Transport of trans-tiliroside (kaempferol-3-β-D-(6"-p-coumaroyl-glucopyranoside) and related flavonoids across Caco-2 cells, as a model of absorption and metabolism in the small intestine

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Abstract

1. Absorption and metabolism of tiliroside (kaempferol 3-β-D-(6''-p-coumaroyl)-glucopyranoside) and its related compounds kaempferol, kaempferol-3-glucoside and p-coumaric acid were investigated in the small intestinal Caco-2 cell model. Apparent permeation (P_{app}) was determined as 0.62×10^{-6} cm/s, 3.1×10^{-6} cm/s, 0 and 22.8×10^{-6} cm/s respectively.

2. Mechanistic study showed that the transportation of tiliroside, kaempferol-3-glucoside and p-coumaric acid in Caco-2 model were transporter(s) involved, while transportation of kaempferol was solely by passive diffusion mechanism.

3. Efflux transporters, multi-drug-resistance-associated protein-2 (MRP2), was shown to play a role in limiting the uptake of tiliroside. Inhibitors of MRP2, (MK571 and rifampicin) and co-incubation with kaempferol (10 μM), increased transfer from the apical to the basolateral side by three-five fold.

4. Metabolites of kaempferol-3-glucoside and p-coumaric acid were not detected in the current Caco-2 model, while tiliroside was metabolised to a limited extent, with two tiliroside mono-glucuronides identified; and kaempferol was metabolised to a higher extent, with three mono-glucuronides and two mono-sulfates identified.

5. In conclusion, tiliroside was metabolised and transported across Caco-2 cell membrane to a limited extent. Transportation could be increased by applying MRP2 inhibitors or co-incubation with kaempferol. It is proposed that tiliroside can be absorbed by human; future pharmacokinetics studies are warranted in order to determine the usefulness of tiliroside as a bioactive agent.

1 Introduction

Tiliroside (kaempferol 3-β-D-(6''-p-coumaroyl)-glucopyranoside; Figure 1) is a flavonol ester present in a number of herbal plants (Nowak, 2003) and medicines (Leitão et al., 2000), and present at a relatively low level in a number of foods, such as strawberry (Itoh et al., 2009), Chinese raspberry (Chen et al., 2009), and rosehip (Kumarasamy et al., 2003). Increasing evidence from in vitro and in vivo animal studies show tiliroside may have health beneficial effects on human health, such as anti-inflammatory (Sala et al., 2003), hepatoprotective (Matsuda et al., 2002), and antidiabetic activity (Ninomiya et al., 2007). In these studies (Matsuda et al., 2002; Ninomiya et al., 2007; Sala et al., 2003), application of tiliroside at high doses (up to 100 mg/kg body wt) was given either orally or by intraperitoneal or subcutaneous injections to mice, and at all doses examined, tiliroside lead to positive effects. In particular, lower levels of orally administered trans-tiliroside (0.1~10 mg/kg body wt/day) inhibited the gain of body weight (especially visceral fat weight), and reduced liver triglyceride, and free fatty acids levels without affecting food intake (Ninomiya et al., 2007). Longer term tiliroside intake (15 days) significantly suppressed the elevation of glucose levels

in plasma after intraperitoneal injection of glucose (1g/kg) in mice (Ninomiya et al., 2007).

It is of paramount importance when investigating bioactivity of flavonoids to understand bioavailability. Tiliroside absorption and metabolism has never previously been studied, and thus we have investigated the potential absorption of this flavonoid conjugate using an intestinal model system. Caco-2 cells are human colon adenocarcinoma cells that undergo spontaneous differentiation into monolayers of enterocytes in culture, with characteristics similar to the human intestinal epithelium (Polli et al., 2004). Caco-2 cells have been the most popular in vitro cell-based model to predict drug permeability. Results from studies of the Caco-2 cell monolayer correlate well with the level of in vivo oral absorption in humans (Artursson and Karlsson, 1991). The aim of our study was to investigate the transport and metabolism of tiliroside and related compounds (kaempferol, kaempferol-3-glucoside, and p-coumaric acid) across the Caco-2 intestinal cell model in order to predict absorption of tiliroside in humans.

2 Materials and Methods

2.1 Chemicals

Dulbelcco's Modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin-streptomycin, amphotericin B, L-glutamine, Hanks' balanced salt solution (HBSS), calcium chloride, and minimum essential medium (MEM), were purchased from Sigma-Aldrich (Exeter, UK). Transwell Polycarbonate semi-permeable membranes of 0.4 μm

pore size and 4.5 cm^2 surface area was obtained from Corning (Loughborough, UK).

p-Coumaric acid was purchased from Sigma-Aldrich (Exeter, UK). Kaempferol and kaempferol-3-glucoside were obtained from Extrasynthese (Genay, France). Tiliroside was kindly provided by Merck KGaA (Darmstadt, Germany). Tiliroside once dissolved in solution is a mixture of trans- and cis- isomers. The cis- isoform accounted for less than 4% of the trans-isoform of tiliroside at the concentration used in this study. Therefore, only trans-tiliroside is considered in this study. If not specified, tiliroside refers to trans-tiliroside in the later text. All flavonoids were HPLC grade, and prepared by first dissolving in DMSO (Sigma-Aldrich) at a high concentration (eg. 10 mM) and were then further diluted in HBSS to the desired concentration (eg. 10 µM; DMSO <0.1% vol.) on the day of experiment. L-Ascorbic acid, rifampicin, sodium hydroxide and hydrochloric acid were purchased from Sigma-Aldrich. MK571 (sodium salt) was purchased from Merck Chemicals Ltd.

2.2 Caco-2 cells

The human colon carcinoma cell line Caco-2 cells (HTB-37) was obtained from the American Type Culture Collection (ATCC, LGC Promochem, Molsheim, France) at passage 21. Caco-2 cells were used between passage 25 and 30. The cells were maintained in 75 cm^2 culture flasks at 37°C under a humidified atmosphere (5% CO_2: 95% O_2) in DMEM supplemented with 584 mg/L of L-glutamine, 1% (v/v) MEM, 100 U/ml of penicillin-streptomycin, 0.25 µg/ml amphotericin B and 15% heat-inactivated fetal calf serum (FCS).

2.3 Cell culture studies

Caco-2 cells were seeded in Transwell plates at 6 x 10^4 cells/cm^2. Cells were allowed to grow and differentiate over a period of 21 d, and were maintained in medium (DMEM containing 10% FCS) which was changed every 2 d. The integrity of the monolayers was evaluated by measurement of the transepithelial electrical resistance (TEER) using a Millicell®-ERS device (Millipore, Zug, Switzerland) before and after the treatments. Only those inserts with TEER values >350 Ωcm^2 were selected for experiments.

2.3.1 Absorption and metabolism studies

The medium was removed from both the apical and basolateral sides and washed with HBSS twice. Each compartment was replaced with 2 ml of the transport solution consisting of HBSS containing 1.8 mM calcium (required for maintaining integrity of the tight junctions), 1 mM ascorbic acid (required to prevent degradation of polyphenols), and either with or without polyphenols at the desired concentration. The pH was adjusted by using less than 1% of HCL or NaOH (3 M) to 6 on the apical side and 7.4 on basolateral side to mimic human intestinal physiological condition. After an incubation of 2 h (37°C, 5% CO_2:95% O_2), the apical and basolateral solutions were collected, and each compartment was gently washed with 2 ml of HBSS three times. Experimental solution (2 ml) and wash solution (6 ml) from each compartment were collected and stored separately. Ascorbic acid (10 mM) was added and samples stored at -80°C until analysed by HPLC.

