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Transport by OATP1B1 and OATP1B3 Enhances the Cytotoxicity of Epigallocatechin 3-O-gallate and Several Quercetin Derivatives[⊥]

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Abstract

Organic anion transporting polypeptide (OATP) 1B1 and 1B3 are transporters that are expressed selectively in human hepatocytes under normal conditions. OATP1B3 is also expressed in certain cancers. Flavonoids such as green tea catechins and quercetin glycosides have been shown to modulate the function of some OATPs. In the present study, the extent to which six substituted quercetin derivatives (**1** – **6**) affected the function of OATP1B1 and OATP1B3 was investigated. Uptake of the radiolabeled model substrates estradiol 17 β -glucuronide, estrone 3-sulfate and dehydroepiandrosterone sulfate (DHEAS) was determined in the absence and presence of compounds **1** – **6** using Chinese hamster ovary (CHO) cells stably expressing either OATP1B1 or OATP1B3. Several of compounds **1** – **6** inhibited OATP-mediated uptake of all three model substrates suggesting that they could also be potential substrates. Compound **6** stimulated OATP1B3-mediated estradiol 17 β -glucuronide uptake by increasing the apparent affinity of OATP1B3 for its substrate. Cytotoxicity assays demonstrated that epigallocatechin 3-O-gallate (EGCG) and most of compounds **1** – **6** killed preferentially OATP-expressing CHO cells. EGCG, **1** and **3** were the most potent cytotoxic compounds, with EGCG and **3** selectively killing OATP1B3 expressing cells. Given that OATP1B3 is expressed in several cancers, EGCG and some of the quercetin derivatives studied might be promising lead compounds for the development of novel anticancer drugs.

Organic anion transporting polypeptides (OATPs) are classified within the *SLCO* superfamily of transporters.¹ The functionally characterized members are multispecific sodium-independent transporters that mediate the uptake of a variety of endo- and xenobiotics including numerous drugs and anticancer agents.^{2, 3} Among the eleven human OATPs, OATP1B1 and OATP1B3 are considered to be liver-specific transporters expressed exclusively in hepatocytes under normal physiological conditions.⁴ However, OATP1B3 has also been detected in several cancers,^{5–7} where it might be involved in the uptake of hormones or growth factors,^{8, 9} or where it potentially could be used to target chemotherapeutic drugs to the cancer.³

[⊥]Dedicated to Dr. Lester A. Mitscher, of the University of Kansas, for his pioneering work on the discovery of bioactive natural products and their derivatives.

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Flavonoids are polyphenolic compounds present in plants and numerous dietary supplements and have been linked to cancer chemoprevention among other potential medicinal benefits.¹⁰ Flavonoids like quercetin and green tea catechins have been shown to have anticancer activity in prostate cancer,¹¹ esophageal squamous cell carcinoma,¹² hepatocellular carcinoma,¹³ pancreatic carcinoma,¹⁴ as well as bladder tumor cell lines.¹⁵ Recently we demonstrated that the green tea catechins, epicatechin 3-O-gallate (ECG) and epigallocatechin 3-O-gallate (EGCG) interact with and are substrates of OATPs.¹⁶ Quercetin and its analogues are structurally similar to the green tea catechins and have recently been shown to have antiviral activity.¹⁷ Furthermore, quercetin has been shown to inhibit OATP1B1-mediated transport.¹⁸ Therefore, the aim of the present study was to investigate to what extent these quercetin derivatives would interact with OATP1B1 and OATP1B3.

