FOOD ADDITIVES AS INHIBITORS OF INTESTINAL DRUG TRANSPORTER OATP2B1

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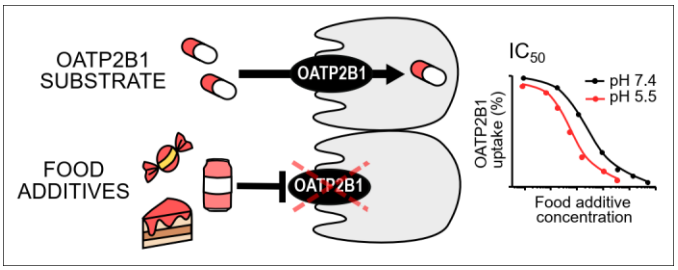

ABSTRACT

Food additives are compounds that are added to food and beverage to improve taste, color, preservation or composition. Generally, food additives are considered safe for human use due to safety evaluation conducted by food safety authorities and high safety margins applied to permitted usage levels. However, the interaction potential of food additives with simultaneously administered medication has not received much attention. Even though many food additives are poorly absorbed into systemic circulation, high concentrations could exist in the intestinal lumen making intestinal drug transporters, such as the uptake transporter organic anion transporting polypeptide 2B1 (OATP2B1), a possible site of food additive–drug interaction. In the present work, we aimed to characterize the interaction of a selection of 25 food additives including colorants, preservatives and sweeteners with OATP2B1 *in vitro*. In HEK293 cells transiently overexpressing OATP2B1 or control, uptake of dibromofluorescein was studied with and without 50 µM food additive at pH 7.4. As OATP2B1 displays substrate- and pH-dependent transport function and the intraluminal pH varies along the gastrointestinal tract, we performed the studies also at pH 5.5 using estrone sulfate as OATP2B1 substrate. Food additives that inhibited OATP2B1-mediated substrate transport with ≥50% were subjected to dose-response studies. Six colorants were identified and validated as OATP2B1 inhibitors at pH 5.5, but only three of these were categorized as inhibitors at pH 7.4. One sweetener was validated as an inhibitor at both assay conditions whereas none of the preservatives exhibited ≥50% inhibition of OATP2B1-mediated transport. Extrapolation of computed inhibitory constants (K_i values) to estimations of intestinal food additive concentrations imply that selected colorants could inhibit intestinal OATP2B1 also *in vivo*. These results suggest that food additives, especially colorants, could alter the pharmacokinetics of orally administered OATP2B1 substrate drugs, although further *in vivo* studies are warranted to understand the overall clinical consequences of the findings.

KEYWORDS

drug transporter; organic anion transporting polypeptide; OATP2B1; food additives; food–drug interaction

ABBREVIATIONS

ABC-transporter, ATP-binding cassette transporter
ADI, acceptable daily intake
BCRP, breast cancer resistance protein (*ABCG2*)
DBF, 4´, 5´-dibromofluorescein
EFSA, European Food Safety Authority
eYFP, enhanced yellow fluorescence protein
GI, gastrointestinal
HEK293, human embryonic kidney 293 cells
$IC_{50,}$ half-maximal inhibitory concentration
K_i, inhibition constant
MRP2, multidrug resistance associated protein 2
OATP2B1, organic anion transporting polypeptide 2B1 (*SLCO2B1)*
P-gp, P-glycoprotein

INTRODUCTION

Food additives are substances widely added to processed food to improve their taste, color, preservation or composition. In the European Union, the use of food additives is controlled by legislation (Regulation (EC) No 1333/2008 of the European Parliament and Council). The European Food Safety Authority (EFSA) evaluates the safety and establishes acceptable daily intake (ADI), defined as the safe amount of food additive to be consumed daily, for each food additive. The evaluation of food additive safety by authorities as well as scientific research has mainly focused on toxicity issues such as acute and chronic toxicity, carcinogenicity, genotoxicity, developmental and reproductive toxicity and hypersensitivity.[1] However, the interaction potential of food additives with concurrently administered medication has not received much attention. Interindividual variability in drug pharmacokinetics is a major concern affecting especially drugs with narrow therapeutic range, and food–drug interactions are known to be a source of variability in many circumstances.[2] Even though many food additives are poorly absorbed and hardly reach the systemic circulation,[1] they could affect drug pharmacokinetics by altering the function of drug transporters they encounter during gastrointestinal (GI) transit.

It is increasingly acknowledged that pharmaceutical excipients used for drug formulation, some of which are also used as food additives, are not pharmacokinetically inert as they can interact with GI phase I and phase II metabolizing enzymes as well as drug transporters.[3,4] For example, in different cell and animal models, the major intestinal ATP-binding cassette (ABC) drug transporters, P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance associated protein 2 (MRP2), are inhibited by excipients such as the surfactants Cremophor EL, Tween 20 or Tween 80.[3] Previously in our group, we identified several colorants capable of inhibiting BCRP and MRP2 *in vitro* with low micromolar IC_{50} values.[5] Also others have reported similar results for the inhibition of BCRP.[6] Even though drug formulations infrequently contain high amounts of these additives, higher concentrations may be present in food products. For example, in extreme cases, the intestinal concentrations after dietary exposure of selected azo dyes could exceed BCRP and MRP2 IC_{50} values more than 100-fold, suggesting that also *in vivo* inhibition of these transporters could occur.[5] In addition to intestinal efflux transporters, cellular uptake transporters localized on the membrane facing the intestinal lumen could be exposed to high food additive concentrations and, thus, be a site of food additive–drug interaction. However, knowledge about the impact of drug or food additives on the function of uptake transporters is limited.[3,4] In a few studies, pharmaceutical excipients, such as solubilizing agents hydroxypropyl-β-cyclodextrin, Solutol HS 15 and Cremophor EL, have been shown to inhibit the main intestinal organic anion transporting polypeptide (OATP), OATP2B1, and other intestinal drug uptake transporters.[7,8]

OATP2B1, encoded by the *SLCO2B1* gene, is expressed widely in many tissues including liver hepatocytes and importantly, along the entire intestine.[9,10] However, there are contradicting findings regarding the apical or basolateral localization of the transporter in enterocytes.[11,12] OATP2B1 accepts a wide range of drugs as its substrate *in vitro*, such as the antihistamine fexofenadine, lipid-lowering agents fluvastatin and rosuvastatin, and anti-inflammatory drug sulfasalazine.[10] Even though the evaluation of the role of OATP2B1 in drug pharmacokinetics has quickly evolved in recent years, this research is relatively new compared to other drug transporters, and currently the evidence of OATP2B1 contribution to drug–drug interactions is limited.[13] However, intestinal OATP2B1 is recognized as a mediator of several clinical food–drug interactions, where inhibition of OATP2B1 by common fruit juices has decreased the oral bioavailability of OATP2B1 substrates, such as aliskiren, celiprolol and fexofenadine, with more than 60%.[14–16] The involvement of OATP2B1 on drug absorption is supported by studies where the genetic variant *SLCO2B1*-c.1457C>T (rs2306168) has been associated to decreased intestinal absorption of fexofenadine and celiprolol.[14,15] In addition, recent knockout studies in mice suggest that the mouse orthologue for OATP2B1, Oatp2b1, is involved in the oral absorption of OATP2B1 substrates fexofenadine and fluvastatin.[17,18] With this background, the International Transporter Consortium (ITC)

introduced OATP2B1 as a clinically emerging transporter for drug development and recommended at least retrospective evaluation of OATP2B1 contribution to drug pharmacokinetics.[19]

Considering the emerging clinical significance of OATP2B1 and the gap in knowledge on food additive–drug interactions, the aim of this study was to characterize the potential of a set of food additives to inhibit OATP2B1 *in vitro*. The selected 25 food additives included 9 colorants, 6 preservatives and 10 sweeteners commonly used in Europe, and have been previously examined for intestinal efflux transporter inhibition.[5] As OATP2B1 displays substrate- and pH-dependent transport function,[12,20,21] we performed studies at pH 5.5 in addition to standard assay conditions at pH 7.4 and used the endogenous OATP2B1 substrate estrone sulfate and fluorescent substrate dibromofluorescein (DBF), respectively, as OATP2B1 probes. Finally, comparison of calculated inhibitory constants with estimations of intestinal food additive concentrations reveal that *in vivo* inhibition of intestinal OATP2B1 cannot be excluded.

MATERIALS AND METHODS

Materials

Food additives and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All food additives had ≥96% purity except betanin, curcumin (≥65 %) and Sunset Yellow FCF (dye content 90%). The food additives were dissolved in DMSO at 4–20 mM. Dulbeccos's modified eagle medium (DMEM; high glucose, GlutaMAX Supplement), Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Waltham, MA, USA). [^{3}H]-Estrone sulfate ammonium salt (specific activity range 40–60 Ci/mmol) and Optiphase Hisafe 3 scintillation liquid were from PerkinElmer (Boston, MA, USA).