Apparent permeability coefficient (P_{app}) was calculated using the following equation (Walgren et al., 1998):

$$P_{app} = \frac{V}{A\,C_0} \times \frac{dC}{dt} = cm \times sec^{-1}$$

Where, V= the volume of the solution in the receiving compartment, A= the membrane surface area, C0 = the initial concentration in the donor compartment, and dC/dT = the change in flavonoid concentration in the receiver solution over time. Data were expressed as the means ± standard deviation of at least three independent repeats. Differences in P_{app} were evaluated using Student's t-test. A P value 0.05 was considered significant.

2.3.2 Time course study

Transport solution (2 ml) containing tiliroside or kaempferol was incubated with Caco-2 cells on either the apical or basolateral side. An aliquot (200 µl) of medium was removed from each side of the compartment at 0, 30, 60, 90 and 120 min after incubation. These solutions were diluted 1:1 (v:v) with methanol and centrifuged (13 300 g, 10 min) before analysed by HPLC. All experiments were individually repeated for at least 3 times.

2.3.3   Inhibition studies

After the HBSS wash, both compartments were replaced with transport solution (2 ml), with the inhibitors MK571 (50 µM) or rifampicin (80 µM) applied to the apical compartment. After

30 min pre-inhibition, solution from both compartments was removed and transport solution (2 ml) was replaced with the inhibitor applied to the apical compartment, and the polyphenol (10 μM) applied to either the apical or basolateral compartment. After incubation (2 h), samples were collected and stored until analysed by HPLC. All experiments were individually repeated for at least 3 times. Differences in P_{app} were evaluated using Student's t-test. A P value 0.05 was considered significant.

A similar study was carried out by co-incubating related polyphenols. The basolateral compartment was replaced with transport solution (2 ml), and the apical compartment had transport solution supplemented with either p-coumaric acid (10 μM), or kaempferol (10 μM), or the mixture of the two compounds (both at 10 μM). After 30 min pre-inhibition, solution from both compartments were removed and replaced with identical solutions, but also including tiliroside (10 μM) in the apical compartment. After incubation (2 h), samples were collected and stored until analysed by HPLC. All experiments were individually repeated for at least 3 times. Differences in P_{app} were evaluated using Student's t-test. A P value 0.05 was considered significant.

2.4 Extraction

Frozen samples from storage were freeze-dried (Scanvac CoolSafeTM Freeze-Drier, Lynge). Each sample was re-dissolved in methanol (2 mL) and vortexed using a Genie 2 vortex mixer (Scientific Industries, USA) operating at full speed for 2 min. Methanol extracts were

sonicated (Clifton Ultrasonic Baths MU-14, UK; 30 min) and centrifuged (10 min, 13 300 g).

The supernatant was analysed by HPLC.

2.5 HPLC analysis

An Agilent 1200 series rapid resolution LC system comprising a thermostatic autosampler, column oven, vacuum degasser, gradient mixer and a binary pump coupled to a diode array detector was used for analysis of all samples. The HPLC was fitted with a C18 Zorbax Eclipse column XDB-C18 (4.6 x 50 mm; internal diameter 1.8 μm; Agilent, Berkshire UK), and held at a constant temperature of 30°C, and the analysis was controlled by ChemStation software. Solvents were A: trifluoroacetic acid (0.1%, HPLC grade) in purified water (Direct Q, Millipore, UK) and B: acetonitrile (100%; HPLC grade), and were run at a flow rate of 0.85 ml/min. The gradient system used was: 15% solvent B at 0 min, increasing to 40% at 3 min, and further increasing to 90% by 5 min. Solvent B was held at 90% for 1 min before decreasing to 15% by 6.5 min, and held at 15% until 8 min. Samples (10 μl) were injected directly on to the column, and the needle was washed with methanol between injections to prevent sample crossover. The eluent was monitored between 200 and 450 nm by diode array. Chromatograms were observed at the optimum wavelength corresponding to each compound.

2.6 LC-MS/MS analysis

The LC system in LC-MS/MS was modified slightly from the HPLC analysis. The solvents were: A: formic acid (0.1%, LC-MS grade) in purified water (Direct Q, Millipore, UK) and B:

acetonitrile (100%; LC-MS grade), and were run at a flow rate of 0.30 ml/min. The gradient system used was: 15% solvent B at 0 min, increasing to 40% at 8 min, and further increasing to 70% by 15 min, then increasing to 90% by 16 min. Solvent B was held at 90% for 1 min before decreasing to 15% by 17.5 min, and held at 15% until 30 min. Samples (5 μl) were injected directly on to the column, and the needle was washed with methanol between injections to prevent sample crossover. The eluent was monitored between 200 and 450 nm by diode array.

MS was performed with an Agilent 6410 triple-quad LC-MS with electrospray source (Agilent Technologies) supplied by a N_2 generator (Peak Scientific, Inchinnan, UK). LC eluent was diverted to waste for the initial 3 min, the eluent then entered the electrospray nebulizer that ejected a N_2 flow (60 psi) into the spray chamber which set in negative ionization mode (ESI-). The solvent was removed with a source gas temperature of 350°C and a gas flow of 11 L/min. The capillary potential was 4000V, and the fragmentor potential was 135V. Quad 1 was set to filter an m/z of the ionized parent compound in unit resolution. The filtered compounds were then subjected to a collision energy set at a potential of 10~20V; quad 2 further filtered an m/z of product ions. Dwell time of quad 2 for each m/z was 200 ms. The m/z monitoring was at: 769 > 593 (tiliroside monoglucuronide [M-H]⁻); 593 > 285 (tiliroside [M-H]⁻); 447 > 285 (kaempferol-3-glucoside [M-H]⁻), 461 > 285 (kaempferol monoglucuronide [M-H]⁻), 365 > 285 (kaempferol monosulfate [M-H]⁻), 541 > 285 (kaempferol glucurono-sulfate [M-H]⁻); 637 > 285(kaempferol di-glucuronides [M-H]⁻);

445 > 285 (kaempferol di-sulfates [M-H]⁻); 163 > 119 (coumaric acid [M-H]⁻).

3 Results

3.1 Transport across Caco-2 cells

The transportation of tiliroside and related compounds (kaempferol, kaempferol- 3-glucoside and p-coumaric acid) following incubation of the compound on either the apical or basolateral side is summarized in Table 1 and the apparent permeation (P_{app}) of the compounds is shown in Figure 2. A concentration of 10 μM was selected for all compounds investigated as it is a relevant physiological concentration of polyphenol in humans (Williamson and Manach, 2005).

As shown in Table 1 and Figure 2, tiliroside had a low absorption (1%) from the apical (A) to basolateral (B) side, while it was approximately 7-fold greater from B to A. Kaempferol had a higher absorption (5%) than tiliroside, and transportation in both directions was equivalent. These results were also demonstrated in the time course studies presented in Figure 3. There was no observable transportation of kaempferol-3-glucoside from the A to B direction for Caco-2 cells, though transport of kaempferol-3-glucoside from B to A (4%) did occur. p-Coumaric acid was found to have a high absorption across the Caco-2 cell model (A to B; 36%), although a lower level of transport occurred in the B to A direction (12%).

The recovery of tiliroside and p-coumaric acid in both chambers was good (~90%), however

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kaempferol-3-glucoside and kaempferol had lower recoveries (<70% and <50%, respectively). The lower recoveries are probably, due at least in part, to insolubility at the concentration used. However kaempferol was also further metabolised.