RESULTS AND DISCUSSION

Effect of Substituted Quercetin Derivatives on OATP-mediated Uptake

Previous data from our laboratory have demonstrated that, among other green tea catechins, EGCG can modulate uptake of OATP1B3 in a substrate-dependent way.¹⁶ Since the differentially substituted quercetin derivatives used herein have similar structures to EGCG (Figure 1), it was considered of interest to test whether these substances would also modulate OATP1B1- and OATP1B3-mediated transport. Estrone 3-sulfate (E3S), estradiol 17 β -glucuronide (E17 β G), and dehydroepiandrosterone sulfate (DHEAS) are three model substrates that have been used in past studies to characterize OATP1B1- and OATP1B3-mediated uptake.¹ In previous work, it was shown that OATP1B3-mediated E3S uptake is stimulated by EGCG,¹⁶ OATP1B3-mediated E17 β G uptake is stimulated by clotrimazole,¹⁹ and OATP1B1-mediated uptake of DHEAS is stimulated by rutin.²⁰ Therefore, the uptake of these three substrates (E3S and E17 β G at 0.1 μ M; DHEAS at 0.5 μ M) was determined in the absence or presence of 50 μ M of compounds **1** – **6**. As can be seen in Figure 2A – 2C, substrate dependent modulation of OATP1B1 and OATP1B3 was seen, with compounds **1**, **3** and **4** found to be strong inhibitors for both OATP1B1- and OATP1B3-mediated uptake while **5** had only an effect on DHEAS. Hence, attachment of the gallate moiety at C-3 or C-3' (**1** or **4**) and a 4-amino-3-hydroxybenzoate at C-3 (**3**) resulted in inhibition, while introduction of 3-aminopropoxy group at C-3' (**5**) did not facilitate a strong inhibitory effect. Interestingly, although **6**, possessing a propoxy group at C-5, inhibited OATP1B1-mediated uptake of all three substrates and OATP1B3-mediated uptake of DHEAS (Figure 2), it stimulated OATP1B3-mediated uptake of E17 β G up to three-fold compared to the control (Figure 2B). Compound **2** also slightly stimulated OATP1B3-mediated uptake of DHEAS (Figure 2C). These results suggest that although quercetin derivatives examined have similar structures, they modulate OATP1B1 and OATP1B3 in different ways and interact at different sites of the protein compared to the known stimulators, EGCG, clotrimazole, and rutin. Since compound **6** resulted in the largest stimulation, it was further characterized and the kinetic parameters of OATP1B3-mediated E17 β G were determined in the absence and presence of two concentrations of the compound **6**. As shown in Figure 3, the $V_{\max}$ value increased by about 1.5 fold from 103 ± 9 to 152 ± 8 pmol/mg protein•min in the presence of 100 μ M **6** while the apparent K_m value decreased by about four-fold from 12.2 ± 3.7 μ M to 2.8 ± 0.8 μ M. At 50 μ M **6**, the $V_{\max}$ was not affected (116 ± 11 pmol/mg protein•min) but the K_m value was decreased to the same degree as in the presence of 100 μ M **6** (2.8 ± 1.4 μ M). These results suggest that **6** affects the substrate affinity similar to what has been observed for clotrimazole.¹⁹ However, these results were different to those observed previously with EGCG and quercetin 3-O- α -L-arabinopyranosyl (1 $\rightarrow$ 2) α -L-rhamnopyranoside, which both stimulated uptake of E3S but not E17 β G by decreasing both the K_m and the $V_{\max}$ values.^{16,21} An explanation for these

different effects is that OATP modulation is substrate dependent. Thus, allosteric stimulators of OATP1B3-mediated uptake of E17 β G affect the substrate affinity and to a lesser extent the maximal transport rate, while allosteric stimulators of E3S uptake increase the substrate affinity and decrease the maximal transport rate. A possible mechanistic explanation for these different effects is that in the case of E17 β G, the off rate of the substrate is not affected. However, in the case of E3S, the increase in apparent affinity leads to a stronger binding of E3S to the transporter with a decreased off rate, ultimately leading to a slower overall transport. Additional experiments are required to further investigate these effects at the molecular level.

Effect of EGCG and Quercetin Derivatives on the Cell Viability of OATP-expressing Cells

