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Gly45 and Phe555 in Transmembrane Domains 1 and 10 are Critical for the Activation of OATP1B3 by Epigallocatechin Gallate

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Abstract

Organic anion transporting polypeptides (OATPs) 1B1 and 1B3 are two highly homologous transporters expressed in the human liver. However, epigallocatechin gallate (EGCG) which is the most predominant catechin in green tea has opposite effects on the function of OATP1B1 and 1B3. In the present study, the critical structural domains and amino acid residues for the activation of OATP1B3 by EGCG have been determined by characterizing the function of a series of OATP1B3-derived chimeric transporters, site-directed mutagenesis, and kinetic studies. Our results showed that G45 and F555 in transmembrane domains (TMs) 1 and 10 are the most important amino acid residues for OATP1B3's activation. Kinetic studies showed that the activation of OATP1B3 by EGCG at low substrate concentration was due to its increased substrate binding affinity. However, EGCG caused increased K_m and decreased V_{max} for 1B3-G45A and 1B3-F555H. The flexibility at position 45 and aromaticity at position 555 might be important for OATP1B3's activation. While 1B3-G45A and 1B3-F555H could not be activated by EGCG, their transport activity for EGCG was comparable to that of wild-type OATP1B3. In conclusion, the present study elucidated the molecular mechanism for OATP1B3's activation by EGCG.

Keywords

OATP; epigallocatechin gallate; transporter; transmembrane domain; activation; chimera

INTRODUCTION

Organic anion transporting polypeptides (OATPs), which belong to the superfamily of solute carriers (*SLC*),[1] are important membrane transport proteins that mediate sodium-

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independent transport of a wide range of amphipathic organic compounds including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides, numerous drugs, and other xenobiotic substances.[2,3] So far eleven human OATPs have been identified.[4] Among them, OATP1B1 and OATP1B3 are two liver-specific transporters expressed exclusively on the basolateral membrane of hepatocytes under normal physiological conditions.[4,5] They mediate transport of many drugs from portal blood into hepatocytes and thus are important for drug disposition. OATP1B1 and 1B3 are two highly homologous transporters who share 80% amino acid sequence identity.[6,7]

A number of drug-drug and drug-food interactions have been associated with OATP1B1 and 1B3, affecting the pharmacokinetics and pharmacodynamics of their substrate drugs.[8–16] It has also been reported that many natural substances present in food or dietary supplements such as flavonoids could intact with OATP1B1 and 1B3 and alter the pharmacokinetics of OATP substrate drugs.[11,15,17–21] As OATP1B1 and 1B3 have high sequence similarity, many natural compounds such as apigenin, kaempferol, quercetin, naringenin, naringin, and rutin, showed the same effect on the function of OATP1B1 and 1B3.[15,18–20] However, some natural compounds showed opposite effects on their function. For instance, green tea catechin epigallocatechin gallate (EGCG) and quercetin 3-O-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside stimulated OATP1B3-mediated estrone-3-sulfate (E3S) uptake, while they inhibited OATP1B1-mediated E3S uptake.[11,19] Quercetin derivative 5-O-propylquercetin stimulated OATP1B3-mediated estradiol-17β-glucuronide (E17βG) uptake, while it inhibited OATP1B1-mediated E17βG uptake.[21] In contrast, the anti-cancer and anti-fungal phytochemical dioscin inhibited OATP1B3-mediated steviol glucuronide (SVG) uptake, while it stimulated OATP1B1-mediated SVG uptake.[15] Among them, EGCG showed the greatest opposite effects on OATP1B1 and 1B3. So far, little is known about the molecular determinants for OATP1B3 that are critical for its activation by the green tea catechin EGCG.

In the present study, the molecular mechanism for the activation of OATP1B3 by EGCG has been investigated by measuring the function of a series of OATP1B3-derived chimeric transporters, site-directed mutagenesis, and kinetic studies. Hydropathy analysis and experimental evidence indicated that all OATPs comprise twelve putative transmembrane domains (TMs).[22,23] Previously, we proposed a topological structure model for OATP1B3 using TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) and constructed twelve chimeras of OATP1B3 by replacing each TM domain together with the loop preceding it with its corresponding region of OATP1B1.[6] Figure 1A shows the predicted topological structure of OATP1B3 with the starting and ending amino acid residues for each TM being labeled and Figure 1B is the schematic representation of the twelve chimeric transporters of OATP1B3. By using these chimeric transporters and site-directed mutagenesis, structural domains and residues critical for the activation of OATP1B3 by EGCG have been determined and the molecular mechanism for OATP1B3 activation has been elucidated.

MATERIALS AND METHODS

Materials.

Radiolabeled [^{3}H]estrone-3-sulfate was purchased from PerkinElmer (Waltham, MA). Unlabeled estrone-3-sulfate (E3S) was obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's medium (DMEM), and trypsin were from Hyclone (Logan, UT). Lipofectamine 2000 and Opti-MEM were purchased from Invitrogen (Carlsbad, CA). Sulfo-N-hydroxysuccinimide-SS-biotin, streptavidin-agarose beads, and the BCA protein assay kit were purchased from Pierce Chemical (Rockford, IL). Antibodies for detecting the 6-His tag and the Na^+/K^+-ATPase α subunit were purchased from Tiangen (Beijing, China) and Abcam (Boston, MA). Horseradish peroxidase-conjugated secondary antibodies were purchased from ProteinTech (Chicago, IL) and Sunshine Biotechnology (Nanjing, China). Immobilon Western blot detection kit was from Millipore (Billerica, MA). Human embryonic kidney (HEK293) cell line was from ATCC (Manassas, VA).

Construction of Chimeric Transporters and Mutants of OATP1B3.

We previously published the basic twelve chimeras of OATP1B3 and additional chimeras were constructed in the present study following the original procedure.[6] All chimeras were constructed with a 6-His tag at the C-terminal end of the open reading frame. OATP1B3 mutants were generated by site-directed mutagenesis with the QuikChange kit (Stratagene). The sequences of all constructs were confirmed by DNA sequencing.

Transporter Expression in HEK293 Cells.

Human embryonic kidney (HEK293) cells were cultured and transfected as described previously.[24] In brief, HEK293 cells were grown at 37°C in a humidified 5% CO_2 atmosphere in DMEM medium supplemented with 10% FBS. HEK293 cells were transiently transfected with Lipofectamine 2000 or Fugene HD (for the kinetics) according to the manufacturer's instructions. Transfected cells were incubated for 24 h at 37°C and then used for surface biotinylation and functional assay.

Cell Surface Biotinylation and Immunoblot Analysis.