3.2 Metabolism in Caco-2 cells

When p-coumaric acid and kaempferol-3-glucoside were incubated, only the parent compounds were observed in the apical and basolateral compartments. Figure 4(a) shows a typical HPLC chromatogram of tiliroside in apical solution after incubation for 2 h with Caco-2 cells. There was evidence of metabolism of tiliroside, as several small new peaks (T1-T8) were observed (see Fig. 4b), however these peaks accounted for less than 4% (based on peak area) of the total tiliroside. Only the main metabolites, T5 and T6, had sufficient signal for UV spectral analysis (Fig. 4c). LC-MS/MS operated in negative mode found these two peaks had an identical molecular weight of 769 (the same mass as tiliroside mono-glucuronides negative ion), and after application of 10 V collision energy the resultant product ions had a molecular weight of 593 (the same mass as tiliroside negative ion). T5 and T6 therefore were identified as tiliroside mono-glucuronides.

Following incubation of kaempferol for 2 h with Caco-2 cells, kaempferol and four new peaks (K1-K4) were identified by HPLC (see Figure 5a) and LC-MS/MS. Based on kaempferol-3-glucoside standard approximately 35% of kaempferol was present as conjugate form. In negative mode, K1, K2, K3 were found to have an identical molecular weight of

461(the same mass as kaempferol mono-glucuronides negative ion) and after application of 10 V collision energy, all the resultant product ions had an identical molecular weight of 285 (the same mass as kaempferol negative ion). Thus, K1, K2 and K3 were identified as kaempferol mono-glucuronides

Peak K4 had a molecular weight of 365 (the same mass as kaempferol mono-sulfate negative ion) and and after application of 10 V collision energy, the resultant product ion had a molecular weight of 285 (the same mass as kaempferol negative ion). Therefore, K4 was identified as kaempferol mono-sulfate. The molecular weights, in selected-ion monitoring (SIM) and product ion mode, for kaempferol glucurono-sulfate, kaempferol di-glucuronide, and kaempferol di-sulfates were assessed, but none of these potential metabolites were observed.

The UV absorption shift of the peak maxima corresponding to Band I of the metabolites, in relation to kaempferol, allows the position of conjugation to be determined (see Figure 5b). Only the 3-, 4′- and 7- positions are available on kaempferol for conjugation, and both the 3- and 4′- would cause a hypsochromic Band I shift. Conjugation at the 4′- position would cause a small shift of 3-5 nm, while conjugation at the 3-position would cause larger shift of 12-17 nm (Day et al., 2000). K1 has a Band I absorption maximum at 348 nm, a large shift in comparison to kaempferol, and hence can be identified as kaempferol-3-glucuronide. K2 has a band I absorption maximum at 368 nm, the same as kaempferol, therefore is likely to be

kaempferol-7-glucuronide. K3 has a Band I absorption maximum at 362 nm (a small shift compared to kaempferol), indicating conjugation at the 4′- position. K4 is a broad peak, and is likely to be a mixture of more than one sulfate. The UV spectra acquired at different positions of the HPLC peak showed different Band I absorption maxima; one at 368 nm and one at 364 nm. Thus, it is likely that kaempferol-7-sulfate and -4′-sulfate were formed by the Caco-2 cells.

3.3 Inhibitors of transport

Known inhibitors of MRP2 efflux transporter, were incubated with either tiliroside or kaempferol. As shown in Figure 6(a), incubation with MK571 resulted in increased transport of tiliroside from the A to B direction and significantly decreased transport from B to A. Incubation with rifampicin gave similar results to incubation with MK571, where transportation of tiliroside from A to B was increased about 5-fold compared with control. Figure 6(b) shows that kaempferol transport from both directions did not significantly change in the presence of any of the inhibitors.

Tiliroside was also co-incubated with its potential breakdown compounds, kaempferol and p-coumaric acid. Figure 7 demonstrates that transfer of tiliroside from the apical to the basolateral compartment was significantly increased (3-fold) when kaempferol was co-incubated on the apical side, with or without p-coumaric acid. On its own p-coumaric acid had no effect on tiliroside transfer.

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4. Discussion

4.1 Transport study

Tiliroside is a relatively hydrophobic compound, despite having a hydrophilic conjugating side chain (coumaroyl-glucoside), it has a Log P of 2.7 (Luo et al., 2011). It is therefore feasible that tiliroside can diffuse across biological membranes. However tiliroside also has a large molecular weight (594), which exceeds the "rule of five" summarized by Lipinski (Lipinski et al., 1997), in that compounds with a molecular mass greater than 500 are likely to be poorly absorbed. Tiliroside did, however, cross the Caco-2 cell model, with approximately 1% being transferred to the basolateral side; a higher rate of transfer was observed from the basolateral to the apical side, and the increase was time-dependent. This suggests that efflux transporter(s) are likely to be involved in pumping tiliroside back out of the intestinal cells, limiting the extent to which apical to basolateral transfer can occur.

For flavonoids, the efflux transporters MRP2 has been shown to contribute to limiting absorption; this transporter has been previously reported to be functionally expressed in Caco-2 cells (Hunter et al., 1993; Maubon et al., 2007). MK571 has been used as an MRP2 inhibitor in many studies (Walgren et al., 2000; O'Leary et al., 2003). Recently, it was reported that MK571 was not specific to MRP2, but could both affect MRP2 and BCRP to different extents (Matsson et al., 2009). By comparing behaviour of 122 drugs, Matsson et al., (2009) reported rifampicin as a highly specific inhibitor to MRP2, with less than 1%

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inhibition to BCRP. Therefore, MK571 and rifampicin were selected to inhibit MRP2.

When tiliroside was incubated with MK571 or rifampicin there was an increase in transfer from the apical to basolateral side of the Caco-2 cells (see Figure 6a). Likewise, for MK571 and rifampicin (91 and 99% specific for MRP2, respectively (Matsson et al., 2009)) the basolateral to apical efflux was significantly reduced. These results suggest that MRP2 is strongly involved in tiliroside efflux which consequently reduces absorption of tiliroside across the Caco-2 cells. Other transporters may also play a role and further investigations would need to address the extent of the non-MRP2 contribution.

Tiliroside could potentially be hydrolysed to either kaempferol-3-glucoside and coumaric acid, or kaempferol, and therefore it was interesting to compare the transfer of these related compounds in the same Caco-2 model. Kaempferol is more hydrophobic (log P = 3.2 (Luo et al., 2011)) than tiliroside, with a lower molecular weight (286) and, thus would be expected to diffuse passively across biological membranes. We found that 5% of kaempferol aglycone was transferred from the apical to basolateral membrane, and that no significant difference in transfers were observed in both directions, at similar constant linear rates (see Figure 2 and 3b). Kaempferol transfer from both directions was not affected by the application of inhibitors of efflux transporters MRP2. This evidence is indicative of passive transport, and is consistent with a previously published study (Barrington et al., 2009).

Kaempferol-3-glucoside was not transported from the apical to basolateral side of the Caco-2 model, and kaempferol-3-glucoside is not a substrate of cytosolic β-glucosidase (Day et al., 1998) but can be hydrolysed to kaempferol by lactase phlorizin hydrolase (LPH (Németh et al., 2003)) at the lumen brush border. LPH is expressed at very low levels in Caco-2 cells (Chantret et al., 1994; Day et al., 2003) and therefore lack of hydrolysis and basolateral transfer of kaempferol-3-glucoside were anticipated. Furthermore, kaempferol-3-glucoside was transported from the basolateral to apical side of the Caco-2 cells, suggesting that efflux transporters are involved. Quercetin-4′-glucoside was a substrate of MRP2 (Walgren et al., 2000), and thus it is likely that kaempferol-3-glucoside is also a substrate.

p-Coumaric acid was found to have a significant apical to basolateral transfer (37%) of p-coumaric acid, and a lower basolateral efflux (12%), which is consistent with previously published work (Konishi and Shimizu, 2003; Konishi and Kobayashi, 2004; Poquet et al., 2008). p-Coumaric acid is hydrophilic (Log P -0.65 (Luo et al., 2011)) and has a relatively low molecular weight (164). It is possible that transportation occurs via paracellular tight junctions rather than by a transcellular route. A related phenolic acid, ferulic acid however, was proposed to be transported by transcellular passive diffusion (Poquet et al., 2008). The degree of phenolic acid transport is associated with the extent of ionisation (Poquet et al., 2008); ionisation creates a concentration gradient across the intestinal cells allowing passive diffusion of the phenolic acid. It has also been proposed that facilitated transport of coumaric acid using a proton pump, which is only active across a concentration gradient (Konishi and

Shimizu, 2003; Konishi and Kobayashi, 2004).

