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Influence of the flavonoids apigenin, kaempferol and quercetin on the function of organic anion transporting polypeptides 1A2 and 2B1

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Abbreviations

OATP	organic anion transporting polypeptide
BSP	sulphobromophthalein
HEK	human embryonic kidney
IC_{50}	concentration with 50% inhibition
K_i	inhibitory constant
SLCO	solute carrier organic anion transporter
VC	vector control

1 Abstract

OATP1A2 and OATP2B1 are uptake transporters of the human organic anion transporting polypeptide (OATP) family with a broad substrate spectrum including several endogenous compounds as well as drugs such as the antihistaminic drug fexofenadine and HMG-CoA reductase inhibitors. Both transporters are localized in the apical membrane of human enterocytes. Flavonoids, abundantly occurring in plants, have previously been shown to interact with drug metabolizing enzymes and transporters. However, the impact of flavonoids on OATP1A2 and OATP2B1 transport function has not been analyzed in detail. Therefore, HEK293 cell lines stably expressing OATP1A2 and OATP2B1 were used to investigate the influence of the Ginkgo flavonoids apigenin, kaempferol, and quercetin on the transport activity of OATP1A2 and OATP2B1. K_i values of all three flavonoids determined from Dixon plot analyses using BSP as substrate indicated a competitive inhibition with quercetin as the most potent inhibitor of OATP1A2 (22.0µM) and OATP2B1 (8.7µM) followed by kaempferol (OATP1A2: 25.2µM, OATP2B1: 15.1µM) and apigenin (OATP1A2: 32.4µM OATP2B1: 20.8µM). Apigenin, kaempferol, and quercetin led to a concentration-dependent decrease of the OATP1A2-mediated fexofenadine transport with IC_{50} values of 4.3µM, 12.0µM, and 12.6µM, respectively. The OATP1A2- and OATP2B1-mediated transport of atorvastatin was also efficiently inhibited by apigenin (IC_{50} for OATP1A2: 9.3µM, OATP2B1: 13.9µM), kaempferol (IC_{50} for OATP1A2: 37.3µM, OATP2B1: 20.7µM) and quercetin (IC_{50} for OATP1A2: 13.5µM, OATP2B1: 14.1µM). These data indicate that

modification of OATP1A2 and OATP2B1 transport activity by apigenin, kaempferol, and quercetin may be a mechanism for food-drug or drug-drug interactions in humans.

2 Keywords

organic anion transporting polypeptide

flavonoids

drug-drug interaction

fexofenadine

inhibition

herbal drugs

3 Introduction

Flavonoids represent the most abundant polyphenols in vegetables, fruits, and plants. They are also ingredients of tea, wine, juices, numerous multivitamin preparations and herbal products (e.g. Ginkgo biloba formulations). In vitro and in vivo studies indicate pharmacological effects of flavonoids on prevention of cardiovascular diseases as well as anticancerogenic and antioxidative effects [1-4].

It has recently been shown that several of these compounds inhibit drug metabolizing enzymes and drug transporters such as cytochrome P450 3A4 and the drug efflux transporter P-glycoprotein (ABCB1) [5], highlighting their potential for food-drug interactions. For example, the flavonoids apigenin, kaempferol, and quercetin which are constituents of Ginkgo biloba formulations, onions, strawberries, and apples [2] were characterized by inhibition and/or induction of CYP3A4, ABCB1, and ABCC2 [6-7]. OATP1A2 and OATP2B1 are members of the human OATP family and are localized in the apical membrane of human enterocytes [8-9]. OATP1A2 mediates the intracellular uptake of drugs such as fexofenadine [10] as well as of endogenous compounds such as taurocholate [11]. OATP2B1 mediates the cellular uptake of drugs such as fluvastatin [12], atorvastatin [13] and of endogenous compounds such as estrone-3-sulfate [14]. The influence of flavonoids on intestinal drug metabolism and transport is of special interest because flavonoids such as apigenin, kaempferol, and quercetin can reach high concentrations in the gut lumen. The inhibition of an OATP-mediated drug uptake in the intestine may decrease the plasma concentration of a substrate of OATPs, due to a reduced absorption.

In vitro studies in HeLa cells and additional in vivo studies showed that the flavonoid naringin, a constituent of grapefruit juice, inhibits the uptake of fexofenadine mediated by OATP1A2 [9, 15-16]. This inhibition leads to a reduced area under the plasma concentration time curve (AUC) of the OATP1A2 substrate fexofenadine when grapefruit juice is coadministered with orally taken fexofenadine. Grapefruit juice or orange juice at a concentration of 5% significantly inhibited the OATP2B1-mediated uptake of estrone-3-sulfate by 82% and 53% in HEK293 cells stably expressing OATP2B1 [17].

Furthermore it was shown in an in vitro study that several herbal extracts, such as Ginkgo biloba extracts, potently inhibited the OATP2B1-mediated uptake of estrone-3-sulfate in HEK293 cells. These results suggest that coadministration of some dietary supplements may decrease the absorption of orally administered OATP2B1 substrates [18].

However, there are no data showing the influence of the flavonoids apigenin, kaempferol, and quercetin, which are chemically related to the grapefruit juice flavonoid naringin, on the function of human intestinal OATPs such as OATP1A2 and OATP2B1. Therefore, we investigated the potential influence of the flavonoids apigenin, kaempferol, and quercetin on the transport function of OATP1A2 and OATP2B1 in order to gain more insights regarding further possible mechanisms of food-drug or drug-drug interactions. Changes in function of the OATP1A2- and OATP2B1-mediated drug uptake by apigenin, kaempferol, and quercetin may influence the absorption and systemic exposure of OATP substrates. Considering the frequent intake of herbal preparations of Ginkgo biloba, vegetables, and fruits containing these flavonoids, the hypothesized interaction with the intestinal uptake transporters OATP1A2 and

OATP2B1 might be an important determinant of intraindividual variability of drug disposition.

4 Methods

4.1 Chemicals

[^{3}H]Sulphobromophthalein (14 Ci/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). Unlabeled sulphobromophthalein was purchased from Applichem GmbH (Darmstadt, Germany). Sodium butyrate was purchased from Merck KGaA (Darmstadt, Germany). Apigenin (purity ≥ 95.0%, HPLC), kaempferol (purity ≥ 96%, HPLC), quercetin (purity ≥ 98%, HPLC), and fexofenadine hydrochloride were purchased form Sigma-Aldrich Chemie GmbH (Munich, Germany). [^{3}H]Atorvastatin (10 Ci/mmol) and unlabeled atorvastatin were obtained from BioTrend Chemikalien GmbH (Cologne, Germany) and Toronto Research Chemicals Inc. (North York, ON, Canada), respectively. All compounds were dissolved in dimethyl sulfoxide (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). All other chemicals and reagents, unless stated otherwise, were obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and were of the highest grade available.