Preparation of *SLCO2B1* carrying vectors

The full length protein coding sequence of *SLCO2B1* (NCBI Reference Sequence: NM_007256.5) was cloned with polymerase chain reaction (PCR) from human adult normal liver tissue (BioChain, San Francisco, CA, USA). The primers were 5'-TGCAGTCGACCAGTCATGGGACCCAG-3' and 5'-GAGGATTCCCGAGTGTGAGAATTCTTGGG-3' and were designed to contain SalI and EcoRI restriction sites, which were used to clone the insert into Gateway pENTR4 dual selection vector with T4 DNA Ligase (Thermo Fisher Scientific). For eukaryotic translation initiation, a kozak consensus sequence (GCC GCC ACC) was added in front of the translation initiation codon with site-directed mutagenesis (Q5 Site-Directed Mutagenesis Kit, New England Biolabs, Ipswich, MA, USA). Eurofins Genomics sequencing service (Edersberg, Germany) was used to verify the correct sequence of *SLCO2B1*.

Preparation of recombinant *SLCO2B1* carrying baculoviruses for transient expression of OATP2B1 in HEK293 cells was conducted according to manufacturer's protocols (Bac-to-Bac Baculovirus Expression System and ViraPower BacMam Expression System from Thermo Fisher Scientific) and as previously described for *ABCG2*.[22] In brief, from the pENTR4 entry vector, the *SLCO2B1* gene was transferred into a modified Bac-to-Bac destination vector with Gateway LR Clonase II enzyme mix (Thermo Fisher Scientific). Purified plasmid construct was transformed into competent DH10Bac *E. Coli* cells for transposition into bacmid. Cellfectin II reagent (Thermo Fisher Scientific) was used to transfect adherent Sf9 cells with recombinant bacmid DNA. The cells were incubated for seven days at 27 °C before harvesting recombinant baculovirus. This P1 viral stock was further amplified to yield P2 and P3 baculovirus stocks by infecting Sf9 cells with the harvested baculovirus and incubating for seven days at 27 °C. Baculovirus containing the gene for enhanced yellow fluorescence protein (eYFP) were produced similarly to be used as negative control in the HEK293 uptake assay.

HEK293 uptake assay

HEK293 cells were routinely cultured in DMEM supplemented with 10% FBS at 37 °C and 5% CO_2. For the uptake assay, HEK293 cells were seeded at 50 000 cells/well on Nunclon Delta Surface 48-well plates (Thermo Fisher Scientific) coated with poly-D-lysine, or CellBind 48-well plates (Corning, NY, USA) and incubated overnight at 37 °C and 5% CO_2. The growth medium was replaced with transduction mix with 5 mM sodium butyrate and recombinant *SLCO2B1* or eYFP (control) P3 baculovirus in DMEM supplemented with 10% FBS.

The uptake assay was initiated approximately 48 hours later by pre-incubating cells with transport buffer with pH 7.4 or pH 5.5 for 5 minutes at 37 °C. The transport buffer was composed of 25 mM HEPES (pH 7.4) or MES (pH 5.5) and 4.17 mM $NaHCO_3$ in HBSS adjusted to the assigned pH with NaOH. After the pre-incubation, transport buffer was replaced with test solution containing OATP2B1 probe substrate in transport buffer, and incubated at 37 °C while keeping the assay plate well-stirred in an orbital shaker. The uptake was terminated by washing cells three times with ice-cold transport buffer. Probe substrates used were 4´, 5´-dibromofluorescein (DBF) at pH 7.4,

and estrone sulfate at pH 5.5. A range of substrate concentrations and incubation times were tested to find the linear part of substrate transport.

The set of food additives was tested for OATP2B1 inhibition at 50 µM concentration. DMSO concentration of test solution was kept below 1.5%. Concentrations and incubation times of OATP2B1 substrates were chosen from the linear transport zone and were 1 µM (5 minutes) and 0.5 µM (2–3 minutes) for DBF and estrone sulfate, respectively. Food additives that inhibited OATP2B1-mediated transport of substrate with ≥50% were selected for dose–response studies for half-maximal inhibitory concentration (IC_{50}) determination.

Sample analysis

DBF samples. Cells were lysed with 0.1 M NaOH for 10 minutes before measuring cellular drug accumulation. DBF lysates were analyzed with fluorescence detection using Varioskan LUX microplate reader (Thermo Fisher Scientific) with 503 nm excitation and 525 nm emission.

Estrone sulfate samples. Inhibition and optimization studies with estrone sulfate were performed with radiolabeled substrate whereas LC-MS/MS detection was used to determine transport kinetics of estrone sulfate due to high consumption of substrate. [^{3}H]-Estrone sulfate containing cells were lysed in 0.1 M NaOH and subsequently neutralized with equivalent moles of 1 M HCl before adding Optiphase HiSafe 3 scintillation liquid and measuring radioactivity of samples with MicroBeta^2 2450 Microplate Counter (PerkinElmer). Estrone sulfate from transport kinetics samples were quantitated with ultra-high pressure liquid chromatography tandem mass spectrometry instrument (UPLC-MS/MS) (Waters, MA, USA). Cells were lysed with 3:1 methanol-water solution containing 25 ng/mL estrone sulfate D5 as internal standard (ISTD). After incubation for 30 minutes, the lysates were centrifuged at 14 000*g* for 10 minutes. Analytes in the supernatant were separated on a liquid chromatography coupled with Waters UPLC HSS T3 column (1.8 µm, 2.1 × 100 mm) at 40 °C, while injection volume was 1 µL. Mobile phase consisted of 0.1% of formic acid (Merck, Darmstadt, Germany) in ultrapure water (A) and 100% of LC-MS grade acetonitrile (Honeywell, Seelze, Germany) (B). Gradient elution started with 20% of B at 0–0.5 minutes, continued with 20–95% B at 0.5–2 minutes, while complete run time was 5 minutes including column wash and equilibration. The flow rate was set to 0.4 mL/min.

Mass spectrometric measurements were carried out using Waters Xevo TQ-S triple quadrupole mass spectrometer coupled with an electrospray ionization (ESI) on a negative mode. Optimized ms-parameters were as follows: capillary 1.5 kV, cone voltage 80 V for estrone sulfate and 86 V for ISTD, source temperature 150 °C and desolvation temperature 600 °C. Nitrogen (AGA, Helsinki, Finland) was used as desolvation gas (600 L h^{-1}) and cone gas (150 L h^{-1}), and argon (AGA) as collision gas. The multiple reaction monitoring mode was employed for quantification. Precursor and fragment ions were for estrone sulfate 349.02 > 269.35 (collision energy (CE) 31 V) and for ISTD 354.05 > 274.36 (CE 32 V). Resulting data was analyzed with Waters MassLynx V4.1 software.

Data analysis

OATP2B1-mediated uptake of substrates was calculated by subtracting background passive uptake obtained from eYFP transduced control wells and normalizing to test solution incubation time and average total protein amount in wells (typically 0.02–0.10 mg/well) measured with Pierce Coomassie (Bradford) Assay kit (ThermoFisher Scientific). The percentage of OATP2B1-mediated uptake inhibited by the food additives was obtained by normalizing the uptake of substrate to control, where cells were incubated with substrate only.

The IC_{50} values were calculated with GraphPad Prism version 6.07 (GraphPad Software, San Diego, CA, USA) by fitting the data to the four parameter dose-response curve (eq. 1).

$$\text{Substrate uptake (\% of control)} = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + \left(\frac{[\text{I}]}{\text{IC}_{50}}\right)^{\text{Hill Slope}}} \quad (1)$$

According to the model, bottom and top values are the maximum and minimum plateaus of the curve. The bottom plateau was constrained to zero, and, if necessary, top value to 100. [I] is the inhibitor concentration, and the Hill Slope describes the steepness of the curve. Inhibition constant (K_i) values were extrapolated from the IC_{50} values according to the Cheng-Prusoff equation (eq. 2) assuming that inhibition mode is competitive and only one substrate/inhibitor binding site is involved.[23,24] However, it should be noted that multiple binding sites on OATP2B1 exist.[21,25]

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}} \quad (2)$$

In eq. 2, [S] is the substrate concentration and K_m is the Michaelis-Menten constant. The K_m values for OATP2B1-mediated transport of DBF at pH 7.4 and estrone sulfate at pH 5.5 were defined with the HEK293 uptake assay by measuring substrate uptake in OATP2B1 and control transduced cells with different substrate concentrations and fitting the data with non-linear regression with Graphpad Prism.

Molecular properties of compounds were calculated with ACD/Labs version 8.0 (Advanced Chemistry Development, Inc., Toronto, ON, Canada).

Assay interference studies

Assay interference studies were performed as previously described[5] to exclude false positive or negative results due to aggregation of food additives, intrinsic fluorescence of food additives or quenching of DBF fluorescence signal. Aggregation of 50 µM food additives in the transport buffer at pH 7.4 and pH 5.5 was tested using Nepheloskan Ascent nephelometer (Thermo Fisher Scientific). When aggregation was observed, further dilutions of the food additives were made to find solubility limits. Quenching of DBF fluorescence or intrinsic fluorescence of food additives were assayed by mimicking the assay conditions by measuring the fluorescence of 0.1 µM DBF with 50 µM food additive in 0.1 M NaOH. These conditions assume that all of the food additive in the inhibition assay would be taken up by the cells and retained in the final sample elute, which is not likely. If fluorescence interference was observed in these conditions, DBF fluorescence was measured together with lower concentrations of food additives assuming that 20% (10 µM), 10% (5 µM) or 2% (1 µM) of the initial 50 µM food additive would be retained in the final fluorescence sample.

