European Journal of Pharmaceutical Sciences 196 (2024) 106740

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

The 2-aminoethyl diphenylborinate-based fluorescent method identifies quercetin and luteolin metabolites as substrates of Organic anion transporting polypeptides, OATP1B1 and OATP2B1

Hana Kaci [a,b], Éva Bakos [a], Paul W. Needs [c], Paul A. Kroon [c], Kateřina Valentová [d], Miklós Poór [e,f,g], Csilla Özvegy-Laczka [a,*]

[a] Institute of Molecular Life Sciences, RCNS, HUN-REN, H-1117 Budapest, Magyar tudósok krt. 2., Hungary
[b] Doctoral School of Biology, Institute of Biology, ELTE Eötvös Loránd University, 1117 Budapest Pázmány Péter sétány 1/C, Hungary
[c] Food, Microbiome & Health Programme, Quadram Institute Bioscience, Norwich Research Park, Norwich, Norfolk NR4 7UQ, UK
[d] Institute of Microbiology of the Czech Academy of Sciences, Vídeňská 1083, Prague CZ-142 00, Czech Republic
[e] Molecular Medicine Research Group, János Szentágothai Research Centre, University of Pécs, Ifjúság útja 20, Pécs H-7624, Hungary
[f] Department of Pharmacology, Faculty of Pharmacy, University of Pécs, Rókus u. 2, Pécs H-7624, Hungary
[g] Department of Laboratory Medicine, Medical School, University of Pécs, Ifjúság útja 13, Pécs H-7624, Hungary

ARTICLE INFO

Keywords:
Fluorescence enhancer
Luteolin metabolite
Quercetin metabolite
Organic anion transporting polypeptide
Fluorescence-based direct transport

ABSTRACT

Organic anion transporting polypeptides (OATPs), OATP1B1 and OATP2B1 are membrane proteins mediating the cellular uptake of chemically diverse organic compounds. OATP1B1 is exclusively expressed in hepatocytes and plays a key role in hepatic detoxification. The ubiquitously expressed OATP2B1 promotes the intestinal absorption of orally administered drugs. Flavonoids are widely found in foods and beverages, and many of them can inhibit OATP function, resulting in food-drug interactions. In our previous work, we have shown that not only luteolin (LUT) and quercetin (Q), but also some of their metabolites can inhibit OATP1B1 and OATP2B1 activity. However, data about the potential direct transport of these flavonoids by OATPs have been incomplete. Hence, in the current study, we developed a simple, fluorescence-based method for the measurement of intracellular flavonoid levels. The method applies a cell-permeable small molecule (2-aminoethyl diphenylborinate, 2-APB), that, upon forming a complex with flavonoids, results in their fluorescence enhancement. This way the direct uptake of LUT and Q, and also their metabolites' could be investigated both by confocal microscopy and in a fluorescence plate reader in living cells. With this approach we identified quercetin-3′-*O*-sulfate, luteolin-3′-*O*-glucuronide, luteolin-7-*O*-glucuronide and luteolin-3′-*O*-sulfate as substrates of both OATP1B1 and OATP2B1. Our results highlight that OATP1B1 and OATP2B1 can be key participants in the transmembrane movement of LUT and Q conjugates with otherwise low cell permeability. In addition, the novel method developed in this study can be a good completion to existing fluorescence-based assays to investigate OATP function.

Abbreviations

APB	2-aminoethyl diphenylborinate,
BBB	blood-brain barrier,
BSP	Bromosulfophthalein,
COMT	catechol-*O*-methyltransferases,
DDI	drug-drug interaction,
EMA	European Medicines Agency,
FDA	The US Food and Drug Administration,
FDI	Food-Drug Interactions,
L3′G	luteolin-3′-*O*-glucuronide,
L3′S	luteolin-3′-*O*-sulfate,
L7G	luteolin-7-*O*-glucuronide,
LUT	luteolin,
N4′S	naringenin-4′-*O*-sulfate,
N7G	naringenin-7-*O*-glucuronide,
NAR	naringenin,
OATPs	Organic anion transporting polypeptides,
Q	quercetin,
Q3′S	quercetin-3′-*O*-sulfate,

* Corresponding author.
E-mail address: laczka.csilla@ttk.hu (C. Özvegy-Laczka).

https://doi.org/10.1016/j.ejps.2024.106740
Received 13 December 2023; Received in revised form 28 January 2024; Accepted 1 March 2024
Available online 2 March 2024

SLC Solute Carrier,
SULTs sulfotransferases,
TX Triton X-100,
UGTs uridine-5′-diphosphate glucuronosyltransferases

1. Introduction

Organic anion-transporting polypeptides (OATPs), encoded by the SLCO genes are solute-carrier (SLC)-type transmembrane proteins expressed in endothelial or epithelial cells of the human body (Murray and Zhou, 2017). OATPs mediate the sodium- and ATP-independent cellular uptake (Hagenbuch and Meier, 2004; Bronger et al., 2005) of a broad range of endo- or exogenously derived organic compounds (Kindla et al., 2009). Typical endogenous substrates of OATPs are bilirubin, bile acids and steroid hormones (Svoboda et al., 2011). In addition to these, several members of the OATP family (OATP1A2, OATP1B1, OATP1B3 and OATP2B1) also recognize compounds of exogenous origin, including various drugs (Tóth et al., 2018), food components (Ali et al., 2020), and toxins (Letschert et al., 2006). Of these drug transporter OATPs, OATP1B1 and OATP1B3 are exclusively expressed in the sinusoidal membrane of hepatocytes (Badée et al., 2015), where they mediate the hepatocellular uptake of a large variety of endogenous substances (e.g., bile acids, bilirubin, and estradiol-17β-d-glucuronide), as well as many clinically applied drugs, such as statins (Hagenbuch and Meier, 2004; Tóth et al., 2018). The other two multispecific OATPs, OATP1A2 and OATP2B1 are ubiquitously expressed throughout the body, present e.g., in enterocytes (Kinzi et al., 2021), in endothelial cells of the blood-brain barrier (BBB) (Schäfer et al., 2021), and in hepatocytes (only OATP2B1) (Kinzi et al., 2021). Similarly to OATP1B1 and OATP1B3, OATP1A2 and OATP2B1 are also involved in the cellular uptake of a wide variety of endogenous metabolites (e.g., estrone-3-sulfate (Kobayashi et al., 2003), dehydroepiandrosterone sulfate (Nozawa et al., 2004) and thyroid hormones (Medwid et al., 2021)) and drugs (e.g., rosuvastatin (Johnson et al., 2017) and atorvastatin (Kashihara et al., 2017)). Owing to their important role in pharmacokinetics, multispecific OATPs can have their function compromised by drug-drug (DDI) or food-drug interactions (FDI), when co-administration of their substrates and/or inhibitors may result in altered pharmacokinetics of OATP substrate drugs. This may then lead to adverse drug effects or even toxicity or, on the other hand, to insufficient efficacy of the drug (Ali et al., 2020). To prevent unwanted drug effects, regulatory authorities (The U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA), and Japan Pharmaceuticals and Medical Devices Agency (PMDA)) recommend the evaluation of potential interactions between new molecular entities and OATP1B1 and OATP1B3 transporters during the pre-clinical phase of