HEK293 cells were cultured and transfected in poly-D-lysine coated 6-well plates. 24 h after transfection, cells were treated with sulfo-N-hydroxysuccinimide-SS-biotin (1 mg/mL in PBS) and washed three times with ice-cold PBS containing 100 mM glycine and incubated for 10 min at 4°C with the same buffer. Then cells were lysed with 700 μL of lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, pH 7.4, containing protease inhibitors). After centrifugation at 10000 × g for 2 min, the supernatants of lysates were incubated with streptavidin-agarose beads for 1 h at room temperature. Beads were then pelleted at 850 × g for 1 min and washed three times with ice cold lysis buffer. Cell surface proteins were recovered from the resin by incubation of the beads with 2 × Laemmli buffer containing 100 mM dithiothreitol at room temperature for 30 min. Cell membrane proteins were then subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. OATP1B3 and its chimeras and mutants were detected with a mouse anti-His

antibody (Tiangen) (1:2000), followed by HRP-conjugated goat anti-mouse IgG (ProteinTech) (1:5000). The plasma membrane marker Na^+/K^+-ATPase was detected with a rabbit anti-Na^+/K^+-ATPase α subunit antibody (Abcam) (1:5000), followed by HRP-conjugated goat anti-rabbit IgG (Sunshine) (1:10000). Immunoblots were developed with a chemiluminescence method and scanned with ChemiDoc MP imaging system (Bio-Rad, Hercules, CA).

Functional Assay of Transporters in HEK293 Cells.

HEK293 cells were seeded in poly-D-lysine coated 24-well plates and transfected with 500 ng of cDNA per well using Lipofectamine 2000 or Fugene HD (for the kinetics). Transport assays were performed 24 h or for the kinetics 48 h post-transfection. The amount of E3S transported into cells was measured by liquid scintillation counting. Initial experiments showed that OATP1B3-mediated uptake of 0.1 μM E3S was linear up to at least 1.5 min. Therefore, uptake and kinetic experiments for E3S were performed at 1 min, and its procedure was the same as described in our previous publication.[8] In all experiments, cells transfected with empty vector were used as background control. Transporter-specific uptake was calculated by subtracting the background uptake from OATP-transfected uptake and normalized with total protein concentration which was determined by the BCA assay.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed to quantify the amount of EGCG transported into cells. Chromatographic separation was achieved with an Agela Venusil C_{18} column (2.1 mm × 50 mm, 5 μm) and the flow rate was set at 0.4 mL/min. The mobile phase consisted of water containing 0.1% acetic acid (A) and acetonitrile containing 0.1% acetic acid (B) with the following gradient: 0–0.5 min, 3% B; 2 min, 28% B; 2.0–8.0 min, 28% B; and 8.1–10.0 min, 3% B. The mass spectrometer was operated in negative electrospray ionization (ESI) mode and quantitation was performed by multiple reaction monitoring (MRM). The ion transitions for EGCG and internal standard (IS) epicatechin gallate (ECG) were selected as m/z 457.3→169.0 and m/z 440.7→168.8, respectively.

Data Analysis.

Uptake experiments were performed in triplicates and data with error bars represent mean ± SD ($n = 3$). Kinetic parameters were calculated using nonlinear regression analysis incorporated in Prism 5 (GraphPad Software, La Jolla, CA). Unpaired t test was used to compare difference between means of two groups. When comparing two or more groups with control, one-way ANOVA was performed followed by Dunnett's test. The p value for statistical significance was set to be < 0.05 at 95% confidence interval.

RESULTS AND DISCUSSION

Green tea is a commonly consumed beverage and has received much attention for its reputed health benefits. Green tea has high content of catechins, which make up 30% to 40% of its dry weight.[11] EGCG, the most predominant catechin in green tea, showed stimulating and inhibitory effects on OATP1B3- and 1B1-mediated E3S transport, respectively, although OATP1B1 and 1B3 have high sequence identity.[11] OATP1B3 is considered to be a liver-

specific transporter under normal physiological conditions. However, it is also expressed in some cancer tissues.[5] Therefore, the activation of OATP1B3 could probably be utilized to enhance drug transport into cancer tissues or into hepatocytes. Elucidation of the molecular mechanism for the activation of OATP1B3 by small molecules could be of great significance. Therefore, in the present study we have identified and characterized the critical structural domains and amino acid residues for the activation of OATP1B3 by EGCG by systematically examining the function of a series of OATP1B3-derived chimeric transporters, site-directed mutagenesis, and kinetic studies.

Effect of EGCG on OATP1B1- and 1B3-mediated Uptake of E3S.

To confirm the opposite effects of EGCG on the function of OATP1B1 and 1B3 reported previoulsy,[11] first we transiently expressed OATP1B1 and 1B3 in HEK293 cells and determined E3S uptake in the absence and presence of EGCG. Cell surface biotinylation and immunoblot analysis showed that OATP1B1 and 1B3 were normally expressed on the plasma membrane of HEK293 cells (Figure 2A). OATP1B1-mediated E3S uptake was inhibited by EGCG, while OATP1B3-mediated E3S uptake was stimulated by EGCG (Figure 2B), which were consistent with the observations reported before with CHO cells stably expressing OATP1B1 and 1B3.[11] These results indicated that our expression and functional assay systems worked well, and OATP1B1 and 1B3 had the same function in transient and stable expression systems.

TM1 and TM10 are Critical for the Activation of OATP1B3 by EGCG.

To determine which transmembrane domain(s) is(are) critical for the activation of OATP1B3 by EGCG, twelve OATP1B3-derived chimeric transporters had been constructed (Figure 1B)[6] and their surface expression and function were examined. As shown in Figure 3A, all twelve OATP1B3 chimeric transporters were properly expressed on the cell surface. While most chimeras had a similar molecular mass as wild-type OATP1B3, chimeras 1B3-T4 and 1B3-T10 had significantly lower molecular masses than OATP1B3, which indicated that OATP1B3 is more glycosylated in extracellular loops 2 and 5 than OATP1B1. Functional studies showed that chimeras 1B3-T1 and 1B3-T10 could not be activated by EGCG, while all other chimeras were activated by EGCG although chimera 1B3-T8 only showed a slight activation (Figure 3B). These results indicated that T1 and T10 regions are critical for the activation of OATP1B3 by EGCG.

To narrow down the region of importance for OATP1B3 activation, T1 and T10 regions were further divided into N-terminus (NT) and transmembrane domain 1 (TM1), and extracellular loop 5 (EL5) and TM10, respectively. Therefore, four additional chimeras, 1B3-NT and 1B3-TM1 based on 1B3-T1, and 1B3-EL5 and 1B3-TM10 based on 1B3-T10, were constructed (Figure 4A). Chimeras 1B3-NT and 1B3-TM1 were constructed by replacing NT (residues 1–24) and TM1 (residues 25–48) of OATP1B3 with their corresponding regions of OATP1B1, respectively. Chimeras 1B3-EL5 and 1B3-TM10 were constructed by replacing EL5 (residues 433–527) and TM10 (residues 528–560) of OATP1B3 with corresponding regions of OATP1B1. Surface biotinylation and Western blot analysis showed that chimeras 1B3-NT, 1B3-TM1, 1B3-EL5, and 1B3-TM10 were properly expressed on the cell surface (Figure 4B). Functional studies showed that for the T1 region, chimera 1B3-NT

could be activated by EGCG, while chimera 1B3-TM1 could not be activated by EGCG. For the T10 region, chimera 1B3-EL5 could be activated by EGCG, while chimera 1B3-TM10 could not be activated by EGCG (Figure 4C). These results indicated that amino acid residues within TM1 (residues 25–48) and TM10 (residues 528–560) but not NT and EL5 were important for the activation of OATP1B3 by EGCG.

