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Research Paper

Quercetin and naringenin transport across human intestinal Caco-2 cells

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Abstract

Objectives Flavonoids are phenolic compounds found in most edible fruits and vegetables. Previous studies have demonstrated their biological and beneficial effects on human health. However, their bioavailability and, in particular, their intestinal absorption mechanism have not yet been clearly identified. The aim of our work was to quantify and to characterize *in vitro* the nature of the transport of two flavonoids distinguished by their physicochemical and pharmacological properties: quercetin, a flavan-3-ol, and naringenin, a flavanone.

Methods Differentiated and polarized Caco-2 human intestinal epithelial cell lines were used for this purpose.

Key findings In our experimental conditions, quercetin and naringenin were poorly absorbed by Caco-2 cells. Quercetin was absorbed by passive diffusion and a pH-dependent mechanism mediated by the organic anion transporting protein B (OATP-B). It was not a multidrug resistance associated protein (MRP)1 substrate, but was substrate of the MRP2 efflux transporter and not P-glycoprotein (P-gp). Intestinal permeability from the apical to the basolateral side was higher for naringenin than for quercetin, which was partly explained by naringenin's physicochemical characteristics. Naringenin, partially absorbed by passive diffusion, was also an ATP-dependent transport substrate mediated by MRP1, but was not an OATP-B substrate. However, naringenin was secreted via active P-gp and MRP2 efflux transporters.

Conclusions The contribution of ATP-dependent efflux transporters (MRP2 and P-gp) to the permeability of these compounds in the apical side could explain their low bioavailability. In conclusion, knowledge of the absorption mechanism of these two flavonoids was used to determine the intake level that has a beneficial effect on human health and their putative role in food–drug interactions.

Keywords absorption; Caco-2 cell; flavonoids; naringenin; quercetin; transporter

Introduction

Flavonoids are phenolic compounds belonging to a superfamily of polyphenols that are produced by the secondary metabolism of plants. Over 4000 different compounds with 2-phenylchromone nuclei as a common feature have been described. They are distinguished by their nucleus modifications, the number and position of the phenol functions and their glycosylation level. Flavonoids are found in many edible fruits and vegetables in their aglycone forms and glycoside derivatives. Among the aglycone forms, several groups of flavonoids are distinguishable: flavones and isoflavones, flavonols, flavanones and chalcones. The main role of flavonoids in plants is to protect them from physical (UVA and UVB radiation) and biological (fungi and predators) aggressors. Their regular consumption may also have beneficial implications for human health.[1,2] Many of the biological effects of these flavonoids have been attributed to their antioxidant properties, which are derived from their ability to scavenge oxygen free radicals. In epidemiologic studies, flavonoid intake has often been linked to a reduced incidence or delayed development of diseases such as cancer, and cardiovascular and other age-related or inflammatory diseases.[3–7]

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Because of this, understanding of flavonoid bioavailability and in particular their absorption mechanisms is essential in determining the level of flavonoid intake that is necessary to provide a beneficial effect to human health. The absorption of flavonoids is rapid but only a small quantity is absorbed. However, the exact transport mechanism of this class of compounds across intestinal epithelial cells is not clearly understood.

For a long time, absorption of xenobiotics was described as a passive diffusion mechanism.[8] More recently, in addition to this 'classical' absorption pathway, other mechanisms involving membrane transport proteins have been described. Two main carrier superfamilies are involved in drug absorption: ATP-binding cassettes (ABC) and solute carriers (SLCs).

The ABC proteins, which were first described in cancer multidrug-resistance studies, are involved in the cellular efflux processes for several drugs. Although there are a large number of ABC transporters, ABCB1 (P-glycoprotein (P-gp) encoded by the multidrug resistance gene MDR1), ABCC (multidrug resistance associated proteins (MRPs)) and ABCG2 (breast cancer resistance protein (BCRP)) are of particular importance due to their involvement in clinically important drug accessibility.

The family of uptake SLCs is less well characterized but seems to play an important control role in cellular trafficking of many drugs. In particular, these proteins act as transport mechanisms for many different kinds of drugs. Examples include organic anion transporting polypeptide (OATP), organic anion transporters (OAT), organic cation transporters (OCT), monocarboxylic acid transporters (MCT) and peptide transporters (PepT).[9]

Few data on flavonoid absorption have been published and although the exact mechanism still remains unclear their absorption is always described as low. Some data indicate that quercetin is preferentially absorbed in a monoglucoside form, such as quercetin-3-glucoside.[10] The aglycone form is more available than complex glycosides.[11–13] Other authors have indicated that phloridzin, a glycosylated flavonoid, seems to be transported by the sodium-dependent glucose transporter 1 (SGLT1).[14–16] Even if flavonoids are not described as efflux carrier substrates, therefore, it seems that some of them are able to modulate the activity of these carriers.[17,18] One study has demonstrated that naringenin, a major compound of grapefruit juice, is able to inhibit P-glycoprotein, thus explaining the well-known interaction of grapefruit and drugs.[19–22]

The aim of our work was to study simultaneously, *in vitro* and under various pH conditions, the transport mechanisms of quercetin and naringenin by human intestinal epithelial Caco-2 cell monolayer in order to better understand their absorption mechanisms and to estimate their putative role in food–drug interactions.

Methods

Chemical reagents

Dulbecco's modified Eagle's medium (DMEM), non-essential amino acid solution, antibiotic solution (50 000 U/l penicillin and 50 000 mg/l streptomycin), Hank's balanced salt solution (HBSS) and phosphate saline buffer (PBS, without calcium and magnesium) were purchased from Invitrogen (Cergy-Pontoise, France). D-[^{3}H]-mannitol, acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid, phosphoric acid, ammonium acetate, fetal bovine serum, sodium orthovanadate, propranolol, verapamil, probenecid, digoxin, estrone-3 sulfate, quercetin and naringenin were obtained from Sigma-Aldrich (St Quentin Fallavier, France). MK-571 was acquired from Cayman Chemicals (Ann Arbor, MI, USA). PSC-833 was kindly donated by Novartis AG (Basel, Switzerland).

