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Research Paper

Coumarins permeability in Caco-2 cell model

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Abstract

Objectives The presence of coumarins in human diet, their multiple pharmacological properties and occurrence in various herbal remedies represent significant reasons to explore their membrane permeability, as a first event contributing to coumarins oral bioavailability. Thus, we evaluated the permeability and cytotoxicity of 18 coumarins, with different substitution patterns involving OH, OCH_3 and CH_3 groups.

Methods A modified Caco-2 permeability model was used, in which the permeability test is performed with a robotic workstation and cells are grown on 96-well plates for 7 days.

Key findings All studied coumarins were highly permeable, with calculated P_{app} values that varied within 4.1×10^{-5} to 2.1×10^{-4} in apical to basolateral studies and within 1.8×10^{-5} to 7.0×10^{-5} in basolateral to apical studies. The efflux ratio remained in all cases below 1. It was demonstrated that the type and position of substituents contributed more to the permeability than the number of substituents.

Conclusions The results allowed us to predict that these coumarins are well absorbed in the gut lumen and efflux is not limiting the absorption. Five coumarins had an influence on the mitochondrial function of Caco-2 cells (1 < 80%, 4 > 120%), according to the WST-1 cytotoxicity test, but this does not seem to affect the permeability of the compounds.

Keywords Caco-2 cells; coumarins; cytotoxicity; permeability

Introduction

Coumarins are molecules consisting of a basic nucleus of benzene joined to an α-pyrone ring. They can occur as the hydroxylated, alkoxylated or alkylated derivatives of the parent compound, coumarin (1,2-benzopyrone), along with their glycosides. A frequent substitution by a hydroxyl group in position 7 generates the 7-hydroxycoumarin umbelliferone, which is also present in more complex coumarin structures. The facts that natural coumarins possess many potent pharmacological activities (e.g. Härmälä *et al.*,[1] Hoult and Paya,[2] Kostova and Mojzis[3]) and that chemical substitution can occur at many sites in the basic structural nucleus have made them interesting molecules for drug discovery.[2] Coumarins are also part of the human diet and an estimate of human exposure to coumarin from the diet has been calculated to be around 0.02 mg/kg per day.[4,5] In addition, their presence in human diet evokes an issue related to the ability of certain coumarins to inhibit cytochrome P450 (CYP) enzymes, as in the well-known example of grapefruit juice, that contains 6′,7′-dihydroxybergamottin, which inhibits CYP3A activity,[6] an enzyme involved in the metabolism of about 50% of all drugs.

Toxicity and therapeutic effects of coumarins are, as in all compounds, dependent on the events taking place when administered to the organisms, and until now, most of these events in humans have been studied for the coumarin itself or the 7-hydroxycoumarin. Indeed, it has been extensively shown that, when orally administered, coumarin is rapidly absorbed from the human gastrointestinal tract, and it is extensively metabolized by the liver in the first-pass, so that only 2–6% of the compound reaches the systemic circulation intact.[5] The major metabolic pathway in humans occurs via 7-hydroxylation, catalysed by a specific cytochrome P450 enzyme, CYP2A6.[7] The resultant products, 7-hydroxycoumarin and its glucuronide conjugates, are non-toxic and represent the majority of urinary metabolites following human oral administration.[4,8] Not much information exists, however, on the membrane permeability and metabolism of other coumarins, but it is expected that the variety of substitutions that can take place in the basic structural core might have influence over these properties. Given the previously illustrated relevance of

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coumarins, significant reasons exist to explore their membrane permeability, as a first event contributing to their oral bioavailability.