Amino Acid Residues G45 in TM1 and F555 in TM10 are Critical for the Activation of OATP1B3 by EGCG.

Amino acid sequence alignment showed that there are five and fourteen amino acid residues different in TM1 and TM10 regions between OATP1B1 and 1B3, respectively (Figure 5A). To determine which amino acid residues in TM1 and TM10 would be important for the activation of OATP1B3 by EGCG, we constructed five and fourteen single-point mutants in TM1 and TM10, respectively, by replacing each residue in OATP1B3 with its corresponding residue in OATP1B1. All nineteen single-point mutants showed proper expression on the cell surface with similar surface expression levels as wild-type OATP1B3 (Figure 5B). Functional studies showed that only mutant 1B3-G45A in TM1 and mutant 1B3-F555H in TM10 could not be activated by EGCG, while all other mutants could be activated by EGCG although mutant 1B3-S545L only showed slight activation (Figure 5C). These results indicated that G45 in TM1 and F555 in TM10 are the most important amino acid residues for OATP1B3's activation by EGCG.

Effect of EGCG on the Kinetics of E3S Uptake Mediated by OATP1B3, 1B3-TM1, 1B3-TM10, 1B3-G45A, and 1B3-F555H.

So far we have identified that TM1 and TM10, and furthermore G45 and F555, are critical for the activation of OATP1B3 by EGCG. In order to further unveil the molecular mechanism underlying their critical role in OATP1B3 activation, kinetic studies of E3S uptake mediated by OATP1B3, 1B3-TM1, 1B3-TM10, 1B3-G45A, and 1B3-F555H in the absence and presence of EGCG were carried out and their kinetic parameters were calculated (Table 1). The determined K_m value for OATP1B3-mediated E3S uptake was 47.3 ± 23.8 μM, which was similar to that obtained with the stable expression system in our previous report.[8] EGCG greatly decreased it to 3.3 ± 1.9 μM. Therefore, the stimulating effect of EGCG on OATP1B3-mediated E3S uptake at low substrate concentrations (such as 0.1 μM E3S) was due to increased binding affinity. EGCG also significantly decreased the maximal rate (V_{max}) of OATP1B3-mediated E3S transport from 282.6 ± 52.6 to 36.1 ± 3.9 pmol/mg protein/min. Thus, at high substrate concentrations (such as 100 μM E3S), EGCG showed inhibitory effect on OATP1B3-mediated E3S uptake.

For chimeras 1B3-TM1, 1B3-TM10, 1B3-G45A, and 1B3-F555H, their K_m values were all increased in the presence of EGCG as compared to their respective K_m values in the absence of EGCG, whereas their V_{max} values were all decreased in the presence of EGCG (Table 1). Therefore, 1B3-TM1, 1B3-TM10, 1B3-G45A, and 1B3-F555H could not be activated by EGCG due to decreased binding affinity for E3S at low substrate concentration. With decreased V_{max}, EGCG also showed inhibitory effect on E3S uptake mediated by 1B3-TM1, 1B3-TM10, 1B3-G45A, and 1B3-F555H at high substrate concentrations.

Functional Characterization of Additional Mutants for G45 and F555 of OATP1B3.

To further characterize the effect of amino acid side chains at positions 45 and 555 on OATP1B3's activation, additional mutants, namely 1B3-G45S, G45D, G45K and G45F at position 45, and 1B3-F555Y, F555W and F555L at position 555, were constructed. As shown in Figure 6A, all additional mutants at both positions were properly expressed on the cell surface. Functional studies showed that only glycine at position 45 were activated by EGCG. All other amino acid residues with nonpolar (alanine), polar and hydrogen-bond forming (serine), negatively charged (aspartate), positively charged (lysine), and aromatic (phenylalanine) side chains at position 45 impaired OATP1B3's activation (Figure 6B). These results demonstrated that the glycine at position 45 was necessary for activation. Because glycine is much more flexible than other amino acids,[25] it seems that flexibility rather than other properties of the amino acid residue at position 45 is critical for OATP1B3's activation by EGCG. Previously we developed a three-dimensional structure model for OATP1B3.[6] In this model, G45 is located at the extracellular half of TM1 and contributes to the extracellular opening of the substrate translocation pathway with residues from other TMs (Figure 7). Its flexibility may be beneficial for OATP1B3 to bind and translocate substrates and/or modulators.

Two additional mutants at position 555, namely 1B3-F555Y and F555W, could be activated by EGCG, while 1B3-F555L could not be activated by EGCG (Figure 6B). Like phenylalanine, tyrosine and tryptophan have aromatic side chains and the function of 1B3-F555Y and F555W was similar to that of wild-type OATP1B3. Among nonaromatic amino acids, leucine is the most similar to phenylalanine.[26,27] However, because mutant 1B3-F555L could not be activated by EGCG, it seems that the aromatic ring at position 555 is quite important for OATP1B3's activation. Given that mutant 1B3-F555H cannot be activated although the side chain of histidine is aromatic, it seems that a positive charge is unfavorable at F555. This position (F555) is located near the intracellular membrane border of TM10 and its side chain is positioned away from the substrate translocation pathway (Figure 7). Therefore, F555 might be important for the overall structure of OATP1B3 instead of interacting with substrate directly.

G45A and F555H did Not Compromise OATP1B3-mediated Uptake of EGCG.

It was reported that EGCG was transported by OATP1B3 but not by OATP1B1.[11] To investigate the relationship between EGCG transport and its stimulating effect on OATP1B3, we measured the uptake of EGCG by OATP1B1, OATP1B3, 1B3-G45A, and 1B3-F555H. As shown in Figure 8, OATP1B3 showed significantly higher uptake for EGCG than OATP1B1. Uptake of EGCG by OATP1B3-G45A and 1B3-F555H was comparable to that of wild-type OATP1B3. These results suggested that transport of EGCG did not necessarily lead to OATP1B3's activation, and E3S and EGCG might have different binding sites in OATP1B3. A recent study indicated that OATP1B3 could form homodimers.[28] Whether substrate and modulator bind to one OATP1B3 molecule or to two respective molecules of a dimer remains to be elucidated.

In the present study, we have elucidated and characterized the critical structural domains and amino acid residues for the activation of OATP1B3 by EGCG. Our results showed that TM1

and TM10 are critical for OATP1B3 to be activated by EGCG (Figures 3 and 4). We further identified that G45 in TM1 and F555 in TM10 are the most important amino acid residues for OATP1B3's activation (Figure 5). Kinetic studies showed that the activation of OATP1B3 by EGCG at low substrate concentration was due to an increased substrate binding affinity (Table 1). However, for chimeras 1B3-TM1 and 1B3-TM10 and mutants 1B3-G45A and 1B3-F555H, the K_m values were increased and the V_{max} values were decreased in the presence of EGCG (Table 1). Therefore, 1B3-TM1, 1B3-TM10, 1B3-G45A, and 1B3-F555H could not be activated by EGCG. The flexibility at position 45 and aromaticity at position 555 might be important for OATP1B3's activation. While 1B3-G45A and 1B3-F555H could not be activated by EGCG, their transport activity for EGCG was comparable to that of wild-type OATP1B3 (Figure 8).