4.2 Generation of a HEK293 cell line stably expressing OATP1A2

The *SLCO1A2* coding sequence (NM_134431.3) was cloned by reverse transcription reaction and subsequent polymerase chain reaction from human brain cDNA (Human Total RNA Master Panel II, Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) into the pcDNA3.1(+) vector (Invitrogen GmbH, Karlsruhe, Germany) and

subcloned into the retroviral vector pQCXIN (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). Human embryonic kidney cells (HEK293) were transfected with the plasmid pQCXIN-OATP1A2 using a retroviral gene transfer and expression kit (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). After geneticin (G-418; 500 µg/ml) treatment, single colonies were selected and characterized for OATP1A2 mRNA and protein expression using real-time PCR, immunofluorescence, and immunoblot analysis as previously described [19-20]. The polyclonal rabbit anti human OATP1A2 antiserum was kindly provided by Professor Richard B. Kim (Division of Clinical Pharmacology, Department of Medicine, Schulich School of Medicine & Dentistry, The University of Western Ontario, London, Ontario, Canada). The primer for real-time PCR analysis of *SLCO1A2* mRNA were forward: 5'-AAGACCAACGCAGGATCCAT -3' and reverse: 5'-GAGTTTCACCCATTCCACGTACA -3' with a resulting amplicon size of 101 base pairs. The primers for the housekeeping gene β-actin were forward: 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' and reverse: 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3' with a resulting amplicon size of 661 base pairs. The secondary antibodies for immunofluorescence and immunoblot analyses were used as previously published [19]. HEK293-VC (VC: vector control) cells were established by the same method using the plasmid lacking the insert for transfection.

4.3 Cell culture

HEK293-OATP1A2 cells were cultured in minimum essential medium containing 10 % heat-inactivated fetal bovine serum, 2mM non essential amino acids, 500 µg/ml geneticin, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5 % CO_2. HEK293-OATP2B1 cells [21-22] were cultured in minimum essential medium containing 10 % heat-inactivated fetal bovine serum, 800 µg/ml geneticin, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C and 5 % CO_2.The cells were routinely subcultured by trypsinization using trypsin (0.05 %)-EDTA (0.02 %) solution. All cell culture media supplements were obtained from Invitrogen GmbH (Karlsruhe, Germany).

4.4 Transport assays

The uptake experiments were performed as previously described [19-20]. Briefly, HEK293-OATP1A2, HEK293-OATP2B1, and the respective HEK293-VC cells were seeded in poly-D-lysine coated 12-well plates at an initial density of $7.5x10^5$ cells / well. After 24 h, cells were treated with sodium butyrate for 24 h prior to the uptake experiments in order to increase the levels of the recombinant protein [23]. First, cells were incubated with prewarmed (37 °C) uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, pH 7.3) containing a mix of radiolabeled (0.01 µM) and nonradiolabeled BSP or atorvastatin. HEK293-OATP1A2 cells were incubated with BSP (concentrations see below) and atorvastatin (5 µM) for five minutes and 10 minutes, respectively. OATP2B1 cells were incubated with BSP and atorvastatin for 10 minutes. HEK293-OATP1A2 and HEK293-VC cells were incubated with unlabeled fexofenadine (5 µM) for 5 minutes. Estimation of

the inhibitory constants (K_i) of flavonoids for OATP1A2 and OATP2B1 were performed using Dixon-Plot analyses [24]. The OATP1A2-mediated BSP net uptake was determined at concentrations of 2.5, 5.0 and 10 µM after an incubation of 5 minutes in absence or presence of increasing concentrations of flavonoids (apigenin: 1, 10, 100 µM; kaempferol: 10, 50, 100 µM for OATP1A2; 1, 10, 100 µM for OATP2B1; quercetin: 25, 50, 100 µM). For OATP2B1, the BSP net uptake was determined using concentrations of 0.5, 1.0 and 2.5 µM at 10 minutes of incubation in absence or presence of increasing concentrations of flavonoids (see above). After incubation cells were lyzed with 0.2% sodium dodecyl sulfate and the intracellular accumulation of radioactivity was determined in 500 µl of cells lysates by liquid scintillation counting (TriCarb 2800, PerkinElmer Life Sciences GmbH). The appropriate protein concentration of each well was determined in 25 µl of cells lysates by bichinchoninic acid assay (BCA Protein Assay Kit; Thermo Scientific). The net uptake was expressed as pmol*mg protein^{-1}*min^{-1}. Furthermore, a concentration dependent inhibition of OATP1A2-mediated fexofeandine transport was investigated at concentration of flavonoids ranging from 1 µM to 750 µM. The influence on the OATP1A2- and OATP2B1-mediated uptake of atorvastatin was investigated at a concentration range between 0,1 µM and 250 µM of flavonoids. Measurements were repeated for each concentration three to six times.

4.5 Quantitation of fexofenadine by LC-MS/MS

The quantitation of fexofenadine by LC-MS/MS was performed as previously described [25]. In brief, 10 µl of samples were prepared by addition of 90 µl of internal standard solution (MDL 026042, kindly provided by Aventis Pharma Deutschland GmbH, Frankfurt a.M., Germany) to 100 µl of the cell lysates. Analysis was performed using the triple quadrupole sytem API 4000™ LC-MS/MS System (Applied Biosystems, Toronto, ON, Canada). The HPLC column used was a Luna 3u CN 100A, New Column 100 x 2.0 mm with SecurityGuard Cartridges, CN 4 x 2.0 mm purchased from Phenomenex (Aschaffenburg, Germany). A mixture of 12 mM ammonium acetate and acetonitril (50:50, v/v) was used as mobile phase. The flow rate was set at 0.25 ml/min. The lower limit of quantitation was 0.5 ng/ml. The peak area ratio of fexofenadine to the internal standard was calculated using Analyst software v1.4.2 (Applied Biosystems, Darmstadt, Germany). The retention time of fexofenadine and of the internal standard was 2.0 min. The calibration curves were linear over the range from 0.5 to 100 ng/ml with a mean correlation coefficient of 0.9992. The mass spectrometer was operated in the selected ion monitoring mode using the respective MH^+ ions, *m/z* 502.3 for fexofenadine and *m/z* 530.3 for the internal standard. Coefficient of variation (CV) of the interassay variability (n = 15; quality controls containing 2.5, 25 and 100 ng/ml of fexofenadine) ranged between 4.0 and 6.6 %. The CV of the intraassay variability (n = 5) ranged between 3.4 and 7.4 %. The appropriate protein concentration of each well was determined in 25 µl of cells lysates by bichinchoninic acid assay (BCA Protein Assay Kit; Thermo Scientific).

4.6 Data and statistical Analysis

The OATP1A2- and OATP2B1- mediated net uptake was obtained by subtracting the uptake in VC cells from that in OATP1A2- and OATP2B1-expressing cells. The percentage of uptake inhibition was calculated from control experiments in the absence of flavonoids (100% uptake). The corresponding IC_{50} values for inhibition of OATP1A2-mediated fexofenadine uptake were calculated by fitting the data to a sigmoid dose-response regression curve (GraphPad Prism 5.00 for Windows; GraphPad Software, San Diego, CA, USA). The IC_{50} value is the concentration at which 50% inhibition of substrate uptake is obtained. To determine the type of inhibitory interaction of flavonoids with the OATP1A2- and OATP2B1-mediated BSP uptake, the uptake of ^{3}[H]BSP was determined in the presence of increasing concentrations of flavonoids. K_i values were calculated by the method of Dixon, in which the reciprocal velocity, 1/V, is plotted against the concentration of the inhibitor. The OATP1A2-mediated uptake of BSP and atorvastatin was analyzed using an unpaired t-test with Welch's correction. The calculations were performed using GraphPad Prism 5.00. All data are presented as means ± S.E.M. A value of $p \leq 0.05$ was considered statistically significant.