Statistical analysis

The single concentration (50 µM) food additive testing was performed once with three replicate wells. The IC_{50} assays were repeated on 3–4 independent occasions with three replicates each (n = 3–4). Kinetic assays were performed once with three replicate wells.

Statistical significance was calculated with unpaired student's *t*-test or when multiple groups were compared, one-way ANOVA with Tukey's or Dunnett's post hoc test. Statistical significance was considered when $p < 0.05$.

RESULTS

The uptake of DBF into OATP2B1 and control (eYFP) transduced HEK293 cells was studied at pH 7.4. OATP2B1-mediated uptake of DBF was time- (Supporting Information Figure S1) and concentration-dependent with a K_m value of 2.7 ± 0.29 µM (Figure 1A). High passive background uptake of DBF (apparent pKa 6.69 [most acidic]; $logD_{5.5}$ 4.87) in control transduced cells at pH 5.5 impeded the use DBF as OATP2B1 substrate at this pH (data not shown). Estrone sulfate (pKa not applicable; $logD_{5.5}$ 0.01) demonstrated time- (Supporting Information Figure S1) and concentration-dependent OATP2B1-mediated uptake with low background uptake and a K_m value of 12.9 ± 3.2 µM at pH 5.5 (Figure 1B). The transport affinity of both substrates was comparable with previously published data.[26–28]

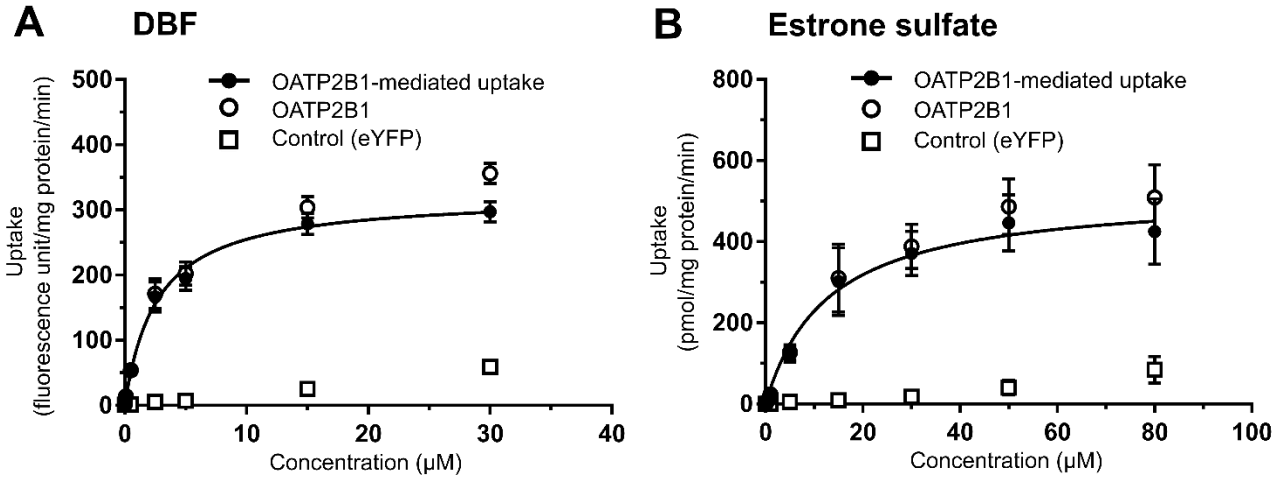

Figure 1. Concentration-dependent uptake of **(A)** dibromofluorescein (DBF) and **(B)** estrone sulfate into HEK293 cells expressing OATP2B1 (○) or control (□). OATP2B1-mediated uptake (●) was obtained by subtracting control uptake from OATP2B1 uptake. Uptake of DBF was studied at pH 7.4 over 5 minutes and uptake of estrone sulfate at pH 5.5 over 2 minutes. The data is presented as mean ± SD from one study with three replicate wells. Data used to create the figures is presented in Supporting Information Table S1 and S2.

The potential to inhibit OATP2B1-mediated uptake of DBF at pH 7.4 and estrone sulfate at pH 5.5 was tested for a set of 25 food additives at 50 µM concentration (Figure 2 and Supporting Information Table S4). Three of the colorants (Allura Red AC, Carmoisine and curcumin) inhibited OATP2B1-mediated transport of DBF and estrone sulfate with ≥50%. Three additional colorants (Brilliant Black BN, Brilliant Blue FCF and Sunset Yellow FCF) were characterized as inhibitors only when estrone sulfate was used as substrate. None of the preservatives inhibited OATP2B1-mediated transport with over 50% compared to the control. Neohesperidin dihydrochalcone (DC) was the only sweetener identified as an OATP2B1 inhibitor at both assay conditions while rebaudioside A inhibited OATP2B1-mediated DBF uptake ≥50%. Sucralose seemed to increase OATP2B1 mediated uptake of DBF as uptake was 153.5 ± 8.9% compared to control.

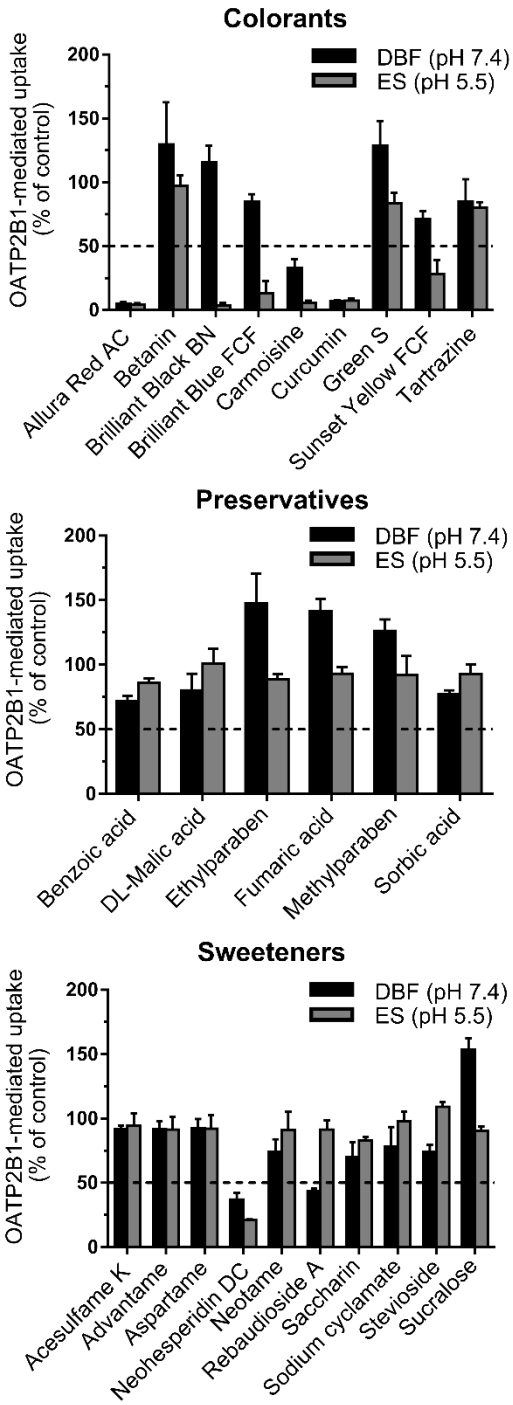

Figure 2. Inhibition of OATP2B1-mediated uptake of 1 µM dibromofluorescein (DBF) at pH 7.4 (black bars) or 0.5 µM estrone sulfate (ES) at pH 5.5 (gray bars) by a selection of 25 food additives including colorants, preservatives and sweeteners. OATP2B1 and control overexpressing HEK293 cells were incubated with 50 µM food additive and substrate to define percent of OATP2B1 uptake inhibited. Food additives that inhibited OATP2B1-mediated uptake more than 50% (dashed line) were selected for dose-response studies. The results are expressed as mean ± SD (in three replicate wells) of OATP2B1-mediated uptake normalized to control without food additive.

Based on the initial single inhibitor concentration assay, potential OATP2B1 inhibitors were selected for dose-response studies to determine IC_{50} (Figure 3 and Table 1), which were then used to extrapolate K_i values according to the Cheng-Prusoff equation. Because substrate concentrations used were well below K_m values, the calculated K_i values were close to the IC_{50} values determined experimentally. Allura Red AC was identified as the strongest inhibitor at both conditions with K_i values 0.6 ± 0.3 µM and 1.5 ± 0.5 µM at pH 7.4 and pH 5.5, respectively. Rebaudioside A inhibited OATP2B1-mediated uptake of DBF (pH 7.4) with ≥50% at 50 µM concentration (Figure 2), but dose-dependent inhibition was not observed (data not shown). The K_i value of Carmoisine was significantly lower at pH 5.5 compared to pH 7.4 ($p = 0.013$), while Allura Red AC, curcumin and neohesperidin DC had comparable inhibitor profiles at pH 7.4 and pH 5.5 ($p > 0.05$).