Cell culture

Caco-2 cells from the European Type Culture Collection were grown to less than 90% confluency at 37°C in a humidified atmosphere of 5% CO_2 in 75 cm^2 T-flasks in DMEM, supplemented by 10% fetal calf serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. After trypsinization, Caco-2 cells were seeded in 12 mm i.d. Transwell inserts (polycarbonate membrane of 0.4 μm pore size; Greiner, France) in 12-well plates at a density of 10^5 cells/cm^2 for around 20 days.

The integrity of the cell monolayer was checked before and after each experiment by measuring transepithelial electrical resistance (TEER) using a voltometer (EVOM, World Precision Instruments, Aston, Stevenage, UK). Only monolayers with a TEER value above 300 Ω/cm^2 were used for transport studies.

As a marker of paracellular transport, D-[^{3}H]-mannitol (4.5 μCi/ml) was used. To quantify cellular fluxes, we used propranolol (100 μM) as a passive transcellular marker and digoxin (30 μM) as the P-gp mediated efflux substrate.

Quercetin and naringenin transcellular transport by Caco-2 cells

In order to be more representative of the in-vivo situation, our experiments were carried out at pH 6.5 on the apical side (the pH of gut lumen), in contrast to standard studies of transport, which are performed at pH 7.4.

All experiments were performed in HBSS buffer containing 20 mM HEPES and 20 mM D-glucose, which was adjusted to pH 6.5 or pH 7.4 on the apical side (0.5 ml) depending on the experiment, and to pH 7.4 on the basolateral side (1.5 ml). After washing the inserts twice with warm HBSS buffer for 30 min, transepithelial permeability was measured for increasing substrate concentrations (10–100 μM). The compounds tested were dissolved in methanol then diluted in HBSS to a 0.5% final concentration of methanol.

Flavonoid transport was studied in the presence of efflux and influx inhibitors like verapamil or PSC833 (P-gp inhibitors),[23,24] MK571 (an MRP1 inhibitor),[25] probenecid (an MRP2 inhibitor)[26] and estrone-3 sulfate (an OATP substrate and inhibitor).[27] Inhibitor stock solutions were dissolved in methanol then diluted in HBSS (with a final concentration of 50 μM, except for PSC833, which was used at 10 μM). Inhibitors were added at both the apical and basolateral sides of Caco-2 cell monolayers. After 45 min of

preincubation of the inhibitor solutions, the flavonoid solution was added to either the apical or basolateral side (0.5 or 1.5 ml respectively) and HBSS buffer was added to the other side. As a control, in each inhibition experiment flavonoid transport was also assessed in the absence of any inhibitor.

Samples of 200 μl were taken after 30, 60, 90 and 120 min from the receiver compartment then immediately replaced. For the influx study, the receiver side was the basolateral chamber, while for the efflux study the receiver side was the apical chamber.

Stability and cytotoxicity of flavonoids for transport studies

The stability of quercetin and naringenin was investigated in an HBSS medium, in which both compounds were stable (data not shown). The toxicity of quercetin, naringenin and the inhibitors (at 0.5% methanol final concentration) was evaluated by flow cytometry. Cytotoxicity was assessed using the Guava Nexin kit on the Guava Easycyte Plus system as detailed elsewhere.[28] At a final methanol concentration of 0.5%, neither the monolayer integrity nor cell survival was affected.

Caco-2 cell concentration was 8×10^4 cells/ml and cells were treated with different concentrations (1–100 μM) of quercetin or naringenin for 3 h. Positive control of apoptosis was obtained by adding 50 μM of Celastrol[28] and negative control with dimethyl sulfoxide (DMSO) at 1% or methanol at 0.5%. Cell samples were stained with annexin V and 7-aminoactinomycin D (7-AAD) for 20 min on ice. Nexin buffer was then added to stop the staining reaction, and data were immediately acquired on the Guava Easycyte Plus system. Cells were gated based on forward and side-scattered light and results reported as the percentage of gated cells that were negative for both annexin V and 7-AAD staining.

Analytical methods

Extraction procedure

A 200 μl sample was added to the same volume of methanol and centrifuged three times at 750*g* for 10 min. The supernatants were then collected and evaporated to dryness and reconstituted with methanol before analysis by liquid chromatography (LC).

LC analysis

The samples were analysed by reversed-phase liquid chromatography coupled to UV detection. The analytical system consisted of an autosampler (Kontron model 360) connected to a pump (Merck–Hitachi, model L6200) and a UV programmable detector (Merck–Hitachi, model L 4200).

Chromatographic quercetin analysis was performed on a Lichrosphere RP-C18 column (125 × 4 mm, 5 μm particle size, Macherey-Nagel, France) protected by a superguard LC-18 precolumn (10 mm × 4 mm, Macherey–Nagel, France) at room temperature. The mobile phase consisted of an acid solution containing a mixture of phosphoric acid, pH 2.3 and acetonitrile (65:35, v/v) at a flow rate of 1 ml/min. Quercetin elution was recorded at a constant wavelength of 365 nm. Quantitation was by comparison of peak area measurements to quercetin standard curves.

Naringenin chromatographic analysis was performed on a Lichrosphere ODS-2 column (250 × 4.6 mm, 5 μm particle size, Macherey–Nagel, France) protected by a superguard LC-18 precolumn (10 mm × 4 mm, Macherey–Nagel, France) at room temperature. The mobile phase consisted of an acid solution containing a mixture of ammonium acetate buffer (pH 4.9) and acetonitrile (79 : 21, v/v) at a flow rate of 1 ml/min. Naringenin elution was recorded at a constant wavelength of 292 nm. Quantitation was by comparison of peak area measurements to naringenin standard curves.

Evaluation of transport rates

Quercetin and naringenin transport was quantified by determination of their amounts in the different containers and their apparent permeability coefficients (*Papp*) across Caco-2 cell monolayers in both apical (A) to basolateral (B) ($Papp_{AB}$) and B to A ($Papp_{BA}$) directions.

