of $P_{app\text{-}PAMPA}$ for eriodictyol because the permeability was too low ($<10^{-8}$ cm/s). The $P_{app\text{-}PAMPA}$ values of paracetamol, antipyrine, ketoprofen and salicylic acid are in agreement with those found in the literature.[21–23] One possible reason for low permeability is that the compound binds to the plastic surfaces and/or is retained in the artificial membrane during the permeability measurement. Values of binding loss (percent) of the flavanones studied here were at similar levels: hesperetin 6.0/7.4 (16.61 ± 9.26%), hesperetin 7.4/7.4 (13.69 ± 4.37%), naringenin 6.0/7.4 (10.25 ± 5.87%), naringenin 7.4/7.4 (11.51 ± 3.85%), eriodictyol 6.0/7.4 (15.48 ± 6.63%), eriodictyol 7.4/7.4 (17.75 ± 5.43%), sakuranetin 6.0/7.4 (20.67 ± 0.50%), sakuranetin 7.4/7.4 (24.03 ± 1.40%), homoeriodictyol 6.0/7.4 (16.27 ± 3.15%), homoeriodictyol 7.4/7.4 (10.91 ± 3.40%).

Transport of flavanones through Caco-2 cell monolayers

The permeability coefficients through Caco-2 cell monolayers are listed in Table 2. We previously reported that the permeation rates (nmol/min/mg protein) of hesperetin, naringenin and eriodictyol in the apical-to-basolateral direction in Caco-2 cell monolayers were greater in the presence of a proton gradient than in its absence.[10] We also showed here that the permeability coefficients ($P_{app\text{-}Caco\text{-}2}$) of homoeriodictyol, hesperetin, naringenin and eriodictyol were greater in the presence of a proton gradient than in its absence (Table 2). The $P_{app\text{-}Caco\text{-}2}$ values of sakuranetin were almost the same in the presence and absence of a proton gradient. The $P_{app\text{-}Caco\text{-}2}$ values of hesperetin and naringenin are in agreement with literature values.[24]

Correlation between $P_{app\text{-}PAMPA}/P_{app\text{-}Caco\text{-}2}$ and LogD

Figure 2a shows a plot of $P_{app\text{-}PAMPA}$ vs LogD between pH 6.0 and 7.4. Because PAMPA corresponds to the passive diffusion permeability, the plot shows a positive correlation. In contrast, a plot of $P_{app\text{-}Caco\text{-}2}$ vs LogD showed no correlation (Figure 2b). Although the LogD values of eriodictyol and naringenin are higher than those of the other compounds, the $P_{app\text{-}PAMPA}$ values were lower. Passive diffusion is also affected by hydrogen bonding.[25] We are not sure why eriodictyol and naringenin showed low permeability in this study.

Temperature dependence and effect of amino acid-modifying reagents

The transcellular transport of flavanones across Caco-2 cell monolayers was markedly reduced when the temperature was lowered to 4°C (Figure 3a). Figure 3b shows the effect of treatment of the apical membrane of Caco-2 cells with DEPC, NEM or DTT on flavanone transport. Treatment with DTT, which reduces protein disulfide bonds, significantly reduced the transport of all flavanones. By contrast, NEM, a reagent for the covalent modification of cysteine residues in proteins, and DEPC, a modification reagent for histidyl and tyrosine residues in proteins, reduced only sakuranetin transport.

Table 2 Effective permeability values in the presence (pH 6/7.4) and absence (pH 7.4/7.4) of a proton gradient in the Caco-2 cells and in the PAMPA models