5 Results

5.1 Characterization of a HEK293 cell line stably expressing OATP1A2

In the present study, we established a HEK293 cell line stably expressing the human uptake transporter OATP1A2. The selected cell clones of the HEK293-OATP1A2 and HEK293-VC cells were investigated regarding their expression, localization, and function of OATP1A2 using real-time PCR, immunoblot and immunofluorescence analysis. The HEK293-OATP1A2 cells were characterized by a significantly higher expression of *SLCO1A2* mRNA and OATP1A2 protein compared to the control cells (figure 1a, b). The immunofluorescence analysis revealed that OATP1A2 is localized in the plasma membrane and to a lower extent in the cytosol of HEK293-OATP1A2 cells. No staining was observed in HEK293-VC cells (figure 1c). The functional characterization showed a 2.4-fold ($p < 0.0001$, $df = 25$) and 6.9-fold ($p < 0.0001$, $df = 5$) higher uptake of BSP (5 µM) and atorvastatin (5 µM), respectively, into the HEK293-OATP1A2 cells compared to HEK293-VC cells (figure 1d, e) demonstrating that both compounds are substrates of OATP1A2.

5.2 Influence of flavonoids on the OATP1A2- and OATP2B1-mediated uptake of BSP

In order to investigate the inhibitory effects of apigenin, kaempferol, and quercetin on the transport function of OATP1A2 and OATP2B1, the K_i values of these flavonoids for inhibition of OATP1A2- and OATP2B1-mediated BSP uptake (measured by liquid scintillation counting) were determined. To evaluate the type of interaction of flavonoids with OATP1A2- and OATP2B1-mediated transport of BSP, Dixon plot analyses were performed (figure 2). The lines drawn for each concentration of substrate intersect at a single point above the x-axis indicating that all flavonoids influence both OATP transporters by competitive inhibition. The K_i values of all three flavonoids determined from the Dixon plots were lower for OATP2B1 compared to OATP1A2 (figure 2). Quercetin was the most potent inhibitor of the OATP1A2-mediated BSP uptake followed by kaempferol and apigenin (22.0 ± 5.9 µM, 25.2 ± 13.0 µM, and 32.4 ± 12.2 µM). For OATP2B1 quercetin was also the most potent inhibitor followed by kaempferol and apigenin (8.7 ± 0.1 µM, 15.1 ± 7.8 µM, and 20.8 ± 8.0 µM).

5.3 Influence of flavonoids on the OATP1A2- and OATP2B1-mediated uptake of drugs

To determine whether apigenin, kaempferol, and quercetin alter the OATP1A2-mediated transport of fexofenadine (measured by LC-MS/MS), inhibition studies were performed (figure 3). Because we previously found that fexofenadine is not a substrate of OATP2B1 [9] only the impact of flavonoids on the OATP1A2-mediated uptake of fexofenadine was investigated. Apigenin was the most potent inhibitor of OATP1A2-mediated fexofenadine transport with the lowest IC_{50} value followed by kaempferol and

quercetin (4.3 ± 1.5 µM, 12.0 ± 1.2 µM, and 12.6 ± 1.3 µM). Secondly, the influence of apigenin, kaempferol and quercetin on the OATP1A2- and OATP2B1-mediated atorvastatin transport (measured by liquid scintillation counting) was investigated (figure 4). Apigenin and quercetin were the most effective inhibitors for the OATP1A2- and OATP2B1-mediated atorvastatin uptake. For OATP1A2 the IC_{50} values of apigenin and quercetin were 9.3 ± 1.5 and 13.5 ± 1.7 µM. The IC_{50} values of apigenin and quercetin for OATP2B1 were with 13.9 ± 1.6 µM and 14.1 ± 1.3 µM very similar. Kaempferol showed the lowest inhibitory potency of the OATP1A2- and OATP2B1-mediated atorvastatin transport compared to apigenin and quercetin (IC_{50} for OATP1A2: 37.3 ± 1.6 µM, IC_{50} for OATP2B1: 20.7 ± 1.8 µM).

6 Discussion

The major findings of the present study were that the flavonoids apigenin, kaempferol and quercetin affect the transport function of OATP1A2- and OATP2B1-mediated uptake of BSP and of the drugs fexofenadine and atorvastatin.

Because most of the drugs used in pharmacotherapy are orally administered, the bioavailability of these compounds can already be influenced by intestinal drug transport and metabolism. Drug uptake transporters such as OATP1A2 expressed in the apical membrane of enterocytes [9] seem to influence the intestinal absorption of drugs such as fexofenadine [9-10, 16]. The absorption of fexofenadine, which is not metabolized in humans, is reduced when fexofenadine is coadministered with grapefruit juice due to an inhibition of OATP1A2-mediated fexofenadine uptake by the flavonoid naringin [15]. Vegetables, fruits, and herbal drugs are rich of flavonoids and therefore, we investigated the influence of the flavonoids apigenin, kaempferol, and quercetin on the transport activity of intestinal transporters OATP1A2 and OATP2B1 which are expressed in the apical membrane of enterocytes [8-9].

The amount of apigenin, kaempferol, and quercetin is different within vegetables and herbal drugs. Interestingly, a study indicated that quercetin is more abundant compared to kaempferol in vegetables and fruits such as onions, lettuce, french bean, and apples [26]. High amounts of quercetin were detected in onions and apples with mean values of 347 and 36 mg / kg of fresh edible part, respectively [26]. Kaempferol showed the highest amounts in leek and endive with values of 30 and 46 mg / kg of fresh edible part, respectively. A further analyses of different beverages revealed that quercetin can

be found in red wine (highest concentration measured: 16 mg/l), apple juice, tomato juice, and different tea brands [2]. Taken the high and regular consumption of these vegetables, fruits, and beverages into consideration, it seems comprehensible that the concomitant intake of such foods with drugs such as fexofenadine or atorvastatin may lead to a reduced absorption of these drugs. Therefore, the inhibition of intestinal OATPs by flavonoids could lead, in addition to the modification of cytochrome P450 enzymes and ABC-transporters, to an altered pharmacokinetic and pharmacodynamic of drugs.

The flavonoids apigenin, kaempferol, and quercetin and their glycosides are also the major constituents of herbal drug preparations of Ginkgo biloba. A study investigating different extracts of commercially available Ginkgo biloba preparations showed that in the analyzed extracts approximately 30 % of the active substances were flavone glycosides [27]. Most of these glycosides will be deglycosylated by a β-glucosidase (lactase-phlorizin hydrolase) localized in the apical membrane of enterocytes [28]. This enzyme may increase the access of the deglycosylated apigenin, kaempferol, and quercetin to OATP1A2 and OATP2B1.