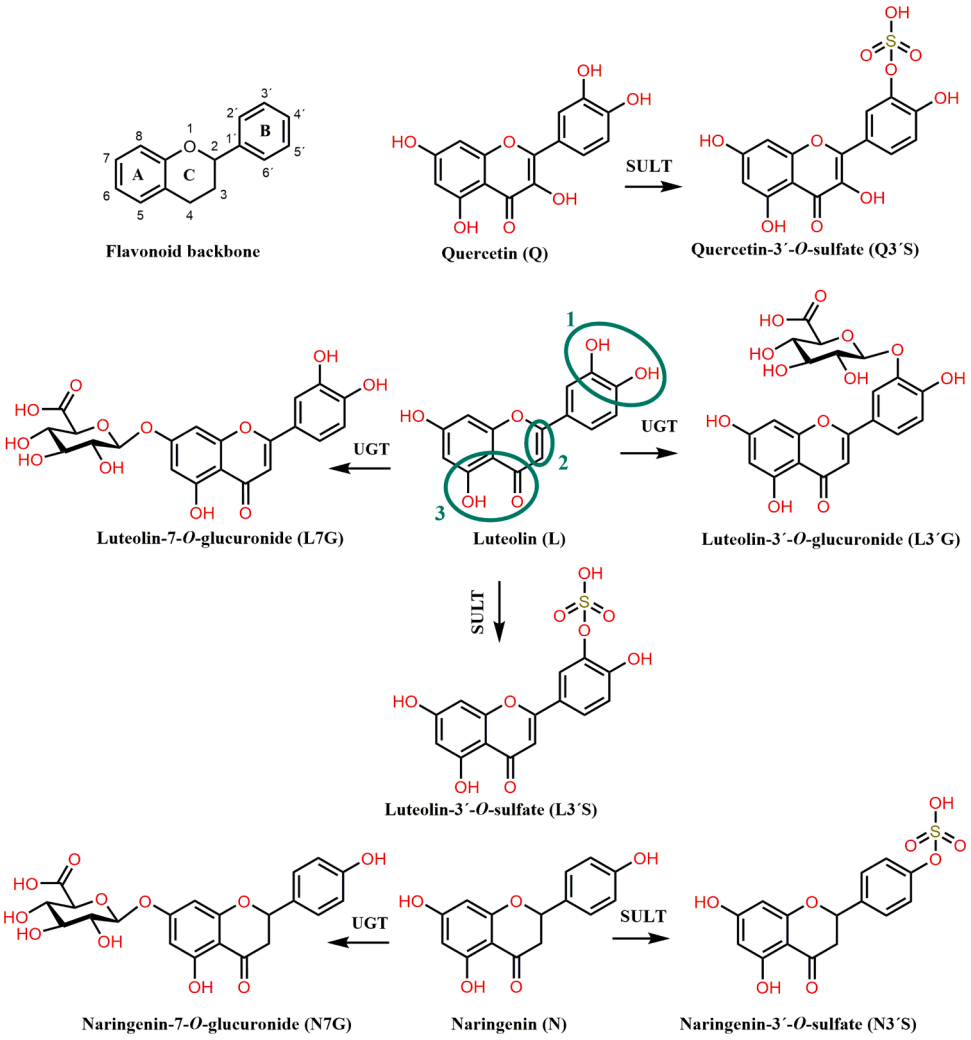

Fig. 1. Chemical structures and conversion of flavonoids investigated in the current study and the possible binding sites of APB. The green circles presented on the chemical structure of luteolin show possible binding sites between APB and flavonoids, which may be on the 5-hydroxy-4-keto sites of ring A-C (Site 3) (De et al., 2022) or the 3′,4′-*O*-diphenolic site of ring B (Site 1) (Matteini et al., 2011). Additionally, the double bond at C2-C3 in flavonoids may also participate in the complex formation (Site 2) (Matteini et al., 2011).

drug development (Koziolek et al., 2019). In addition, investigation of OATP2B1 inhibition may also be warranted according to the guidance released by the International Transporter Consortium (Zamek-Gliszczynski et al., 2018).

Flavonoids are a class of polyphenols widely present in human food and drink, including tea, wine, grains, fruits and vegetables (Xing et al., 2021). The average daily intake of flavonoids in the United States has been estimated to be 200–250 mg (Vieux et al., 2020). The chemical structure of flavonoids includes two benzene rings (A and B), coupled with a heterocyclic pyran ring (C) (Kumar and Pandey, 2013) (Fig. 1). Multiple research showed that flavonoids have broad biological activities including antioxidant, anti-inflammatory, anticancer and antimicrobial effects, with no or low toxicity (Jucá et al., 2020; Ullah et al., 2020). Flavonoids undergo extensive biotransformation mostly by uridine-5′-diphosphate glucuronosyltransferases (UGTs), sulfotransferases (SULTs), and/or catechol-*O*-methyltransferases (COMT) in the intestine and/or in the liver, during which *O*-glucuronides, sulfate esters and/or *O*-methyl ether metabolites are formed (Chen et al., 2014). Therefore, the parent flavonoids have low oral bioavailability, e.g., the C_{max} of total quercetin (Q) was 0.29–2.26 μM in adults after the peroral administration of 500 mg Q (Kaushik et al., 2012). Flavonoids are long-established inhibitors of OATPs (Stieger and Hagenbuch, 2014), potentially resulting in OATP-mediated food-drug interactions. Moreover, the pharmacokinetics of flavonoids can also be influenced by OATPs, since some of them, including epigallocatechin gallate, epicatechin gallate, Q and luteolin-3′-*O*-glucuronide (L3'G) are transported substrates of OATPs (Kondo et al., 2017; Roth et al., 2011; Kobayashi et al., 2011; Zhi et al., 2020). In addition, recent studies demonstrated that not only the parent flavonoids, but also their metabolites can inhibit OATP function (Kaci et al., 2023; Mohos et al., 2020). However, with few exceptions (e.g., L3'G) (Zhi et al., 2020), it is unclear whether flavonoid conjugates are just plain inhibitors or they are also transported substrates of OATPs.