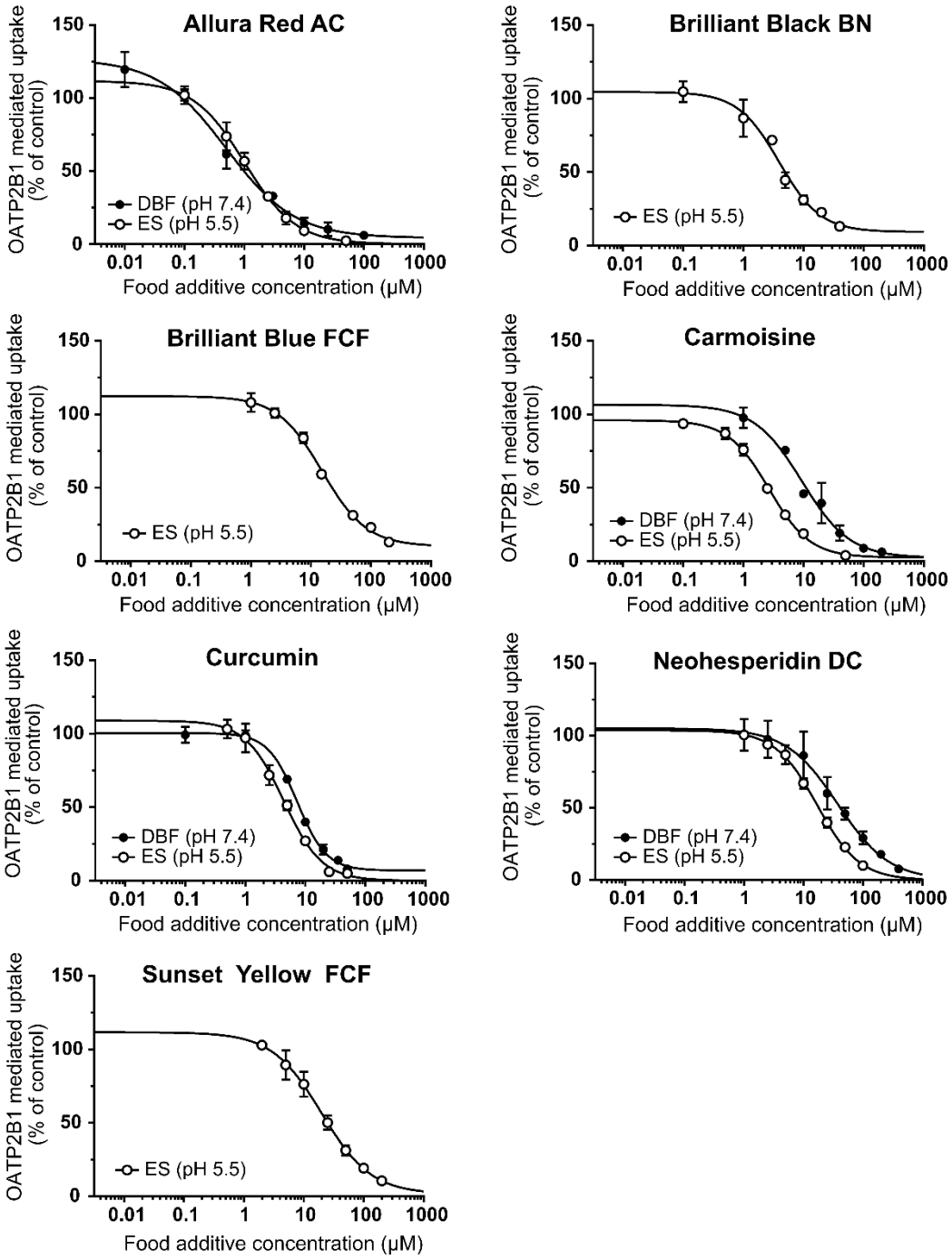

Figure 3. Inhibitory curves of OATP2B1-mediated uptake of 1 μM dibromofluorescein (DBF) at pH 7.4 (●) and 0.5 μM estrone sulfate at pH 5.5 (○) by selected food additives in HEK293 overexpression system. Brilliant Black BN, Brilliant Blue FCF and Sunset Yellow FCF were characterized as inhibitors only at pH 5.5. Data is presented as mean ± SD from a representative study with three replicate wells.

Table 1. Half-maximal inhibitory concentrations (IC_{50}) and extrapolated inhibition constant (K_i) values for selected food additives at pH 7.4 (DBF as substrate) and pH 5.5 (estrone sulfate). IC_{50} are expressed as mean ± SD calculated from values obtained from 3–4 individual experiments (n = 3–4) with three replicate wells. K_i values were calculated from the IC_{50} values with the Cheng-Prusoff equation.[23,24]

Food additive		**pH 7.4** (μM)	**pH 5.5** (μM)
Allura Red AC	IC_{50}	0.8 ± 0.4	1.6 ± 0.6
	K_i	0.6 ± 0.3	1.5 ± 0.5
Brilliant Black BN	IC_{50}	[a]	3.9 ± 0.4
	K_i	[a]	3.8 ± 0.3
Brilliant Blue FCF	IC_{50}	[a]	18.4 ± 2.6
	K_i	[a]	17.7 ± 2.5
Carmoisine	IC_{50}	9.7 ± 2.6	2.9 ± 0.3
	K_i	7.0 ± 1.9*	2.7 ± 0.3*
Curcumin	IC_{50}	6.9 ± 1.0	5.4 ± 1.0
	K_i	5.0 ± 0.7	5.3 ± 1.0
Neohesperidin DC	IC_{50}	48.6 ± 17.4	15.5 ± 1.6
	K_i	35.4 ± 12.7	14.9 ± 1.5
Sunset Yellow FCF	IC_{50}	[a]	20.3 ± 1.0
	K_i	[a]	19.6 ± 1.0

[a] IC_{50} is above 50 μM according to the single inhibitor concentration assay and, therefore, IC_{50} (and K_i) was not determined
*$p < 0.05$, significant difference between K_i values at pH 7.4 and pH 5.5

To evaluate whether the difference in OATP2B1 inhibitory activity at pH 7.4 and pH 5.5 observed for Brilliant Black BN, Brilliant Blue FCF and Sunset Yellow FCF was due to the different extracellular pH or different substrate used, we tested their OATP2B1 inhibition of estrone sulfate transport also at pH 7.4 at 50 µM concentration (Figure 4). Brilliant Black BN displayed pH-dependent activity as no inhibition was observed at pH 7.4 regardless of the substrate. Brilliant Blue FCF demonstrated ≥50% transport inhibition only at pH 5.5, but decrease in transport function was observed also at pH 7.4 for both substrates. The inhibitory activity of Sunset Yellow FCF seemed to be mostly substrate-dependent since inhibition of estrone sulfate at pH 7.4 and pH 5.5 were comparable but differed from DBF uptake inhibition at pH 7.4 ($p < 0.05$).

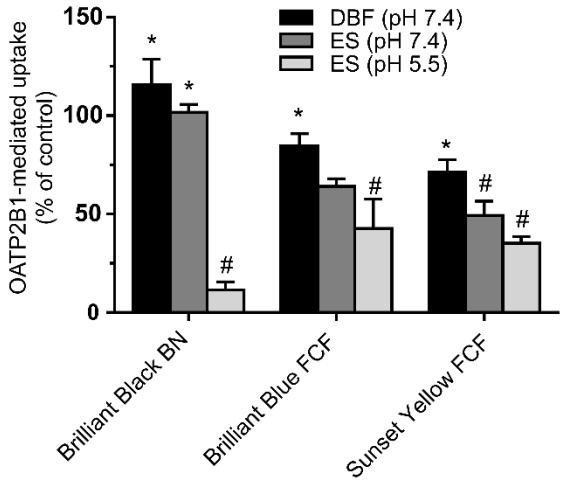

Figure 4. Substrate- and pH-dependent inhibition of OATP2B1-mediated uptake of dibromofluorescein (DBF; 1 µM) at pH 7.4, and estrone sulfate (ES; 0.5 µM) at pH 7.4 and pH 5.5 by selected food additives. Concentration of food additive was 50 µM (40 µM for Brilliant Black inhibition of estrone sulfate transport). Results are expressed as mean ± SD from one study with three replicates except for estrone sulfate (pH 5.5), where results are expressed as mean ± SD from three independent studies with three replicates. Data was partly integrated from inhibitor identification assays (Figure 2) and dose-response studies (Figure 3). *$p < 0.05$ compared to ES (pH 5.5), #$p < 0.05$ compared to DBF (pH 7.4).

To rule out false positive or negative results due to fluorescence interference of test compounds or poor aqueous solubility of food additives, assay interference studies were performed. Quenching of DBF fluorescence signal was observed for nine compounds when DBF fluorescence was measured together with 50 µM food additive (Supporting Information Figure S2). Four of these compounds (Allura Red AC, Carmoisine, curcumin and rebaudioside A) were identified as OATP2B1 inhibitors at pH 7.4 in the initial inhibition assay. However, as it is very unlikely that all of the food additive is taken up into the cells during the uptake assay, the fluorescence interference was then tested with lower concentrations for these four compounds. When it was assumed that 10% (5 µM) or less of the food additive is retained in the final sample elute in the uptake assay, quenching of DBF fluorescence was less than 10% for all four compounds (Figure 5A).