	$P_{app\text{-}PAMPA}$ (10^{-6}) (cm/s)		$P_{app\text{-}Caco\text{-}2}$ (10^{-6}) (cm/s)	
Compound	6/7.4	7.4/7.4	6/7.4	7.4/7.4
Hesperetin	3.97 ± 1.02	1.85 ± 0.43	71.44 ± 5.34	33.86 ± 2.86
Naringenin	1.43 ± 0.08	0.09 ± 0.07	80.44 ± 5.11	52.75 ± 2.16
Eriodictyol	nd	nd	38.81 ± 1.36	13.36 ± 0.55
Homoeriodictyol	0.65 ± 0.28	0.23 ± 0.01	78.61 ± 1.90	57.06 ± 1.40
Sakuranetin	1.74 ± 0.25	1.33 ± 0.09	33.28 ± 2.21	34.00 ± 0.17
Paracetamol	0.40 ± 0.00	0.43 ± 0.00	68.67 ± 2.88	78.03 ± 4.66
Antipyrine	0.82 ± 0.02	0.86 ± 0.00	66.24 ± 1.02	66.17 ± 1.47
Ketoprofen	1.34 ± 0.11	0.05 ± 0.01	179.35 ± 4.58	51.38 ± 2.23
Salicylic acid	0.013 ± 0.001	0.002 ± 0.00	198.61 ± 1.27	6.01 ± 0.38

Data are presented as the mean ± SD of three experiments. PAMPA, parallel artificial membrane permeation assay.

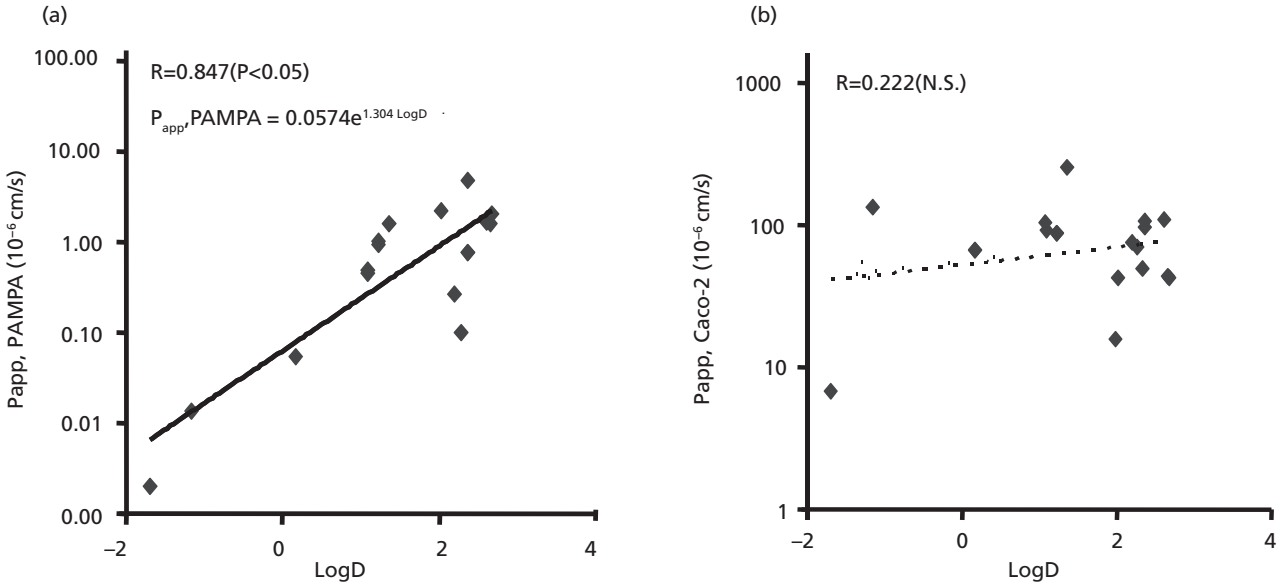

Figure 2 Correlation between $P_{app\text{-}PAMPA}$/$P_{app\text{-}Caco\text{-}2}$ and LogD. (a) Comparison of parallel artificial membrane permeation assay (PAMPA)/LogD correlation. (b) Caco-2/LogD correlation.