The transport activity of OATPs is most frequently investigated by measuring the cellular accumulation of radioactively-labeled substrates (Sakurai et al., 2021). Alternatively, non-labeled substrates can also be detected with mass spectrometry (MS) (Bednarczyk and Boiselle, 2016). Flavonoid uptake by OATPs was also investigated by MS (Kondo et al., 2017; Chabane et al., 2010; Glaeser et al., 2014). An alternative approach for measuring OATP transport activity and identifying OATP inhibitors is the application of substrates with intrinsic fluorescence or labeled with a fluorescent tag (Kaci et al., 2023; Mohos et al., 2020; Mandery et al., 2010). These fluorescence-based methods may provide a cheaper and/or simpler alternative to radioligand- or MS-based OATP activity assays (Bednarczyk, 2010). At physiologically relevant concentrations, most of the flavonoids have low fluorescence quantum yield (De et al., 2022; Ferrara and Thompson, 2019) and hence cannot be directly detected by measuring fluorescence. However, with fluorescence enhancers (e.g., aluminum chloride or 2-aminoethyl diphenylborinate (APB)) resulting in a complex with increased fluorescence, visualization of flavonoids may be feasible (De et al., 2022) even in living cells (De et al., 2022; Rozanski et al., 2019). In the current study, we aimed to investigate direct transport of previously identified OATP inhibitors (Kaci et al., 2023; Mohos et al., 2020) Q, luteolin (LUT), naringenin (NAR) and their metabolites, by OATP1B1 and OATP2B1 in living cells. For this aim, we optimized the APB-based flavonoid detection method to monitor OATP-mediated cellular accumulation. Whereupon several sulfated and glucuronidated LUT and Q conjugates were identified as novel substrates of OATP1B1 and OATP2B1.

2. Materials and methods

2.1. Materials

Quercetin (Q), naringenin (NAR), 2-aminoethyl diphenylborinate (APB), bovine serum albumin (BSA), and bromosulfophthalein (BSP) were from Merck (Darmstadt, Germany). Luteolin (LUT) was purchased from Extrasynthese (Genay, France). Quercetin-3′-*O*-sulfate (Q3'S) was synthesized as has been previously reported (Needs and Kroon, 2006). Luteolin-3′-*O*-sulfate (L3'S) and naringenin-4′-*O*-sulfate (N4′S) were synthesized chemo-enzymatically using aryl-sulfotransferase from *Desulfitobacterium hafniense* as has been described earlier (Kaci et al., 2023; Káňová et al., 2020). Luteolin-3′-O-glucuronide (L3'G), luteolin-7-O-glucuronide (L7G) and naringenin-7-O-glucuronide (N7G) were obtained from Carbosynth (Berkshire, UK). Flavonoid stock solutions (each 10 mM) were prepared in dimethyl sulfoxide (DMSO) and stored at –20 °C. APB was dissolved in DMSO to a concentration of 125 mg/mL, and then stored at –20 °C. BSA was prepared at a concentration of 1 mg/mL, Triton X-100 (TX) 0.35 % (v/v), and paraformaldehyde (PFA) 4 % in phosphate-buffered saline (PBS, pH 7.4). BSP was dissolved in distilled water at a final concentration of 10 mM.

2.2. Generation of OATP overexpressing cell lines

A431 and HEK-293 cell lines overexpressing human OATPs, 1B1 or 2B1 and their mock transfected controls were generated as has been described earlier (Patik et al., 2018). All cell lines were cultured in DMEM (Gibco, Thermo Fischer Scientific, Waltham, MA, US) supplemented with 10 % fetal bovine serum, 2 mM L-glutamine (Merck (Darmstadt, Germany)), 100 U/mL penicillin, and 100 μg/mL streptomycin (Merck (Darmstadt, Germany)) at 37 °C, 5 % CO_2, humidified atmosphere.

2.3. Flavonoid detection by fluorescence measurement

2.3.1. Cell-free detection of quercetin fluorescence

Q was diluted in PBS from the stock solution to the desired concentration in a final volume of 200 μL without or with APB (at a final concentration of 250 mg/mL), or TX (at a final concentration of 0.35 % (v/v)), or BSA (at a final concentration of 1 mg/mL), or PFA (paraformaldehyde, 1 % final concentration), or with a combination of APB/TX, APB/BSA or APB/PFA. The fluorescence was determined using an Enspire plate reader (PerkinElmer, Waltham, MA, US) with excitation/emission wavelengths of 485/550 nm.

2.3.2. Cell-free determination of flavonoid spectra

Each flavonoid was dissolved in PBS from the stock solution to the desired quantity 25 *p*mol in a final volume of 200 μL with APB (at a final concentration of 250 mg/mL), and BSA (at a final concentration of 1 mg/mL). Fluorescence spectra of the flavonoids were determined using an Enspire plate reader (PerkinElmer, Waltham, MA, US) by setting excitation wavelength range to 500–700 nm and emission wavelength range to 400–500 nm. Excitation and emission maxima are summarized on Table 1.

2.3.3. Measurement of cellular flavonoid uptake in living cells using a microplate reader

The direct uptake of Q, LUT and their metabolites' was investigated in A431 or HEK-293 cells overexpressing OATP1B1 or OATP2B1, or

Table 1
Excitation and emission wavelength optima of flavonoids in PBS containing APB and BSA.

Flavonoid	Excitation (nm)	Emission (nm)
Quercetin	480	545
Quercetin-3′-*O*-sulfate	450	510
Luteolin	450	510
Luteolin-3′-*O*-sulfate	460	510
Luteolin-7-*O*-glucuronide	485	550
Luteolin-3′-*O*-glucuronide	460	510

Excitation and emission spectra of the flavonoids was determined in a cell-free environment, in PBS (pH 7.4), containing 250 mg/mL APB and 1 mg/mL BSA, as described in Section 2.3.1.

their mock transfected controls. 16–24 h before the transport measurement, A431 cells were seeded onto 96-well plates at a density of 8 × 10^4/well in 200 μL cell culture medium. HEK-293 cells were seeded in 96-well plates coated with poly-l-lysine (0.01 mg/mL, Merck (Darmstadt, Germany)) at a density of 80,000 cells/well 16–24 h before the transport assay. Next day, cell culture medium was removed, and the cells were washed three times with 200 μL PBS (pH 7.4) and preincubated with 50 μL HBSS (Hank's Balanced Salt Solution, pH 7.4) for 5 min at 37 °C. The uptake was initiated by the addition of 50 μL HBSS containing Q, LUT or their metabolites at a final concentration of 0.5–10 μM. After 2–15 min of incubation (specified in Figure legends) at 37 °C, the reaction was stopped by removing the supernatant and washing the cells three times with ice-cold PBS. Finally, 200 μL of PBS buffer containing APB (250 μg/mL) and BSA* (1 mg/mL) was added to each well, and the fluorescence was measured at room temperature using an Enspire plate reader (PerkinElmer, Waltham, MA, US) at wavelengths indicated in Table 1. Experiments were repeated in at least three biological replicates. Average ± SD values are shown.