Flavonoids such as EGCG, have been shown to be antiproliferative and to inhibit the growth of certain cancer cell lines.^{11–15} Based on previous results that demonstrated that EGCG modulates and is transported by OATP1B3,¹⁶ combined with the presented findings that the structurally similar quercetin compounds interact with OATP1B3, it was tested whether the expression of OATP1B1 and/or OATP1B3 would affect EGCG- and quercetin analogue-mediated cytotoxicity in Chinese hamster ovary (CHO) cells. As shown in Figure 4, EGCG killed wild-type CHO cells at relatively high concentrations ($IC_{50} = 271 \pm 1 \mu M$). However, with the expression of OATP1B1 or OATP1B3 the cytotoxicity of EGCG increased by 35- or 85-fold, respectively (Table 1). In order to preserve the normal cell culture conditions, the medium was supplemented with 10% FBS during the whole 48 h incubation period. Using the reported average affinity constants for flavonoids and assuming a similar binding affinity to bovine serum albumin as to human serum albumin,²² it was estimated that similar as in the *in vivo* situation, in the experiments at a concentration of $1 \mu M$ about 99% of EGCG and the quercetin derivatives were protein bound and at a concentration of $10 \mu M$ this decreased to about 85%. Thus, given that after a single dose of 1600 mg of EGCG in healthy volunteers a peak plasma concentration between 5.8 and $11.3 \mu M$ was reached,²³ the IC_{50} values of less than $10 \mu M$ are well within this range and it is conceivable that OATP-expressing cancer cells could potentially be targeted using high-dose EGCG supplements. However, the feasibility of such a treatment will have to be confirmed using established cancer cell lines or primary tumor cells expressing OATP1B1 or OATP1B3. Because hepatocytes, which express these OATPs, are not rapidly dividing cells, they should be less sensitive to compounds that target rapidly dividing cancer cells. Similarly, as summarized in Figure 4, all of the quercetin derivatives showed toxicity to OATP-expressing cells but to different extents and in three different patterns. These were: (1) Compound **6** was toxic to all three cell lines. However, toxicity was increased when OATP1B1 or OATP1B3 were expressed; (2) Compounds **1** and **4** exhibited little to no toxicity to wild-type CHO cells but were toxic to OATP1B1- and OATP1B3-expressing cells, respectively; and (3) Compounds **2**, **3**, and **5** were preferentially toxic to OATP1B3-expressing cells but had little to no effect on wild-type and OATP1B1-expressing cells. With respect to toxicity, compounds **1** and **3** are the most potent compounds with IC_{50} values below $5 \mu M$, as summarized in Table 1 (**1**: IC_{50} for OATP1B1: $3.5 \pm 1.4 \mu M$; for OATP1B3: $3.8 \pm 1.3 \mu M$; **3**: IC_{50} for OATP1B3: $4.1 \pm 1.3 \mu M$). Since toxicity increased in all cases when an OATP was expressed it was concluded that these quercetin derivatives are substrates of OATP1B1 and/or OATP1B3. Compounds **2**, **3**, **5** and **6** seem to be selective substrates of OATP1B3, while **1** and **4** appear to be equally good substrates for both transporters. Based on the above mentioned assumptions for protein binding these IC_{50} values suggest that the compounds are very efficiently taken up by OATP1B1 and OATP1B3 and/or are very toxic once inside cells.

To further investigate whether EGCG and the quercetin derivatives would kill cells by apoptosis, flow cytometry analysis was performed with EGCG and the two most potent analogues, **1** and **3**. Notably, both these compounds contain a 3,4,5-trihydroxy- or a 4-

amino-3-hydroxybenzoyl function at C-3, implying that further modification at C-3 of quercetin may enhance cytotoxicity. Since OATP1B3 has been shown to be expressed in several different tumors, this transporter was focused on for the rest of the study. Figure 5 shows that EGCG and the quercetin compounds kill cells by inducing apoptosis. EGCG increased the apoptotic cells from 9 to 15%, **1** increased them from 10 to 32% and **3** from 12 to 70%. These experiments were performed at 10 μ M for compounds **1** and **3** but at 1 μ M for EGCG because at 10 μ M EGCG cell killing was too complete and flow cytometry did not yield reproducible numbers. After synchronizing the cells into the G0 phase, it was analyzed whether EGCG and the quercetin derivatives selected would arrest the cells in a certain cell cycle. No cell cycle arrest was observed for compounds **1** and **3** but for EGCG the cells were arrested in G0/G1 phase (Supplemental Figure 1). This is consistent with a previous report showing that incubation of the NBT-II bladder cancer cell line with EGCG resulted in G0/G1 arrest¹⁵ and suggests that NBT-II bladder cancer cells potentially express a transporter for EGCG given that they could be killed with 10 μ M EGCG while CHO cells were not (Figure 4). Messenger RNA for OATP6A1, one of the OATP members that has so far not yet been functionally characterized, was detected in bladder cancers²⁴ and therefore might be a candidate mediating uptake of EGCG in these cells. However, further experiments are needed to prove that EGCG and the quercetin compounds could be lead compounds to develop cytotoxic compounds that would kill OATP-expressing cancer cells.