A recent study showed that TM1 is important for maintaining proper function of OATP2B1.[29] However, no study examining the role of TM1 for the function of OATP1B3 has been reported so far. In the present study, our results showed that G45 in TM1 is critical for OATP1B3's activation by EGCG. However, the role of other amino acid residues in TM1 for OATP1B3's function such as substrate selectivity remains to be elucidated.

Our previous studies showed that TM10 was critical for substrate selectivity for both OATP1B1 and 1B3.[6,7] For OATP1B3, Y537, S545, and T550 in TM10 were the most important amino acid residues for cholecystokinin octapeptide (CCK-8) transport.[6] In the present study, our results showed that F555 in TM10 was the most important amino acid residue for OATP1B3's activation by EGCG. Therefore, it seems that TM10 is important for both substrate translocation and modulator action for OATP1B3. However, as the specific amino acid residues critical for substrate transport and activation were different, the binding sites for substrate and modulator in OATP1B3 might be different.

In conclusion, our present study showed that G45 in TM1 and F555 in TM10 are critical for the activation of OATP1B3 by green tea catechin EGCG. G45 is irreplaceable by any other amino acid residues, while F555 can be replaced by other aromatic amino acids without positive charge.

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ABBREVIATIONS USED

DMEM	Dulbecco's Modified Eagle's medium
E17βG	estradiol-17β-glucuronide
E3S	estrone-3-sulfate
ECG	(−)-epicatechin gallate
EGCG	(−)-epigallocatechin gallate

ESI	electrospray ionization
FBS	fetal bovine serum
HEK293	human embryonic kidney cells
IS	internal standard
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MRM	multiple reaction monitoring
OATP	organic anion transporting polypeptide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
SLC	solute carrier
TM	transmembrane domain

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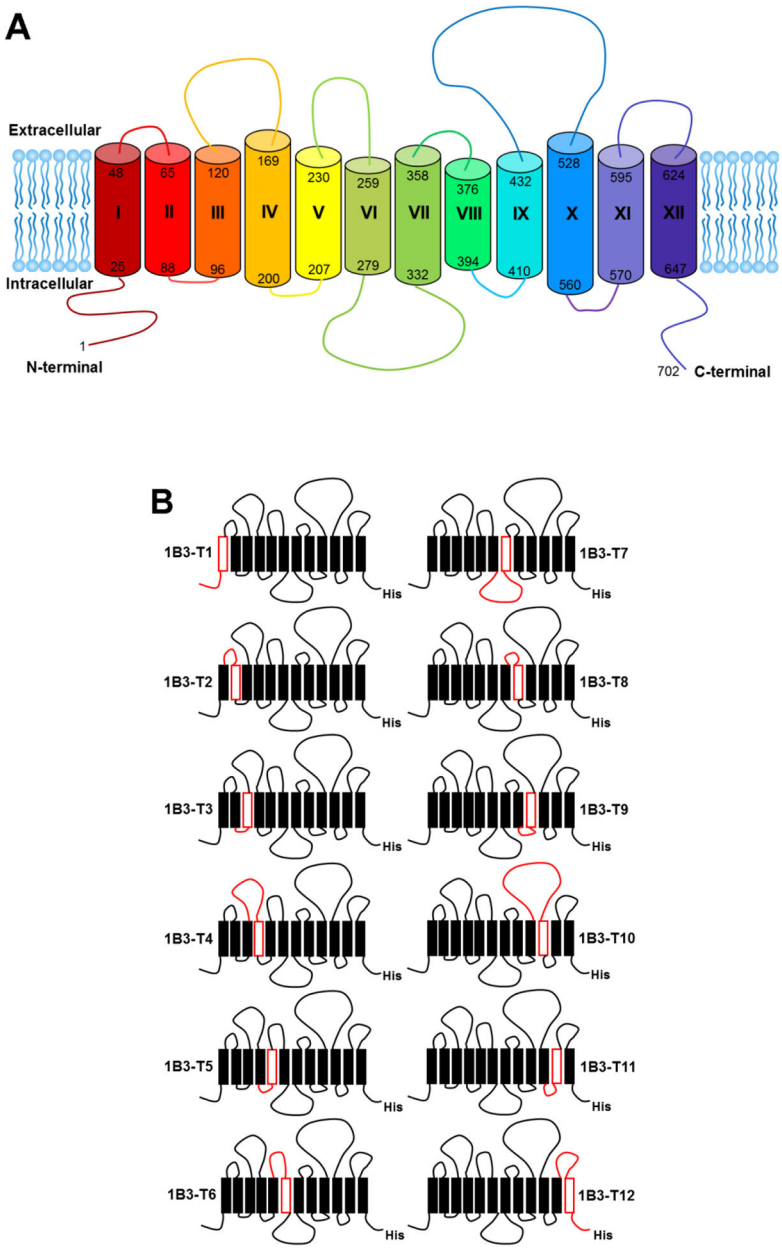

Figure 1.

Topological structure of human OATP1B3 and twelve OATP1B3-derived chimeric transporters. (A) Predicted topological structure of human OATP1B3 with twelve transmembrane domains. The starting and ending amino acid residues for each transmembrane domain were indicated. (B) Schematic representation of twelve human OATP1B3-derived chimeric transporters. The chimeric transporters were constructed by replacing each TM domain together with the loop preceding it with its corresponding region of OATP1B1. For instance, chimera 1B3-T1 was obtained by replacing amino acid residues 1–48 of OATP1B3 with amino acid residues 1–48 of OATP1B1. A His tag was incorporated into the C-terminal end of all constructs for convenient detection of all chimeras with an anti-His antibody.