*: BSA was included in the buffer only to have the same condition as used for the calibration. In intact cells low BSA uptake is expected and high protein concentration in the cytoplasm itself may result in APB-flavonoid fluorescence enhancement.

2.3.4. Measurement of cellular flavonoid uptake in living cells by confocal imaging

A431 cells expressing OATP1B1 or OATP2B1 or their mock transfected controls were seeded on 8-well μ-slide chambers (Ibidi, Germany) with a density of 1 × 10^5 / well in 400 μL of cell culture medium. The following day, the growth medium was removed, and the cells were washed three times at room temperature with 200 μL PBS buffer (pH 7.4). The cells were then preincubated for 5 min at 37 °C with 50 μL HBSS (pH 7.4) with or without BSP (20 μM), a potent inhibitor of OATPs (Izumi et al., 2013). L7G transport was started by the addition of 50 μL μL HBSS containing 5 μM of L7G. After 5 min incubation at 37 °C, the reaction was stopped by washing the cells three-times with 200 μL ice-cold PBS. Finally, 200 μL PBS containing APB (250 μg/mL) and BSA (1 mg/mL) per well was added to the cells. Images were obtained using a Zeiss LSM-710 inverted confocal microscope with 63x Plan Apochromat oil immersion objective (Carl Zeiss, Jena, Germany). All images were processed by the Zen 2010 software (Carl Zeiss microscopy, Jena, Germany).

2.4. Calculation of transport kinetics

All experiments were performed at least in triplicates. OATP-mediated transport was calculated by subtracting the fluorescence measured in mock controls. All data are expressed as the mean ± SD. K_m values were calculated by Michaelis Menten fitting using the Origin software (version 2018, OriginLab Corporation, Northampton, MA, USA)

3. Results

3.1. Optimization of fluorescence-based detection of flavonoids in cell-free environment

APB is a small (<250 Da), cell-permeable (Rozanski et al., 2019; Chokshi et al., 2012) fluorescence enhancer that can form a complex with flavonoids containing a catechol or combined 4-carbonyl + 5-hydroxyl groups (De et al., 2022; Matteini et al., 2011) (Fig. 1). Compared to the original flavonoid, the resulting flavonoid-APB complex has a strongly increased fluorescence. However, in a cell-free environment, various additives (e.g., BSA, Triton-X and/or PFA) should be applied to result in further fluorescence enhancement allowing fluorescence-based detection of the flavonoid-APB complex (Grootaert et al., 2016; Papadopoulou et al., 2005; Liu and Guo, 2006). To check which of these additives is ideal for the flavonoids tested in the current study, we investigated the fluorescence of Q in solutions supplemented with APB, TX, BSA or PFA alone and APB in combination with TX, BSA or PFA (Fig. 2A).

As shown on Fig. 2A, we found the highest Q fluorescence in the presence of APB and BSA (final concentrations 250 μg/mL and 1 mg/mL, respectively). Therefore, in further experiments, a PBS buffer containing both APB and BSA was used to visualize the flavonoids.

Next, we determined the fluorescence spectra of a set of flavonoids (already described as OATP inhibitors (Kaci et al., 2023; Mohos et al., 2020)), in the presence of APB and BSA in a cell-free environment. The excitation and emission maxima obtained in these measurements are summarized on Table 1 (spectrum shown only for Q, Fig. 2B). In further experiments, these optimal wavelengths were used. As shown on Fig. 2C, we found that Q and LUT, and their sulfated and/or glucuronidated derivatives have a well-detectable fluorescence in the presence of APB and BSA. On the other hand, NAR and its sulfate or glucuronic acid conjugates showed only weak fluorescence.

3.2. Uptake of Q, LUT and their metabolites uptake in A431-OATP1B1 and A431-OATP2B1 cells

Next, we evaluated the cellular accumulation of the flavonoids in A431 cells overexpressing OATP1B1 or OATP2B1, and also in their respective mock controls. These cell lines have been previously established and characterized in our laboratory (Patik et al., 2018). We observed high uptake of Q and LUT in each cell line, regardless of the presence of OATPs (Fig. 3). This can be explained by high passive uptake of the parent flavonoids that could not be further enhanced by OATPs', 1B1 or 2B1 functions. Another explanation to apparently high Q and LUT uptake can be transport mediated by other transporters not investigated here. However, when the cells were incubated with the metabolites Q3'S, L3'S, L3'G or L7G, we detected low fluorescence in mock control cells and, compared to this, an enhanced fluorescence both in OATP1B1- and OATP2B1-overexpressing cells (Fig. 3).

To further investigate whether the observed difference in the fluorescence signal (OATP-expressing cells versus mock controls) was indeed due to OATP-mediated uptake of the flavonoid metabolites, we investigated the concentration-dependent uptake of these compounds (Fig. 4). In these measurements, we observed a concentration-dependent, saturable uptake of the metabolites in A431-OATP cells (K_m values are summarized in Table 2), compared to which fluorescence in mock controls did not significantly change with increasing amount of the flavonoids. Furthermore, in additional experiments, flavonoid metabolite accumulation could be prevented by the addition of the OATP inhibitor BSP (Fig. 5). These results clearly indicate an OATP-mediated uptake of the LUT and Q metabolites.

3.3. Investigation of L7G uptake in A431-OATP1B1 and A431-OATP2B1 cells by confocal microscopy

Localization of one of the flavonoid metabolites with the highest cellular fluorescence signal, L7G was investigated by confocal microscopy in A431-OATP and mock cells. Fig. 6 shows that in A431-OATP1B1 and A431-OATP2B1 cells L7G was accumulated in the cytoplasm. This accumulation was largely prevented in the presence of the OATP inhibitor BSP and was absent in mock control cells.