In conclusion, the present results demonstrate that quercetin derivatives are able to modulate OATP-mediated substrate transport and in particular that compound **6** can allosterically stimulate OATP1B3-mediated E17 β G transport. Furthermore, the results also indicate that EGCG and certain quercetin derivatives are cytotoxic to OATP1B3-expressing cells and might be valuable lead compounds for the development of analogues to eventually improve chemotherapy of OATP-expressing cancers.

EXPERIMENTAL SECTION

General Experimental Procedures

Radiolabeled [³H]estrone 3-sulfate (45.6 Ci/mmol), [³H]estradiol 17 β -glucuronide (34.3 Ci/mmol) and [³H]dehydroepiandrosterone sulfate (79.5 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). The synthesis of the substituted quercetins used in the present study was recently described¹⁷ and the purity of the compounds determined by HPLC analysis is >95%. The structures are shown in Figure 1¹⁷ and their nomenclatures are: **1**: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl 2,5-dihydroxybenzoate; **2**: 5,7-dihydroxy-2-(3,4-dihydroxyphenyl)-4-oxo-4*H*-chromen-3-yl 4-hydroxy-3-aminobenzoate; **3**: 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl 4-amino-3-hydroxybenzoate; **4**: 2-hydroxy-5-(3,5,7-trihydroxy-4-oxo-4*H*-chromen-2-yl)phenyl 3,4,5-trihydroxybenzoate; **5**: 3,5,7-(trihydroxy)-2-[4-hydroxy-3-(3-aminopropoxy)phenyl]-4-oxo-4*H*-chromene; and **6**: 3,7-(dihydroxy)-2-(3,4-dihydroxyphenyl)-5-propyloxy-4-oxo-4*H*-chromene. The CellTiter-Glo® assay kit was purchased from Promega (Madison, WI, USA), the FITC Annexin-V Apoptosis Detection Kit I from BD Biosciences (San Jose, CA, USA), Dulbecco's modified Eagle's medium from Caisson Laboratories (North Logan, UT, USA), and Fetal bovine serum (FBS) from HyClone (Logan, Utah, USA). All other materials were purchased from Sigma-Aldrich or Invitrogen.

Cell Culture

Chinese hamster ovary (CHO) cells stably transfected with OATP1B1*1b or OATP1B3 haplotype 1 were described previously,¹⁹ and were grown at 37 °C in a humidified 5% CO₂ atmosphere in Dulbecco's Modified Eagle Medium, containing 1 g/L D-glucose, 2 mM L-

glutamine, 25 mM HEPES and 110 mg/L sodium pyruvate, supplemented with 10% FBS, 50 µg/mL L-proline, 100 U/mL penicillin and 100 µg/mL streptomycin. For OATP expressing cell lines, 50 µg/mL geneticin was added. Cells were passaged twice a week and used up to passage 65.

Transport Assays

Substrate uptake with wild-type and OATP-expressing CHO cells was performed as previously described 24-well plates.¹⁹ Uptake of estradiol 17β-glucuronide (E17βG) and estrone 3-sulfate (E3S) was determined at 20 sec while uptake of dehydroepiandrosterone sulfate (DHEAS) was measured at 5 min.

Cell Viability Assays

Wild-type and OATP-expressing CHO cells were seeded in 384-well plates at 1600 cells per well. After 24 h in culture, medium was replaced with medium containing 5 mM sodium butyrate to induce non-specific gene expression¹⁹ and another 24 h later, medium was replaced with medium containing different concentrations of the test compounds. After 48 h of incubation, CellTiter-Glo® reagent was added and fluorescence was determined using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Flow Cytometry Analysis

