Human organic anion transporter 2 is an entecavir, but not tenofovir, transporter.

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Entecavir (ETV) and tenofovir (TFV) are essential nucleoside analogues in current hepatitis B virus (HBV) treatments. Since these drugs target the HBV polymerase that is localized within human hepatocytes, determining of their cellular uptake process is an important step in fully understanding their pharmacological actions. However, the human hepatic transporters responsible for their uptake have remained unidentified. Therefore, this study aimed at identifying the primary ETV and TFV uptake transporter(s) in human hepatocytes. In transport assays, temperature-sensitive ETV and TFV uptake by human hepatocytes were observed, and their uptake were strongly inhibited by bromosulfophthalein, which is an inhibitor of organic anion transporters/organic anion transporting polypeptides (OATs/OATPs). Given these results, ETV and TFV uptake activities in several human OAT/OATP expression systems were examined. The results showed that, among the transporters tested, only OAT2 possessed ETV transport activity. On the other hand, none of the transporters showed any TFV uptake activity. To summarize, our results identify that human OAT2 is an ETV transporter, thereby suggesting that it plays an important part in the mechanisms underlying ETV antiviral activity. Furthermore, although the hepatic TFV transporters remain unknown, our results have, at least, clarified that these two anti-HBV drugs have different hepatocyte entry routes.
Key words: entecavir, tenofovir, organic anion transporter, human hepatocytes, hepatitis B
Entecavir (ETV) and tenofovir (TFV), which are essential nucleoside analogues in current chronic hepatitis B treatment (see Fig. s1 for their structures), have shown better clinical efficacy than the classical anti-hepatitis B virus (HBV) drugs. However, it is also true that their effectiveness levels are highly variable, depending on the patients, and that a number of patients (roughly 10 to 40% in 48-week treatment) do not receive sufficient levels of benefit from these antiviral drugs [1-3]. In addition, there are currently no established guidelines instructing clinicians as to which drugs should be administered first for naïve patients. Nevertheless, since strong suppression of HBV activity is critically important for the incident rate reduction of liver cancer development, efficacy improvements for ETV- and TFV-based therapy (e.g., through the development of a manipulated personalized therapy method) are urgent issues that need to be addressed. Toward this end, detailed characterization of the mechanisms underlying ETV and TFV pharmacological actions should provide fundamental information.

Both ETV and TFV are converted into ETV-triphosphate and TFV-diphosphate intracellularly, which then act as HBV polymerase inhibitors. Therefore, it is very likely that ETV or TFV uptake by hepatocytes is a prerequisite step prior to exerting their antiviral activity, and that certain transporters, due to their significant hydrophilic natures, facilitate this step.
However, the uptake of ETV or TFV by human hepatocytes has not been characterized. On the other hand, human organic anion transporter 1 (OAT1) and OAT3 have been identified as ETV or TFV uptake transporters [4,5], and it has also been reported recently that concentrative nucleoside transporter 2 (CNT2) and CNT3 can transport ETV [6]. While these transporters are abundantly expressed in the kidney, they are only expressed marginally, if at all, in the human liver.

In light of the above-mentioned background, in this study, we sought to identify key ETV and TFV uptake transporters in human hepatocytes.
2. Materials and Methods

2.1. Transport assay using human primary hepatocytes

Pooled human primary hepatocytes (5-donor) were purchased from BioreclamationIVT (Baltimore, MD, USA). The Ethics Committee of the Chiba University Graduate School of Pharmaceutical Sciences approved the use of human samples in this study.

[\textsuperscript{3}H]-ETV (American Radiolabeled Chemicals, St. Louis, MO, USA) or [\textsuperscript{3}H]-TFV (Moravek Biochemicals, Brea, CA, USA) uptake by hepatocytes was measured using a previously described centrifugal filtration technique (see the supplemental information). Bromosulfophthalein (BSP 200 µM) (Sigma, St. Louis, MO, USA), which is an OAT/organic anion transporting polypeptide (OATP) inhibitor, and indomethacin (IDM 50 µM) (Sigma), which is an OAT2 substrate/inhibitor, were used in our inhibition assays. All assays were performed at 37°C and 4°C, with the data calculated by subtracting the 4°C activity from that at 37°C.