3.4. Investigation of Q, LUT and their metabolites' uptake in HEK-OATP1B1 and HEK-OATP2B1 cells

Finally, in order to prove that the observed OATP-mediated uptake is not a cell line specific phenomenon, we investigated Q, LUT, Q3'S, L3'S, L3'G and L7G uptake in HEK-293 cells engineered to overexpress either OATP1B1 or OATP2B1, and also in the respective mock-transfected controls. These cell lines were also generated and characterized in our

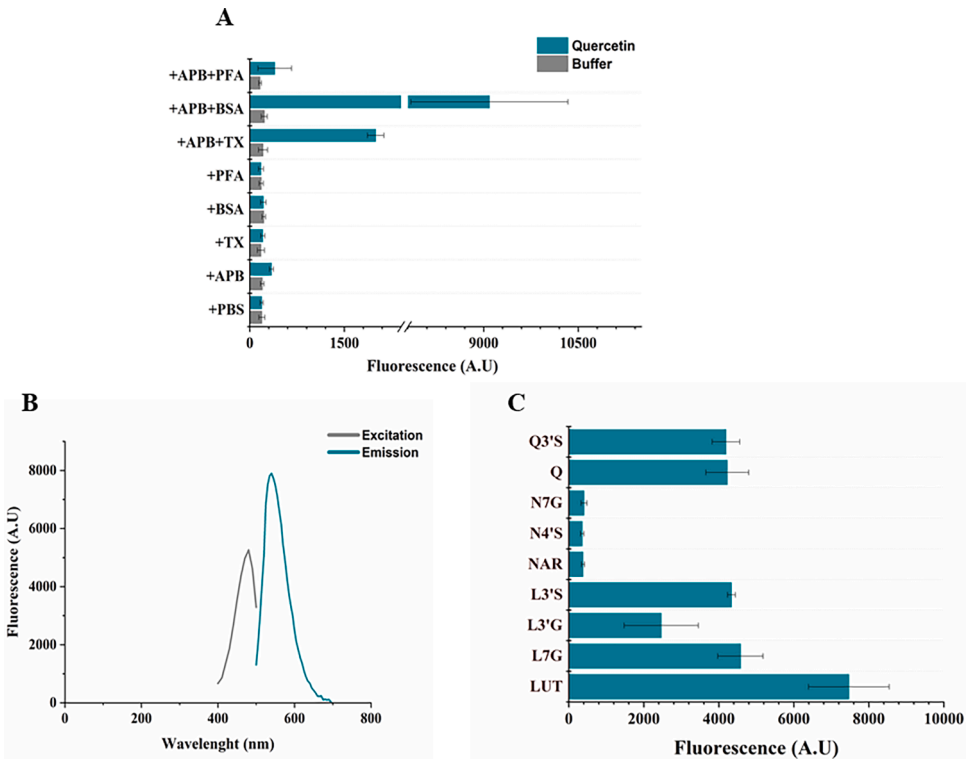

Fig. 2. Fluorescence of flavonoids in cell-free environment. (A) Fluorescence of Q in various solutions. The fluorescence of 25 *p*mol Q was measured in different solutions at excitation/emission wavelengths of 485/550 nm (n = 3; mean ± SD). **(B) Characterization of quercetin-APB/BSA complex spectra.** Fluorescence excitation and emission spectra of Q was recorded in PBS buffer containing 250 μg/mL APB, 1 mg/mL BSA and 25 *p*mol Q. **(C) Fluorescence of flavonoids.** The fluorescence of 10 *p*mol of Q, LUT, NAR and their metabolites was determined in PBS buffer containing 250 μg/mL APB and 1 mg/mL BSA (n = 3; mean ± SD is shown). Q: quercetin, BSA: bovine serum albumin (1 mg/mL), TX: tritonX-100 (0.35 % (v/v)), PFA: paraformaldehyde (1 % final concentration), Q3'S: quercetin-3′-*O*-sulfate, LUT: luteolin, L3'G: luteolin-3′-*O*-glucuronide, L7G: luteolin-7-*O*-glucuronide, L3'S: luteolin-3′-*O*-sulfate, NAR: naringenin, N4′S: naringenin-4′-*O*-sulfate, N7G: naringenin-7-O-glucuronide.

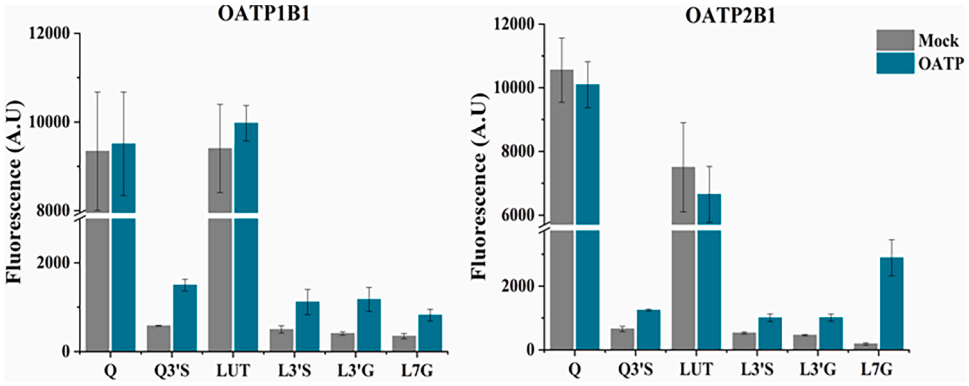

Fig. 3. Cellular uptake of Q, LUT and their metabolites in A431 cells overexpressing OATP1B1 or OATP2B1. Cells plated on 96-well plates were incubated with 10 μM of flavonoids for 15 min at 37 °C. After washing, PBS buffer containing 250 μg/mL APB and 1 mg/mL BSA was added to the cells and the fluorescence was determined at Ex/Em wavelengths indicated on Table 1. Average of means ± SD values obtained in three biological replicates each performed with technical triplicates are shown. .

earlier work (Patik et al., 2018). Consistent with the results observed in A431 cells, we observed high uptake of Q and LUT in the mock and the OATP-expressing HEK-293 cells (Fig. 7), indicating again their high passive uptake. On the other hand, we observed BSP-sensitive uptake of Q3'S, L3'S, L3'G and L7G in HEK-OATP1B1 and HEK-OATP2B1 cells (Fig. 7), but uptake was not observed in the mock controls.