One of the in-vitro regulatory accepted and a widely used system for permeability studies in humans is the Caco-2 cell monolayer (reviewed by Bailey *et al.*,[9] Artursson *et al.*[10]). These cells, originating from a human colonic adenocarcinoma, differentiate spontaneously morphologically and functionally to resemble intestinal enterocytes.[11] To obtain these monolayers, cells are traditionally grown on 12- or 24-well plates for 21–28 days, which is expensive, laborious and time consuming. To adapt the model for higher-throughput applications we have built and optimized an automated 96-well plate Caco-2 permeation model with reduced growth time.[12,13]

Thus, in this contribution, the modified Caco-2 permeability model was used to evaluate the permeability of 18 coumarins with different numbers of OH, OCH_3, and CH_3 substituents, including coumarin and 7-hydroxycoumarin as reference compounds. Permeation tests were performed in both apical to basolateral (AP-BL) as well as basolateral to apical (BL-AP) directions. Our experiments were intended to gain a general understanding of the contribution of the substituents (type, position or number) on the membrane permeability properties of the coumarins, which in turn could help in predicting their human oral bioavailability. Additionally, toxicity studies were conducted in Caco-2 cells to generate a more complete predictive scenario of the effects of coumarins in humans.

Materials and Methods

Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Hank's balanced salt solution (HBSS) and HEPES solution were obtained from Gibco Invitrogen Corporation (Paisley, UK). Phosphate-buffered saline (PBS), non-essential amino acids (NEAA), L-glutamine, antibiotic mixture (10 000 IU/ml penicillin G, 10 000 μg/ml streptomycin) and trypsin were purchased from Cambrex Bio Science (Verviers, Belgium). Acetonitrile and tetrahydrofuran were obtained from Rathburn (Walkerburn, UK) and methanol (MeOH) from J.T. Baker (Deventer, Holland). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) was obtained from Roche Diagnostics GmbH (Mannheim, Germany) and Triton X-100 from Sigma (St Louis, MO, US). Coumarins 1–7, 10, 13–14, 16–18, were purchased from Extrasynthèse (Genay, France) and coumarins 8, 9, 11, 12 and 15 from Sigma Chemical Co (St Louis, MO, US) (Table 1).

Cell culture

Caco-2 cells (originating from American Type Culture Collection) were kindly donated by Professor Arto Urtti, from the Drug Discovery and Development Technology Center, at the Faculty of Pharmacy, University of Helsinki, Finland. Cells were grown in DMEM with 4.5 g/l glucose, which was supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% Minimum Essential Medium (MEM) non-essential amino acids. Cell cultures were maintained at 37°C in an incubator (BB 16 Gas Incubator; Haeraeus Instruments GmbH, Hanau, Germany) in an atmosphere of 95% air and 5% CO_2 at 95% humidity. Cells were harvested with trypsin-EDTA and seeded onto polycarbonate filter membranes with pore size of 0.4 μm and growth areas of 0.11 cm^2 in clusters of 96 wells (MultiScreen Caco-2; Millipore, Billerica, MA, US) at a density of 2.1×10^5 cells/cm^2. The growth medium was changed every second day. Cells from passage number 50–72 were used for the experiments.

Permeability experiments

Coumarins were first dissolved in DMSO, then diluted in HBSS (pH 7.4) to yield a final concentration of 250 μM. Coumarin 18 was prepared in a similar manner with the exception that MeOH was used instead of DMSO. The final DMSO or MeOH concentration did not exceed 1%.

The permeability experiments were performed in an incubator (Stuart, orbital incubator S150; Bibby Sterilin Ltd, Stafford, UK), in humified atmosphere at 37°C while shaking at 75 rev/min. Transepithelial electrical resistance (TEER) was measured with an EVOMX voltohmmeter (EVOM, World Precision Instruments Inc., Sarasota, FL, US) to evaluate the integrity of the monolayers. Cells were washed twice with HBSS solution. After washing, cell monolayers were allowed to equilibrate for 30 min before experiments. TEER values were measured again and the apical solution was changed to HBSS containing the coumarins. Samples were obtained after 0, 20 and 120 min by moving the cell monolayer into a new receiver well containing fresh HBSS (AP-BL experiments) or by taking a sample from the receiver side and replacing the volume with fresh HBSS (BL-AP experiments). After 120 min monolayers were washed again with HBSS solution and TEER values were measured to assure the monolayer integrity. Samples were kept at −20°C until analysed. The apparent permeability coefficients, P_{app} (cm/s), for both AP-BL and BL-AP experiments were calculated according to the following equation:

$$P_{app} = (dQ/dt)/(A \times 60 \times C_0) \quad (1)$$

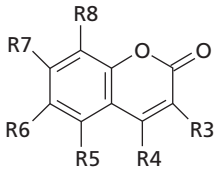
where dQ/dt is the amount of compound transported within a given time period, A is the surface area of the monolayer (cm^2), 60 is the time unit (1 min, conversion from minutes to seconds) and C_0 is the initial drug concentration in the donor compartment (μg/ml).

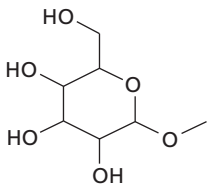
Efflux ratio (ER) was calculated according to the following equation:

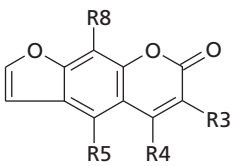
$$ER = P_{app}\ \text{BL-AP}/P_{app}\ \text{AP-BL} \quad (2)$$

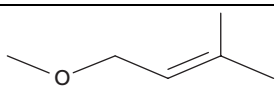
Paracellular marker Lucifer Yellow was used to assess monolayer integrity during experiments. The permeated amount was determined with a Varioskan scanning spectrofluorometer (Thermo Fisher Scientific, Waltham, MA, US) with excitation-λ 430 nm and emission-λ 535 nm.

Table 1 Coumarin and furanocoumarin core structures and substituents of the studied coumarins



No.	Coumarin	R4	R5	R6	R7	R8
1	4-Methylumbelliferone	CH_3			OH	
2	Scoparone			OCH_3	OCH_3	
3	Isoscopoletin			OH	OCH_3	
4	Fraxidin			OCH_3	OCH_3	OH
5	Daphnetin-7-methylether				OCH_3	OH
6	Scopoletin			OCH_3	OH	
7	Umbelliferone				OH	
8	6-Methylcoumarin			CH_3		
9	6-Methoxy-4-methylcoumarin	CH_3		OCH_3		
10	7-Methoxy-4-methylcoumarin	CH_3			OCH_3	
11	Coumarin					
12	5,7-Dihydroxy-4-methylcoumarin	CH_3	OH		OH	
13	6,7-Dihydroxy-4-methylcoumarin	CH_3		OH	OH	
14	7,8-Dihydroxy-4-methylcouarin	CH_3			OH	OH
15	Herniarin				OCH_3	
16	Esculin sesquihydrate				OH	
17	Citropten		OCH_3		OCH_3	



No.	Furanocoumarin	R4	R5	R6	R7	R8
18	Imperatorin					

Sample HPLC analysis

Coumarin samples from receiver compartments were analysed using a reversed-phase D-7000 HPLC system (LaChrom, Merck, Hitachi, Japan) consisting of L-7100 pump, L-7455 diode array detector, L-7300 column oven, L-7200 autosampler, L-7612 solvent degasser and HSM-Sys 1 chromatographic workstation. A Gemini 5 μm C18 column 150 mm $\times$ 4.6 mm (Phenomenex, Torrance, CA, US) with a SecurityGuard C18 guard column (Phenomenex, Torrance, CA, US) was used with a flow rate of 1 ml/min. Mobile phase consisted of 69% MilliQ water, 22% tetrahydrofuran, 5% acetonitrile and 4% MeOH. The injection volume for the samples was 10 μl except for sample 18 for which it was 15 μl. The separations were performed by isocratic elution. Wavelengths and retention times are presented in Table 2.