2.2. Preparation of transporter expression systems

The OAT2 cDNA (encoding 546 amino acids, NM_006672) and the organic cation
transporter 1 (OCT1) cDNA was cloned from human liver cDNA and subcloned into the pcDNA3.1/Neo(-) (Thermo Fisher Scientific, Waltham, MA USA). OAT2/pcDNA or OCT1/pcDNA was transfected into human embryonic kidney 293 (HEK293) cells (Human Science, Tokyo, Japan), and the cells stably expressing OAT2 or OCT1 at the highest level were isolated and termed OAT2/HEK or OCT1/HEK, respectively.

Na\(^{+}\)-taurocholate cotransporting polypeptide (NTCP) expression plasmid was kindly provided by Dr. Watashi (The National Institute of Infectious Diseases, Tokyo, Japan). As above, the HEK293 cells stably expressing NTCP were developed (NTCP/HEK).

Preparation of the HEK293 cells expressing OATP1B1, OATP1B3, or OATP2B1, (which are OATP1B1/HEK, OATP1B3/HEK, and OATP2B1/HEK, respectively), the second segment of the proximal tubule (S2) cells expressing OAT7 (OAT7/S2), and their control cells (Mock/HEK and Mock/S2) were reported previously (see the supplemental information).

2.3. Transport assay using transporter expression systems

Uptake studies using transporter expression systems were conducted according to the method described previously (see the supplemental information). Transporter activities of each system were validated using authentic substrates and inhibitors as shown in the figure legend.
The ETV and TFV uptake incubation times for each transporter were set at the same values employed for classical substrate uptake.

2.4. Other

Please see the supplemental information for detailed materials and methods, including their references.
3. Results and discussion

Considering that ETV and TFV are nucleoside analogues, it is possible that they might be substrates for equilibrative nucleoside transporters (ENTs). Therefore, we first examined this possibility using HepG2 cells, where ENTs are functionally expressed. However, the results showed that neither ETV nor TFV were taken up by ENTs (Fig. S1), thus indicating that other uptake systems are involved in their uptake by human hepatocytes.

It has also been reported that several members of OATP and OAT families are abundantly expressed in human hepatocytes, and that they facilitate the uptake of various drugs into the hepatocytes by taking advantage of their broad substrate specificities [7]. To clarify whether such organic anion transporters are involved in ETV and TFV uptake, transport assays using pooled (5-donor) human hepatocytes were carried out (Fig. 1). The results showed that temperature-sensitive ETV and TFV uptake activities were clearly observed, and that these activities were strongly repressed by BSP.

In order to identify the key transporters that contribute significantly to hepatic ETV uptake, we prepared in vitro expression systems of the primary BSP-sensitive hepatic uptake transporters OATP1B1, OATP1B3, OATP2B1, OAT2, OAT7, and NTCP. Additionally, we prepared OCT1/HEK to further test its possible involvement in ETV uptake. When ETV uptake
was examined using these systems, the results clearly showed that while all transporter activities were validated (Fig. 2A), only OAT2 demonstrated remarkable ETV transport activity (Fig. 2B and Fig. s2). Then, the kinetic analysis of OAT2-mediated ETV uptake was also performed using OAT2/HEK. As the results, the transport pathway appeared to be the low affinity system, where apparent $K_m$ and $V_{max}$ values were estimated to be $150 \pm 44$ (µM) and $1,559 \pm 276$ (pmol/mg protein/min), respectively (Fig. s2). In order to evaluate contribution of OAT2 to ETV uptake by human hepatocytes, we examined inhibitory effects of IDM on the hepatic ETV uptake. The results showed that, in the presence of IDM, the hepatocyte ETV uptake level decreased to less than 35% of that obtained in the absence of IDM (Fig. s3). This is in agreement with the report describing involvement of rat Oat2 in ETV uptake by rat liver [8].

Therefore, even though another unidentified transporter may also be involved, the circumstantial evidence in our results suggests that OAT2 plays an important role in hepatic ETV uptake by human hepatocytes. This notion is imminently reasonable when considering that the target cell type of ETV is hepatocyte, and that OAT2 is known as a liver-enriched transporter in the OAT family. Since it has been reported that drugs containing a guanine moiety (such as acyclovir and ganciclovir [9]) are substrate of OAT2, presumably OAT2 transports ETV through a similar substrate recognition site.