4.2 Metabolism study

Tiliroside was metabolised in our experiments, but to a very limited extent. The main two metabolites were identified as mono-glucuronides. Kaempferol was metabolised to mono-sulfate(s) and mono-glucuronides by the Caco-2/HTB37 cell model, with approximately 35% being metabolised as assessed in the apical transport solution after 2 h. Metabolism of kaempferol has been studied previously in Caco-2/TC7 cells (Barrington et al., 2009), and here five metabolites were identified, including a kaempferol glucurono-sulfate. Differences in results may be accounted for by the different Caco-2 clonal lines (Maubon et al., 2007), and different passage numbers used, as these may result in altered expression of phase II enzymes isoforms and transporters. No metabolism was observed for kaempferol-3-glucoside and p-coumaric acid in the current Caco-2 model.

4.3 Co-incubation

Co-incubation of tiliroside with kaempferol significantly increased the transfer of tiliroside from the apical to the basolateral side, suggesting that kaempferol may improve tiliroside absorption. As absorption of tiliroside is mediated by MRP2, it is possible that kaempferol is indirectly inhibiting this transporter. Kaempferol itself is not a substrate of MRP2, though kaempferol metabolites are effluxed by MRP2. It is therefore logical to propose that the

mechanism of increased absorption of tiliroside after co-incubation with kaempferol is a result of competitive inhibition of MRP2 efflux transporters by kaempferol metabolites.

Tiliroside, in the absence of inhibitors, is transferred from the apical to basolateral membranes with an apparent permeation of 0.62×10^{-6} cm/s. By comparing 20 drugs with apparent permeability ranging from 5×10^{-6} to 5×10^{-8} cm/s (Artursson and Karlsson, 1991), and 55 drugs with known human absorption value (Irvine et al., 1999), a good correlation between oral absorption in human and the results in Caco-2 model was reported. Drugs that were absorbed to >1% but <100% had apparent permeability $0.1{\sim}1.0 \times 10^{-6}$ cm/s, and drugs that are absorbed to <1% had apparent permeability $\leq 1 \times 10^{-7}$ (Artursson and Karlsson, 1991). Thus, tiliroside would be expected to be absorbed in humans.

Several factors may limit absorption of tiliroside in the Caco-2 cells compared to the intact intestine. The relatively homogenous nature of enterocytes in the model system means that there is lack of mucus-producing goblet cells (Polli et al., 2004). Consequently, there is an absence of a mucus layer which may limit the predicted absorption of hydrophobic compounds such as tiliroside. The mucus layer allows hydrophobic compounds to have more intimate contact with the intestinal epithelium and increase residence time at absorption sites (Schipper et al., 1999; Behrens et al., 2002). Furthermore, although most influx and efflux transporters are all functionally expressed in Caco-2 cells, they are expressed to a different degree compared to the in vivo situation. Maubon (2007) reported that there was at least a five

times higher mRNA expression of MRP2 efflux transporters between Caco-2 (HTB-37) and the human small intestine. As MRP2 is strongly involved in efflux of tiliroside, the Caco-2 cell model may significantly underestimate the degree of potential absorption in humans.

6. Conclusions

The present study provides evidence for absorption and metabolism of tiliroside in Caco-2 cells. Assuming a good correlation between apparent permeation in Caco-2 cells and oral absorption, tiliroside would be expected to be absorbed in the small intestine in humans. The efflux transporter, MRP2, appears to limit uptake, but as tiliroside often co-exists with kaempferol in foods, absorption of tiliroside may be increased. Tiliroside metabolism appears to be limited. Hence, tiliroside should be present in plasma after intake of tiliroside-rich foods or supplements. A human pharmacokinetic intervention study on the absorption of tiliroside is required to confirm the hypothesis.

Declaration of Interest

The authors report no declarations of interest.

Figure Legends.

Figure 1:   Chemical structure of tiliroside and its related polyphenols

Figure 2:   Transport of polyphenols across Caco-2 cells. White bars represent transportation in absorptive direction (apical to basolateral; A to B) and grey bars represent transportation in secretive direction (B to A). T: tiliroside; K: kaempferol; K3G: kaempferol-3-glucoside; CA: p-coumaric acid. Error bars are standard deviation of minimum three transwell plates. * denotes A to B transportation is significant different from B to A transportation ($P < 0.01$).

Figure 3:   Transportation of (a) tiliroside and (b) kaempferol across Caco-2 cells over time. Filled triangle-solid line = the amount of compound in an aliquot (200 μl) removed from apical media after apical loading; filled triangle-dashed line = the amount of compound in an aliquot (200 μl) removed from basolateral media after apical loading; filled circle-dashed line = the amount of compound in an aliquot (200 μl) removed from apical media after basolateral loading; and filled circle-solid line = the amount of compound in an aliquot (200 μl) removed from basolateral media after basolateral loading. Error bars are standard deviations of a minimum of three transwell plates.

Figure 4:   Representative HPLC Chromatogram (a), its enlargement (b) and UV spectra (c) of tiliroside and its potential metabolites in apical media after Caco-2 incubation. In (c), UV spectra of tiliroside and its potential metabolites, solid line = tiliroside standard; dashed line = T6; double dotted-dashed line= T5.

Figure 5:   Representative HPLC Chromatogram (a) and UV spectra (b) of kaempferol and its potential metabolites in apical media after Caco-2 incubation. In (b), UV spectra of kaempferol and its potential metabolites, Solid line = kaempferol standard; dashed line = K2; dotted-dashed line = K3; double dotted-dashed line = K1.

Figure 6:   Effect of ABC transporter inhibitors on the transportation of (a) tiliroside and (b) kaempferol. White bars represent A to B transportation; grey bars represent B to A transportation. Error bars are standard deviations of a minimum of three transwell plates. ** denotes significant difference compare with control A to B transportation ($P* < 0.01$). # denotes significant difference compare with control B to A transportation ($P^{\#} < 0.05$, $P^{\#\#} < 0.01$).