The permeability coefficient expressed in centimetres per second (cm/s) was calculated using the equation of Artursson and Karlsson.[29]

$$Papp = (\Delta Q/\Delta t) \times [1/(A \times Co)]$$

where $\Delta Q/\Delta t$ is the rate at which the compound appears in the receiver chamber, *Co* is the initial concentration of the compound in the donor chamber and *A* represents the surface area of cell monolayer.

The efflux ratio *ER* (i.e. the net efflux of our compounds) was determined by calculating the ratio of *Papp* in the secretory (BA) direction divided by that in the absorptive (AB) direction, according to the following equation:[30]

$$ER = Papp_{BA} / Papp_{AB}.$$

At equilibrium, a similarity between the *Papp* in two directions ($ER = 1$) indicated that the molecular transport through the intestinal epithelium was a passive diffusion mechanism. A difference between the two *Papp* values could be explained by either facilitated or active transport mechanism via membrane carriers. $Papp_{AB}$ greater than $Papp_{BA}$ ($ER < 2$) highlighted an involvement of a polarized influx transport. In contrast, $Papp_{AB}$ lower than $Papp_{BA}$ ($ER > 2$) indicated a polarized efflux transport.

Statistical analysis

Data were collected from the three separate experiments, each being performed in triplicate. The differences between the mean values were analysed for significance using one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered as being statistically significant.

Results

Permeability coefficient determination of cellular flux markers in Caco-2 cells

The transport of drugs can be estimated by in-vitro experiments using a Caco-2 cell monolayer, which resembles the intestinal barrier. Prior to using this model for the

assessment of individual compounds, validation studies have to be conducted to confirm the suitability of the model under the selected experimental conditions. Permeability of mannitol, a paracellular transport marker, was $1.02 \pm 0.08 \times 10^{-6}$ cm/s. Permeability of propranolol, a transcellular transport marker, was higher, with a $Papp_{AB}$ (absorption) equivalent to the $Papp_{BA}$ (secretion) ($Papp_{AB} = Papp_{BA} = 41.9 \pm 3.30 \times 10^{-6}$ cm/s). Our data showed that the cell monolayer was intact and that cells were polarized. The values of apparent permeability coefficients (*Papp*) of cellular flux markers in Caco-2 cells were equivalent to the values described in the literature.[31]

Digoxin transport showed a very low permeability from apical to basolateral side, with a $Papp_{AB}$ value of $0.70 \pm 0.08 \times 10^{-6}$ cm/s, whereas the BA flux value was higher ($Papp_{BA} = 57.7 \pm 5.1 \times 10^{-6}$ cm/s). In the presence of 10 μM of PSC833, a P-gp inhibitor, AB flux was restored ($Papp_{AB} = 5.7 \pm 0.1 \times 10^{-6}$ cm/s) and BA flux was decreased ($Papp_{BA} = 27.7 \pm 2.4 \times 10^{-6}$ cm/s). Our results showed that the active efflux transport mediated by P-gp was functional in our Caco-2 cells model.

Cytotoxicity assay of flavonoids

In our experimental conditions and for all tested compounds, no significant cytotoxicity was revealed since 90% of the cells were viable up to 100 μM of quercetin or naringenin (Figure 1).

Transport of quercetin through Caco-2 cells

The transcellular influx of quercetin was determined after apical incubation at 25, 50 and 100 μM (Figure 2a) with sampling on the basolateral side every 30 min throughout the 120 min incubation period. The quercetin $Papp_{AB}$ value at 100 μM concentration was about fivefold higher than for mannitol, a marker of paracellular transport (quercetin $Papp_{AB} = 5.53 \pm 0.32 \times 10^{-6}$ cm/s versus mannitol $Papp_{AB} = 1.02 \pm 0.08 \times 10^{-6}$ cm/s), suggesting an oral absorption of quercetin by the transcellular route. Moreover, quercetin permeability was lower than that of propranolol, a trancellular flux marker (quercetin $Papp_{AB} = 5.53 \pm 0.32 \times 10^{-6}$ cm/s versus propranolol $Papp_{AB} = 41.9 \pm 3.30 \times 10^{-6}$ cm/s), suggesting that the quercetin was poorly absorbed.

The determination of the exact mechanisms involved in this transport required the comparison of quercetin absorption (AB flux) and secretion (BA flux) at pH 7.4 and pH 6.5 on the apical side. A pH of 6.5 on the apical side was chosen since it is close to that of the in-vivo intestinal lumen and hence the experiment should be more representative of the in-vivo situation. Quercetin permeability from apical to

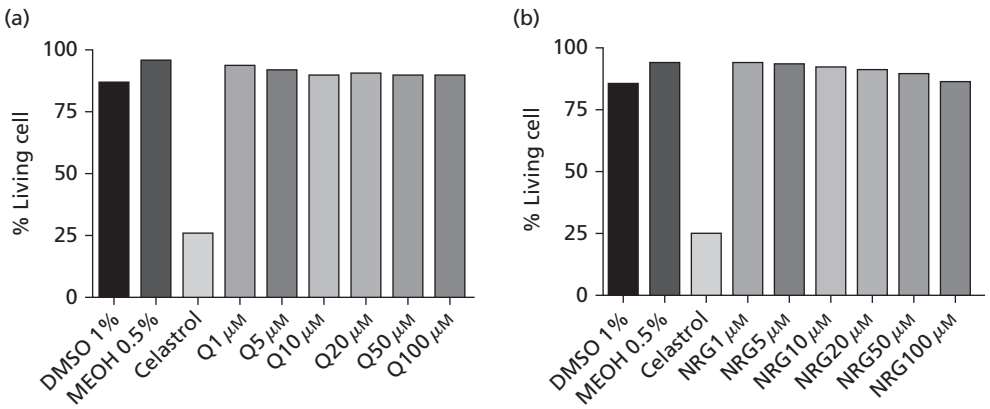


Figure 1 Cytotoxicity assay of (a) quercetin and (b) naringenin across human intestinal Caco-2 cells. Percentage of living cells after incubation of different concentrations of quercetin and naringenin (1–100 μM). Celastrol: positive control and DMSO 1%, MeOH 0.5%. DMSO, dimethyl sulfoxide; NRG, naringenin; Q, quercetin.