In addition, it should be briefly mentioned that, while rat Oct1 has been considered to
be involved in ETV uptake by rat liver [8], our results suggest that human OCT1 is unlikely to contribute to hepatic ETV uptake significantly. The reason for this apparent difference is currently unknown, but this might result from species difference.

Having identified OAT2 as an ETV transporter, the next likely question is whether the functional variability of OAT2 is directly related to ETV’s pharmacological action. Previously, we have shown that the efficacy of ribavirin, which is an anti-hepatitis C virus nucleoside analogue, is clearly dependent on its ENT1-mediated uptake [10]. Therefore, a similar narrative is suspected in the case of ETV, and it is worth investigating this possibility in future studies.

On the other hand, we also attempted to identify hepatic TFV uptake transporters utilizing the same set of transporter expression systems, but none of the transporters showed TFV uptake activity. Since OAT1-mediated TFV uptake was clearly observed (Fig. s4) and that each expression system shows clear classical substrate uptake activity (Fig. 2A) (please also see Fig. s5B, where OAT2 expression level in OAT2/HEK293 appears to be comparable to that of the human primary hepatocytes used in this study), the results were unlikely due to inappropriate experimental conditions or considerable insufficiency of the transporter expression levels. In addition, TFV concentration used in this study (1 μM) is similar to the $C_{\text{max}}$ after taking a single dose of 300 mg of tenofovir disoproxil fumarate (0.73-1.03 μM, TENOZET Interview Form). Therefore, although the possibility that OAT2 and the other transporters might
show TFV uptake activity at its higher concentrations cannot be fully excluded, their TFV uptake levels in clinical settings are assumed to be very low, if any.

Nevertheless, even though the hepatic TFV transporters remain unclear, our results provide at least one important finding, which is that ETV and TFV have distinctly different hepatocyte entry routes. This also indicates that while ETV and TFV share the structural similarities to target HBV polymerase, there is a clear difference between the molecular steps regarding their pharmacological actions. Therefore, clarification of the different hepatocyte entry routes between these two anti-HBV drugs may be one of the basic findings contributing to the development of a drug selection methodology in clinical settings, and the identification of the hepatic TFV uptake transporter will remain an important challenge in our future study.

To summarize, our results clearly show that OAT2 is a key ETV uptake transporter in human hepatocytes, and that ETV and TFV are distinctly different in terms of their entry routes into human hepatocytes. Since detailed characterization of the anti-HBV drug uptake profile into hepatocytes will provide various clues that contribute to treatment improvements, continuous research efforts, including additional attempts to identify hepatic TFV transporters, will be necessary.
Acknowledgements

We would like to thank Dr. Koichi Watashi of the National Institute of Infectious Diseases, Tokyo, Japan for providing us with the NTCP expression plasmid. This work is funded by a Ministry of Health, Labor and Welfare Grant-in-Aid for Scientific Research (Emergency Research Project to Conquer Hepatitis), Japan.
Conflict of Interest

None declared.
References


tenofovir by SLC22A family members (hOAT1, hOAT3, and hOCT2), Pharm. Res. 54 (2007) 811-815.


222 1407-1413.
ETV (1 μM, left) and TFV (1 μM, right) uptake by pooled (5-donor) primary human hepatocytes were examined using suspension transport assays. BSP (200 μM) was used as an inhibitor of drug transporters. The assays were also performed at 4°C, and each data was calculated by subtracting the 4°C activity from that at 37°C. The experiments were repeated five times, each performed in duplicate, and the value represents the mean ± S.D. N.D., not detected.