Figure 7:   Transportation of tiliroside in the presence or absence of kaempferol and p-coumaric acid. Control T represents A to B transportation of tiliroside on its own, T+K, T+C, and T+K+C represents A to B transportation of tiliroside in the

presence of kaempferol, or p-coumaric acid, or both, respectively. All compounds were incubated at 10 μM. Error bars are standard deviations of a minimum three of transwell plates. ** denotes significant difference compare with control tiliroside A to B transportation (P < 0.01).
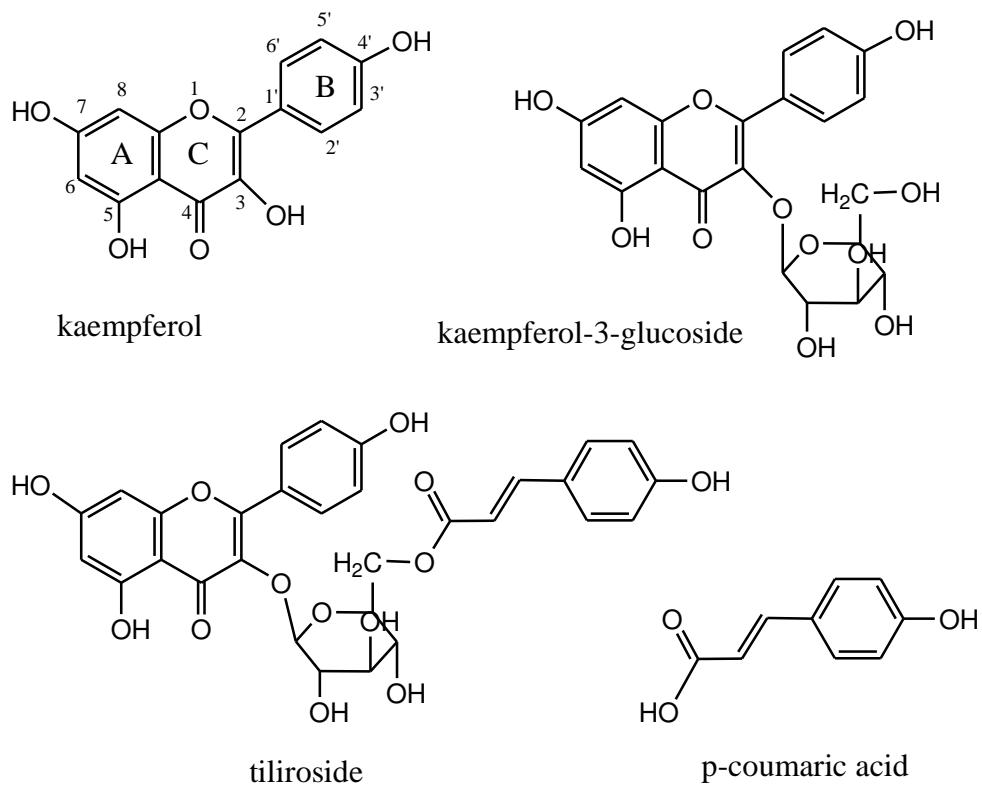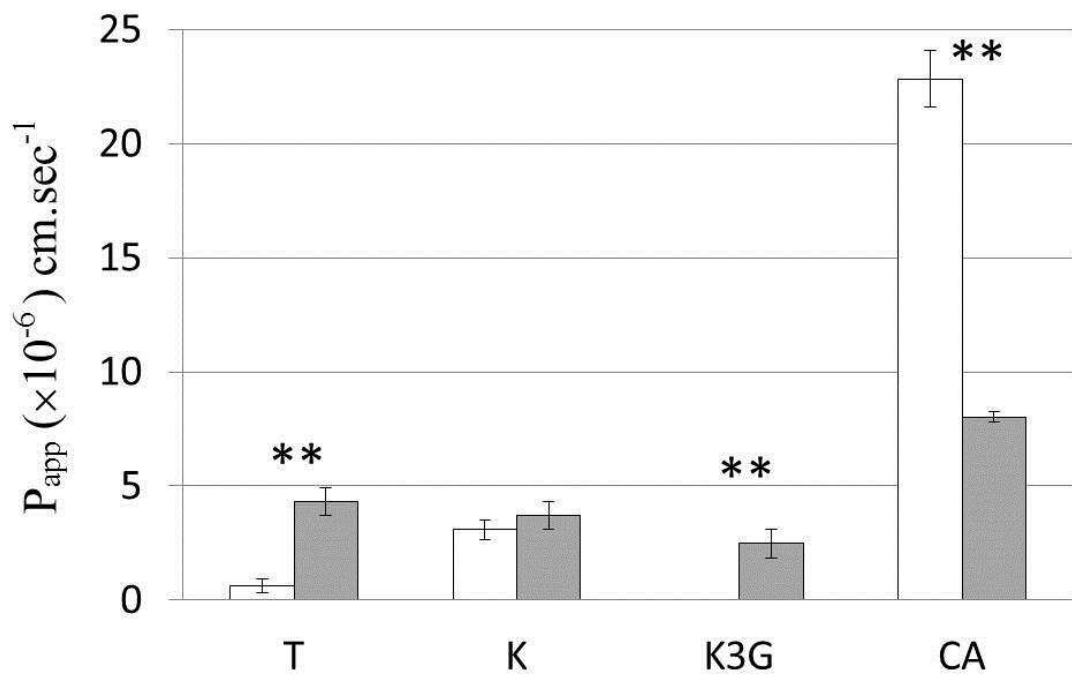
kaempferol
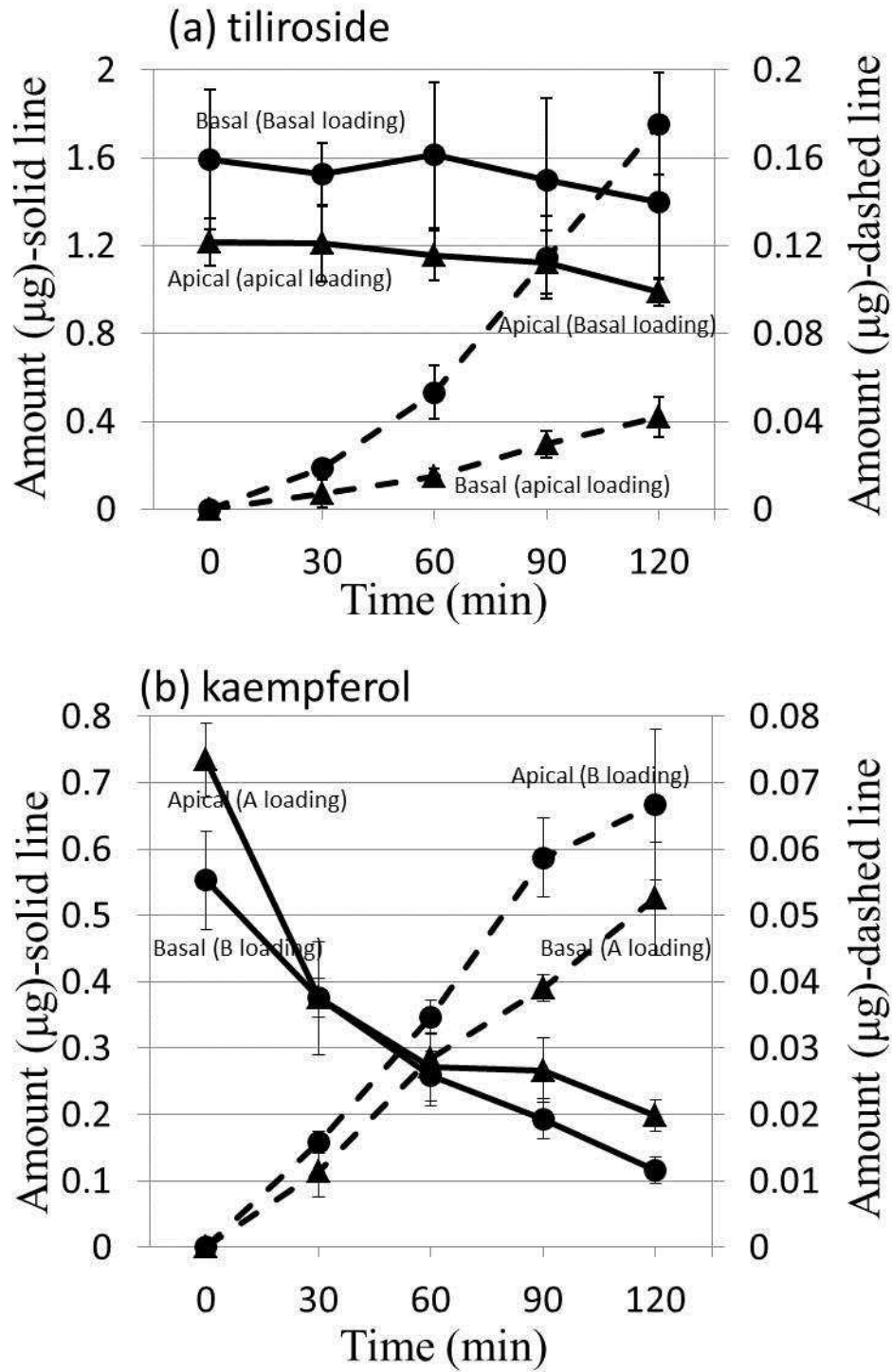
kaempferol-3-glucoside