WST-1 cytotoxicity assay

Cytotoxicity of the compounds was evaluated using a commercial colorimetric WST-1 cell proliferation assay according to the manufacturer's instructions. The assay is based on the cleavage of the WST-1 tetrazolium salt by mitochondrial dehydrogenases in viable cells.[14] Cells were harvested and coumarin solutions were prepared as described before. Cells (4.5×10^4 cells/well in 100 μl) were incubated with test compounds for 2 h at a concentration of 250 μM. Triton X-100 was used as a positive control and 1% DMSO was used as a negative control. Absorbance data were

Table 2 Retention times, wavelengths, LogP and pKa values and efflux ratios of the studied coumarins

No.	Name	Retention time (min)	Wavelength (nm)	LogP	pKa	ER (efflux ratio)
1	4-Methylumbelliferone	4.8	320	2.37 ± 0.73	9.12 ± 0.4	0.29
2	Scoparone	3.2	320	1.6 ± 0.8		0.53
3	Isoscopoletin	3.1	345	0.83 ± 0.79	9.24 ± 0.20	0.45
4	Fraxidin	3.2	305	0.56 ± 0.81	7.81 ± 0.20	0.45
5	Daphnetin-7-methylether	3	320	0.62 ± 0.38	8.21 ± 0.20	0.44
6	Scopoletin	3.4	320	1.28 ± 0.87	9.13 ± 0.20	0.43
7	Umbelliferone	4.4	320	1.58 ± 0.49	9.11 ± 0.20	0.55
8	6-Methylcoumarin	7.3	275	1.85 ± 0.26		0.54
9	6-Methoxy-4-methylcoumarin	6	275	2.49 ± 0.39		0.66
10	7-Methoxy-4-methylcoumarin	6.5	320	2.37 ± 0.39		0.51
11	Coumarin	4.9	275	1.39 ± 0.26		0.44
12	5,7-Dihydroxy-4-methylcoumarin	5.8	320	2.58 ± 0.77	8.24 ± 0.40	0.31
13	6,7-Dihydroxy-4-methylcoumarin	3.3	320	2.08 ± 0.8	8.76 ± 0.40	n.d.
14	7,8-Dihydroxy-4-methylcouarin	3.6	320	1.95 ± 0.8	8.00 ± 0.40	0.33
15	Herniarin	5.6	320	1.78 ± 0.36		0.47
16	Esculin sesquihydrate	2.3	340	−1.52 ± 0.88	8.22 ± 0.2	0.26
17	Citropten	8	320	2.06 ± 0.62		0.44
18	Imperatorin	3.3	245	3.81 ± 0.67		0.34

n.d., not determined.

collected using Varioskan scanning spectrophotometer (Thermo Fisher Scientific, Waltham, MA, US) at 440 nm. Viability (%) was calculated according to the following equation:

$$\text{Viability } (\%) = \frac{(\text{signal} - \text{background})}{(\text{negativecontrol} - \text{background})} \times 100\% \quad (3)$$

The compound was considered to affect cell viability if viability value was lower than 80% or higher than 120%.

Statistical analysis

Proper assay performance in terms of repeatability was studied by comparing the P_{app} values ($n = 4$) obtained for each compound on different days using a paired comparison test (paired two-tailed Student *t*-test) assuming equal variances (Microsoft Excel software). Similarly, P_{app} values ($n = 4$) registered in AP-BL direction for all tested compounds, were compared with values registered in BL-AP direction, using the same statistical test. $P < 0.05$ was considered statistically significant.

Results

Permeability of coumarins

Permeability of 18 coumarins (structures presented in Table 1) was evaluated by using a miniaturized and accelerated Caco-2 model. TEER measurements and Lucifer Yellow permeation were used as references for monolayer formation. TEER values remained at 625 ± 220 Ω ($n = 270$) and 99.2 ± 0.58% ($n = 10$) of the Lucifer Yellow remained in the apical side. These values indicated tightness of the paracellular space and proper Caco-2 cells monolayer formation. It has been calculated that the surface area of the brush border membranes in this system is more than 1000-fold larger than the paracellular surface area[15]. This is a particularly important feature, since it can be satisfactorily assumed that compounds in the Caco-2 cell model are exclusively transported by the passive transcellular route.