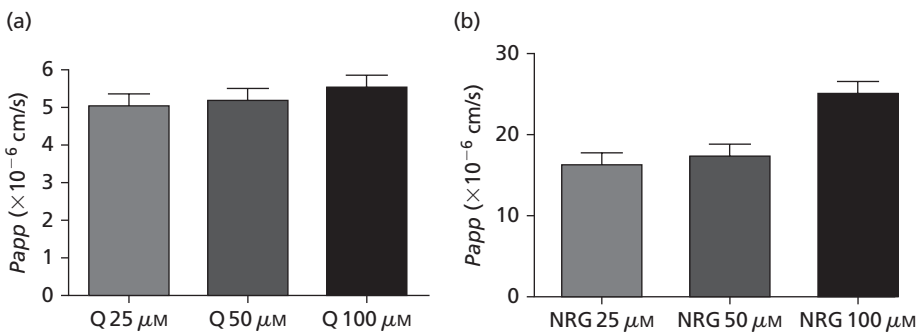


Figure 2 Apparent permeability coefficients of quercetin and naringenin at pH 7.4 and at various concentrations. (a) Quercetin (Q) permeability at 25 μM, 50 μM and 100 μM. (b) Naringenin (NRG) permeability at 25 μM, 50 μM and 100 μM.

basolateral side was higher at pH 6.5 ($Papp_{AB}$ = 9.20 ± 0.34 × 10^{-6} cm/s) than at pH 7.4 ($Papp_{AB}$ = 5.53 ± 0.32 × 10^{-6} cm/s), suggesting a pH-dependent transport mechanism.

Quercetin transport was carried out in glucose-free buffer. At both pHs, quercetin permeability in a glucose-free buffer is similar to its permeability in a HBSS buffer containing 20 mM (Figure 3). Quercetin permeability in the basolateral-to-apical direction ($Papp_{BA}$) is higher than its permeability from apical to basolateral ($Papp_{AB}$). The quercetin efflux ratios were 1.98 and 2.67 at pH 7.4 and 6.5, respectively (Figure 3). Hence this difference in transport between the two directions under both pH conditions suggests the existence of a polarized transport mechanism, and suggests a protein carrier-mediated transport efflux. Our results imply that quercetin may be a substrate of a primary active efflux pump.

In the presence of sodium orthovanadate, an ATPase Na^+/K^+-dependent inhibitor, quercetin transport was totally inhibited ($Papp_{AB}$ = 0.2 ± 0.1 × 10^{-6} cm/s). This implies that quercetin transport is ATP-dependent (active co-transport ATP dependent).

Figure 4a and b shows the quercetin transport experiments in the presence of various selective efflux and influx transporters inhibitors at pH 7.4 and pH 6.5, respectively. In the presence of verapamil, a competitive P-gp inhibitor, quercetin absorption was not affected ($Papp_{AB}$ quercetin + verapamil = 4.58 ± 0.48 × 10^{-6} cm/s and 8.53 ± 0.47 × 10^{-6} cm/s at pH 7.4 and pH 6.5, respectively, versus *Papp* quercetin alone = 5.53 ± 0.32 × 10^{-6} cm/s and 9.20 ± 0.34 × 10^{-6} cm/s). However, the permeability coefficient of quercetin from basolateral to apical side increased in the presence of verapamil ($Papp_{BA}$ quercetin = 10.94 ± 0.3 × 10^{-6} cm/s and 24.56 ± 0.5 × 10^{-6} cm/s at pH 7.4 and pH 6.5 respectively versus $Papp_{BA}$ quercetin + verapamil = 21.94 ± 0.45 × 10^{-6} cm/s and 54.56 ± 0.9 × 10^{-6} cm/s).

Quercetin was not a P-gp substrate in our experimental conditions but could be a P-gp modulator. In the presence of probenecid, an MRP2 inhibitor,[32] quercetin permeability in the apical-to-basolateral direction was greatly enhanced ($Papp_{AB}$ = 13.26 ± 0.82 × 10^{-6} cm/s and 25.53 ± 2.42 × 10^{-6} cm/s at pH 7.4 and pH 6.5, respectively) and quercetin permeability from basolateral to apical was decreased ($Papp_{BA}$ = 5.85 ± 0.42 × 10^{-6} cm/s and 10.8 ± 0.82 × 10^{-6} cm/sec at pH 7.4 and pH 6.5, respectively). These results suggest that quercetin secretion involves an efflux MRP-2 transporter. These latter findings are in full accordance with our previous study on the polarized transport of quercetin ($Papp_{BA}$ > $Papp_{AB}$). In the presence of estrone-3 sulfate, an OATP inhibitor, quercetin absorption was decreased at pH 6.5 ($Papp_{AB}$ 3.36 ± 0.42 × 10^{-6} cm/s), suggesting that quercetin is an OATP substrate. This is in