Discussion

We have already reported that hesperetin, naringenin and eriodictyol are efficiently absorbed via both proton-coupled active transport and transcellular passive diffusion in Caco-2 cells.[9,10] However, Neuhoff *et al*. reported that the pH-dependent transport in Caco-2 cell monolayers can be misinterpreted as active drug transport.[12] Flavonoid aglycones have long been considered to be absorbed via passive diffusion.[8,26,27] Our data confirmed that passive diffusion plays a considerable role in the permeation of hesperetin, naringenin, eriodictyol and homoeriodictyol. Brand *et al*. reported the hesperetin is absorbed by passive diffusion and subsequently conjugated to afford glucuronide and sulfated metabolites, which are transported to the apical side.[27] Other authors have indicated that naringenin is likely to be transported by multidrug resistance associated protein 1.[8] Thus, several mechanisms may influence the absorption of flavanones. Therefore, we used PAMPA and Caco-2 cell monolayers to study the nature of the mechanisms underlying pH-dependent passive transport of hesperetin, naringenin, eriodictyol and homoeriodictyol. These compounds are weakly acidic and consequently they are expected to be transported effectively by passive diffusion according to the pH-partition hypothesis. Hesperetin, naringenin, eriodictyol and homoeriodictyol are expected to be 94.9% and 42.2–42.7% non-ionized at pH 6.0 and pH 7.4, respectively (Table 1). Non-ionized flavanone passes through the lipid membrane very quickly and then dissociates in part to the ionized form after being transported to the acceptor (basolateral) side, depending on the pH (7.4). The concentration gradient of non-ionized flavanone across the PAMPA or Caco-2 cell monolayer is thus maintained until the equilibrium state, where the concentration of the non-ionized form is equal on the donor (apical) and acceptor (basolateral) sides. For hesperetin, naringenin, eriodictyol and homoeriodictyol, the values of C_{bl}/C_{ap} (Equation 1) calculated in the presence of a pH gradient suggest that 70% of these flavanones would be transported to the acceptor (basolateral) side in the equilibrium state.

Thus, hesperetin, naringenin, eriodictyol and homoeriodictyol are absorbed efficiently in the presence of a pH gradient. However, sakuranetin, paracetamol and antipyrine were not ionized at pH 6.0 and 7.4, and thus $P_{app\text{-}PAMPA}$ and $P_{app\text{-}Caco\text{-}2}$ are almost the same in the presence or absence of the proton gradient. Paracetamol and antipyrine are model compounds for transcellular passive diffusion.

$P_{app\text{-}PAMPA}$ values of hesperetin, naringenin, eriodictyol and homoeriodictyol were different, even though these compounds have the same p*K*a and similar Log*P*. Passive diffusion is affected by such underlying physicochemical properties as lipophilicity (Log*P*), p*K*a, molecular weight and hydrogen bonding,[25] which might influence the permeability of flavanones in a very complex manner. It has been reported that $P_{app\text{-}PAMPA}$ was affected by the type of membrane lipid.[24] In this study, soy lecithin was chosen as the phospholipidic component of the membrane, because it has been reported to afford the best correlation with human jejunal permeability.[24] The $P_{app\text{-}PAMPA}$ and $P_{app\text{-}Caco\text{-}2}$ of paracetamol, antipyrine, ketoprofen and salicylic acid are consistent with