To gather information on the physico-chemical properties relevant to our approach, the coumarins were assessed with ACD Labs ACD/LogD Sol Suite 8.0 (Advanced Chemistry Development, Inc, Toronto, Canada). pKa and LogP were calculated and values are presented in Table 2. The calculation was based on the atomic contribution, fragmental contribution and intramolecular contribution. The studied compounds contain OH, OCH_3 and CH_3 groups, which are well characterized in the ACD database and the database also contains experimental values.

The permeation tests of coumarins were performed in both AP-BL and BL-AP directions on two different days. As seen in Figure 1, all studied coumarins were highly

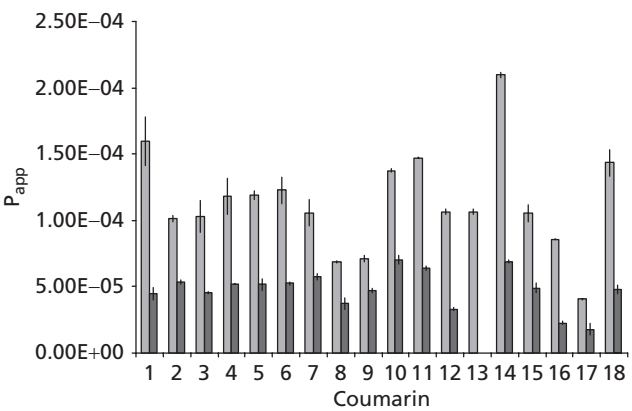


Figure 1 Permeation of studied coumarins (at 250 μM) in apical-to-basolateral (AP-BL) and basolateral-to-apical (BL-AP) directions. Values are presented as permeation coefficients P_{app} ± SD, $n = 4$. First column of each pair, AP-BL; 2nd column of each pair, BL-AP.

permeable. Coumarin **11**, the parental reference compound with no substituents, was found to easily permeate Caco-2 monolayers with P_{app} values well over 10^{-6}. AP-BL permeation of coumarin was higher than its main metabolite, 7-hydroxycoumarin (umbelliferone) (**7**), but BL-AP permeation was at the same level. In all coumarins, P_{app} values varied in AP-BL studies within the range of 4.1×10^{-5} to 2.1×10^{-4} and they were statistically significantly higher than values obtained in BL-AP studies, which varied within the range of 1.8×10^{-5} to 7.0×10^{-5}. The efflux ratio remained in all cases below 1 (Table 2), thus also illustrating that all coumarins were more permeable in the AP-BL direction than the BL-AP direction. These results indicated that these compounds are well absorbed from the gut lumen and efflux is not limiting the absorption. Mass balance, defined as the ratio of the sum of the cumulative amount transported plus the amount remaining in the donor compartment in relation to the initial amount in the donor compartment,[16] showed that coumarins were not excessively retained in cellular structures or adsorbed to the plastic device.

The contribution to the coumarins permeability of different substitution patterns using three main substituents (OH, OCH_3 and CH_3) was studied in detail. 4-Methylumbelliferone (**1**), 5,7-dihydroxy-methylcoumarin (**12**), 6,7-dihydroxy-methylcoumarin (**13**) and 7,8-dihydroxy-methylcoumarin (**14**) contain both methyl (CH_3) and hydroxyl (OH) substituents. Compound **1** has only one OH group while compounds **12**, **13** and **14** have two OH groups with one CH_3 group. The efflux ratio (ER) of these compounds was ~ 0.3, which indicates that AP-BL permeation is about 3 fold that of BL-AP permeation. It seems that the number of OH groups does not affect the permeation. LogP value and retention time were higher, and thus lipophilicity, when OH groups were not attached to adjacent carbons, as in **12**. It was interesting to note that compound **14** had much greater AP-BL permeation than compound **13** with the same substituents in adjacent carbons. Compound **13** acted more like compound **12**, having the same groups further from each other. The presence of adjacent phenolic hydroxyl groups has been indicated as an important factor for antioxidant and apoptosis-inducing activity of dihydroxy-4-methylcoumarins,[17,18] and 7,8-dihydroxy-methylcoumarin has been found to be the most effective molecule. Umbelliferone (**7**), with an OH group but no CH_3 group, and 6-methylcoumarin (**8**), with a CH_3 group but no OH group, had much higher ER values (0.55 and 0.54, respectively) than compounds **1**, **12** and **14**, which have both of these groups. Of the studied coumarins, only esculin (**16**), glucoside of esculetin, and furanocoumarin imperatorin (**18**) had as low ER values as compounds **1**, **12** and **14**.