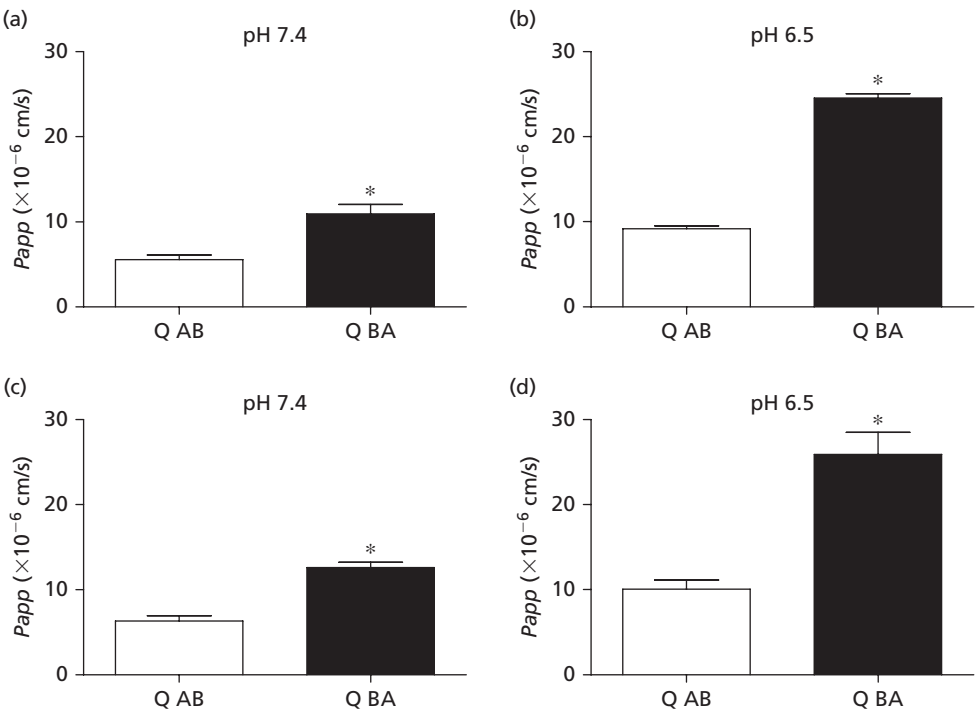


Figure 3 Quercetin transport across Caco-2 cells monolayer. (a) Bidirectional transport of quercetin (Q) across Caco-2 cells monolayer in glucose buffer (apical pH 7.4, basolateral pH 7.4) in the absorptive (AB) and secretory (BA) directions. (b) Bidirectional transport of quercetin across Caco-2 cells monolayer in glucose buffer (apical pH 6.5, basolateral pH 7.4) in the AB and BA directions. (c) Bidirectional transport of quercetin across Caco-2 cells monolayer in glucose-free buffer (apical pH 7.4, basolateral pH 7.4) in the AB and BA directions. (d) Bidirectional transport of quercetin across Caco-2 cells monolayer in glucose-free buffer (apical pH 6.5, basolateral pH 7.4) in the AB and BA directions. AB denotes apical to basolateral flux (□) and BA denotes basolateral to apical flux (■). Each set of data represents the mean ± SD of three experiments. *Significantly greater than $Papp_{AB}$ of quercetin ($P < 0.05$).

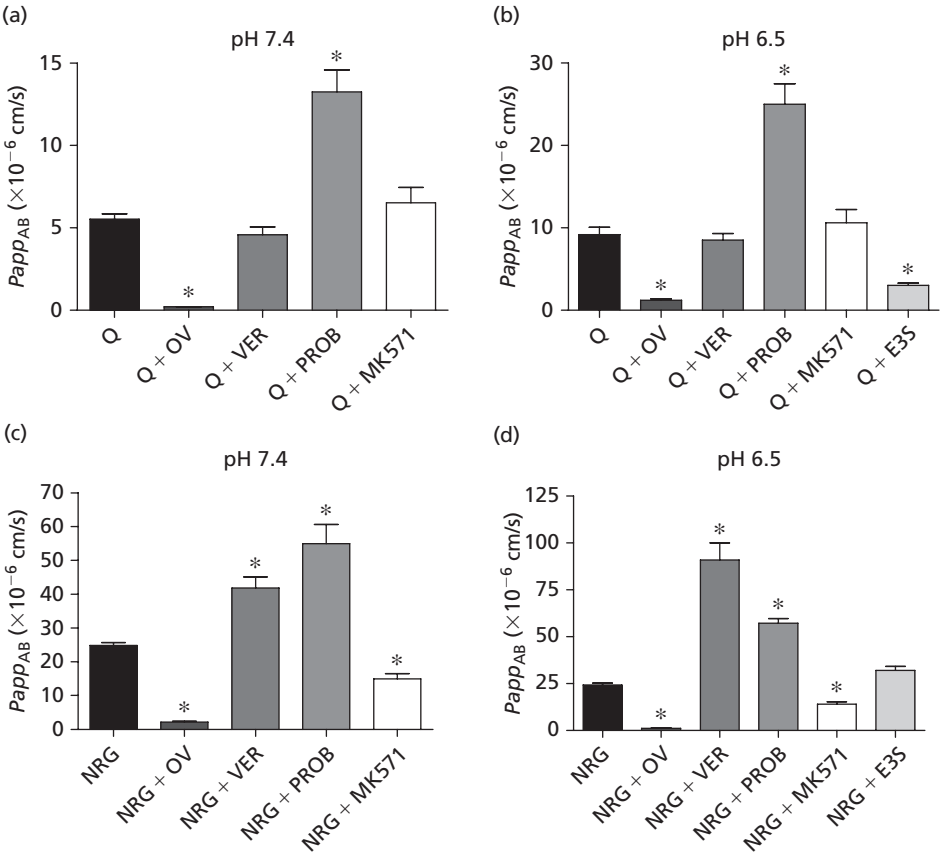


Figure 4 Quercetin and naringenin transport across Caco-2 cells monolayer with various transport inhibitors. (a) Apparent permeability of quercetin (Q) with various transport inhibitors in the absorptive direction (AB) at apical pH 7.4. (b) Apparent permeability of quercetin (Q) with various transport inhibitors in the absorptive direction (AB) at apical pH 6.5. (c) Apparent permeability of naringenin with various transport inhibitors in the absorptive direction (AB) at apical pH 7.4. (d) Apparent permeability of naringenin with various transport inhibitors in the absorptive direction at apical pH 6.5. In each case, the bar indicates mean ± SD (n = 3 experiments). OV, sodium orthovanadate; VER, verapamil; PROB, probenecid; E3S, estrone-3-sulfate. *Significantly greater and/or lower than $Papp_{AB}$ of naringenin ($P < 0.05$).

agreement with the $Papp_{AB}$ values of quercetin, which were higher at pH 6.5 than at pH 7.4, since the transport via OATP is pH dependent. In the presence of MK-571, an MRP1 inhibitor, quercetin permeability was not modified ($Papp_{AB}$ $6.53 \pm 0.93 \times 10^{-6}$ cm/s and $10.6 \pm 0.52 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively).