4. Discussion

The list of flavonoids reported as *in vitro* inhibitors of OATP transport activity is long. For example, baicalein, biochanin A, fisetin, silibinin, genistein, and epigallocatechin-3-gallate, along with three pairs of flavonoids and their respective glycosides (hesperitin/hesperidin, naringenin/naringin, and phloretin/phloridzin) all inhibit OATP1B1 transport activity (Wang et al., 2005; Kawasaki et al., 2020). It has been reported that the flavonoids apigenin, kaempferol, and Q inhibit BSP transport mediated by intestinal OATPs, 1A2 and 2B1 (Mandery et al., 2010). OATP2B1-mediated 4′−5′-dibromofluorescein uptake was also blocked by apigetrin, scutellarein, and scutellarin (Peng et al., 2023). In addition to these, in our previous study, we found that the sulfate and

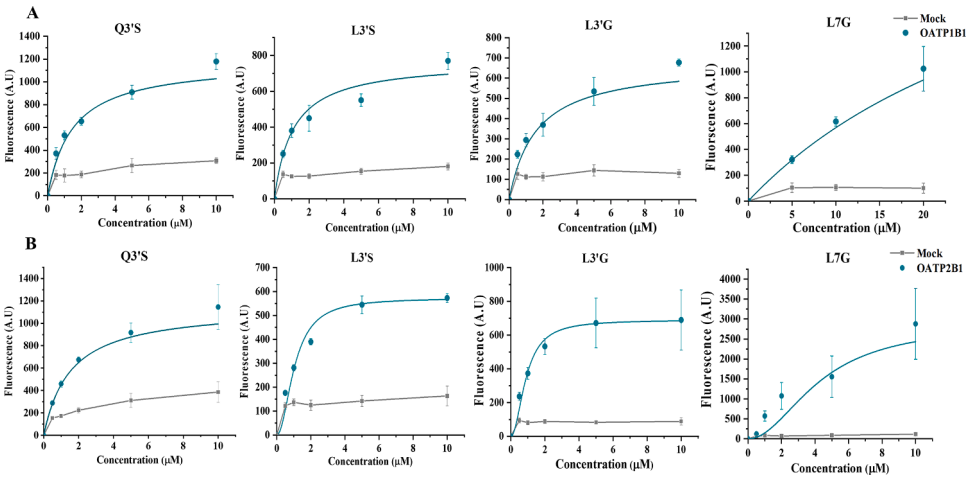

Fig. 4. Concentration-dependent uptake of Q3'S, L3'S, L3'G, L7G in A431-OATP cells. A431-OATP1B1 and A431-OATP2B1 cells (and their mock controls) were incubated with increasing concentrations of the flavonoid metabolites at 37 °C in buffer (pH 7.4) in the linear phase of uptake (2 min for OATP1B1 and 5 min for OATP2B1). After washing, PBS buffer containing 250 μg/mL APB and 1 mg/mL BSA was added to the cells and the fluorescence was determined at Ex/Em wavelengths indicated on Table 1. Each point represents the mean value obtained in three independent biological experiments with technical triplicates in each ± SD. .

Table 2
K_m values of OATP1B1 and OATP2B1-mediated flavonoid uptake.

Flavonoids	K_m (μM) OATP1B1	OATP2B1
Q3'S	2.32 ± 0.40	2.21 ± 0.85
L3'S	2.73 ± 0.60	2.89 ± 0.76
L3'G	2.50 ± 0.94	1.59 ± 0.72
L7G	9.08 ± 1.14	5.3 ± 3.00

Flavonoid uptake was performed as described at Fig. 4. K_m values were calculated by Michaelis Menten fitting using the Origin software.

glucuronide conjugates of LUT and NAR possess inhibitory effect on OATP1B1 and OATP2B1 functions (Kaci et al., 2023). Moreover, both Q and its conjugates (including Q3'S) exhibited potent inhibition of multispecific OATPs (1B1, 1B3, and 2B1) (Mohos et al., 2020). Some of these inhibitions can result in OATP-mediated FDI, as it has been confirmed by *in vivo* studies (Bailey et al., 2007; Glaeser et al., 2007; Dresser, 2002).

Besides inhibiting OATP activity and hence the potential to cause OATP-mediated FDI, flavonoids themselves can be transported by OATPs. Direct transport of epigallocatechin gallate and epicatechin gallate by OATP1A2 and OATP1B3 was reported (Roth et al., 2011), and according to (Wong et al., 2012), OATP4C1 was able to mediate the transport of the Q derivatives (e.g., Q-3′-*O*-glucuronide and Q3'S) in HepG2 cells. Another study showed the involvement of OATP1B1 and OATP1B3 in the cellular uptake of L3'G (Zhi et al., 2020). These previous investigations applied radioactively labeled flavonoids or determined intracellular flavonoid levels using LC-MS. Recently, APB (a cell-permeable small molecule fluorescence enhancer) was shown to be applicable for measuring certain flavonoids in living cells (Rozanski et al., 2019). De and colleagues demonstrated the uptake of Q, myricetin, morin, azaleatin and fisetin in neuroblastoma cells using APB (De et al., 2022). In another study, Rosanski and colleagues also utilized APB, to measure real-time efflux of Q, LUT and kaempferol from MDCKII cells expressing the ATP Binding Cassette multidrug transporter, ABCG2 (Rozanski et al., 2019). However, in cell-free environment, further additives are needed to achieve a sufficient increase in fluorescence quantum yield of the APB-flavonoid complex that allows its detection by fluorescence microscopy or using a fluorescence plate reader (Rozanski et al., 2019). Earlier, TX, BSA and PFA have been applied for such

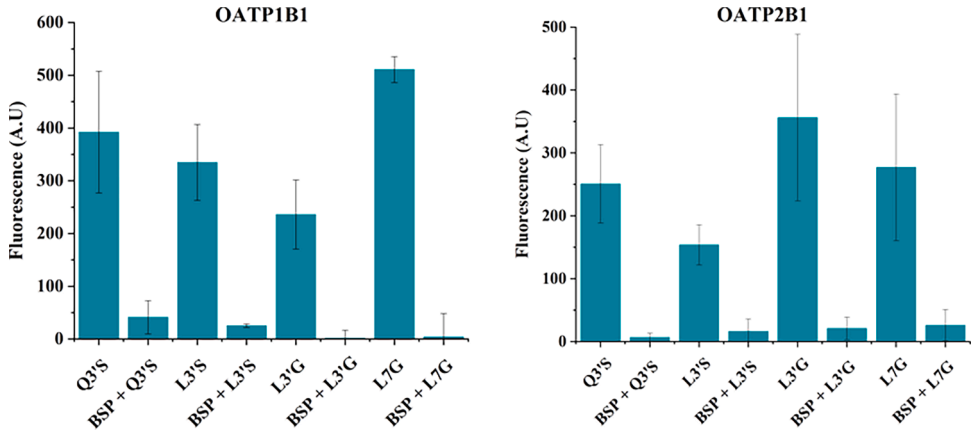

Fig. 5. Inhibition of Q3'S, L3'S, L3'G and L7G uptake in A431-OATP1B1 or A431-OATP2B1 cells. Transport of Q3'S, L3'S, L3'G, L7G (2 μM) or L7G (10 μM, OATP1B1) uptake was measured with or without 20 μM BSP for 2 min (OATP1B1) and 5 min (OATP2B1) at 37 °C on A431 cells seeded on 96-well plates. After washing, PBS buffer containing 250 μg/mL APB and 1 mg/mL BSA was added to the cells and the fluorescence was read at Ex/Em wavelengths indicated on Table 1. Fluorescence was measured after the addition of PBS containing 250 mg/mL APB and 1 mg/mL BSA using an EnSpire plate reader at wavelengths indicated on Table 1. Data represent the mean values obtained in three independent measurements ± SD values.