The solubility of the food additives in transport buffer was tested to rule out OATP2B1 inhibition by aggregates. Only curcumin displayed clear aggregation at the 50 µM test concentration at pH 7.4 and pH 5.5 and was subjected to further studies (Figure 5B; Supporting Information Figure S3). At both pH conditions, curcumin had a similar solubility profile, with significant aggregation observed at concentrations over 1 µM ($p < 0.05$).

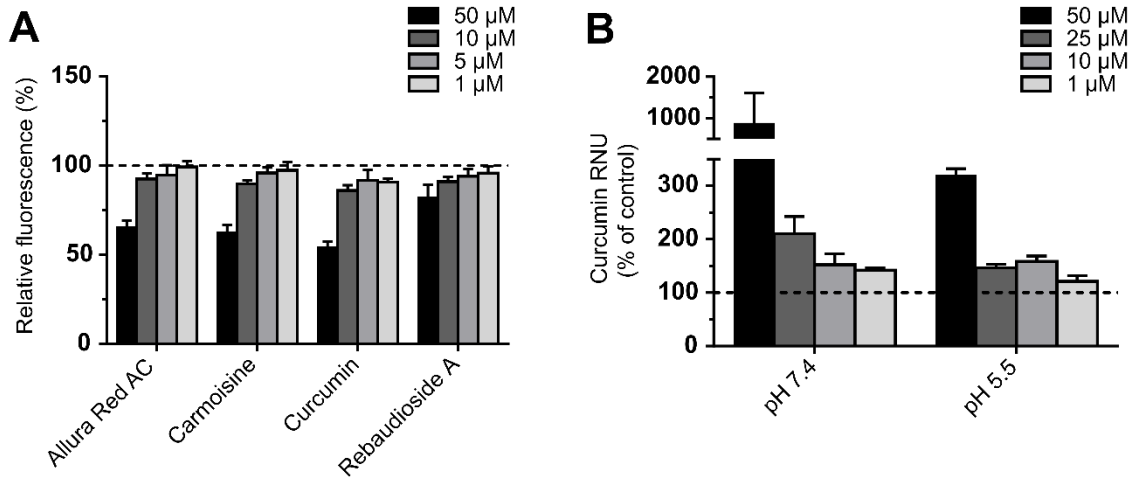

Figure 5. **A)** Fluorescence interference of selected food additives with 0.1 µM dibromofluorescein (DBF) assuming that 100% (50 µM), 20% (10 µM), 10% (5 µM) or 2% (1 µM) of the initial food additive in the HEK293 uptake assay would be retained in final samples. Results are expressed as relative fluorescence of 0.1 µM DBF with food additive compared to DBF fluorescence alone. **B)** Solubility of curcumin in transport buffer (pH 7.4 and pH 5.5) measured with nephelometry. Results are expressed as mean ± SD (three replicates) relative nephelometric unit (RNU) of curcumin in transport buffer normalized to RNU of transport buffer alone.

DISCUSSION

In this study, we identified seven food additives as OATP2B1 inhibitors from a selection of 25 commonly used colorants, preservatives and sweeteners. With the exception of the sweetener neohesperidin DC, all OATP2B1 inhibitors were color additives. Moreover, the inhibitors were mostly azo dyes as Allura Red AC, Brilliant Black BN, Carmoisine and Sunset Yellow FCF share the functional group diazenyl R-N=N-R' (Supporting Information Figure S4). In general, high molecular weight and, conversely, both lipophilicity and polarity are characteristic for OATP inhibitors.[29] Among the studied food additives, most OATP2B1 inhibitors were rather polar and of high molecular weight, as they had calculated $logD_{7.4}$ and $logD_{5.5}$ between -7.64 to -0.17 and molecular weights above 350 Da (Supporting Information Table S3). The calculated $logD_{7.4}$ and $logD_{5.5}$ of neohesperidin DC and curcumin were higher, ranging from 2.59 to 3.08. Assay interference studies indicated that curcumin inhibitor properties could have been affected by aggregates in the test solution. However, clear curcumin dose-dependent inhibition of OATP2B1 was observed notwithstanding the inhibition mechanism by curcumin specifically or its aggregates. Our findings regarding the inhibitory potential of curcumin are also in agreement with a previous study where 10 µM curcumin significantly inhibited OATP2B1-mediated transport of estrone sulfate at pH 7.4.[30]

During the review process of this manuscript, a study investigating food and drug additives as OATP2B1 inhibitors was published.[31] Similar to our study, OATP2B1 inhibitors identified in this study were mostly color additives and, furthermore, enriched in azo dyes. When DBF was used as substrate, comparable K_i values were obtained for Allura Red AC and neohesperidin DC in both studies, and the K_i value of Sunset Yellow FCF (68.4 µM) is also in agreement with our screening result with DBF at pH 7.4. However, we defined lower K_i values for Allura Red AC (1.5 µM versus 10.5 µM[31]) and Sunset Yellow FCF (19.6 µM versus 54.2 µM[31]) when estrone sulfate was used as substrate, which is possibly explained by our acidic assay condition compared to neutral pH used by Zou et al.[31] We did not observe significant OATP2B1 inhibition of DBF uptake by 50 µM Brilliant Blue FCF (uptake 84.7% of control), but Zou et al.[31] reported marked decrease of DBF transport by 200 µM Brilliant Blue FCF and defined a K_i value of 13.0 µM, which is comparable with the K_i value we obtained for estrone sulfate at pH 5.5. We used only a single concentration (50 µM) in the initial inhibitor identification assay, and advanced food additives inhibiting OATP2B1 transport ≥50% for dose-response studies, which could have underestimated the prevalence of relevant inhibitors among food additives that are present in high amounts in food products. However, ten of the inactive food additives in our study were included in the study by Zou et al.,[31] and were not identified as OATP2B1 inhibitors even though Zou et al. used mostly higher concentrations (200–1000 µM) for the initial single concentration inhibitor screen. Comparison of these results from our study and the study by Zou et al.[31] are summarized in Supporting Information Table S4.

The function of OATP2B1 is known to be largely substrate- and perpetrator-dependent, a phenomenon explained at least partly by multiple binding sites on OATP2B1 and best described for estrone sulfate transport.[21,25] OATP2B1 has low- and high affinity sites for estrone sulfate, which, however, are not necessarily completely common with binding sites for other substrates.[25] Also perpetrators may alter the function of either the low- or high-affinity site or both, and yet the possibility of allosteric binding sites cannot be excluded.[25,32,33] Furthermore, perpetrators can either stimulate or inhibit these binding sites independently, as progesterone stimulates the uptake of estrone sulfate by the high-affinity site but rather inhibits the low-affinity site.[25] In addition to significant OATP2B1 inhibition by food additives, we observed an apparent OATP2B1 stimulating effect for sucralose, which increased OATP2B1 mediated uptake of DBF with ≥50% at pH 7.4 but not uptake of estrone sulfate at pH 5.5. These findings may be an artifact of screening with a single concentration, as Zou et al.[31] did not observe any effect on OATP2B1-mediated transport of DBF with 1000 µM sucralose. However, due to the complicated nature of OATP2B1 modulation described above, the stimulation of OATP2B1 by sucralose could be substrate- and also concentration-dependent similar to progesterone, which stimulates OATP2B1 at lower concentrations but the

stimulatory effect fades out with higher concentrations.[32] The apparent stimulatory effect of food additives require further experiments dedicated for this purpose, as well as studies addressing the overall clinical relevance of OATP2B1 stimulation, which is currently lacking.[33,34]

OATP2B1 has maximal functional activity at an acidic extracellular environment,[20,26] which can be relevant in the intestine, where orally administered drugs are exposed to a pH gradient. Therefore, the International Transporter Consortium has suggested that incorporation of acidic assay conditions to standard OATP2B1 *in vitro* studies at neutral pH could improve translation of *in vitro* results to the clinical situation.[19] The luminal pH can vary from about 4.5 to 8.0 in different intestinal segments, but the pH-microclimate at the epithelial cell surface is typically between 5.2 and 6.7.[35,36] Considering the pH- and substrate-dependent function of OATP2B1, we included studies with estrone sulfate at pH 5.5 to accompany findings from neutral assay conditions with DBF, and identified three colorants that inhibited OATP2B1 at pH 5.5 but not at pH 7.4. However, as these observations could be due to change in extracellular pH and/or different substrates between the two conditions, we then tested the inhibition of estrone sulfate also at pH 7.4 for the three additives that were identified as inhibitors of estrone sulfate transport at pH 5.5 but not DBF at pH 7.4 (Figure 4). An apparent pH-dependent inhibitory effect of Brilliant Black BN was observed as no inhibition was evident at pH 7.4 regardless of the substrate, but the inhibitor activity of Brilliant Blue FCF and Sunset Yellow FC was more diverse. These findings could be explained by the multiple binding sites on OATP2B1 as only the low affinity estrone sulfate binding site shows pH-dependent substrate transport.[21] The effect of extracellular pH on OATP2B1 inhibitor activity has not been comprehensively studied. Varma et al.[20] studied the inhibitor properties of rifamycin SV on OATP2B1-mediated uptake of estrone sulfate and rosuvastatin and observed similar profiles at pH 7.4 and pH 6.0. Our results suggest that OATP2B1-inhibition is pH-, substrate- and perpetrator-dependent, which complicates the extrapolation of results from one substrate and perpetrator to another and that care should be taken also to choose *in vitro* assay conditions carefully to imitate the *in vivo* site of inhibition.