A, Functional validation of each transporter expression system was conducted. Substrates are: E2G (100 nM) for OATP1B1, CCK-8 (10 nM) for OATP1B3, E1S (50 μM and 1 μM) for OATP2B1 and OAT7, respectively, cGMP (2 μM) for OAT2, MPP+ (10 μM) for OCT1, and TCA (1 μM) for NTCP, while inhibitors are: RIF (10 μM) for OATP1B1, BSP (100 μM) for OATP1B3, OATP2B1 and OAT7, IDM (100 μM) for OAT2, TEA (20 mM) for OCT1, and CA
(100 μM) for NTCP. Since transporter activities of the OATP1B1, OATP1B3, OATP2B1 expression systems have been tested in previous reports (please see supplemental file), experiments were conducted twice for confirmation, with the results shown as the mean values. For OAT2, OCT1, OAT7, and NTCP expression systems, validation experiments were performed three times, each performed in duplicate. The results are shown as the mean ± S.D. B and C, ETV (1 μM) and TFV (1 μM) uptake were examined using the same systems and inhibitors. Each value is the mean ± S.D. of transport activity for three independent experiments, each performed in duplicate.
**Supplemental materials and methods**

**Materials**

[$^3$H]-labelled entecavir (ETV), [$^3$H]-labelled estradiol-17β-D-glucuronide (E$_2$G), [$^3$H]-labelled taurocholic acid (TCA), and [$^3$H]-labelled 1-methyl-4-phenylpyridinium (MPP$^+$) were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). [$^3$H]-labelled estrone-3-sulfate (E$_1$S) and [$^3$H]-labelled cholic acid (CA) were obtained from Sigma (St. Louis, MO, USA). [$^3$H]-labelled cyclic GMP (cGMP), and [$^3$H]-labelled adenine (Ado) were purchased from Moravek Biochemicals (Brea, CA, USA). ETV and TFV were purchased from Toronto Research Chemicals (Toronto, ON, Canada). E$_2$G, E$_1$S, cGMP, bromosulfophthalein (BSP), indomethacin (IDM), TCA, cholic acid (CA), Ado, nitrobenzylmercaptopurine riboside (NBMPR), MPP$^+$, tetraethylammonium (TEA), and G418 disulfate were purchased from Sigma (St. Louis, MO, USA). CCK-8, Rifampicin (RIF), and Zeocin were purchased from Peptide Institute (Osaka, Japan), Wako (Osaka, Japan), and Invivogen (San Diego, CA, USA), respectively.

The pcDNA3.1/Neo(-), the pcDNA3.1/Zeo(-), and insulin-transferrin-selenium supplement was provided by Thermo Fisher Scientific (Waltham, MA USA). Human embryonic kidney 293 (HEK293) cells and human hepatoma HepG2 cells were obtained from the Human Science (Tokyo, Japan) and the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), respectively. Dulbecco's Modified Eagle's Medium (DMEM), DMEM/Ham’s F-12, and recombinant human epidermal growth factor (Lonza, Walkersville, MD, USA) were obtained from Wako (Osaka, Japan).

Transport assays using hepatocytes

Pooled human hepatocytes (5-donor) were purchased from BioreclamationIVT (Baltimore, MD, USA). The Ethics Committee of the Chiba University Graduate School of Pharmaceutical Sciences approved the use of human samples in this study. [$^3$H]-ETV or [$^3$H]-TFV uptake by hepatocytes was measured using a previously described centrifugal filtration technique [1]. BSP (200 µM, an organic anion transporter (OAT)/organic anion transporting polypeptide [OATP] inhibitor) and IDM (50 µM, an OAT2 substrate/inhibitor) were used in our inhibition assays. All assays were performed at 37°C and 4°C, with the data calculated by subtracting the 4°C activity from that at 37°C.
Preparation of transporter expression systems

The OAT2 cDNA (encoding 546 amino acids, NM_006672) or the OCT1 cDNA (NM_003057) was cloned from human liver cDNA using polymerase chain reaction (PCR) with primers, as shown in Table S1. Subsequently, OAT2 or OCT1 cDNA was subcloned into the pcDNA3.1/Neo(-). Similarly, OAT1 cDNA (NM_153276) was cloned from human kidney cDNA (Table S1), and transferred into pcDNA3.1/Neo(-). Their cDNA sequences were confirmed by DNA sequencing.

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics in 5% CO$_2$ at 37°C. OAT2/pcDNA or OCT1/pcDNA was transfected into HEK293 cells, after which the cells stably expressing OAT2 or OCT1 were selected by G418 (400 µg/mL). The clonal cell line stably expressing OAT2 or OCT1 at the highest level was isolated and termed OAT2/HEK or OCT1/HEK, respectively. In addition to their functions, OAT2 or OCT1 mRNA expression in the cells was confirmed by reverse-transcription PCR (RT-PCR) using the primers shown in Table S1, the result of which was shown in Fig. sS.5.