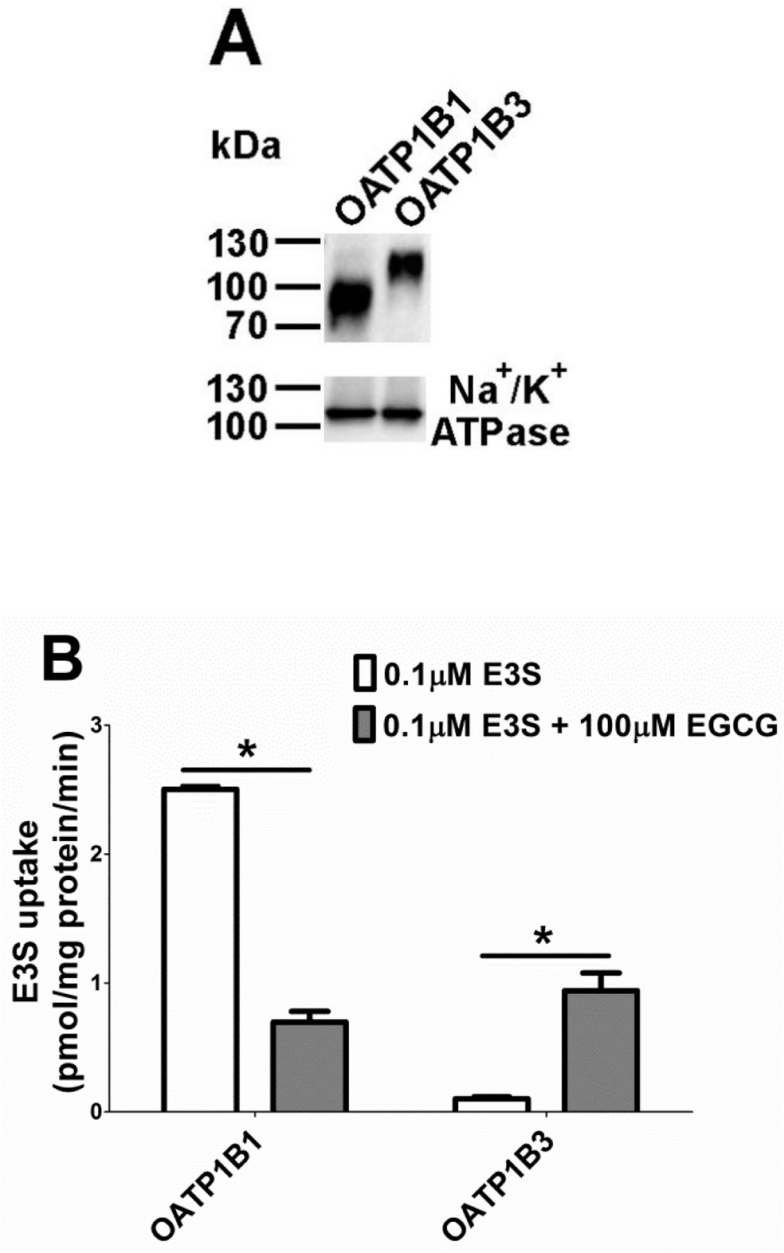

Figure 2.

Surface expression and functional characterization of OATP1B1 and 1B3 in HEK293 cells. (A) Surface expression of OATP1B1 and 1B3. OATP1B1 and 1B3 expressed on the cell surface were isolated by surface biotinylation and detected by immunoblot analysis with an anti-His antibody. The plasma membrane marker Na^+/K^+-ATPase α subunit was used as protein loading control. (B) Functional characterization of OATP1B1 and 1B3. Uptake of 0.1 μM E3S in the absence and presence of 100 μM EGCG was measured at 37°C for 1 min with empty vector and OATP-transfected cells. The net uptake was obtained by subtracting the uptake of empty vector-transfected cells from the uptake of OATP-transfected cells. Data were presented as mean ± SD ($n = 3$). Asterisks indicated a $p < 0.05$ level of significant difference between uptakes in the absence and presence of EGCG.

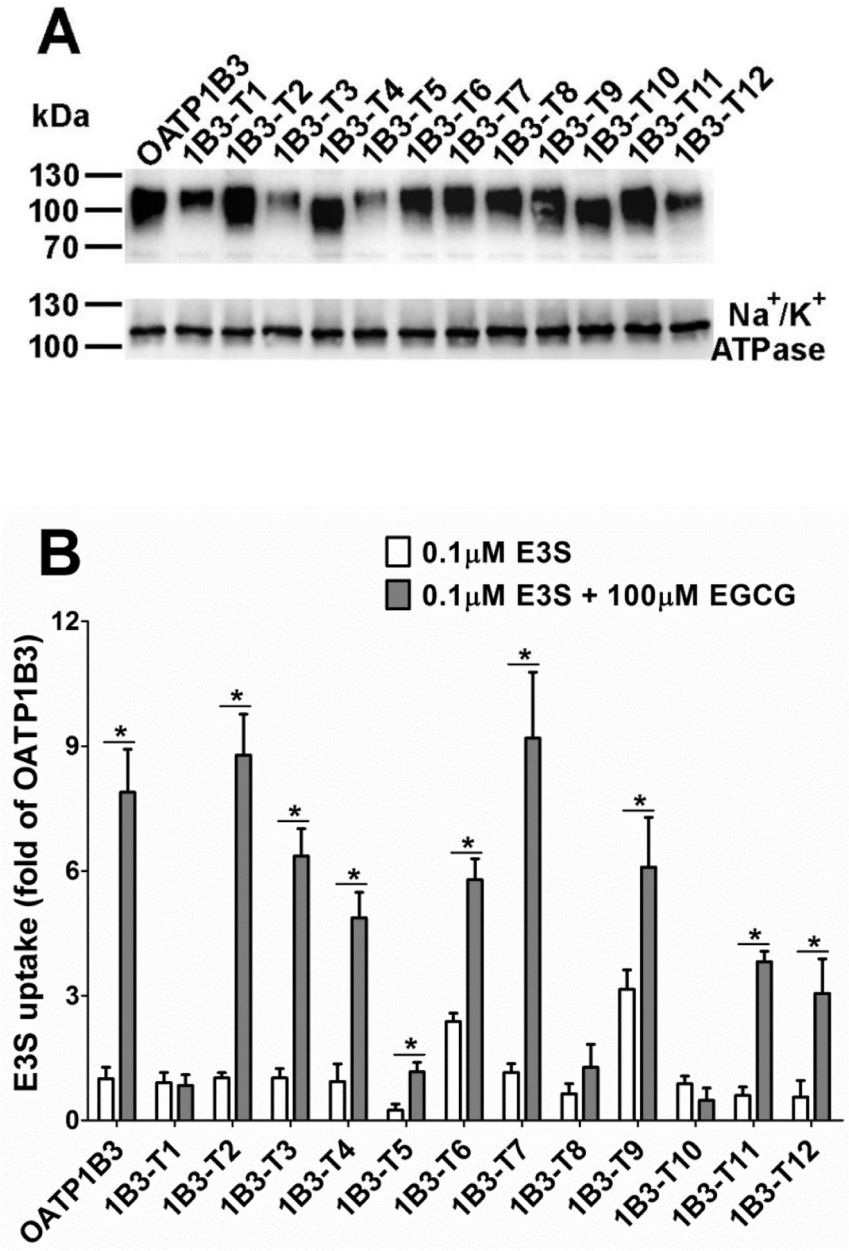

Figure 3.

Surface expression and functional characterization of twelve OATP1B3-derived chimeric transporters. (A) Surface expression of twelve chimeric transporters derived from OATP1B3. Chimeras expressed on the cell surface were isolated by surface biotinylation and detected by immunoblot analysis with an anti-His antibody. The plasma membrane marker Na^+/K^+-ATPase α subunit was used as protein loading control. (B) Functional characterization of twelve OATP1B3 chimeric transporters. Uptake of 0.1 μM E3S in the absence and presence of 100 μM EGCG was measured at 37°C for 1 min with empty vector and OATP-transfected cells. The net uptake was obtained by subtracting the uptake of empty vector-transfected cells from the uptake of OATP-transfected cells. Data were presented as mean ± SD ($n = 3$).