A pure preparation of 500 mg quercetin (Quercetin-500 Plus®), available as dietary supplement (http://supplementspot.com), taken with 300 ml of water could lead to a maximal intestinal concentration of 5514 µM. This shows that these supplements could lead to concentrations of quercetin in the intestine high enough to influence the transport activity of OATP1A2 and OATP2B1.

The presented data indicate that the flavonoids apigenin, kaempferol, and quercetin inhibit the OATP1A2- and OATP2B1-mediated uptake of substrates into HEK293 cells by a competitive mechanism. Therefore, these flavonoids could be also substrates of

OATP1A2 and OATP2B1. A study with Caco-2 cells provided some in vitro evidence that OATP2B1 may be involved in the translocation of quercetin from the apical to the basal compartment [29]. Quercetin seems to be a potent inhibitor of the OATP1A2- and OATP2B1-mediated transport represented by the determined K_i values in this study. Interestingly, quercetin is also a potent inhibitor of CYP3A4 and P-glycoprotein [30]. Considering that quercetin is also one of the most abundant flavonoids, foods and herbal preparations containing quercetin may be therefore associated with a high risk for food-drug or drug-drug interactions by influencing the intestinal drug uptake, metabolism, and excretion. Furthermore, the potency of inhibition of OATP1A2 and OATP2B1 by apigenin and quercetin was different for the investigated substrates BSP, fexofenadine and atorvastatin. Quercetin was the most potent inhibitor of the OATP1A2- and OATP2B1-mediated BSP transport, whereas apigenin was the strongest inhibitor of the OATP1A2-mediated atorvastatin and fexofenadine transport. For the OATP2B1-mediated atorvastatin transport no difference in the potency between apigenin and quercetin was observed.

Some in vivo studies indicate an interaction between herbal drugs such as Ginkgo biloba preparations and the transporter-mediated absorption of drugs. A study investigated the influence of Ginkgo biloba extract on the pharmacokinetics of fexofenadine [31]. After an intake of Ginkgo biloba extract (120 mg bid) for two weeks a non significant reduction by 20 % in fexofenadine AUC was observed. However, it is not stated whether fexofenadine (120 mg) and Ginkgo biloba extract (120 mg) were given simultaneously. Therefore, it could be speculated that the lack of significance is caused by a non simultaneous administration of Ginkgo biloba extract and fexofenadine.

Our data are in line with a previous publication showing that Ginkgo leaf extracts and quercetin-3-glucoside, quercetin-3-rutinoside, kaempferol-3-glucoside, and kaempferol-3-rutinoside inhibited the OATP2B1-mediated transport of estrone-3-sulfate in HEK293-cells stably expressing OATP2B1 [18].

In the present study we also established HEK293 cells stably expressing OATP1A2. In previous studies investigating the function of OATP1A2, Xenopus laevis oocytes or Hela cells transiently expressing OATP1A2 were used [10-11]. To the best of our knowledge, this is the first study using HEK293 cells stably expressing OATP1A2. In addition, we were able to show that atorvastatin is a substrate of OATP1A2 which was already published previously [32]. Because atorvastatin is a frequently used drug, the inhibition of OATP1A2-mediated atorvastatin transport by flavonoids should be considered as a possible new mechanism for food-drug or drug-drug interactions. However, atorvastatin is also a substrate of CYP3A4, P-glycoprotein, and OATP2B1. Considering all the possible changes in the pharmacokinetics of atorvastatin due to the modulation of CYP3A4, P-glycoprotein, OATP1A2 and OATP2B1 and also the pharmacogenomics of P-glycoprotein, OATP1A2, and OATP2B1 it becomes obvious that in vivo studies are necessary to clarify the clinical relevance of flavonoid-mediated drug interactions with atorvastatin. An in vivo study investigating the interaction between grapefruit juice and atorvastatin or pravastatin showed an increase in T_{max} of atorvastatin and pravastatin [33]. Grapefruit juice was ingested for two days and on day 3 with the drugs, 0.5 h and 1.5 h after drug intake. It could be speculated that the inhibition of OATP1A2- and OATP2B1-mediated uptake of atorvastatin (OATP1A2 and OATP2B1) and pravastatin (OATP2B1) by grapefruit juice may be responsible for the observed effects.

Taken together, our study shows that the inhibition of OATP1A2- and OATP2B1-mediated transport by apigenin, kaempferol, and quercetin should be considered as a potential, new mechanism for food-drug or drug-drug interactions. The clinical relevance of the observed interactions needs to be elucidated in clinical interactions studies.

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8 References

[1] Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 1993;342:1007-11.

[2] Hertog MGL, Hollman PC, van de Putte B. Content of Potentially Anticarcinogenic Flavonoids of Tea Infusions, Wines, and Fruit Juices. J Agric Food Chem 1993;41:1242-46.

[3] Jagtap S, Meganathan K, Wagh V, Winkler J, Hescheler J, Sachinidis A. Chemoprotective mechanism of the natural compounds, epigallocatechin-3-O-gallate, quercetin and curcumin against cancer and cardiovascular diseases. Curr Med Chem 2009;16:1451-62.

[4] Boots AW, Haenen GR, Bast A. Health effects of quercetin: from antioxidant to nutraceutical. Eur J Pharmacol 2008;585:325-37.

[5] Pal D, Mitra AK. MDR- and CYP3A4-mediated drug-drug interactions. J Neuroimmune Pharmacol 2006;1:323-39.

[6] Alvarez AI, Real R, Perez M, Mendoza G, Prieto JG, Merino G. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. J Pharm Sci 2010;99:598-617.

[7] Pal D, Mitra AK. MDR- and CYP3A4-mediated drug-herbal interactions. Life Sci 2006;78:2131-45.

[8] Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, Tamai I. Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent

transport across intestinal apical membrane. J Pharmacol Exp Ther 2003;306:703-8.

[9] Glaeser H, Bailey DG, Dresser GK, Gregor JC, Schwarz UI, McGrath JS, et al. Intestinal drug transporter expression and the impact of grapefruit juice in humans. Clin Pharmacol Ther 2007;81:362-70.

[10] Cvetkovic M, Leake B, Fromm MF, Wilkinson GR, Kim RB. OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. Drug Metab Dispos 1999;27:866-71.

[11] Kullak-Ublick GA, Hagenbuch B, Stieger B, Schteingart CD, Hofmann AF, Wolkoff AW, et al. Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. Gastroenterology 1995;109:1274-82.

[12] Kopplow K, Letschert K, König J, Walter B, Keppler D. Human hepatobiliary transport of organic anions analyzed by quadruple-transfected cells. Mol Pharmacol 2005;68:1031-8.

[13] Grube M, Kock K, Oswald S, Draber K, Meissner K, Eckel L, et al. Organic anion transporting polypeptide 2B1 is a high-affinity transporter for atorvastatin and is expressed in the human heart. Clin Pharmacol Ther 2006;80:607-20.