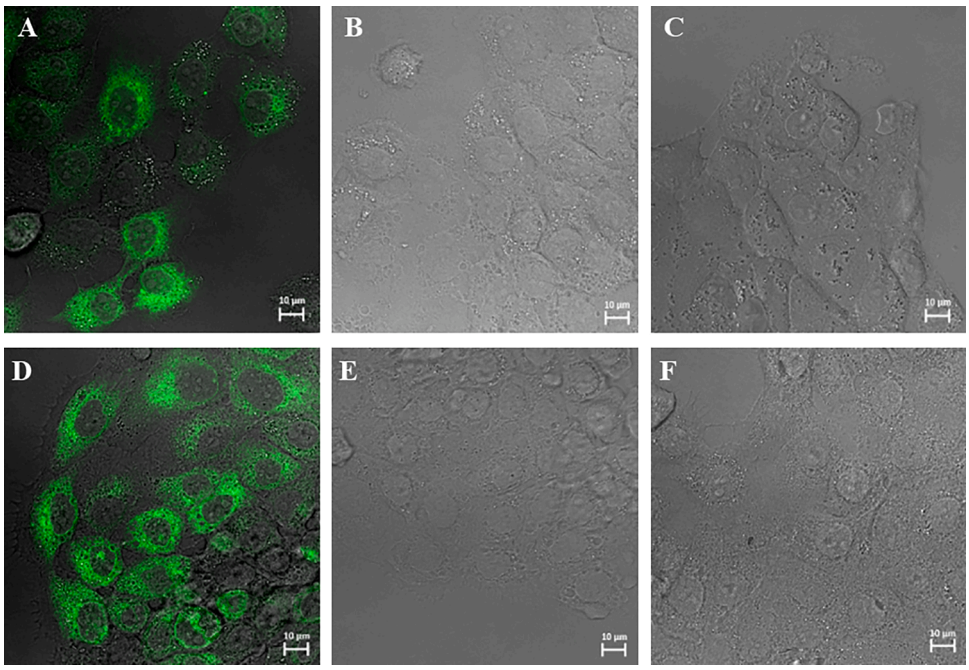

Fig. 6. Visualization of L7G uptake in OATP1B1- and OATP2B1-expressing A431 cells by confocal microscopy. The uptake of L7G, with a concentration of 5 µM and a duration of 5 min, was assessed in A431 cells overexpressing OATP1B1 (A) or OATP2B1 (D), or their mock transfected controls (B and E, respectively). These investigations were comprehensively performed under two discrete conditions; in the absence or the presence (C, F) of 20 µM BSP, a potent inhibitor of OATPs. After washing, PBS buffer containing 250 µg/mL APB and 1 mg/mL BSA was added to the cells. The acquired images were captured using a Zeiss microscope equipped with a 63 × oil-immersion objective lens, enabling enhanced magnification and resolution. Images were processed by the Zen 2010 software (Carl Zeiss microscopy, Jena, Germany).

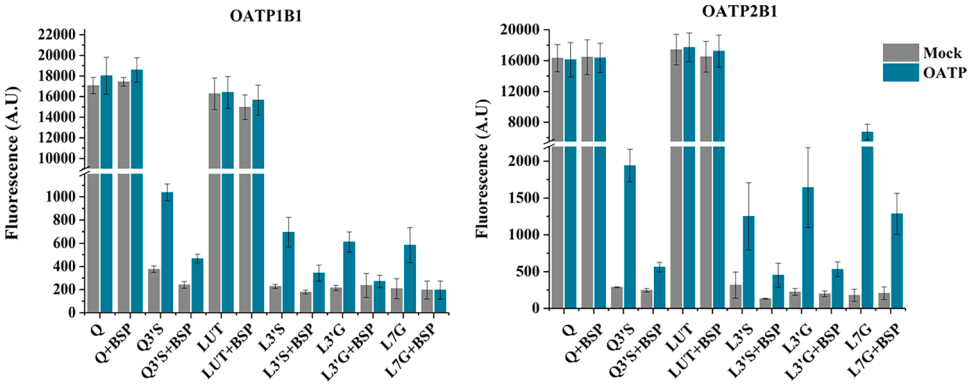

Fig. 7. Uptake of Q, LUT, and their metabolites in HEK-293 cells overexpressing OATP1B1 or OATP2B1. Uptake of 10 µM of Q, LUT, and their metabolites was measured for 15 min at 37 °C on HEK-OATP1B1, HEK-OATP2B1 or their mock control cells with or without 20 µM of BSP. Fluorescence was measured in an EnSpire plate reader at wavelengths Ex/Em indicated on Table 1, after adding 200 µL of PBS containing 250 µg/mL APB and 1 mg/mL BSA per each well. Average of at least three independent biological replicates ± SD are shown. .

purposes (Grootaert et al., 2016; Papadopoulou et al., 2005; Liu and Guo, 2006). Here, by assessing all these additives in various combinations, we found that the addition of BSA together with APB results in the highest fluorescence enhancement of Q compared to TX or PFA in a cell-free environment (Fig. 2A).

Based on earlier studies, the catechol structure (two hydroxyl groups on C3′ and C4′ in ring B) and the C2-C3 double bond (in ring C) can be involved in the complexation with APB (Matteini et al., 2011). Furthermore, the simultaneous presence of 4-carbonyl and 5-hydroxyl groups may also take part in the formation of fluorescent flavonoid-APB complexes (De et al., 2022; Matteini et al., 2011). After the optimization of flavonoid detection, we investigated the ABP-induced fluorescence signals of flavonoids. Q and LUT (possessing the catechol moiety in ring B and the C2-C3 double bond in ring C) showed well-measurable fluorescence (Fig. 2C). However, in agreement with earlier studies (Ferrara and Thompson, 2019), NAR, N4'S and N7G (without these structures) had only weak fluorescence with APB-BSA (Fig. 2C). Interestingly, although they also miss the OH group on C3', Q3'S, L3'S and L3'G maintained their APB-induced fluorescence. For the deeper understanding of the structural requirements of the ABP-mediated enhancement in the fluorescence of flavonoids, we also tested the impacts of ABP on the following flavonoids: 7,8-dihydroxyflavone, baicalein, taxifolin, 7,3′,4′-trihydroxyflavone, fisetin, isorhamnetin, tamarixetin, chrysoeriol and diosmetin (Supplementary Table S1). Baicalein and 7,8-dihydroxyflavone possess a catechol structure but in ring A (instead of ring B); however, these flavonoids did not exert fluorescence in the presence of ABP+BSA. Despite the presence of 5-hydroxy-4-keto structures in baicalein and taxifolin, we did not observe