Asterisks indicated a $p < 0.05$ level of significant difference between uptakes in the absence and presence of EGCG.

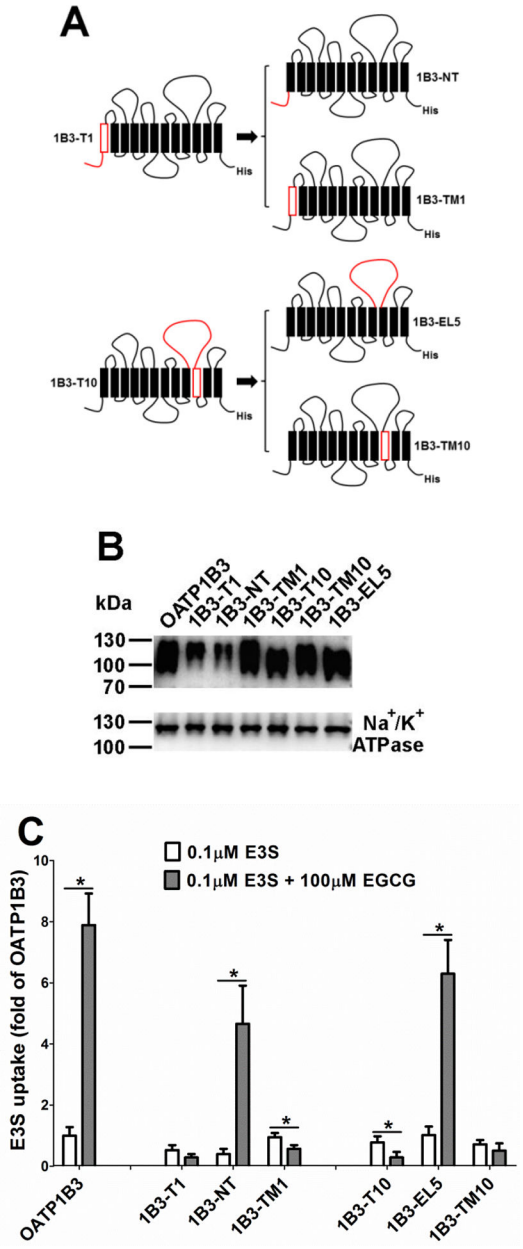

Figure 4.

Schematic representation of chimeras 1B3-NT, 1B3-TM1, 1B3-EL5, and 1B3-TM10, and their surface expression and functional characterization. (A) Chimeras 1B3-NT and 1B3-TM1 were derived from 1B3-T1 and constructed by replacing the N-terminus (residues 1–24) and TM1 (residues 25–48) of OATP1B3 with their corresponding regions of OATP1B1, respectively. Chimeras 1B3-EL5 and 1B3-TM10 were derived from 1B3-T10 and constructed by replacing extracellular loop 5 (residues 433–527) and TM10 (residues 528–560) of OATP1B3 with corresponding regions of OATP1B1, respectively. (B) Surface expression of chimeras 1B3-NT, 1B3-TM1, 1B3-EL5, and 1B3-TM10. Chimeras expressed on the cell surface were isolated by surface biotinylation and detected by immunoblot analysis with an anti-His antibody. The plasma membrane marker Na^+/K^+-ATPase α subunit

was used as protein loading control. (C) Functional characterization of chimeras 1B3-NT, 1B3-TM1, 1B3-EL5, and 1B3-TM10. Uptake of 0.1 μM E3S in the absence and presence of 100 μM EGCG was measured at 37°C for 1 min with empty vector and OATP-transfected cells. The net uptake was obtained by subtracting the uptake of empty vector-transfected cells from the uptake of OATP-transfected cells. Data were presented as mean ± SD ($n = 3$). Asterisks indicated a $p < 0.05$ level of significant difference between uptakes in the absence and presence of EGCG.

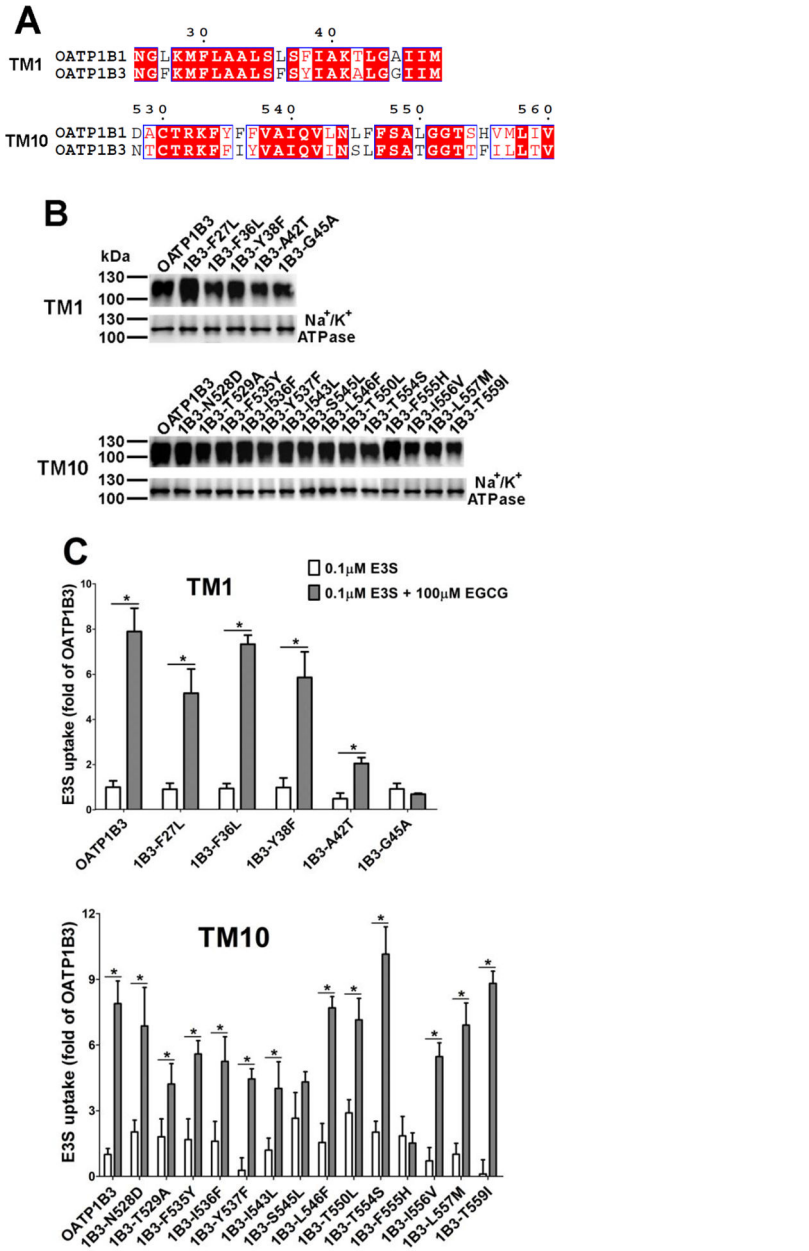

Figure 5.