[14] Tamai I, Nezu J, Uchino H, Sai Y, Oku A, Shimane M, et al. Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. Biochem Biophys Res Commun 2000;273:251-60.

[15] Bailey DG, Dresser GK, Leake BF, Kim RB. Naringin is a major and selective clinical inhibitor of organic anion-transporting polypeptide 1A2 (OATP1A2) in grapefruit juice. Clin Pharmacol Ther 2007;81:495-502.

[16] Dresser GK, Bailey DG, Leake BF, Schwarz UI, Dawson PA, Freeman DJ, et al. Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to decrease the oral availability of fexofenadine. Clin Pharmacol Ther 2002;71:11-20.

[17] Satoh H, Yamashita F, Tsujimoto M, Murakami H, Koyabu N, Ohtani H, et al. Citrus juices inhibit the function of human organic anion-transporting polypeptide OATP-B. Drug Metab Dispos 2005;33:518-23.

[18] Fuchikami H, Satoh H, Tsujimoto M, Ohdo S, Ohtani H, Sawada Y. Effects of herbal extracts on the function of human organic anion-transporting polypeptide OATP-B. Drug Metab Dispos 2006;34:577-82.

[19] Glaeser H, Mandery K, Sticht H, Fromm MF, König J. Relevance of conserved lysine and arginine residues in transmembrane helices for the transport activity of organic anion transporting polypeptide 1B3. Br J Pharmacol 2010;159:698-708.

[20] Mandery K, Bujok K, Schmidt I, Wex T, Treiber G, Malfertheiner P, et al. Influence of cyclooxygenase inhibitors on the function of the prostaglandin transporter organic anion-transporting polypeptide 2A1 expressed in human gastroduodenal mucosa. J Pharmacol Exp Ther 2010;332:345-51.

[21] Bachmakov I, Glaeser H, Fromm MF, König J. Interaction of oral antidiabetic drugs with hepatic uptake transporters: focus on organic anion transporting polypeptides and organic cation transporter 1. Diabetes 2008;57:1463-9.

[22] Kraft M, Glaeser H, Mandery K, König J, Auge D, Fromm MF, et al. The prostaglandin transporter OATP2A1 is expressed in human ocular tissues and transports the antiglaucoma prostanoid latanoprost. Invest Ophthalmol Vis Sci 2009.

[23] Cui Y, König J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 1999;55:929-37.

[24] Dixon M. The graphical determination of K m and K i. Biochem J 1972;129:197-202.

[25] Hofmann U, Seiler M, Drescher S, Fromm MF. Determination of fexofenadine in human plasma and urine by liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2002;766:227-33.

[26] Hertog MGL, Hollman PC, Katan MB. Content of Potentially Anticarcinogenic Flavonoids of 28 Vegetables and 9 Fruits Commonly Consumed in The Netherlands. J Agric Food Chem 1992;40:2379-83.

[27] Kressmann S, Muller WE, Blume HH. Pharmaceutical quality of different Ginkgo biloba brands. J Pharm Pharmacol 2002;54:661-9.

[28] Nemeth K, Plumb GW, Berrin JG, Juge N, Jacob R, Naim HY, et al. Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur J Nutr 2003;42:29-42.

[29] Nait Chabane M, Al Ahmad A, Peluso J, Muller CD, Ubeaud G. Quercetin and naringenin transport across human intestinal Caco-2 cells. J Pharm Pharmacol 2009;61:1473-83.

[30] Patel J, Buddha B, Dey S, Pal D, Mitra AK. In vitro interaction of the HIV protease inhibitor ritonavir with herbal constituents: changes in P-gp and CYP3A4 activity. Am J Ther 2004;11:262-77.

[31] Robertson SM, Davey RT, Voell J, Formentini E, Alfaro RM, Penzak SR. Effect of Ginkgo biloba extract on lopinavir, midazolam and fexofenadine pharmacokinetics in healthy subjects. Curr Med Res Opin 2008;24:591-9.

[32] Knauer MJ, Urquhart BL, Meyer Zu Schwabedissen HE, Schwarz UI, Lemke CJ, Leake BF, et al. Human skeletal muscle drug transporters determine local exposure and toxicity of statins. Circ Res 2010;106:297-306.

[33] Lilja JJ, Kivistö KT, Neuvonen PJ. Grapefruit juice increases serum concentrations of atorvastatin and has no effect on pravastatin. Clin Pharmacol Ther 1999;66:118-27.

[34] Lee W, Glaeser H, Smith LH, Roberts RL, Moeckel GW, Gervasini G, et al. Polymorphisms in human organic anion-transporting polypeptide 1A2 (OATP1A2): implications for altered drug disposition and central nervous system drug entry. J Biol Chem 2005;280:9610-7.

9 Web references

(http://supplementspot.com) last date accessed: 20 May 2010

10 Figure captions

Figure 1: Characterization of the HEK293-OATP1A2 cell line overexpressing OATP1A2. a) Elevated expression of *SLCO1A2* mRNA in HEK293 cells stably expressing OATP1A2 compared with HEK293 cells transfected with the empty vector. b) Immunoblot of OATP1A2 in HEK293-OATP1A2, HEK293-VC, and HEK293 parental cells. At the molecular masses of approximately 60 and 80 kDa, specific signals were detected in HEK293-OATP1A2 cells, which were not detectable in HEK293-VC and HEK293 parental cells. The lower band represents a deglycosylated form of OATP1A2 [34] c) Immunofluorescence analysis of HEK293-OATP1A2 cells (left) and HEK293-VC cells (right) using confocal laser scanning microscopy. OATP1A2 was visualized using a Cy3-conjugated antibody (red fluorescence). The localization of OATP1A2 is shown in the x-y, x-z, and y-z layers. OATP1A2 was localized in the plasma membrane and cytosol of the HEK293-OATP1A2 cells, whereas no staining was detectable in the HEK293-VC cells. The nuclei were stained green (original magnification, 400x with 4-fold zoom). d) Uptake of BSP (5 µM) into HEK293-OATP1A2 and HEK293-VC cells. The HEK293-OATP1A2 cells showed a significantly higher uptake (2.4-fold) of BSP (measured by liquid scintillation counting) compared to HEK293-VC cells (*** $p < 0.0001$, unpaired t-test with Welch's correction). e) Uptake of atorvastatin (5 µM) into HEK293-OATP1A2 and HEK293-VC cells. The HEK293-OATP1A2 cells showed a significantly higher uptake (6.9-fold) of atorvastatin (measured by liquid scintillation counting) compared to HEK293-VC cells (*** $p < 0.0001$, unpaired t-test with Welch's correction). All data are presented as means ± S.E.M.