Transport of naringenin through the Caco-2 cells

The transcellular influx of naringenin was determined after apical loading of 25, 50 and 100 μM (Figure 2b) and sampling on the basolateral side was every 30 min and throughout the 120 min incubation period. The naringenin permeability value through Caco-2 cells at 100 μM was about 25-fold higher than for mannitol ($Papp_{AB}$ naringenin = $24.81 \pm 0.91 \times 10^{-6}$ cm/s versus $Papp_{AB}$ mannitol = $1.02 \pm 0.08 \times 10^{-6}$ cm/s).

Our results show that naringenin was transported through the human intestinal epithelium with a higher intestinal permeability than quercetin.

Naringenin transport was carried out in glucose-free buffer. The naringenin permeability in a glucose-free buffer is similar to its permeability in an HBSS buffer containing 20 mM of glucose (Figure 5). At both pH levels, the comparison of naringenin fluxes in the apical-to-basolateral and the basolateral-to-apical directions showed that naringenin permeability in the basolateral-to-apical direction ($Papp_{BA}$) was higher than for the apical-to-basolateral direction ($Papp_{AB}$). The naringenin efflux ratio was 2.89 and 2.50 at pH 7.4 and 6.5, respectively.

In contrast, naringenin's $Papp_{AB}$ value was independent of the pH ($Papp_{AB}$ naringenin = $24.81 \pm 0.91 \times 10^{-6}$ cm/s and $24.33 \pm 0.95 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively).

Consequently, our results suggest the existence of a polarized transport mechanism, and therefore a protein carrier-mediated transport efflux. Naringenin seems to be the substrate of a primary active efflux pump.

In the presence of sodium orthovanadate, naringenin absorption was inhibited ($Papp_{AB} = 2.53 \pm 0.96 \times 10^{-6}$ cm/s), suggesting that naringenin also uses an energy–ATP-dependent system.

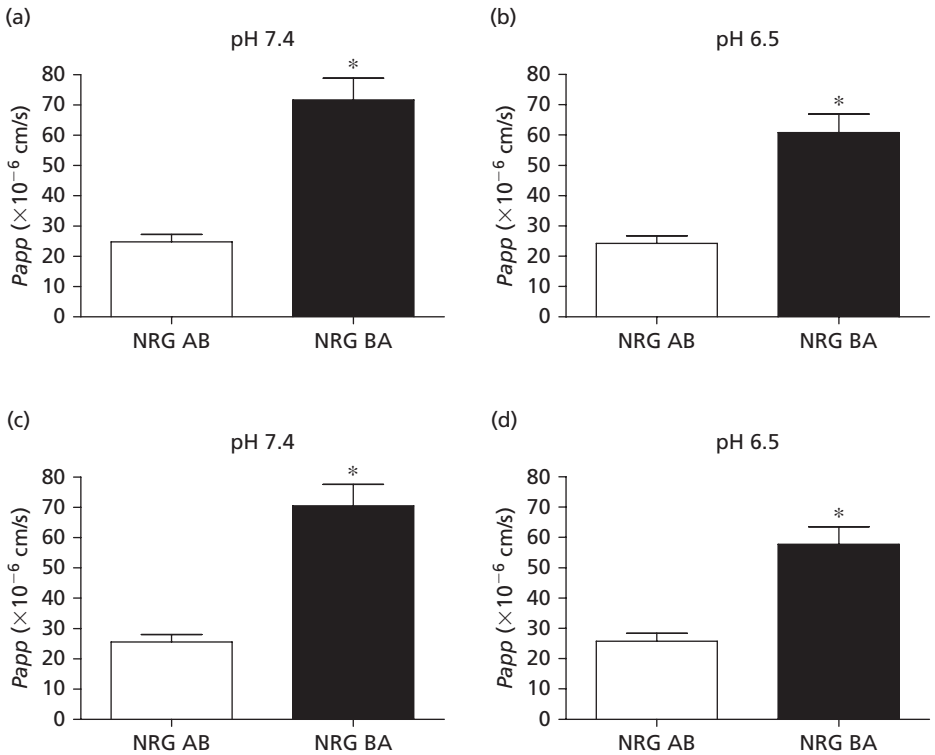


Figure 5 Naringenin transport across Caco-2 cells monolayer. (a) Bidirectional transport of naringenin (NRG) across Caco-2 cells monolayer in glucose buffer (apical pH 7.4, basolateral pH 7.4) in the absorptive (AB) and secretory (BA) directions. (b) Bidirectional transport of naringenin across Caco-2 cells monolayer in glucose buffer (apical pH 6.5, basolateral pH 7.4) in the AB and BA directions. (c) Bidirectional transport of naringenin across Caco-2 cells monolayer in glucose-free buffer (apical pH 7.4, basolateral pH 7.4) in the AB and BA directions. (d) Bidirectional transport of naringenin across Caco-2 cells monolayer in glucose-free buffer (apical pH 6.5, basolateral pH 7.4) in the AB and BA directions. AB denotes apical to basolateral transport (□) and BA denotes basolateral to apical transport (■). Each set of data represents the mean ± SD of three experiments. *Significantly greater than $Papp_{AB}$ of naringenin ($P < 0.05$).

Figure 4c and d shows naringenin transport experiments in the presence of various selective efflux and influx transport inhibitors at pH 7.4 and pH 6.5, respectively.

The naringenin intestinal permeability increases in the presence of verapamil ($Papp_{AB}$ naringenin + verapamil = $95.97 \pm 9.31 \times 10^{-6}$ cm/s and $41.81 \pm 3.31 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively). However, the permeability coefficient of naringenin from basolateral to apical was decreased in the presence of verapamil ($Papp_{BA} = 60.82 \pm 3.04 \times 10^{-6}$ cm/s and $71.71 \pm 7.17 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively, versus $Papp_{BA}$ naringenin + verapamil = $26.06 \pm 1.5 \times 10^{-6}$ cm/s and $20.93 \pm 1.9 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively). Consequently, naringenin is a P-gp substrate.