(A) Amino acid sequence alignment of TM1 (residues 25–48) and TM10 (residues 528–560) between OATP1B1 and 1B3. Five amino acid residues are different in TM1 between OATP1B3 and 1B1, namely (1B3)F27L(1B1), F36L, Y38F, A42T, and G45A. Fourteen amino acid residues are different in TM10, namely N528D, T529A, F535Y, I536F, Y537F, I543L, S545L, L546F, T550L, T554S, F555H, I556V, L557M, and T559I. (B) Surface expression of five single-point mutants of OATP1B3 in TM1 and fourteen single-point mutants in TM10. OATP1B3 mutants expressed on the cell surface were isolated by surface biotinylation and detected by immunoblot analysis with an anti-His antibody. The plasma membrane marker Na^+/K^+-ATPase α subunit was used as protein loading control. (C) Functional characterization of OATP1B3 mutants in TM1 and TM10. Uptake of 0.1 μM E3S

in the absence and presence of 100 μM EGCG was measured at 37°C for 1 min with empty vector and OATP-transfected cells. The net uptake was obtained by subtracting the uptake of empty vector-transfected cells from the uptake of OATP-transfected cells. Data were presented as mean ± SD ($n = 3$). Asterisks indicated a $p < 0.05$ level of significant difference between uptakes in the absence and presence of EGCG.

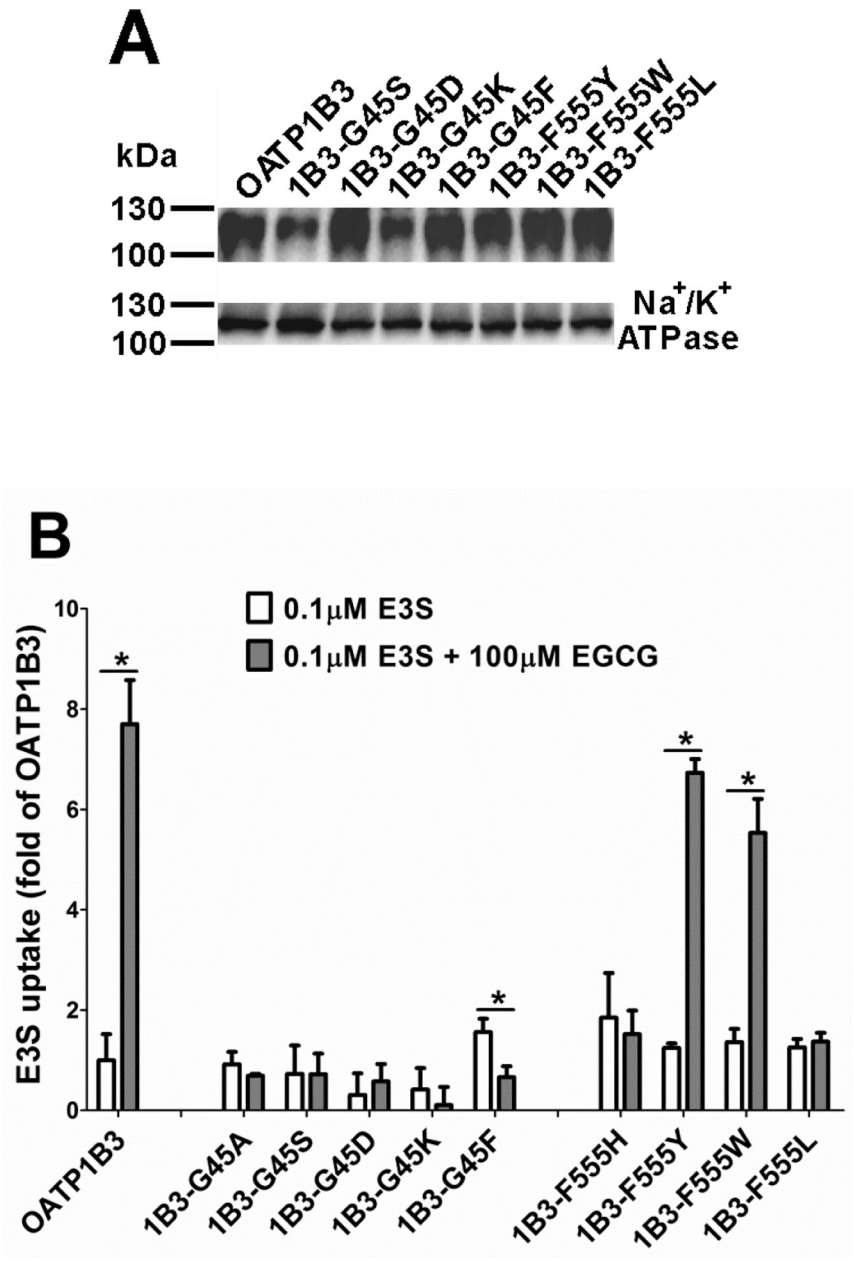

Figure 6.

Surface expression and functional characterization of additional mutants for G45 and F555 of OATP1B3. (A) Surface expression of additional mutants for G45 and F555 of OATP1B3, namely, mutants 1B3-G45S, G45D, G45K, G45F, F555Y, F555W, and F555L. OATP1B3 mutants expressed on the cell surface were isolated by surface biotinylation and detected by immunoblot analysis with an anti-His antibody. The plasma membrane marker Na^+/K^+-ATPase α subunit was used as protein loading control. (B) Functional characterization of additional mutants for G45 and F555 of OATP1B3. Uptake of 0.1 μM E3S in the absence and presence of 100 μM EGCG was measured at 37°C for 1 min with empty vector and OATP-transfected cells. The net uptake was obtained by subtracting the uptake of empty vector-transfected cells from the uptake of OATP-transfected cells. Data were presented as

mean ± SD ($n = 3$). Asterisks indicated a $p < 0.05$ level of significant difference between uptakes in the absence and presence of EGCG.

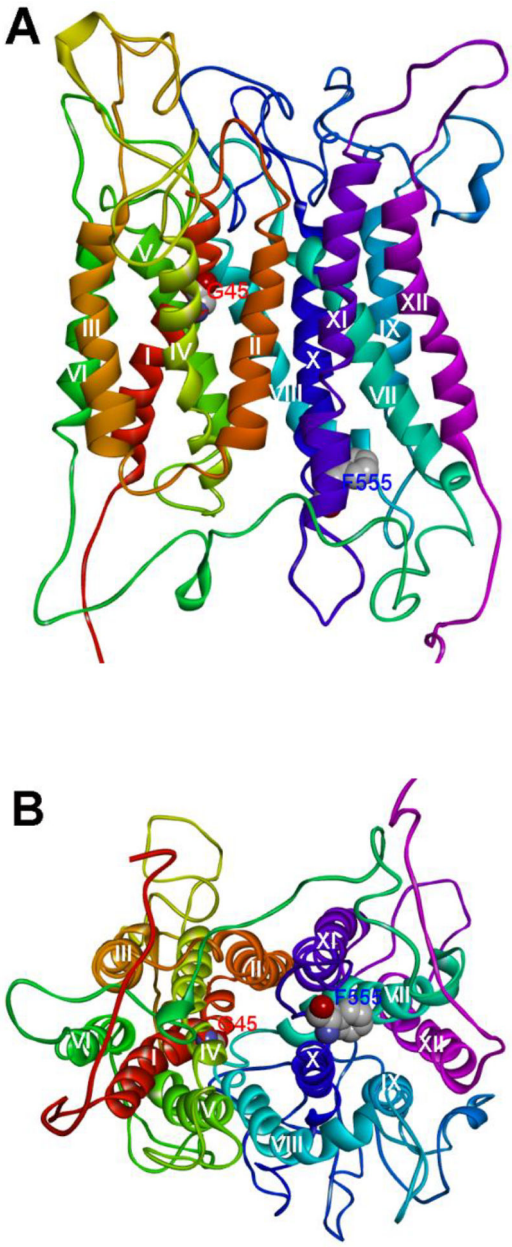

Figure 7.