Isoscopoletin (**3**), fraxidin (**4**), daphnetin-7-methylether (**5**) and scopoletin (**6**) contain methoxy groups (OCH_3) in addition to OH groups. Compounds **3**, **5** and **6** have one OCH_3 group, while compound **4** has two OCH_3 groups with one OH group. As seen in Figure 1, permeability of these compounds was very similar in both AP-BL and BL-AP directions. It seems that the position of the OH or OCH_3, or the increase in OCH_3 groups does not influence permeability in these particular compounds. In addition, their ER values were clearly higher than in CH_3- and OH-group-containing analogues. OCH_3- and OH-group-containing coumarins (**3**, **4**, **5** and **6**) possess lower LogP values than CH_3- and OH-group-containing coumarins (**1**, **12**, **13** and **14**), indicating that compounds **3**, **4**, **5** and **6** are more hydrophilic than compounds **1**, **12**, **13** and **14**. This is additionally seen in shorter retention times of compounds **3**, **4**, **5** and **6**.

6-Methoxy-4-methylcoumarin (**9**) and 7-methoxy-4-methylcoumarin (**10**) possess high logP values and retention times. These compounds contain OCH_3 as well as CH_3 groups. The ER values of these compounds were high (0.66 and 0.51, respectively). Compound **10** was more permeable in both directions than its analogue compound **9**, indicating that permeability is higher when the OCH_3 group is far from the CH_3 group.

Scoparone (**2**), herniarin (**15**) and citropten (**17**) contain only OCH_3 groups. Compound **15** has only one OCH_3 group, while compounds **2** and **17** have two OCH_3 groups. The addition of OCH_3 does not seem to have an effect on permeability or ER values in these compounds, which corresponds to our previous observations with compounds **3**, **4**, **5** and **6**. The lowest permeation of all studied compounds in both directions was observed in compound **17**, bearing two OCH_3 groups in positions **5** and **7**. Compound **2** with similar substituents in positions **6** and **7** had clearly higher permeability values pointing to the importance of the position in the substitution, at least for the OCH_3 group.

The lowest ER was exhibited by compound **16**, esculin, which contains a glucoside substituent. This compound has a very low LogP value and it is likely that this kind of substituent is a substrate for active transport, which enables the transportation in the AP-BL direction in contrast to the BL-AP direction. The highest ER value was registered for compound **9** with CH_3 and OCH_3 groups close to each other. In this compound, a relatively low permeation value in the AP-BL direction was found, in comparison with the rest of the studied coumarins, but similar values were not obtained for compounds having the same chemical substituents (i.e. compound **10**). In fact, the permeability behaviour of compound **9** is clearly different from that exhibited by compound **10**, as was indicated above. Thus, at this stage, no clear explanation of this high ER can be given for compound **9**.

Assay performance

P_{app} values on different days were statistically compared to evaluate the method performance. No significant differences were observed between days ($P > 0.01$). Slight differences from day-to-day measurements were observed with AP-BL permeability of isoscopoletin (**3**) and fraxidin (**4**) and BL-AP permeability of daphnetin-7-methylether (**5**) ($0.01 < P < 0.05$). All of these compounds have both OH and OCH_3 substituents and thus it could be speculated that these particular substituents could account for the permeability differences. Nevertheless, results indicate that repeatability of our modified permeation model is good and it provides accurate and reliable results.