In the presence of probenecid, naringenin permeability was increased ($Papp_{AB} = 57.25 \pm 3.75 \times 10^{-6}$ cm/s and $54.9 \pm 5.67 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively) and naringenin $Papp_{BA}$ in the presence of this MRP-2 inhibitor was decreased ($15.06 \pm 0.75 \times 10^{-6}$ cm/s and $17.77 \pm 0.67 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively). These results suggest that naringenin absorption involves an MRP-2-dependent efflux transport.

In the presence of estrone-3 sulfate, naringenin absorption was increased at pH 6.5 ($Papp_{AB} = 32.06 \pm 5.05 \times 10^{-6}$ cm/s), suggesting that naringenin is not a substrate but an OATP modulator, the mechanism probably involving interaction with estrone-3 sulfate.

In the presence of MK-571, naringenin permeability was decreased ($Papp_{AB} = 14.98 \pm 1.5 \times 10^{-6}$ cm/s and $13.98 \pm 2.01 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively), showing naringenin to be an MRP1 substrate.

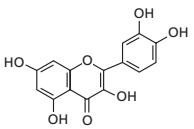
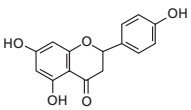
However, in the presence of phloridzin, the naringenin permeability was highly enhanced ($Papp_{AB}$ $57.27 \pm 3.27 \times 10^{-6}$ cm/s and $44.51 \pm 1.91 \times 10^{-6}$ cm/s at pH 7.4 and pH 6.5, respectively), suggesting that naringenin is not an SGLT1 substrate.

Discussion

The aim of this work was to quantify and characterize *in vitro*, on the Caco-2 human intestinal epithelial cell line, the nature of the intestinal transport of two flavonoids – quercetin, a flavan-3-ol, and naringenin, a flavanone – distinguished by their physicochemical and pharmacological properties.

The differences in their lipophilicity and water solubility could explain in part the differences in their ability to be transported through the human intestinal epithelial Caco-2 cell monolayer model. The physicochemical properties of the two flavonoids are presented in Table 1. Quercetin and naringenin are weakly acidic with low water solubility and

Table 1 Physicochemical properties of quercetin and naringenin

	Quercetin	Naringenin
Chemical structure		
Molar mass (g/mol)	302.2	272.2
log P	1.48[47,48]	2.52[49]
clog P	2.64	2.47
log D (pH 6.5)	1.00	ND
pKa	8.25	7.95
Water solubility	60 μg/ml (16°C)	475 μg/ml (25°C)
Percentage of non-ionized forms pH 6.5	98.26	96.62
Percentage of non-ionized forms pH 7.4	87.61	77.94

transported by a passive diffusion mechanism. The theoretical quantity of non-ionized forms can be calculated by taking into account their pKa and physiological pH (pH 7.4 corresponding to the blood circulation and pH 6.5 corresponding to the jejunum part of the intestine). The quantities of non-ionized forms are significant at pH 6.5 and pH 7.4 (77–99%), with higher levels at pH 6.5. However, these two compounds have low oral bioavailability *in vivo*. Quercetin exhibits lower water solubility and lower lipophilicity than naringenin. To best characterize their absorption mechanism, transport of quercetin and naringenin was studied here across human intestinal Caco-2 cells in the presence of various membrane transport inhibitors. This experimental model has been extensively used in a large number of in-vitro studies investigating drug transport across the intestinal epithelium.[33,34] The Caco-2 cell line is derived from human colonic adenocarcinoma, but exhibits many morphological and functional similarities to the normal human small intestinal epithelial cells when they are grown as polarized cells. Moreover, these cells express several membrane transporters such as P-gp, MRPs and OATP. P-gp and MRPs are ATP-dependent efflux transporters. P-gp and MRP-2 are expressed at the apical membrane and MRP-1 is expressed at the basolateral side. OATP-B, a pH-sensitive carrier, is expressed at the apical membrane of cells.[35] Caco-2 cells were standardized and fully validated in our experimental conditions. Our results show that the Caco-2 monolayer cell was intact and polarized with a functional active efflux transport. This latter activity was correlated with P-gp and MRP2 expression (data not shown). Our results are in accordance with literature, showing that P-gp and MRP2 expression in Caco2 cells is comparable to normal human jejunum.[36]

The intestinal permeability of quercetin and naringenin was evaluated in the presence of several transporter inhibitors. Quercetin was poorly absorbed by a transcellular diffusion through Caco-2 cells ($Papp_{AB}$ quercetin < $Papp_{AB}$ propranolol). This result could be explained by its low water solubility and by its secretion involving an active efflux with MRP-2 mediation. Quercetin contains five hydroxyl groups and its planar configuration could also contribute to its poor permeability because of intercalation into cell membranes.[37]

Interestingly, quercetin absorption is pH dependent due to its acid characteristics.[38] Quercetin permeability in the absorptive direction was highly enhanced at lower pH. Quercetin uses an energy-dependent system when it is absorbed since its absorption is prevented by the presence of sodium orthovanadate. Thus, our data suggest that quercetin may be absorbed from the apical to the basolateral side by two mechanisms: (i) passive diffusion and (ii) pH-dependent transport mediated by influx protein carriers belonging to the SLC family, in particular OATP. This observation is due to there being higher quercetin absorption in the presence of estrone-3 sulfate. At low pH, OATP transporters were active, suggesting that quercetin may be acting as a high-affinity substrate of OATP in order to enter the cell. At higher pH values, quercetin was absorbed by a passive diffusion pathway.