tiliroside

p-coumaric acid

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

(a) tiliroside

(b) kaempferol
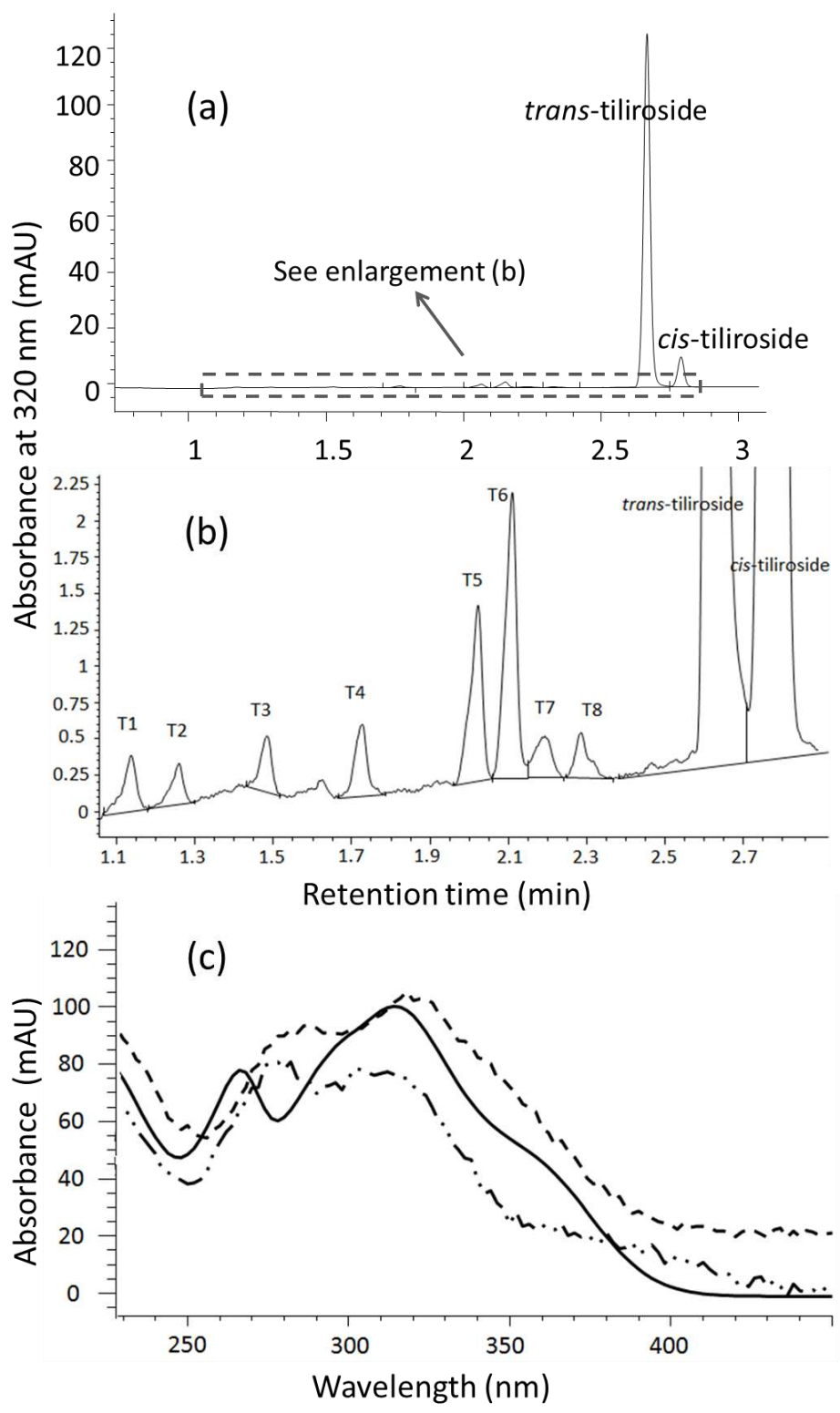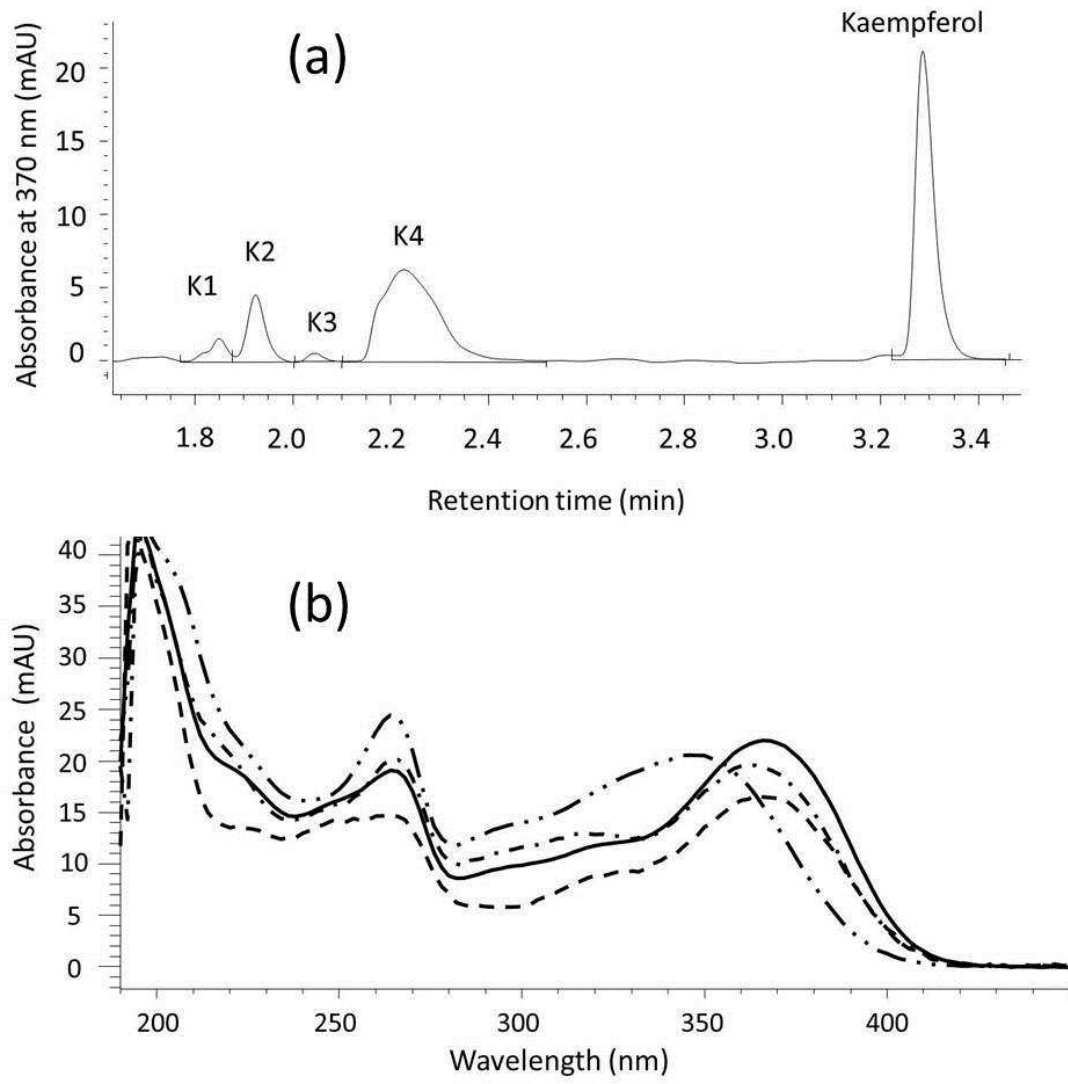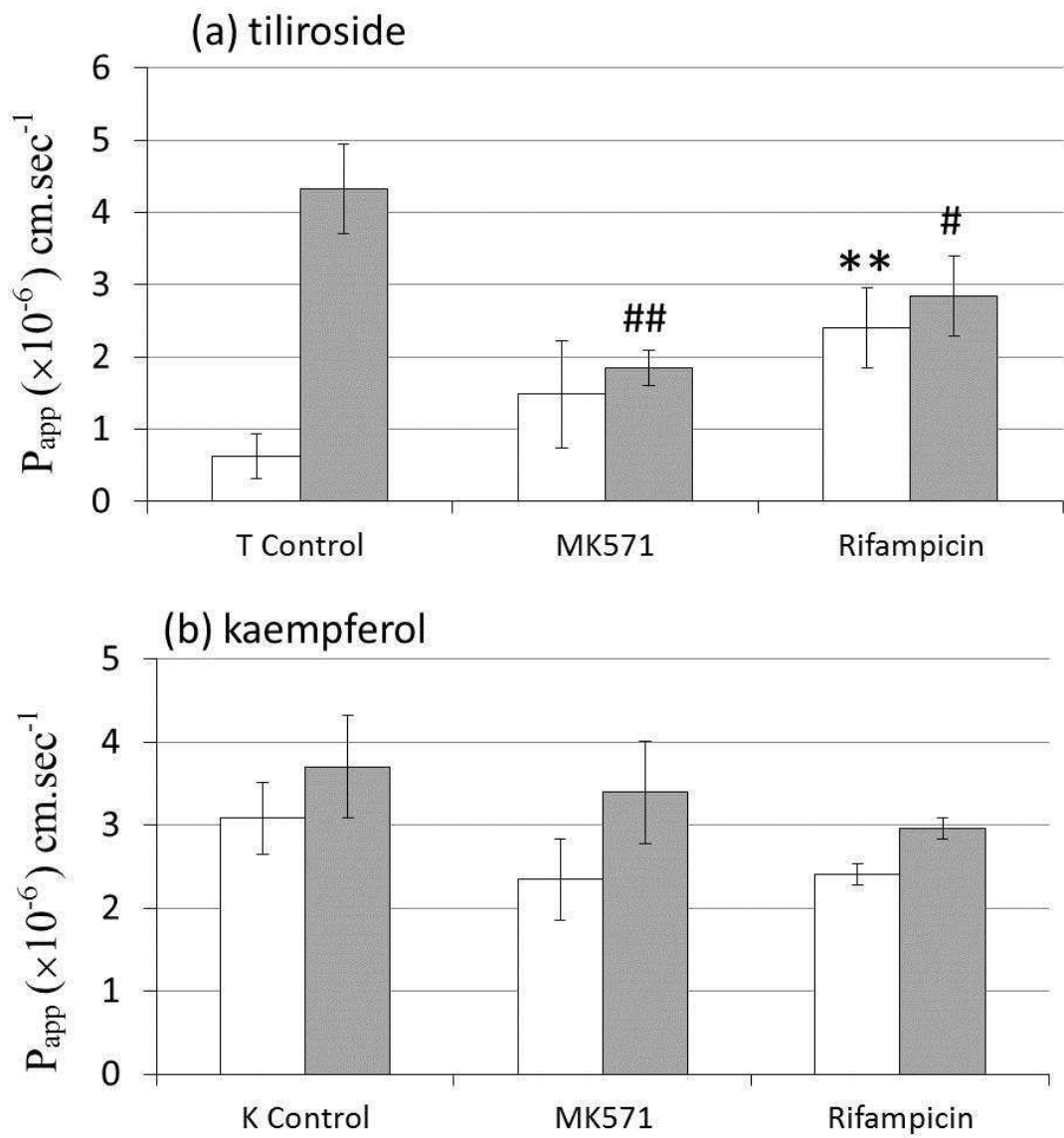
Figure 6