Although systemic absorption of especially many colorants is negligible,[1] concentrations in the intraluminal space of the GI tract can be high due to high amounts of additives in food and beverage. For example, detected concentrations of Allura Red AC in drinks and juices can vary from 0.1 to over 2000 mg/kg.[5] According to European Medicines Agency (EMA) guidance on drug interactions, *in vivo* inhibition of intestinal transporters cannot be ruled out if observed K_i values are lower than maximum expected concentration in the intestinal lumen, which is defined as 0.1-fold the maximum dose on one occasion/250 ml.[37] However, the maximum single doses of food additives are difficult to estimate, because additive amounts in food can vary from product to product, consumption patterns are individual, and the daily additive intake is not typically ingested as a single bolus. To evaluate the possibility of *in vivo* inhibition of OATP2B1 by selected food additives, we decided to use EFSA estimates of maximum daily dietary exposure for a high-level consumer (if available) as the maximum food additive dose (Table 2). Of the OATP2B1 inhibitors identified, neohesperidin DC was the only additive where *in vivo* inhibition could be ruled out based on these calculations. For all other food additives, the resulting intestinal threshold concentrations for *in vivo* inhibition (0.1-fold the maximum dose/250 ml) are higher than the defined K_i values. For example, the concentrations calculated for Allura Red AC are 113-fold and 45-fold higher than the K_i values obtained at pH 7.4 (DBF) and pH 5.5 (estrone sulfate).

Table 2. Estimated dietary exposure and estimation of intestinal concentrations [I] of selected food additives.

Food additive	Dietary exposure[a] (mg/kg bw/day)		[I][c] (μM)
	Mean	High level[b]	
Allura Red AC K_i (pH 7.4) = 0.6 μM K_i (pH 5.5) = 1.5 μM	0.1–0.4	0.4–1.2	68
Brilliant Black BN K_i (pH 5.5) = 3.8 μM	0.05–0.30	0.23–0.69	22
Brilliant Blue FCF K_i (pH 5.5) =17.7 μM	0.6	3.0	106
Carmoisine K_i (pH 7.4) = 7.0 μM K_i (pH 5.5) =2.7 μM	0.04–0.3	0.2–0.9	50
Curcumin K_i (pH 7.4) = 5.0 μM K_i (pH 5.5) = 5.3 μM	0.2–0.6	0.4–1.5	114
Neohesperidin DC K_i (pH 7.4) =35.4 μM K_i (pH 5.5) = 14.9 μM	0.02[d]	-	1
Sunset Yellow FCF K_i (pH 5.5) = 19.6 μM	0.01–0.1	0.1–0.4	25

[a] Data from EFSA (European Food Safety Authority) exposure assessments (References [38–44]). Data for maximum reported use level or brand-loyal scenario in adults, if not otherwise stated

[b] 95th or 97th percentile

[c] [I] Estimated intestinal concentration calculated according to EMA guidance (0.1-fold the maximum dose on one occasion/250 ml).[37] The daily dietary exposure (maximum for high-level consumer, if available) for a 70 kg person was used as the maximum dose

[d] Calculated from modified theoretical added maximum daily intake

Based on the relationships between K_i values and intestinal exposure to food additives, *in vivo* studies would be justified to elucidate the impact of OATP2B1 inhibition by selected food additives.[37] Considering that OATP2B1 is the main OATP isoform in the intestine and it transports a broad range of drugs, *in vivo* inhibition of OATP2B1 would be expected to alter the intestinal absorption of OATP2B1 substrate drugs given that OATP2B1 is a major contributor to absorption of the substrate. It would not be unforeseen if food additives would cause clinical drug interactions via OATP2B1 inhibition, as dietary and natural products, especially fruit juices, have shown to decrease the exposure of OATP2B1 substrate drugs.[13] On the other hand, the lack of clinical OATP2B1-mediated drug–drug interactions can be considered rather surprising, as *in vitro* OATP2B1 inhibitors are not particularly rare among clinically used drugs.[28] The reason could be that OATP2B1 is not the predominant mechanism in drug absorption *in vivo* as other contributing pathways may exist. Other reasons could be that OATP2B1 has been understudied and more clinical interactions appear when systematic research becomes common. As we demonstrated here, more OATP2B1 inhibitors were identified at acidic assay conditions, suggesting that potential intestinal OATP2B1 inhibitors could be overlooked if studied only at neutral pH 7.4.

As we have previously demonstrated, food additives can inhibit other intestinal transporters as well.[5] All food additives identified as OATP2B1 inhibitors in this study have been previously identified also as BCRP and/or MRP2 inhibitors.[5,6] Considering that the consequences of intestinal apical efflux transporter inhibition are opposite to the effects of OATP2B1 uptake inhibition, the concurrent inhibition of both transporters could overrule the

estimated clinical effects for individual transporters taken that the substrate is also transported by both transporters. For example, curcumin can inhibit many intestinal drug transporters relevant for drug absorption (OATP2B1, BCRP, MRP2 and P-gp) as well as drug metabolizing enzymes *in vitro*,[5,45] making extrapolation of *in vivo* effects complicated based on separate *in vitro* assays. However, inhibition of efflux transporters and drug metabolizing enzymes require inhibitor access to the transporter from intracellular side, which may not be achievable for food additives with limited absorption. Food additives can also be extensively metabolized during GI transit. For example, azo dyes are susceptible to bacterial azo reduction,[46] and sweeteners neohesperidin DC, stevioside and rebaudioside A to bacterial deglycosylation.[47,48] Notably, Zou et al.[31] demonstrated that the reduced metabolites of azo dyes did not inhibit OATP2B1 *in vitro*, and that high (25 mg/kg) but not low (2.5 mg/kg) dose Allura Red AC decreased the exposure of fexofenadine in P-gp-deficient (*mdr1a/b−/−)* mice. These results suggest that high doses of azo dyes have the potential to saturate the azoreductase capacity of the gut microbiome, which then can result in intact dye concentrations high enough to cause observable intestinal OATP2B1 inhibition.[31]

In conclusion, this study demonstrates that in addition to previously recognized inhibitory effects on ABC-efflux transporters,[5,6] commonly used food additives can inhibit the intestinal uptake transporter OATP2B1 *in vitro*. Out of the 25 tested food additives, six colorants and one sweetener were validated as OATP2B1 inhibitors. Several colorants were found to inhibit OATP2B1 with inhibitory constants well below estimated intestinal food additive concentrations. We also demonstrated substrate- and pH-dependent inhibition of OATP2B1, as more inhibitors were identified when estrone sulfate was used as OATP2B1 substrate at pH 5.5 mimicking the acidic intestinal environment. Altogether, the results of this study suggest that high exposure to food additives could cause interindividual variability on the absorption of orally administered drugs, but considering the complexity of OATP2B1 modulation, further studies evaluating the overall *in vivo* effect of food additive–drug interactions are required.

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SUPPORTING INFORMATION

Figure S1: Time-dependent uptake of DBF and estrone sulfate
Tables S1 and S2: Data used to analyze concentration-dependent uptake of DBF and estronesulfate
Figure S2: DBF fluorescence interference with food additives (50 µM)
Figure S3: Solubility of food additives (50 µM) in transport buffer
Figure S4: Structural formulas of the food additives that were identified as OATP2B1 inhibitors
Table S3: Molecular properties of the tested food additives
Table S4: Inhibition of OATP2B1-mediated uptake by food additives (50 µM) and comparison with results from study by Zou et al.[31]

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FOOD ADDITIVES AS INHIBITORS OF INTESTINAL DRUG TRANSPORTER OATP2B1

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SUPPORTING INFORMATION

Figure S1: Time-dependent uptake of DBF and estrone sulfate
Tables S1 and S2: Data used to analyze concentration-dependent uptake of DBF and estrone sulfate
Figure S2: DBF fluorescence interference with food additives (50 µM)
Figure S3: Solubility of food additives (50 µM) in transport buffer
Figure S4: Structural formulas of the food additives that were identified as OATP2B1 inhibitors
Table S3: Molecular properties of the tested food additives
Table S4: Inhibition of OATP2B1-mediated uptake by food additives (50 µM) and comparison with results from study by Zou et al.[1]