Figure 2: Inhibition of OATP1A2- and OATP2B1-mediated BSP transport (measured by liquid scintillation counting) by flavonoids. Dixon plots of inhibition of OATP1A2- (a, b, c) and OATP2B1- (d, e, f) mediated uptake of BSP by apigenin (a, d), kaempferol (b, e) and quercetin (c, f) in HEK293 cells stably expressing OATP1A2 and OATP2B1, respectively. BSP transport (5 min for OATP1A2, 10 min for OATP2B1) at three different concentrations was determined in the absence or presence of increasing concentrations of flavonoids. The reciprocal velocity is plotted against the inhibitor concentration. Data points represent the mean ± S.E.M. □, 2.5 µM BSP; ∆, 5 µM BSP; ○, 10 µM BSP; ■, 0.5 µM BSP; ▲, 1 µM BSP; ●, 5 µM BSP.

Figure 3: Inhibition of OATP1A2-mediated fexofenadine transport (measured by LC-MS/MS) by flavonoids. Concentration-dependent effects of apigenin (a), kaempferol (b), and quercetin (c) on OATP1A2-mediated fexofenadine (5 µM) uptake (5 min). The data are expressed as percentage of control (uptake without flavonoids). All data are presented as means ± S.E.M.

Figure 4: Inhibition of OATP1A2- and OATP2B1-mediated atorvastatin (5 µM and 0.5 µM, respectively) transport (10 min, measured by liquid scintillation counting) by flavonoids. Concentration-dependent effects of apigenin (a, d), kaempferol (b, e), and quercetin (c, f) on OATP1A2- and OATP2B1-mediated atorvastatin uptake. The data are expressed as percentage of control (uptake without flavonoids). All data are presented as means ± S.E.M.