Three-dimensional structure model of OATP1B3. (A) Side view and (B) intracellular view are presented. Protein is represented in solid ribbon and the 12 transmembrane domains are labeled. G45 and F555 are shown in CPK mode.

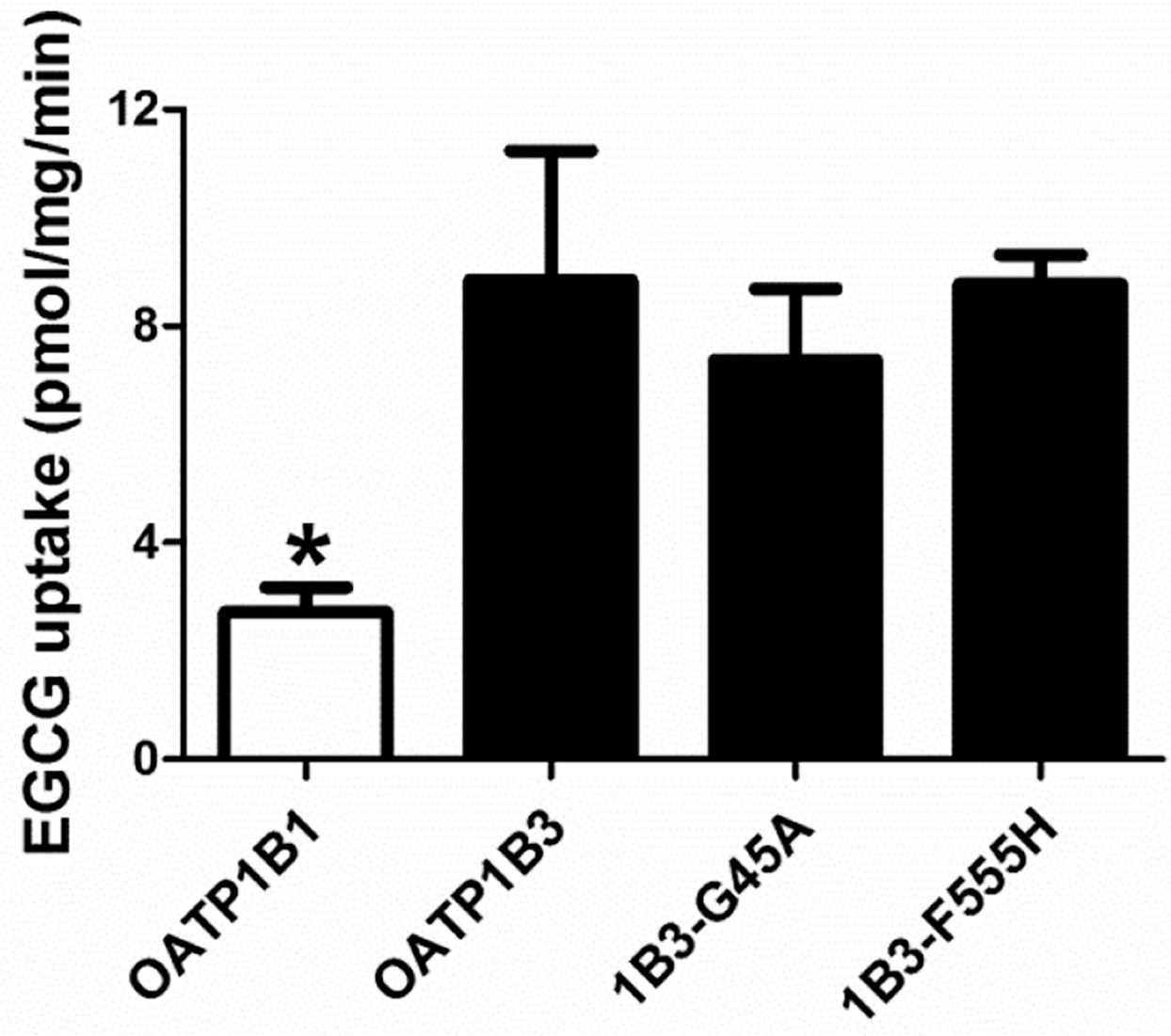

Figure 8.

Uptake of EGCG by OATP1B1, OATP1B3, 1B3-G45A, and 1B3-F555H. Uptake of 100 μM EGCG in the presence of 0.1 μM E3S was measured at 37°C for 1 min with empty vector and OATP-transfected cells. The net uptake was obtained by subtracting the uptake of empty vector-transfected cells from the uptake of OATP-transfected cells. Data were presented as mean ± SD ($n = 3$). Asterisk indicated a $p < 0.05$ level of significantly different uptake compared with that of OATP1B3.

Table 1.

Effect of EGCG on the kinetics of E3S uptake mediated by OATP1B3, 1B3-TM1, 1B3-TM10, 1B3-G45A, and 1B3-F555H[a]

	without EGCG		with EGCG	
	K_m (μM)	V_{max} (pmol/mg protein/min)	K_m (μM)	V_{max} (pmol/mg protein/min)
OATP1B3	47.3 ± 23.8	282.6 ± 52.6	3.3 ± 1.9	36.1 ± 3.9
1B3-TM1	45.0 ± 8.8	521.8 ± 45.8	56.9 ± 28.4	129.6 ± 25.6
1B3-TM10	46.3 ± 17.0	356.2 ± 48.0	132.3 ± 46.5	317.4 ± 58.9
1B3-G45A	32.6 ± 11.5	287.4 ± 33.1	43.9 ± 32.5	105.2 ± 32.5
1B3-F555H	25.8 ± 10.1	290.1 ± 33.8	46.0 ± 7.7	109.8 ± 6.8

[a]Uptake of increasing concentrations of E3S in the absence and presence of 100 μM EGCG was measured at 37°C for 1 min with empty vector and OATP-transfected cells. The net uptake was obtained by subtracting the uptake of empty vector-transfected cells from the uptake of OATP-transfected cells and was normalized to its surface expression level. Normalized net uptake was used to fit the Michaelis-Menten equation to calculate K_m and V_{max} values.