In the equilibrium state, quercetin permeability from apical to basolateral was lower than from basolateral to apical. These results suggest a polarized transport mechanism and involvement of a protein carrier mediated active efflux transport belonging to the ABC family. Furthermore, quercetin absorption seems to be energy dependent, its transport being inhibited by sodium orthovanadate. In addition, the differences between $Papp_{AB}$ and $Papp_{BA}$ were more important at pH 6.5 than at pH 7.4, suggesting more significant activity of efflux membrane proteins at pH 6.5. Our results show that quercetin is an MRP-2 substrate and not a P-gp substrate. However, it may still be a P-gp modulator since its $Papp_{BA}$ increased in the presence of verapamil. These data are in agreement with results in the literature, where flavonoids have been reported to inhibit drug efflux by interacting with various sites of P-gp and MRP2.[39–41] Quercetin has been also reported to have multiple P-gp independent mechanisms. It has been shown to stimulate P-gp and also to inhibit P-gp mediated efflux of rhodamine 123 in a dose-dependent manner.[42–44] It has also been reported that quercetin decreased the efflux of ciclosporin A (a P-gp substrate) but did not alter the efflux permeability of digoxin, another P-gp substrate.[39]

The permeability of quercetin through Caco-2 cells under our experimental conditions is summarized in Figure 6. Quercetin is absorbed by passive diffusion and secondarily mediated by OATP, but not MRP1. Moreover, quercetin is an MRP2 substrate and not a P-gp substrate.

The intestinal absorption of naringenin is higher than for quercetin. This may be due partially to their physicochemical properties. Naringenin is a more lipophilic compound and has better water solubility than quercetin (Table 1). Moreover, naringenin, with three hydroxyl groups, possesses a more tilted configuration, making it less prone to intercalate into the membrane but enhancing its permeability compared to flavonol.

Interestingly, naringenin absorption is not pH dependent and it seems to use an energy-dependent system to enter into cells. It is transported via an MRP1 carrier ($Papp_{AB}$ for naringenin was decreased in the presence of MK571).

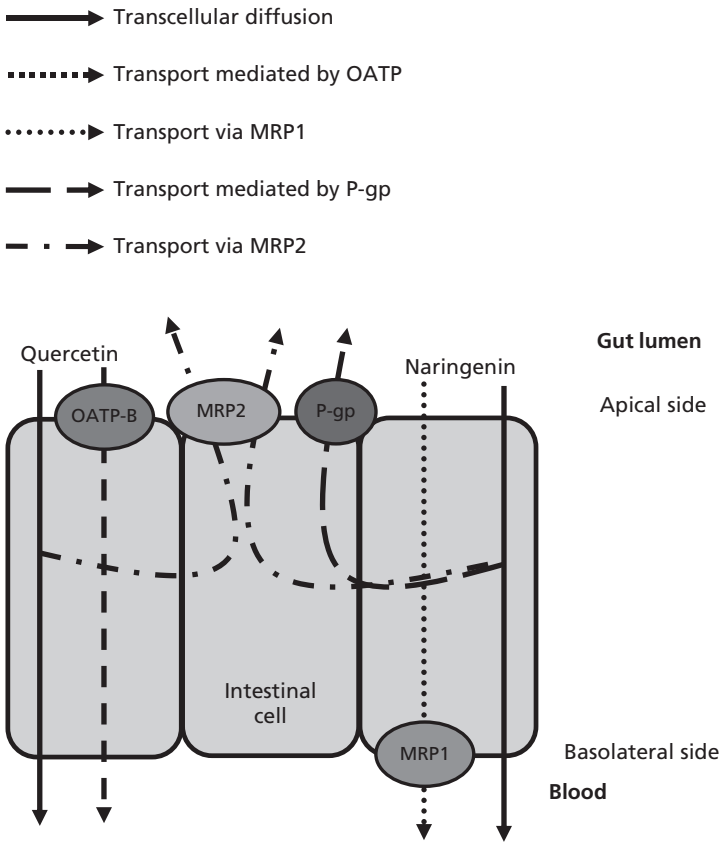


Figure 6 Predictive schema of quercetin and naringenin intestinal transport pathway. Quercetin and naringenin are absorbed through the human intestinal epithelium by a passive transcellular diffusion. But quercetin also seems also to use OATP carriers to enter into the cells and is transported only by MRP2. Naringenin seems to use an active ATP system, mediated by MRP1 expressed at the basolateral side of intestinal cell and is transported by active efflux protein carriers (P-gp and MRP2). MRP, multidrug resistance associated protein; OATP, organic anion transporting polypeptide; P-gp, P-glycoprotein.

In our experimental conditions, naringenin absorption was polarized with $Papp_{BA}$ superior to $Papp_{AB}$, suggesting that naringenin is transported by active efflux protein carriers. Our results show that naringenin is a P-gp and MRP2 substrate. This confirms results previously described, suggesting that naringenin, a grapefruit juice component, can inhibit P-gp mediated efflux of vincristine in the blood–brain barrier.[19,44] Moreover, in the literature naringenin is reported to inhibit drug efflux by directly interacting with various sites of P-gp as well as interacting with multidrug-resistance-associated proteins (MRP1 and MRP2).[45,46]

In summary, naringenin is not a substrate of OATP-B but could modulate this protein carrier since intestinal permeability was increased in the presence of its inhibitor.

Conclusions

In conclusion, our results suggest that quercetin and naringenin are absorbed through the human intestinal epithelium by a passive diffusion mechanism. Quercetin also seems to use a pH-dependent and, directly or indirectly, an active ATP system to enter cells, a mechanism that is mediated by OATP carriers. Naringenin seems to use an active ATP system mediated by MRP1, which is expressed at the basolateral side of the intestinal cell. Moreover, these two flavonoids are transported by active efflux protein carriers (P-gp and MRP2), explaining their role in the interactions with many drugs and substrates of these proteins. Our results are summarized in Figure 6. Further experiments will be necessary to verify if other influx and efflux carriers (OAT, PepT1, MRP3 and BCRP, for example) are involved in the transport of these two flavonoids. Our data contribute to better understanding of the absorption mechanisms of quercetin and naringenin. Knowledge of these mechanisms will help us to determine the level of intake necessary to generate beneficial effects and so to explain their putative role in food–drug interactions.

Declarations

Conflict of interest

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

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