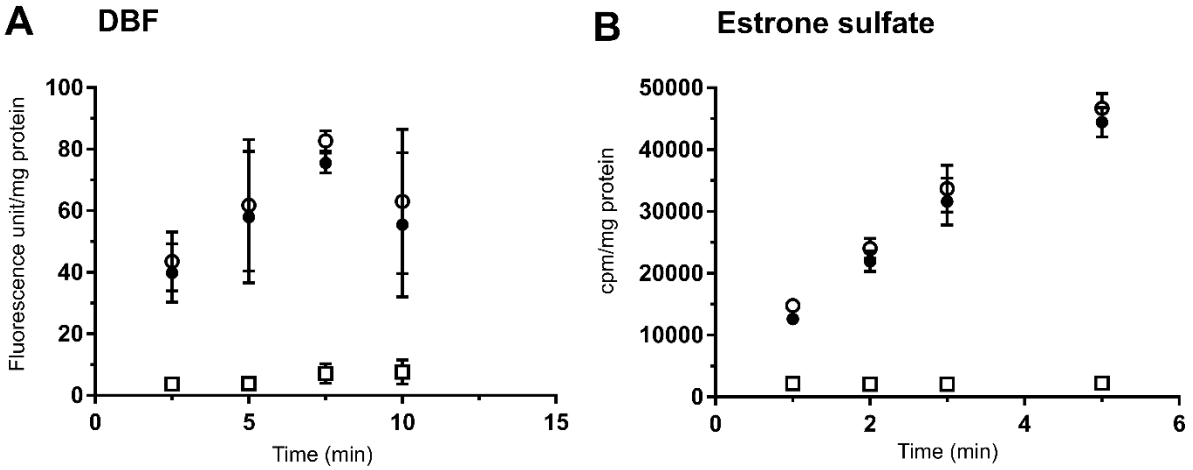


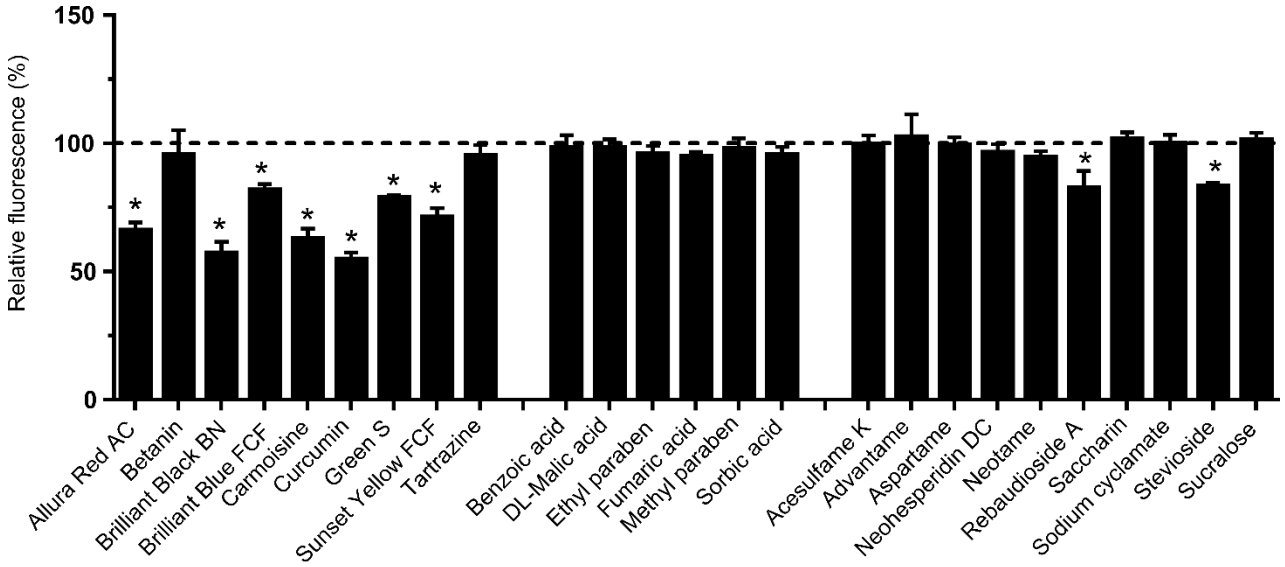
Figure S1. Time-dependent uptake of (A) 1 µM dibromofluorescein (DBF) and (B) 0.5 µM estrone sulfate into HEK293 cells expressing OATP2B1 (○) or control (□). OATP2B1-mediated transport (●) was obtained by subtracting control uptake from OATP2B1 uptake. Uptake of DBF and estrone sulfate were studied at pH 7.4 and pH 5.5, respectively. The data is presented as mean ± SD from one study with three replicate wells.

Table S1. Data used to analyze concentration-dependent uptake of DBF into OATP2B1 and control (eYFP) expressing HEK293 cells. OATP2B1-mediated uptake was obtained by subtracting control uptake from OATP2B1 uptake. The data is presented as mean ± SD from one study with three replicate wells.

DBF concentration (µM)	**OATP2B1 uptake** (fluorescence unit/ mg total protein/min)	**Control (eYFP) uptake** (fluorescence unit/ mg total protein/min)	**OATP2B1-mediated uptake** (fluorescence unit/ mg total protein/min)
0.01	1.72 ± 0.91	1.15 ± 0.68	0.56 ± 1.13
0.1	13.89 ± 0.31	1.60 ± 0.93	12.29 ± 0.98
0.5	54.13 ± 6.53	1.43 ± 0.24	52.70 ± 6.53
2.5	171.65 ± 23.15	5.30 ± 0.84	166.36 ± 23.17
5	202.29 ± 18.00	7.37 ± 0.75	194.92 ± 18.01
15	303.79 ± 16.36	25.17 ± 0.44	278.61 ± 16.37
30	356.22 ± 15.50	58.85 ± 9.98	297.37 ± 18.43

Table S2. Data used to analyze concentration-dependent uptake of estrone sulfate into OATP2B1 and control (eYFP) expressing HEK293 cells. OATP2B1-mediated uptake was obtained by subtracting control uptake from OATP2B1 uptake. The data is presented as mean ± SD from one study with three replicate wells.

Estrone sulfate concentration (µM)	**OATP2B1 uptake** (pmol/mg total protein/min)	**Control (eYFP) uptake** (pmol/mg total protein/min)	**OATP2B1-mediated uptake** (pmol/mg total protein/min)
0.1	0.63 ± 0.09	0.10 ± 0.02	0.53 ± 0.09
0.5	9.52 ± 0.18	0.65 ± 0.23	8.88 ± 0.18
1	23.66 ± 4.61	1.12 ± 0.06	22.54 ± 4.61
5	127.10 ± 18.58	4.77 ± 0.54	122.33 ± 18.58
15	310.56 ± 83.48	8.71 ± 1.53	301.85 ± 83.48
30	388.16 ± 54.89	17.37 ± 4.78	370.78 ± 54.89
50	486.40 ± 69.01	40.15 ± 18.11	446.24 ± 69.01
80	508.95 ± 80.73	84.26 ± 32.57	424.69 ± 80.73



* p<0.05, compared to control

Figure S2. Interference of food additives with dibromofluorescein (DBF) fluorescence assuming that food additives are completely retained in the final fluorescence sample. DBF (0.1 µM) fluorescence was measured with 50 µM food additive in 0.1 M NaOH. Results are expressed as mean ± SD of three replicate measurements normalized to control without food additive.

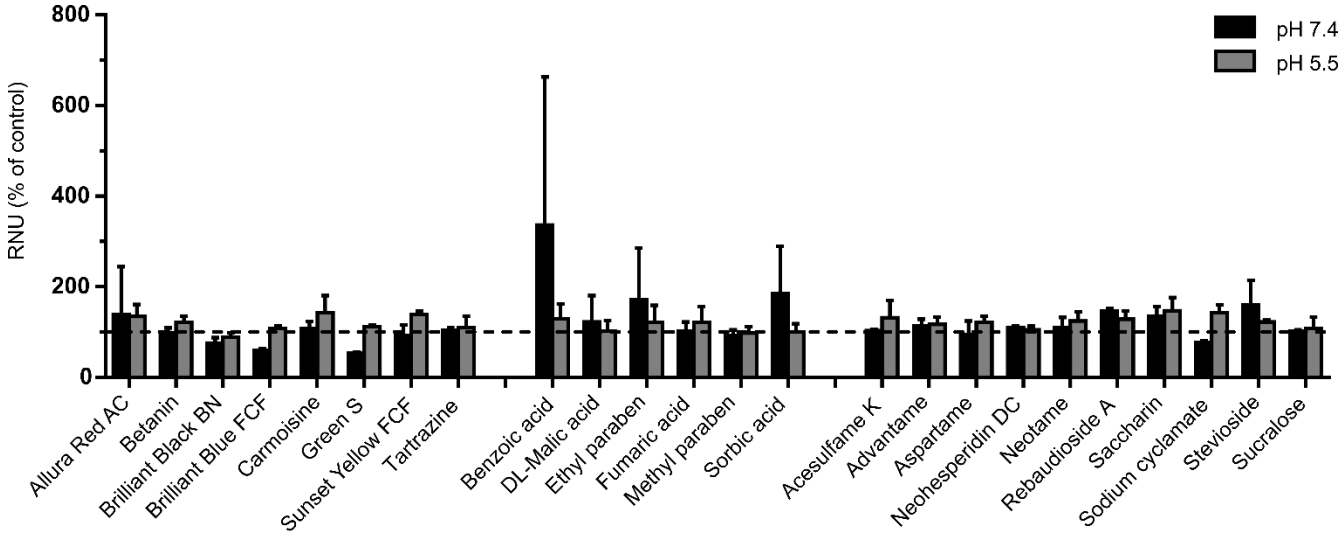
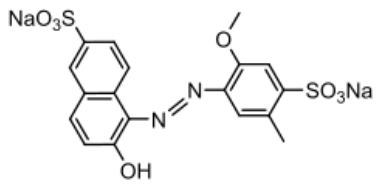
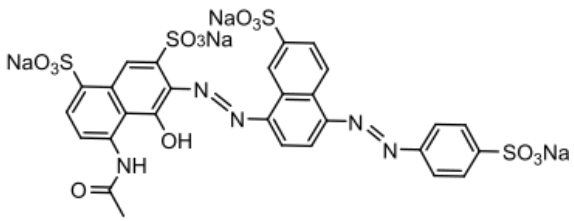


Figure S3. Solubility of food additives (50 µM) in transport buffer pH 7.4 or pH 5.5 measured with nephelometry. Results are expressed as mean ± SD (three replicates) relative nephelometric unit (RNU) of food additives in transport buffer normalized to RNU of transport buffer alone.