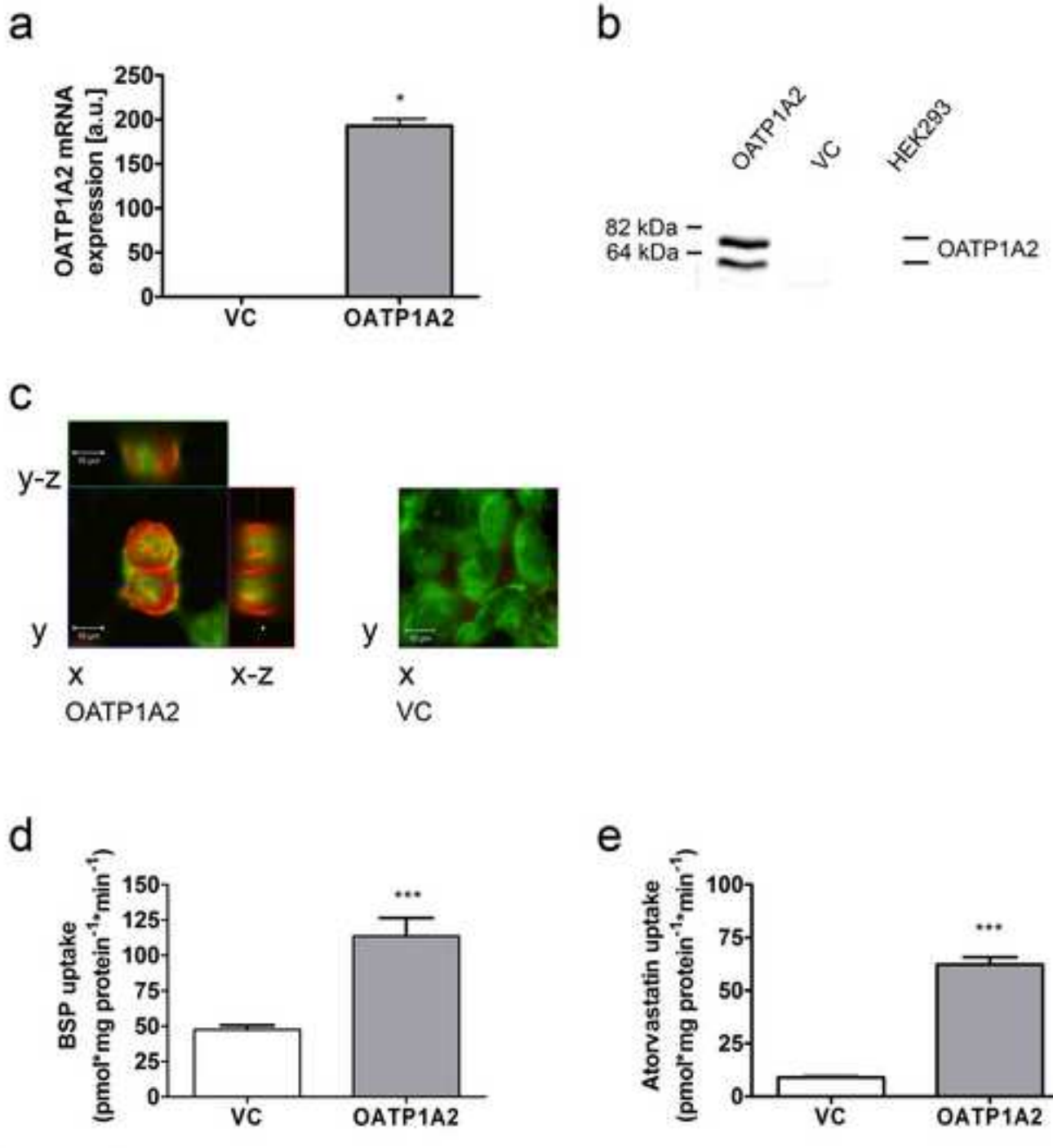

figure 1

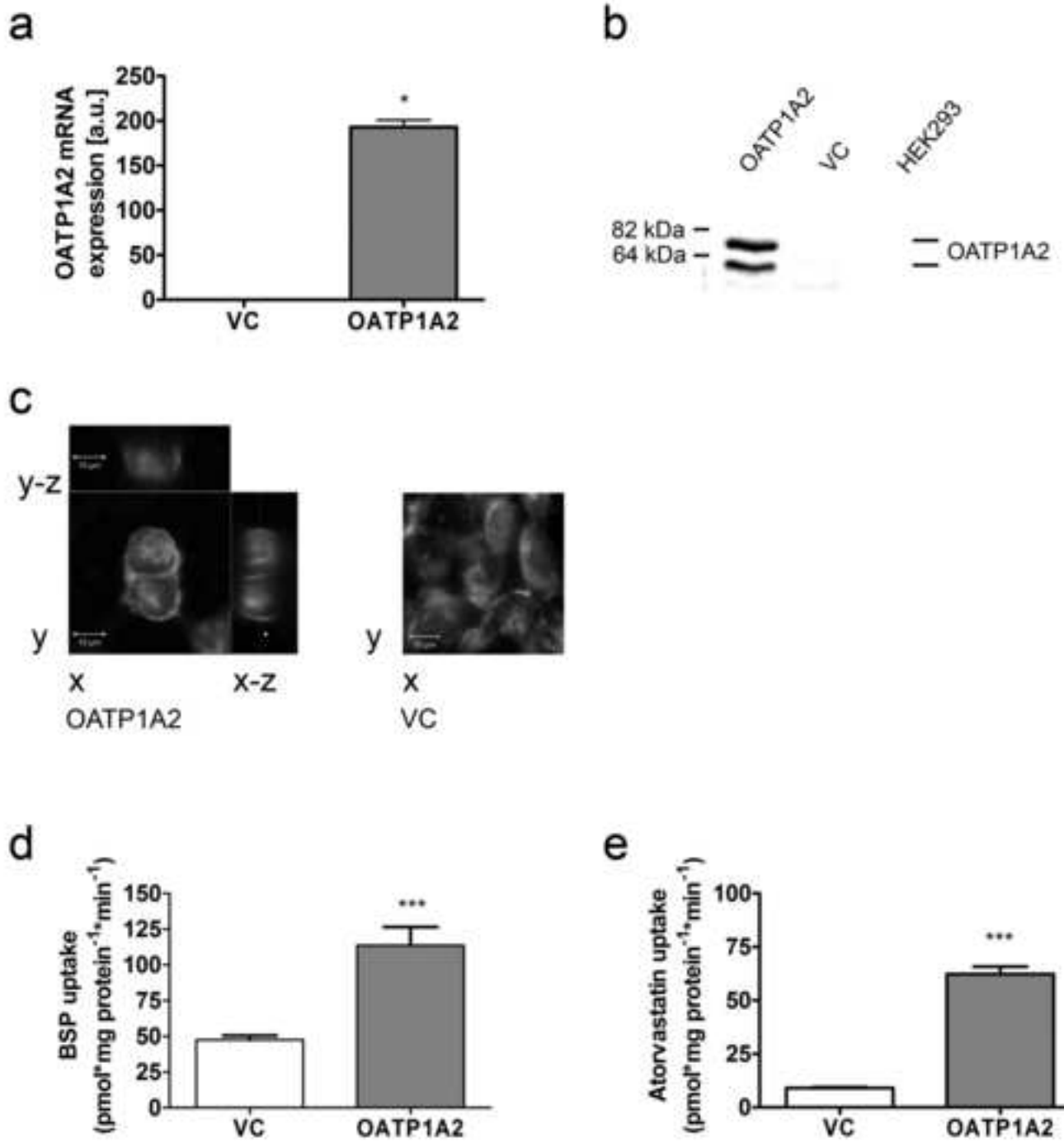

figure 1

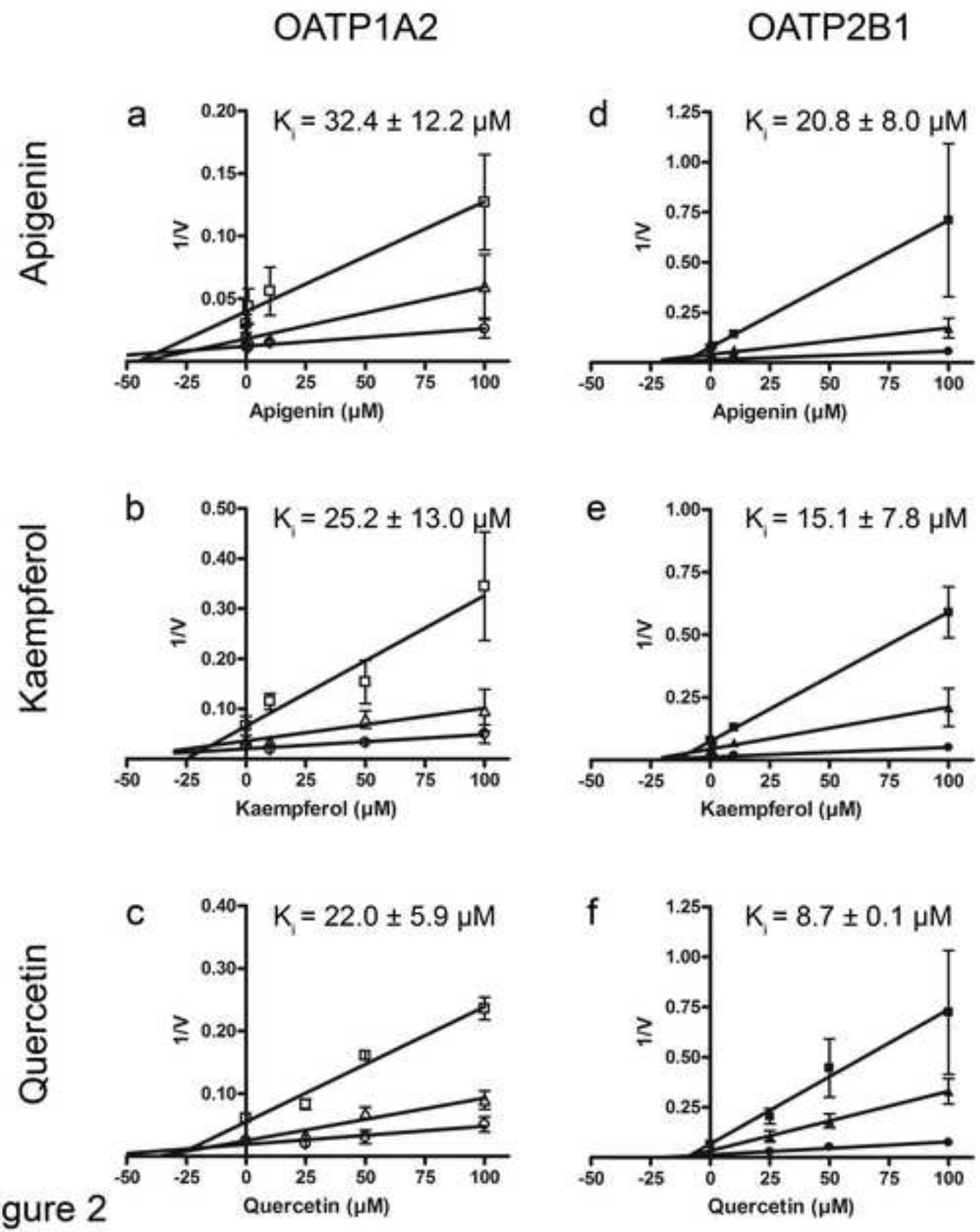

figure 2

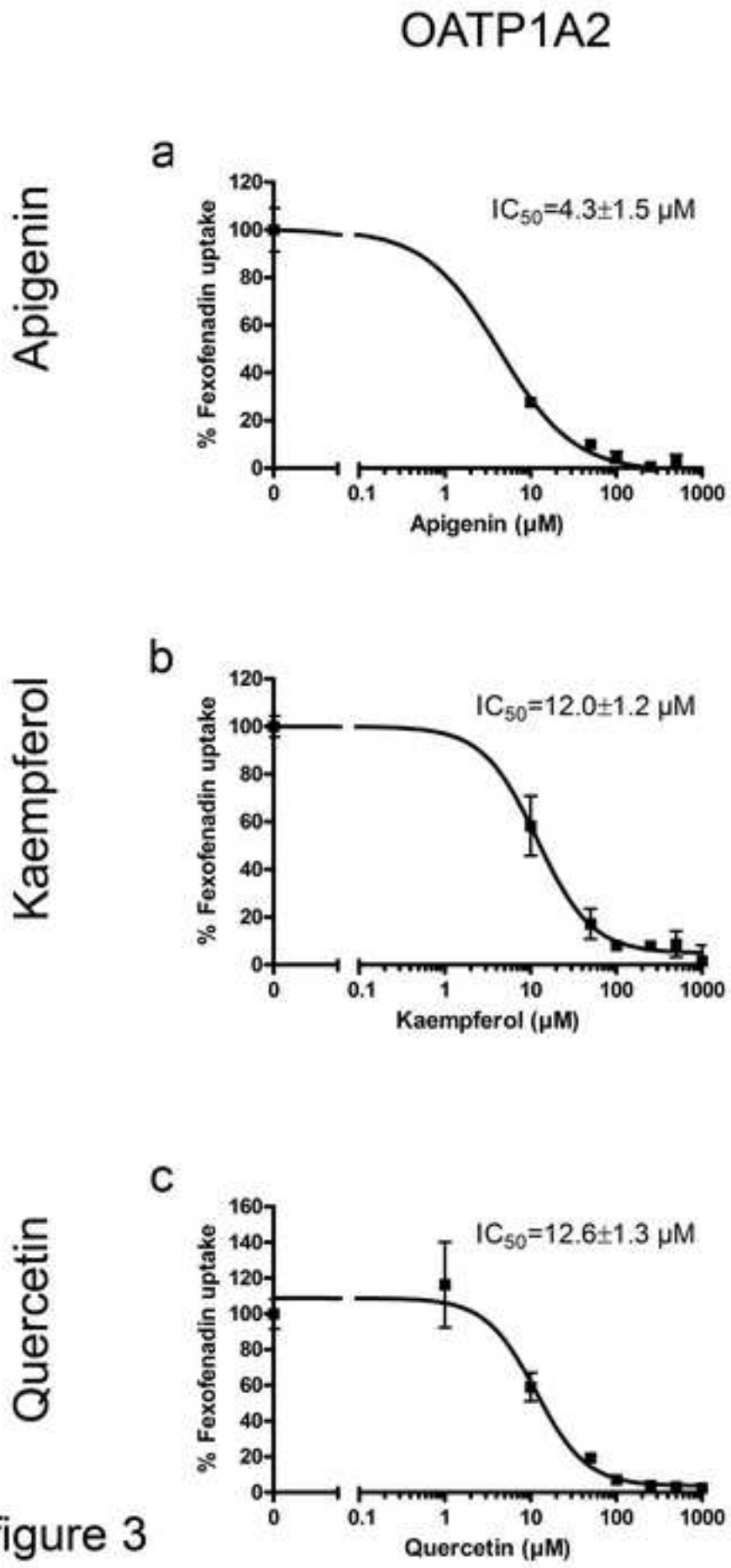

figure 3

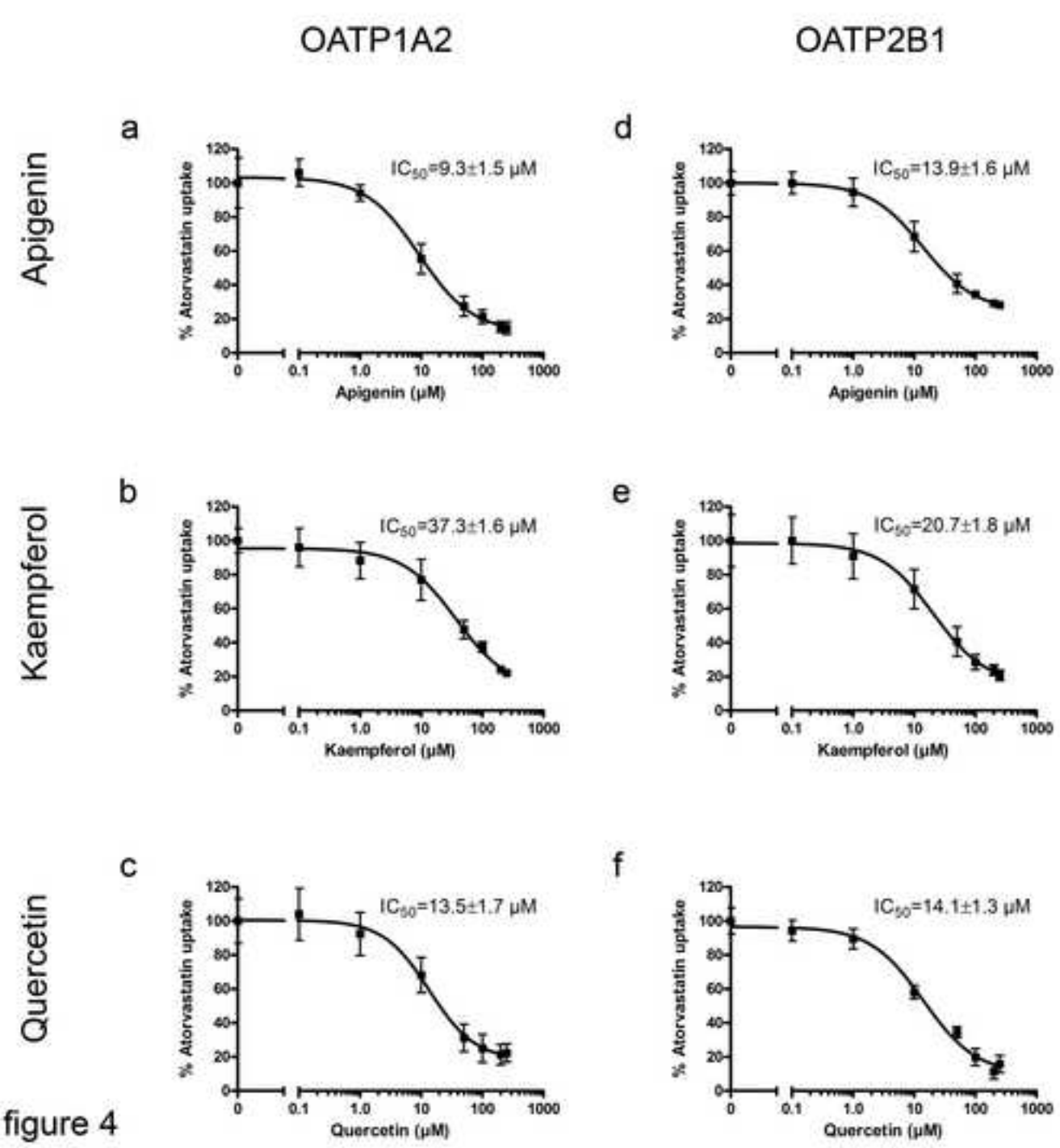
OATP1A2
OATP2B1
Apigenin
Kaempferol
Quercetin
a
b
c
d
e
f
IC50=9.3±1.5 μM
IC50=37.3±1.6 μM
IC50=13.5±1.7 μM
IC50=13.9±1.6 μM
IC50=20.7±1.8 μM
IC50=14.1±1.3 μM
% Atorvastatin uptake
Apigenin (μM)
Kaempferol (μM)
Quercetin (μM)
0
20
40
60
80
100
120
0.1
1.0
10
100
1000

figure 4

*Graphical Abstract