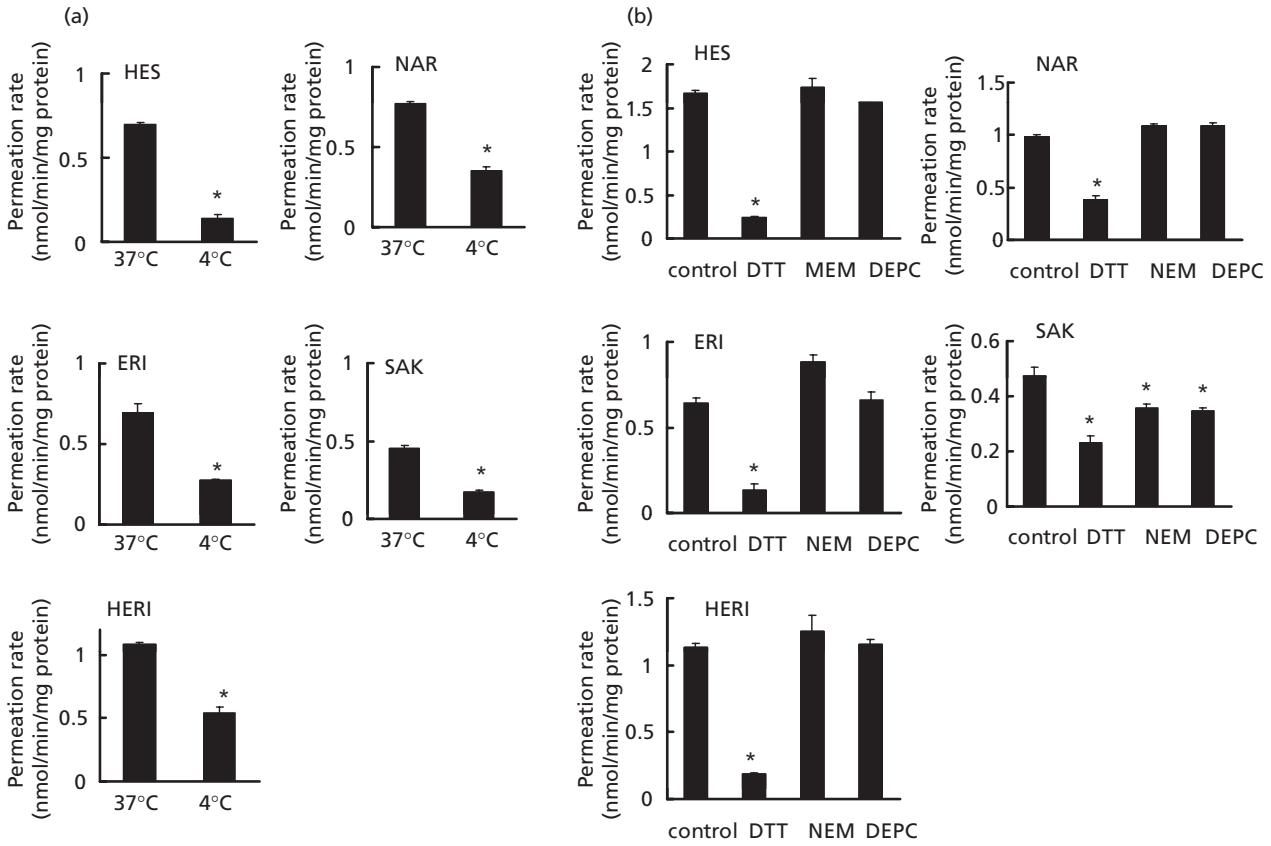

Figure 3 Effects of temperature (a) and amino acid-modifying reagents (b) on the transport of flavanones across Caco-2 monolayers. (a) Permeation rate of flavanones was measured at 37°C and 4°C. (b) After pre-incubation with 0.2 mM diethylpyrocarbonate (DEPC), 10 mM dithiothreitol (DTT) or 0.5 mM *N*-ethylmaleimide (NEM), transport of flavanones was measured at 37°C by Caco-2 cells at an apical-side pH of 6.0 and at a basolateral-side pH of 7.4. ERI, eriodictyol; HERI, homoeriodictyol; HES, hesperetin; NAR, naringenin. Each value represents the mean ± SD of three experiments. Significant differences from the control value were identified by using Student's *t*-test ($P < 0.05$).

the literature values, supporting the validity of the PAMPA and Caco-2 assays in this study. Kerns *et al*.[22] have reported that compounds with molecular weight below 200 had quite low PAMPA permeability. Paracetamol, antipyrine and salicylic acid (MW <200) had high $P_{app\text{-}Caco\text{-}2}$ values, but low $P_{app\text{-}PAMPA}$ values in this study. Furthermore, $P_{app\text{-}Caco\text{-}2}$ values of hesperetin, naringenin and homoeriodictyol were high and similar, but the $P_{app\text{-}PAMPA}$ of homoeriodictyol showed by far the lowest value. Homoeriodictyol had a particularly high Caco-2 permeability value. The value of $P_{app\text{-}Caco\text{-}2}$ increased in the following order: naringenin > hesperetin > eriodictyol. These observations might be attributed to the greater efficiency of absorption or bioavailability of naringenin over hesperetin, which has been reported in several studies.[28,29] Although the PAMPA method is used in drug discovery to predict intestinal absorption, our data suggested that additional Caco-2 assay would be useful for the better prediction of intestinal absorption.