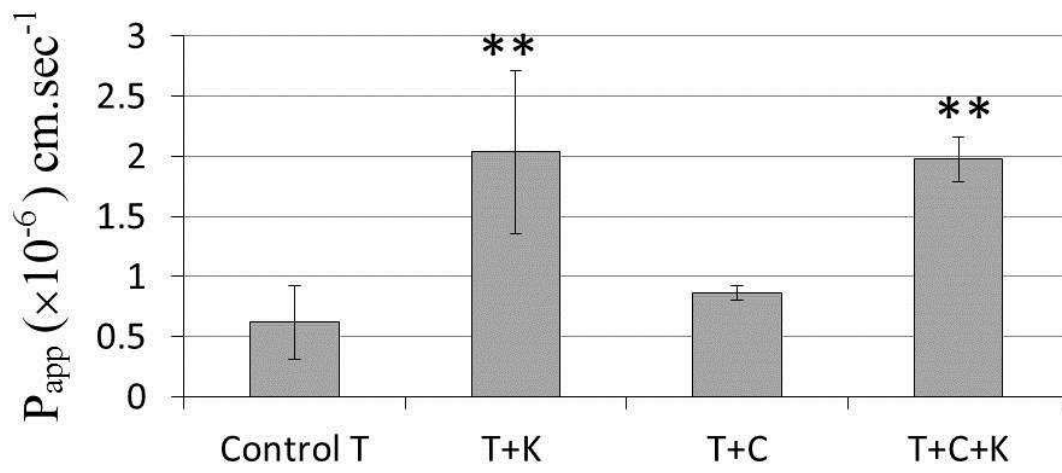Figure 7

Table 1: Transfer of tiliroside and related polyphenols across Caco-2 cells

(A) Transfer from the apical to the basolateral compartment

| Polyphenol | Amount (µg) | | | | Transfer (%) | Recovery (%) |
	Apical	Apical wash	Basal	Basal wash		
Tiliroside	9.9 ± 0.6	1.1 ± 0.02	0.1 ± 0.06	-	1	93
Kaempferol	2.0 ± 0.2	0.6 ± 0.1	0.3 ± 0.04	-	5	51*
K3G	5.5 ± 0.6	-	-	-	0	61
p-Coumaric acid	1.9 ± 0.03	-	1.2 ± 0.07	-	36	94

(B) Transfer from the basolateral to the apical compartment

| Polyphenol | Amount (µg) | | | | Transfer (%) | Recovery (%) |
	Apical	Apical wash	Basal	Basal wash		
Tiliroside	0.8 ± 0.1	0.4 ± 0.1	8.2 ± 0.4	0.8 ± 0.4	7	86
Kaempferol	0.4 ± 0.06	0.3 ± 0.1	1.6 ± 0.2	0.3 ± 0.2	7	46*
K3G	0.4 ± 0.09	-	5.7 ± 0.4	-	4	68
p-Coumaric acid	0.4 ± 0.01	-	2.6 ± 0.1	-	12	91

Polyphenols (10 µM) were applied to Caco-2 cells on the apical (pH 6) or basolateral side (pH 7.4) and transfer to opposite side was monitored by HPLC after a 2 h incubation, 37°C. Standard deviation is for a minimum of three transwell plates.* Value for kaempferol does not include kaempferol metabolites. 10µM of tiliroside, kaempferol, K3G, and p-coumaric acid is equivalent to 11.9 µg, 5.7 µg, 9.0 µg and 3.3 µg respectively under current condition applied. Transfer % = amount of compound in receiving compartment divided by total compound applied × 100%.