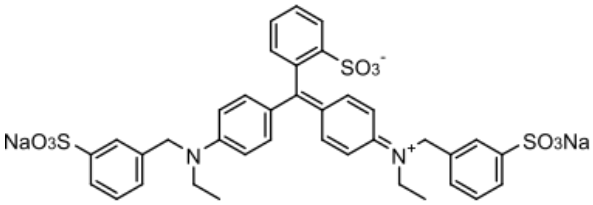
ALLURA RED AC



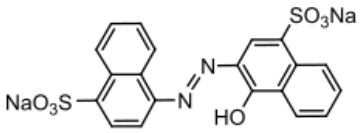
BRILLIANT BLACK BN



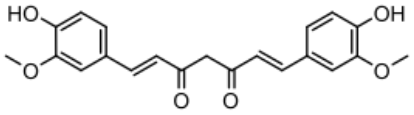
BRILLIANT BLUE FCF



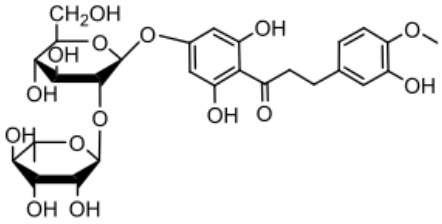
CARMOISINE



CURCUMIN



NEOHESPERIDIN DC



SUNSET YELLOW FCF

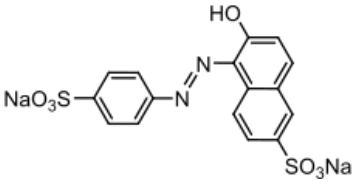


Figure S4. Structural formulas of the food additives that were identified as OATP2B1 inhibitors.

Table S3. Molecular properties of the tested food additives. The properties were calculated with ACD/Labs version 8.0 (Advanced Chemistry Development, Inc., Toronto, ON, Canada) for the parent compound.

Food additive	**Catalog number**	**E number**	**MW** (g/mol)	**PSA**	**LogD$_{7.4}$**	**LogD$_{5.5}$**
Acesulfame K (potassium salt)	47134	E 950	163	81	-2.32	-2.32
Advantame (monohydrate)	80054	E 969	459	134	-0.25	-0.03
Allura Red AC (sodium salt)	38213	E 129	452	180	-1.64	-1.64
Aspartame	47135	E 951	294	119	-1.49	-1.34
Benzoic acid	33047	E 210	122	37	-0.98	0.58
Betanin (red beet extract diluted with dextrin)	CDS000584	E 162	551	247	-6.59	-6.42
Brilliant Black BN (sodium salt)	11220	E 151	780	350	-7.64	-7.64
Brilliant Blue FCF (sodium salt)	80717	E 133	750	195	-0.18	-0.17
Carmoisine (sodium salt)	52245	E 122	458	170	-3.1	-3.07
Curcumin	C1386	E 100	368	93	2.84	2.92
DL-Malic acid	240176	E 296	134	95	-5.99	-3.9
Ethylparaben	111988	E 214	166	47	2.34	2.39
Fumaric acid	47910	E 297	116	75	-4.75	-3.09
Green S (sodium salt)	06737	E 142	556	152	0.98	1.25
Methylparaben	H5501	E 218	152	47	1.81	1.86
Neohesperidin dihydrochalcone	75041	E 959	613	245	2.59	3.08
Neotame	49777	E 961	378	105	1.07	1.17
Rebaudioside A	01432	E 960	967	374	-1.12	-1.12
Saccharin	109185	E 954	183	72	-1.09	-1.08
Sodium cyclamate (sodium salt)	47827	E 952	179	75	-2.52	-2.4
Sorbic acid	S1626	E 200	112	37	-1.41	0.39
Stevioside (hydrate)	S3572	E960	805	295	1.19	1.19
Sucralose	PHR1342	E 955	398	129	0.68	0.68
Sunset Yellow FCF (sodium salt)	465224	E 110	408	170	-3.3	-3.3
Tartrazine (sodium salt)	03322	E 102	468	220	-6.63	-6.63

MW, molecular weight; PSA, polar surface area

Table S4. Inhibition of OATP2B1-mediated uptake of dibromofluorescein (DBF; 1 µM) at pH 7.4, and estrone sulfate (ES; 0.5 µM) at pH 5.5 by food additives at 50 µM concentration. Results are expressed as mean ± SD of OATP2B1-mediated uptake relative to control with DBF or estrone sulfate alone from one experiment with three replicates. For comparison, results from the study by Zou et al.[1] are presented.

Food additive	**DBF uptake (pH 7.4)** (% of control)	**ES uptake (pH 5.5)** (% of control)	**Zou et al.[1] DBF uptake[b]** (% of control)	**K_i (DBF pH 7.4)** (µM)	**K_i (ES pH 5.5)** (µM)	**Zou et al.[1] K_i** (µM)
Acesulfame K	91.7 ± 2.8	94.2 ± 9.8	101 (10 µM)	[a]	[a]	[a]
Advantame	91.5 ± 6.5	91.3 ± 9.9	[a]	[a]	[a]	[a]
Allura Red AC	5.3 ± 1.0	4.4 ± 1.1	3.5 (200 µM)	0.6	1.5	2.59
Aspartame	92.5 ± 7.1	92.0 ± 10.8	103 (200 µM)	[a]	[a]	[a]
Benzoic acid	71.9 ± 4.0	86.0 ± 3.4	[a]	[a]	[a]	[a]
Betanin	129.7 ± 32.9	97.3 ± 8.0	[a]	[a]	[a]	[a]
Brilliant Black BN	115.5 ± 13.2	3.6 ± 2.0	[a]	[a]	3.8	[a]
Brilliant Blue FCF	84.7 ± 6.0	13.1 ± 9.6	19.5 (200 µM)	[a]	17.7	13.0
Carmoisine	33.0 ± 6.9	5.5 ± 1.9	[a]	7.0	2.7	[a]
Curcumin	7.0 ± 0.8	7.4 ± 1.9	[a]	5.0	5.3	[a]
DL - Malic acid	80.3 ± 12.9	101.0 ± 11.4	D-(+) = 96.6 (200 µM) L-(−) = 92.5 (200 µM)	[a]	[a]	[a]
Ethyl paraben	147.7 ± 22.7	88.6 ± 4.1	74.6 (200 µM)	[a]	[a]	[a]
Fumaric acid	141.4 ± 9.7	93.1 ± 4.9	88.9 (200 µM)	[a]	[a]	[a]
Green S	128.9 ± 19.2	83.6 ± 8.4	[a]	[a]	[a]	[a]
Methyl paraben	126.3 ± 8.7	91.9 ± 14.8	92.2 (200 µM)	[a]	[a]	[a]
Neohesperidin DC	37.0 ± 5.4	20.9 ± 0.6	14.7 (200 µM)	35.4	14.9	20.1
Neotame	74.2 ± 9.8	91.3 ± 13.8	67.1 (200 µM)	[a]	[a]	[a]
Rebaudioside A	43.5 ± 2.2	91.5 ± 7.3	[a]	[a]	[a]	[a]
Saccharin	69.9 ± 11.8	82.9 ± 2.7	97.1 (200 µM)	[a]	[a]	[a]
Sodium cyclamate	78.1 ± 15.3	98.0 ± 7.3	[a]	[a]	[a]	[a]
Sorbic acid	77.1 ± 3.1	92.8 ± 7.5	[a]	[a]	[a]	[a]
Stevioside	73.8 ± 5.8	109.1 ± 3.6	[a]	[a]	[a]	[a]
Sucralose	153.5 ± 8.9	90.2 ± 3.5	102 (1000 µM)	[a]	[a]	[a]
Sunset Yellow FCF	71.3 ± 6.3	28.5 ± 10.6	45.5 (50 µM)	[a]	19.6	68.4
Tartrazine	84.9 ± 17.5	80.0 ± 4.6	97.8 (200 µM)	[a]	[a]	[a]

[a] Not determined

[b] Screening concentration in parenthesis

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