$P_{app\text{-}PAMPA}$ correlated with LogD, indicating the involvement of passive transport, although $P_{app\text{-}Caco\text{-}2}$ did not correlate (Figure 2a and 2b). In our previous study, the permeation of flavanones was shown to be inhibited by addition of benzoic acid or CCCP.[9,10] However, because passive transport of weakly acidic compounds is inhibited by organic acids, including benzoic acid,[11] this may be insufficient evidence for involvement of a specific transporter. Since flavanones are poorly soluble in HBSS, concentration-dependence studies above a concentration of 0.5 mM could not be carried out. Therefore, we performed temperature-dependence studies and examined the effects of amino acid-modifying reagents. The transcellular transport of flavanones across Caco-2 cell monolayers was markedly reduced when the temperature was lowered to 4°C (Figure 3a). The permeability coefficient was significantly reduced when Caco-2 cells were treated with amino acid-modifying reagents (Figure 3b). The permeability coefficients of hesperetin, naringenin, eriodictyol and homoeriodictyol were decreased by treatment with DTT, which reduces protein disulfide bonds. It is probable that intramolecular disulfide bonds, not free thiol groups, have an important role in the transport of these compounds, and His

and Tyr residues are not essential. By contrast, NEM, a reagent for the covalent modification of Cys residues in proteins, DEPC, a modification reagent for His and Tyr residues in proteins, and DTT all reduced sakuranetin transport. These results suggested that His and Tyr and/or thiol residues have an important role in the transport of sakuranetin. In contrast, the permeability of eriodictyol was increased by treatment with NEM. Thus, eriodictyol might be an efflux transporter substrate, and Cys residue(s) may be important for the transporter function. The reducing agent DTT has been reported to alter Na^+/glucose cotransporter and H^+-coupled amino acid transporter 1 function.[30,31] Studies with oocytes or cells expressing mutants in which extracellular Cys was replaced with Ala or Ser have demonstrated that a specific extracellular disulfide bridge is essential for full transport function.[29,30] A disulfide bridge may also be important for flavanone transport function. The average ratios of flavanone permeability coefficient in Caco-2 cells at 4°C versus at 37°C (38.51 ± 11.42%) and after DTT treatment versus untreated (27.99 ± 15.21%) were similar (Figure 3a and 3b), and might reflect the loss of carrier-mediated transport of flavanones. Sodium azide also significantly reduced the permeability of sakuranetin and homoeriodictyol (data not shown), as well as other flavanones.[9,10] These data also suggest that flavanones are transported in part by an active transporter in Caco-2 cell monolayers, though we could not clarify the reason for the effect of H^+.

Conclusions

The results of our comparison of the permeability characteristics of flavanones in Caco-2 cells with their behaviour in PAMPA support the view that flavanones are absorbed through human intestinal epithelium via both passive diffusion and an active transport mechanism. Hesperetin, naringenin and homoeriodictyol are absorbed effectively by passive diffusion according to the pH-partition hypothesis. Further experiments will be necessary to identify the active transporter(s) in Caco-2 cells.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. Arts IC, Hollman PC. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr* 2005; 81: 317S–325S.
2. Khan N, Mukhtar H. Tea polyphenols for health promotion. *Life Sci* 2007; 81: 519–533.
3. Kobayashi S, Tanabe S. Evaluation of the anti-allergic activity of *Citrus unshiu* using rat basophilic leukemia RBL-2H3 cells as well as basophils of patients with seasonal allergic rhinitis to pollen. *Int J Mol Med* 2006; 17: 511–515.
4. Erlund I *et al*. Plasma kinetics and urinary excretion of the flavanones naringenin and hesperetin in humans after ingestion of orange juice and grapefruit juice. *J Nutr* 2001; 131: 235–241.
5. Bredsdorff L *et al*. Absorption, conjugation and excretion of the flavanones, naringenin and hesperetin from alpha-rhamnosidase-treated orange juice in human subjects. *Br J Nutr* 2010; 103: 1602–1609.
6. Kottra G, Daniel H. Flavonoid glycosides are not transported by the human Na^+/glucose transporter when expressed in Xenopus laevis oocytes, but effectively inhibit electrogenic glucose uptake. *J Pharmacol Exp Ther* 2007; 322: 829–835.
7. Makino T *et al*. Enzymatically modified isoquercitrin, alpha-oligoglucosyl quercetin 3-O-glucoside, is absorbed more easily than other quercetin glycosides or aglycone after oral administration in rats. *Biol Pharm Bull* 2009; 32: 2034–2040.
8. Nait Chabane M *et al*. Quercetin and naringenin transport across human intestinal Caco-2 cells. *J Pharm Pharmacol* 2009; 61: 1473–1483.
9. Kobayashi S *et al*. Transepithelial transport of hesperetin and hesperidin in intestinal Caco-2 cell monolayers. *Biochim Biophys Acta* 2008; 1778: 33–41.
10. Kobayashi S, Konishi Y. Transepithelial transport of flavanone in intestinal Caco-2 cell monolayers. *Biochem Biophys Res Commun* 2008; 368: 23–29.
11. Takagi M *et al*. A new interpretation of salicylic acid transport across the lipid bilayer: implications of pH-dependent but not carrier-mediated absorption from the gastrointestinal tract. *J Pharmacol Exp Ther* 1998; 285: 1175–1180.
12. Srirangam R, Majumdar S. Passive asymmetric transport of hesperetin across isolated rabbit cornea. *Int J Pharm* 2010; 394: 60–67.
13. Neuhoff S *et al*. pH-Dependent passive and active transport of acidic drugs across Caco-2 cell monolayers. *Eur J Pharm Sci* 2005; 25: 211–220.
14. Shiau YF *et al*. Mechanisms maintaining a low-pH microclimate in the intestine. *Am J Physiol* 1985; 248: G608–G617.
15. Kansy M *et al*. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. *J Med Chem* 1998; 41: 1007–1010.
16. Di L *et al*. High throughput artificial membrane permeability assay for