CHO wild-type cells and CHO cells expressing OATPs were cultured in 6-well plates at 90,000 cells per well for 24 h and then cells were induced with 5 mM sodium-butyrate. After another 24 h incubation period the cells were treated with the different test compounds, and, 48 h later, flow cytometry analysis was performed. For cell cycle analysis, cells were plated in 6-well plates at 90,000 cells per well for 24 h and then synchronized into the G0 phase by cultivating them for 36 h in serum-free medium. After this synchronization, gene expression was induced with 5 mM sodium-butyrate for 24 h and then the cells were treated with the different test compounds for 48 h, before cell cycle analysis was performed. To analyze cells by flow cytometry, cell culture medium was collected; cells were washed twice with phosphate buffered saline (PBS) and then trypsinized. All washes were combined with the medium and the trypsinized cells. For analysis of cell death, cells were centrifuged for 5 min at 300 × g and washed by PBS. Binding buffer from the Apoptosis Detection Kit I with 4 µg/mL propidium iodide and FITC Annexin V was added to the tubes and cells were resuspended. After a 20 min incubation in the dark, cells were analyzed using a BD LSR II flow cytometer. For cell cycle analysis, cells were fixed with 70% iced-cold ethanol, and stored at 4 °C overnight. Before analysis, cells were suspended in PI Staining Buffer (25 µg/mL RNase and 12.5 µg/mL propidium iodide in PBS), and incubated for 40 min.

Statistical Analysis

All results are expressed as means ± S.E.M. Data were analyzed for statistically significant differences using one-way ANOVA followed by the Dunnett post-test, and *p* values less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of quercetin derivatives used in this study.

Figure 2.

Effect of quercetin derivatives on OATP-mediated estrone-3-sulfate (E3S), estradiol-17 β -glucuronide (E17 β G) and dehydroepiandrosterone sulfate (DHEAS) uptake. Uptake of 0.1 μ M E3S (A), 0.1 μ M E17 β G (B) or 0.5 μ M DHEAS (C) was determined at 37 °C for 20 sec (5 min for DHEAS) in the absence or presence of 50 μ M of EGCG (E), rutin (R), or any of the quercetin derivatives. As control (C), 1% DMSO was used. OATP-mediated uptake was calculated by subtracting uptake into wild-type CHO cells. Means $\pm$ S.E.M. of one representative of three independent experiments are given as percent of DMSO control. Asterisks represent values that are significantly different from the DMSO control ($p < 0.05$).

Figure 3.

Effect of compound **6** on the kinetics of OATP1B3-mediated uptake of estradiol 17β-glucuronide. CHO wild-type and OATP1B3-expressing CHO cells were incubated with increasing concentrations of estradiol 17β-glucuronide (E17βG) for 20 sec in the absence (filled circles) and presence of **6** at 50 μM (filled squares) or 100 μM (filled triangles). After uptake into wild-type cells was subtracted net-OATP1B3-mediated uptake was fitted to the Michaelis-Menten equation to calculate K_m and V_{max} values.

Figure 4.

Cytotoxicity of EGCG and quercetin derivatives on OATP expressing cell lines. CHO wild-type (circles) and OATP1B1- (squares) or OATP1B3-expressing CHO cells (triangles) were treated with increasing concentrations of EGCG or quercetin derivatives in cell culture medium for 48 h. Cytotoxicity was then determined using the CellTiter-Glo® assay, and results are expressed as a percent of DMSO control; each value is the mean $\pm$ S.E.M. of at least three independent experiments.

Figure 5. EGCG, compound **1**, and compound **3** induce apoptosis in OATP1B3-expressing CHO cells. Wild-type and OATP1B3-expressing CHO cells were incubated with DMSO (control), 1 μ M EGCG or 10 μ M of compound **1** or **3** for 48 h before flow cytometric analysis was performed. Figures show one representative experiment of two (for EGCG) or three (for compounds **1** and **3**) independent experiments. Values are the average of the individual experiments.

Table 1Summary of IC₅₀ Values (in μ M) for Cytotoxicity by EGCG- and Compounds 1 – 6

Compound	wild-type cells	OATP1B1 -expressing cells	OATP1 B3-expressing cells
EGCG	271 $\pm$ 1	7.7 $\pm$ 1.3	3.2 $\pm$ 1.3
1	NC ^a	3.5 $\pm$ 1.4	3.8 $\pm$ 1.3
2	NC	NC	NC
3	NC	NC	4.1 $\pm$ 1.0
4	NC	7.9 $\pm$ 1.5	6.8 $\pm$ 1.0
5	NC	NC	NC
6	111 $\pm$ 4	42.6 $\pm$ 1.4	11.8 $\pm$ 1.4

^aNC, not calculated