Cytotoxicity of coumarins

Cytotoxicity of coumarins was evaluated by means of the WST-1 cytotoxicity test, which assesses the mitochondrial function of the cells, and results are presented in Figure 2. The lower viability value (< 80%) indicates reduced

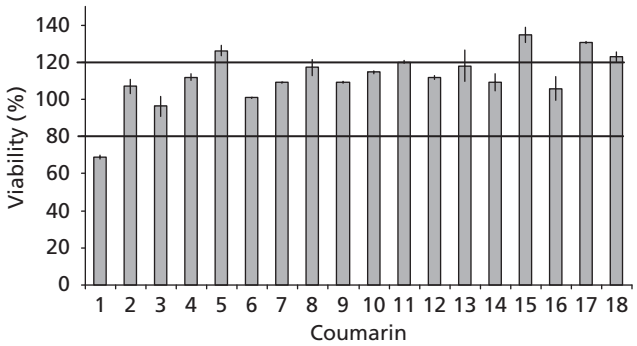


Figure 2 Cytotoxicity of studied coumarins (at 250 μM) in Caco-2 cells, as assessed using WST-1 toxicity test. Values are presented as viability % ± SD, $n = 3$. Compound was regarded to affect the cells viability if the registered value was < 80% or > 120% (toxicity hit limits).

mitochondrial activity and the higher viability value (< 120%) could be a sign of activity caused by cells trying to survive, or increased proliferation of the cells. As seen in Figure 2, only in the case of 4-methyl umbelliferone (**1**) was registered viability below 80%. Compounds **12**, **13** and **14**, with a similar structure but bearing only one more OH group, caused no reduction of Caco-2 cells viability. Daphnetin-7-methylether (**5**), herniarin (**15**), citropten (**17**) and imperatorin (**18**) increased viability values over 120%. 4-Methyl umbelliferone has shown itself to inhibit the cell proliferation in human keratinocytes by 50% at a concentration of 200 μM.[19] Imperatorin has previously also shown to affect energy transfer in rabbit mitochondria at a concentration of 300 μM.[20] In this study, a toxicity test was included to elucidate whether differences in permeability were due to toxic effects, but results show that this kind of conclusion cannot be drawn. Detected effects on mitochondrial Caco-2 cell function produced by certain compounds do not seem to correlate with changes in their permeability. Also, in all cases, effects on mitochondrial function do not induce membrane detectable changes, since Caco-2 cell monolayers remained intact during the experiments.

Discussion

The Caco-2 permeability model system used in this approach to study coumarins permeability is based on the growing of the cells in 96-well plates for seven days and the use of a robotic liquid handling workstation. The reliability of the assay was previously measured by comparison with the traditionally (12 wells, 21 days) grown monolayers using five FDA model compounds.[12,13,21] Expression and functionality of efflux protein P-glycoprotein (P-gp) in these cells was visualized with confocal laser microscopy and bi-directional permeation studies with P-gp substrates rhodamine-123 and verapamil. Confocal laser microscopy images showed that antibody stained P-glycoprotein was localized on the plasma membrane. Rhodamine-123 permeation was higher from the BL-AP direction than the AP-BL direction and this difference was reduced in the presence of verapamil, the P-gp inhibitor.[13] This finding was in good correlation with the studies of Troutman and Thakker.[22]