blood-brain barrier. *Eur J Med Chem* 2003; 38: 223–232.
17. Da Violante G *et al*. Evaluation of the cytotoxicity effect of dimethyl sulfoxide (DMSO) on Caco2/TC7 colon tumor cell cultures. *Biol Pharm Bull* 2002; 25: 1600–1603.
18. Miyamoto Y *et al*. Identification of histidyl and thiol groups at the active site of rabbit renal dipeptide transporter. *J Biol Chem* 1986; 261: 16133–16140.
19. Turner RJ *et al*. Evidence for a $Na^+/K^+/Cl^-$ cotransport system in basolateral membrane vesicles from the rabbit parotid. *J Membr Biol* 1986; 94: 143–152.
20. Guo C *et al*. High-performance liquid chromatography coupled with coulometric array detection of electroactive components in fruits and vegetables: relationship to oxygen radical absorbance capacity. *J Agric Food Chem* 1997; 45: 1787–1796.
21. Fujikawa M *et al*. Relationships between structure and high-throughput screening permeability of diverse drugs with artificial membranes: application to prediction of Caco-2 cell permeability. *Bioorg Med Chem* 2005; 13: 4721–4732.
22. Kerns EH *et al*. Combined application of parallel artificial membrane permeability assay and Caco-2 permeability assays in drug discovery. *J Pharm Sci* 2004; 93: 1440–1453.
23. Chen X *et al*. A novel design of artificial membrane for improving the PAMPA model. *Pharm Res* 2008; 25: 1511–1520.
24. Serra H *et al*. Prediction of intestinal absorption and metabolism of pharmacologically active flavones and flavanones. *Bioorg Med Chem* 2008; 16: 4009–4018.
25. Cruciani G *et al*. In silico pKa prediction and ADME profiling. *Chem Biodivers* 2009; 6: 1812–1821.
26. Nielsen IL *et al*. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *J Nutr* 2006; 136: 404–408.
27. Brand W *et al*. Metabolism and transport of the citrus flavonoid hesperetin in Caco-2 cell monolayers. *Drug Metab Dispos* 2008; 36: 1794–1802.
28. Gardana C *et al*. Flavanoe plasma pharmacokinetics from blood orange juce in human subjects. *Br J Nutr* 2007; 98: 165–172.
29. Kanaze FI *et al*. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. *Eur J Clin Nutr* 2007; 61: 472–477.
30. Dorn M *et al*. Identification of a disulfide bridge essential for transport function of the human proton-coupled amino acid transporter hPAT1. *J Biol Chem* 2009; 284: 22123–22132.
31. Gagnon DG *et al*. Identification of a disulfide bridge linking the fourth and the seventh extracellular loops of the Na^+/glucose cotransporter. *J Gen Physiol* 2006; 127: 145–158.