fluorescence, suggesting the importance of the catechol structure in ring C and the presence of C2-C3 double bond. Furthermore, 7,3′,4′-trihydroxyflavone and fisetin have both the catechol moiety in ring C and the C2-C3 double bond while they have no 5-hydroxyl group (thus the 5-hydroxy-4-keto structure is missing). Nevertheless, we noticed a strong APB- induced fluorescence of 7,3′,4′-trihydroxyflavone and fisetin. Interestingly, in the presence of ABP+BSA, isorhamnetin (3′-*O*-methyl-quercetin), tamarixetin (4′-*O*-methyl-quercetin) and chrysoeriol (3′-*O*-methyl-luteolin) exerted strong fluorescence, while diosmetin (4′-*O*-methyl-luteolin) remained non-fluorescent. Our observations highlight, that the C2-C3 double bond and the catechol structure in ring B are important requirements for the APB-BSA-induced fluorescence enhancement; and even if the 3′- or 4′-hydroxyl groups are conjugated, the metabolites can still interact with ABP. These findings also indicate that the assay developed in our study may be used for only a limited number of flavonoids, to those that form a fluorescent complex with APB.

In the following experiments performed on OATP-expressing and mock cells, we detected high fluorescence of Q and LUT in mock-transfected A431 and HEK-293 cells, which could not be further enhanced by the presence of OATPs, 1B1 and 2B1 (Figs. 3 and 7). These results indicate that the uptake of Q and LUT occurs in a passive way or alternatively, it is mediated by other transporters not investigated in this study. Interestingly, in an earlier report (Glaeser et al., 2014) the OATP2B1-mediated uptake of low concentrations (0.02–0.5 μM) of Q was demonstrated. Therefore, in additional experiments, we also investigated Q and LUT uptake by OATP2B1 and OATP1B1 at lower concentrations (0.2–5 μM) (Figure S1). In these experiments, we still could not detect any differences in LUT-uptake between OATP-expressing and mock control cells. However, we observed a slightly increased fluorescence of Q in OATP2B1-expressing cells (at 1–2 μM of Q). Though, this slight uptake could only be prevented at a very high dose of the OATP inhibitor, BSP (100 μM which is almost 100-fold of its IC_{50} value (Patik et al., 2018)) (Figure S2). Therefore, we can conclude that OATP2B1 may contribute to the uptake of Q only at lower concentrations, while at higher levels the passive uptake or a transport by a non-BSP sensitive transporter dominates.

Contrary to the parent compounds, Q3'S, L3'S, L3'G and L7G showed low passive uptake and a typical OATP-mediated uptake in A431-OATP1B1 and A431-OATP2B1 cells (Figs. 3-6). Uptake of the metabolites was concentration dependent (Fig. 4) and sensitive to inhibition by BSP (Fig. 5). In addition, as a further proof of OATP-mediated uptake, we confirmed the intracellular localization of L7G by confocal microscopy (Fig. 6). Moreover, using HEK-293 cells engineered to express OATP1B1 and OATP2B1, we showed that OATP-mediated uptake of Q3'S, L3'S, L3'G and L7G is not cell-line specific (Fig. 7).

Finally, we would like to emphasize that the limitation of the method developed in our study is that not all types of flavonoids will fluoresce even in the presence of APB and BSA (Supplementary Table S1). Therefore, other methods, e.g., LC-MS/MS may be needed for their investigation. However, one of the main advantages of our assay compared to LC-MS/MS, besides its simplicity, is that it can be used in living cells. Another advantage of the APB-based method above the LC-MS/MS is that it is also suitable for investigating the localization of the flavonoids within the cells.

In summary, our results demonstrate that Q and LUT metabolites, Q3'S, L3'S, L3'G and L7G are not only inhibitors of OATP1B1 and OATP2B1 functions, but their intestinal absorption (OATP2B1) and hepatic elimination (OATP1B1) can also be influenced by these solute carriers. Though to evaluate OATPs physiological evidence still needs further investigation. For example, Kaushik et al. (2012) suggests low bioavailability of Q, hence other factors (besides OATP2B1 mediated re-absorption) should be taken into account when estimating *in vivo* fate of the flavonoid metabolites.

Until now, with the exception of 4′,5′-dibromofluorescein (Kawasaki et al., 2020), all previously identified fluorescent OATP2B1 probes (Patik et al., 2018; Ungvári et al., 2021) could only be used at acidic pH 5.5, since their OATP2B1-mediated uptake at neutral pH is negligible. This can be explained by earlier investigations showing that extracellular acidic pH may enhance the activity of OATPs (Patik et al., 2018). Although these fluorescent probes are seemingly appropriate to identify OATP2B1 inhibitors, one may always question how these results relate to *in vivo* conditions. In the assay described here, we were able to measure OATP2B1 function at neutral pH using naturally occurring substrates. Therefore, although it needs further testing and validation, the fluorescence-based method for flavonoid metabolites described in this study may provide a novel tool to identify OATP2B1 interacting molecules at physiological pH.

Funding

This work has been supported by the National Research Development and Innovation Office (NKFIH, OTKA) [grant numbers FK 128751 (Cs. Ö-L.), FK138184 (M. P.) and K 138518 (Cs. Ö-L.)] and by the Biotechnology and Biological Sciences Research Council (BBSRC) via Institute Strategic Programme Grants (Food Innovation and Health [BB/R012512/1] and its constituent project [BBS/E/F/000PR10343] and Food Microbiome and Health [BB/X011054/1] and its constituent project [BBS/E/F/000PR13630]. The work of M.P. was also supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences [grant number BO/00381/21]. K.V. was supported by Czech Science Foundation [grant number 23–04654S].

CRediT authorship contribution statement

Hana Kaci: Writing – original draft, Methodology, Data curation. **Éva Bakos:** Writing – original draft, Conceptualization. **Paul W. Needs:** Writing – review & editing. **Paul A. Kroon:** Writing – review & editing. **Kateřina Valentová:** Writing – review & editing. **Miklós Poór:** Writing – review & editing, Conceptualization. **Csilla Özvegy-Laczka:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization.

Data availability

Data will be made available on request.

Acknowledgements

The help of Natália Tőkési in confocal microscopy is greatly appreciated.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106740.

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