Permeability values obtained in the system were related to physico-chemical properties (pKa, logP) of the tested coumarins. Since experimental values for all studied coumarins were not available, it was assumed that these ACD calculated values provide sufficient ranking order for this set of compounds. The pKa value (dissociation constant) is a measure of the strength of an acid or a base and indicates in which pH half of the molecules of certain compounds are ionized. Acidic compounds are mostly in ionized form if the pH is more than their pKa value. For bases it is the opposite. Ionization of the compounds increases their water solubility but lowers their lipophilicity and thus decreases the absorption of the compounds. At pH 7.4 used in this study all the coumarins are in the molecular form. Drug candidates are often also screened according to logP, to help guide drug selection and analogue optimization, since lipophilicity is a major determining factor in a compound's absorption, distribution in the body and penetration across vital membranes. In conjunction with logP, a reliable prediction of absorbed drug after oral administration can be achieved using P_{app} values. Drugs that are completely absorbed in humans have high P_{app} values ($> 1 \times 10^{-6}$ cm/s) in the Caco-2 monolayer system.[23]

Coumarin (**11**), the parent reference compound of the tested group, and 7-hydroxycoumarin (umbelliferone) (**7**), its main metabolite, were found to easily permeate Caco-2 monolayers. A recent report by Yang *et al.*[15] indicated high P_{app} values in the Caco-2 system for umbelliferone, in the order of 10^{-5} in both directions, thus in line with our permeability findings. An extensive body of in-vivo evidence has shown the rapid absorption of these molecules from the gastrointestinal tract and the values obtained here correspond to those previous findings (reviewed by Pelkonen *et al.* 1997[24] and Felter *et al.* 2006[5]), thereby supporting the reliability of this accelerated Caco-2 system.

Our results show that coumarins exhibit high permeability values in the AP-BL direction compared with the BL-AP direction, which supports the fact that these compounds are likely substrates for active transporters. However, in-vitro studies with everted and non-everted guts of guinea-pigs and rats have indicated that active transport is not associated with the permeability of coumarin and 7-hydroxycoumarin.[25] Coumarin-based prodrugs are also shown to cross the Caco-2 cell monolayers passively.[26] The assay used here is intended to provide a first-tier, general information on the permeability of pharmacologically active compounds, but detailed information on the mechanisms involved cannot be obtained, since absorptive transporters in this Caco-2 cell system are not fully characterized.[27] Also, results obtained here provide an initial overall picture of the permeability of coumarins and some of the structural determinants that influence these permeation properties. From such findings, rational and comprehensive decisions on further studies can be taken concerning, for example, elucidation of transportation mechanisms involved in coumarins permeability.

In addition, cytotoxic effects of the coumarins on these Caco-2 cells were studied and found to be, in overall terms, not significant. Variations were detected on the mitochondrial function of the cells when some compounds were used, but no relation to changes of the permeability of such compounds was detected. The evaluation of the possible

toxicity associated with coumarins exposure is of utmost importance when conducting coumarin-based research. In the past, coumarin was regarded as a hepatotoxic agent due to its effects in certain animal models (e.g. Hazleton *et al.*[28] and Mukherji[29]) and its use was banned in the USA in 1954 and the UK in 1965. However, further studies with coumarin and its derivatives have shown that toxicity in rats is related to the 3,4-epoxidation metabolic pathway, in which the formation of an unstable coumarin 3,4-epoxide intermediate occurs.[30] In contrast, coumarin metabolism in humans preferably takes place by the 7-hydroxylation route, thus implicating fewer toxicological risks.[5]

Conclusions

In this study we evaluated absorption and cytotoxicity of certain coumarins using Caco-2 cells monolayers, a validated model that provides important predictive information regarding intestinal absorption of drugs. Coumarins were selected by their structure with the aim of knowing how nature, position and number of different substituents could impact their permeability. Our results provided a general view of the permeability of the coumarins collection, by showing that all studied coumarins rapidly permeated across Caco-2 cell monolayers, the type and position of substituents being more critical to that property than the amount of substituents on the structure. All compounds were significantly more permeable in the apical to basolateral direction than in the basolateral to apical direction. Consequently, these results allow us to predict that all studied coumarins are well absorbed in the gut lumen and that efflux is not limiting the absorption. Some variations on the mitochondrial function of Caco-2 cells were registered after the exposure of coumarins, but this does not seem to affect the compounds' permeability.

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Conflict of interest

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

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