References

Artursson P, Karlsson J. (1991). Correlation between oral-drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. Biochem Biophys Res Commun 175: 880-5.

Barrington R, Williamson G, Bennett RN, Davis BD, Brodelt JS, Kroon PA. (2009). Absorption, conjugation and efflux of the flavonoids, kaempferol and galangin, using the intestinal CaCo-2/TC7 cell model. J Funct Foods 1: 74-87.

Behrens I, Pena AIV, Alonso MJ, Kissel T. (2002). Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: The effect of mucus on particle adsorption and transport. Pharm Res 19: 1185-93.

Chantret I, Rodolosse A, Barbat A, Dussaulx E, Brotlaroche E, Zweibaum A, Rousset M. (1994). Differential expression of sucrose-isomaltase in clones isolated from early and late passages of the cell-line Caco-2-evidence for glucose-dependent negative regulation. J Cell Sci 107: 213-25.

Chen J, Lu YH, Wei DZ, Zhou XL. (2009). Establishment of a fingerprint of raspberries by LC. Chromatographia 70: 981-5.

Day AJ, DuPont MS, Ridley S, Rhodes M, Rhodes MJC, Morgan MRA, Williamson G. (1998). Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver beta-glucosidase activity. FEBS Lett 436: 71-5.

Day AJ, Bao YP, Morgan MRA, Williamson G. (2000). Conjugation position of quercetin glucuronides and effect on biological activity. Free Radic Biol Med 29: 1234-43.

Day AJ, Gee JM, DuPont MS, Johnson IT, Williamson G. (2003). Absorption of quercetin-3-glucoside and quercetin-4 '-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. Biochem Pharmacol 65: 1199-206.

Hu M, Chen J, Lin H. (2003). Metabolism of flavonoids via enteric recycling: mechanistic studies of disposition of apigenin in the Caco-2 cell culture model. J Pharmacol Exp Ther 307: 314-21.

Hunter J, Jepson MA, Tsuruo T, Simmons NL, Hirst BH. (1993). Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells kinetics of vinblastine secretion and interaction with modulators. J Biol Chem 268: 14991-7.

Irvine JD, Takahashi L, Lockhart K, Cheong J, Tolan JW, Selick HE, Grove JR. (1999).

MDCK (madin-darby canine kidney) cells: a tool for membrane permeability screening. J Pharm Sci 88: 28-33.

Itoh T, Ninomiya M, Yasuda M, Koshikawa K, Deyashiki Y, Nozawa Y, Akao Y, Koketsu M. (2009). Inhibitory effects of flavonoids isolated from Fragaria ananassa Duch on IgE-mediated degranulation in rat basophilic leukemia RBL-2H3. Bioorg Med Chem 17: 5374-79.

Konishi Y, Shimizu M. (2003). Transepithelial transport of ferulic acid by monocarboxylic acid transporter in Caco-2 cell monolayers. Biosci Biotechnol Biochem 67: 856-62.

Konishi Y, Kobayashi S. (2004) Transepithelial transport of chlorogenic acid, caffeic acid, and their colonic metabolites in intestinal Caco-2 cell monolayers. J Agric Food Chem 52: 2518-26.

Kumarasamy Y, Cox PJ, Jaspars M, Rashid MA, Sarker SD. (2003). Bioactive flavonoid glycosides from the seeds of Rosa canina. Pharm Biol 41: 237-42.

Leitão GG, Soares SSV, Brito TDM, Delle MF. (2000). Kaempferol glycosides from Siparuna apiosyce. Phytochemistry 55: 679-82.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. (1997). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Delivery Rev 23: 3-25.

Luo Z, Murray BS, Yusoff A, Morgan MRA, Povey MJW, Day AJ. (2011). Particle-stabilizing effects of flavonoids at the oil-water interface. J Agric Food Chem 59: 2636-45.

Matsuda H, Ninomiya K, Shimoda H, Yoshikawa M. (2002). Hepatoprotective principles from the flowers of Tilia argentea (Linden): Structure requirements of tiliroside and mechanisms of action. Bioorg Med Chem 10: 707-12.

Matsson P, Pedersen JM, Norinder U, Bergstrom CAS, Artursson P. (2009). Identification of novel specific and general inhibitors of the three major human ATP-binding cassette transporters P-gp, BCRP and MRP2 among registered drugs. Pharm Res 26: 1816-31.

Maubon N, Le Vee M, Fossati L, Audry M, Le Ferrec E, Bolze S, Fardel O. (2007). Analysis of drug transporter expression in human intestinal Caco-2 cells by real-time PCR. Fundam Clin Pharmacol 21: 659-63.

Németh K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, Williamson G, Swallow DM, Kroon PA. (2003). Deglycosylation by small intestinal epithelial cell beta-glucosidases is a

critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur J Nutr 42: 29-42.

Nowak R. (2003). Separation and quantification of tiliroside from plant extracts by SPE/RP-HPLC. Pharm Biol 41: 627-30.

Ninomiya K, Matsuda H, Kubo M, Morikawa T, Nishida N, Yoshikawa M. (2007). Potent anti-obese principle from Rosa canina: Structure requirements and mode of action of trans-tiliroside. Bioorg Med Chem Lett 17: 3059-64.

O'Leary KA, Day AJ, Needs PW, Mellon FA, O'Brien NM, Williamson G. (2003). Metabolism of quercetin-7-and quercetin-3-glucuronides by an in vitro hepatic model: the role of human beta-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. Biochem Pharmacol 65: 479-91.

Polli JE, Balakrishnan A, Seo PR. (2004). Human intestinal cellular characteristics and drug permeability. In: Lu DR, Øie S, editors. Cellular drug delivery principles and practice, New Jersey: Humana Press Inc, 163-80.

Poquet L, Clifford MN, Williamson G. (2008). Transport and metabolism of ferulic acid through the colonic epithelium. Drug Metab Dispos 36: 190-7.

Sala A, Recio MC, Schinella GR, Manez S, Giner RM, Cerda-Nicolas M, Rosi JL. (2003). Assessment of the anti-inflammatory activity and free radical scavenger activity of tiliroside. Eur J Pharmacol 461: 53-61.

Schipper NGM, Varum KM, Stenberg P, Ocklind G, Lennernas H, Artursso P. (1999). Chitosans as absorption enhancers of poorly absorbable drugs 3: Influence of mucus on absorption enhancement. Eur J Pharm Sci 8: 335-43.

Walgren RA, Walle UK, Walle T. (1998). Transport of quercetin and its glucosides across human intestinal epithelial Caco-2 cells. Biochem Pharmacol 55: 1721-27.

Walgren RA, Karnaky KJ, Lindenmayer GE, Walle T. (2000). Efflux of dietary flavonoid quercetin 4 '-beta-glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2. J Pharmacol Exp Ther 294: 830-6.

Williamson G, Manach C. (2005). Bioavailability and bioefficacy of polyphenols in human. II. Review of 93 intervention studies. Am J Clin Nutr 81(suppl): 243S-55S.