Na$^+$-taurocholate cotransporting polypeptide (NTCP) expression plasmid (NTCP/pEF4) was kindly provided by Dr. Koichi Watashi of the National Institute of Infectious Diseases, Tokyo, Japan. As above, NTCP/pEF4 was transfected into HEK293 cells, after which the cells stably expressing NTCP were selected by Zeocin (300 µg/mL). The resultant cells stably expressing NTCP (NTCP/HEK) were used hereafter. As with the case of OAT2, NTCP mRNA expression was confirmed by RT-PCR (Fig. s5).

Preparation procedures of the HEK293 cells expressing OATP1B1, OATP1B3, or OATP2B1 (which are OATP1B1/HEK, OATP1B3/HEK, or OATP2B1/HEK, respectively), as well as those for their control cells (pcDNA3.1/Neo(-) or pcDNA3.1/Zeo(-) empty vector-transfected cells, Mock/HEK), were reported previously [2-4]. Likewise, the second segment of the proximal tubule (S2) cells expressing OAT7 (OAT7/S2), as well as those for their control cells (pcDNA3.1/Neo(+) empty vector-transfected cells, Mock/S2), was described previously [5].

S2 cells were cultured in DMEM/Ham’s F-12 supplemented with 5% fetal bovine serum, insulin-transferrin-selenium supplement, recombinant human epidermal growth factor, G418 (400 µg/mL), and antibiotics in 5% CO$_2$ at 33°C.

Quantitative real-time PCR (qPCR)

qPCR was performed using the previously described SYBR green-based method [6] to determine the OAT2 mRNA expression levels in OAT2/HEK and pooled human hepatocytes.
used in this study (Fig. s5). The primers used for qPCR are described in Table S1, and the amplification efficiency of each PCR was confirmed to be close to one. Data was calculated using the delta-delta-CT method, where GAPDH was used as a control.

### Table S1. Primer sequences used in this study

<table>
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<tr>
<th>Experiments</th>
<th>Direction</th>
<th>Primer sequences</th>
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<tr>
<td>OAT1 cDNA cloning</td>
<td>Sense</td>
<td>5'-ACA GAC AGA GGT CCT GGG A-3'</td>
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<td></td>
<td>Anti-sense</td>
<td>5'-CAC ACT TGG GTC ACC ATT T-3'</td>
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<tr>
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<td>Sense</td>
<td>5'-TGA AGC ATT TGG GTG AGC AGC AT-3'</td>
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<td></td>
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<td>5'-TGG ATG AGC AGA GGG AGC GGG TAC TG-3'</td>
</tr>
<tr>
<td>OCT1 cDNA cloning</td>
<td>Sense</td>
<td>5'-AAG AAT TCG CCA TCA TGC CCA CCG TGG ATG-3'</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-AAG GAT CCT CAG GTG CCC GAG GGT TCT GAG GTT-3'</td>
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<tr>
<td>OAT2 RT-PCR</td>
<td>Sense</td>
<td>5'-CTA TCC CCA GGC TCT CCC CAA CAC-3'</td>
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<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-TGA GCC AGT AAG GGT GCG GGT GA-3'</td>
</tr>
<tr>
<td>OCT1 RT-PCR</td>
<td>Sense</td>
<td>5'-GAA CCT CTA CCT GGA TTT CC-3'</td>
</tr>
<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-CCA GTG CAG GTC AGG TGA GAT AAA A-3'</td>
</tr>
<tr>
<td>NTCP RT-PCR</td>
<td>Sense</td>
<td>5'-TTG TTC TTC ATC ATG CTC TCG C-3'</td>
</tr>
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<td></td>
<td>Anti-sense</td>
<td>5'-CCA AGG GCA CAG AAG GTG GAG C-3'</td>
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<tr>
<td>GAPDH RT-PCR</td>
<td>Sense</td>
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<td></td>
<td>Anti-sense</td>
<td>5'-GGA TGC AGG GAT GAT GTT C-3'</td>
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<td>5'-TAG CTG TCA CCC CTC TGT GT-3'</td>
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<td>GAPDH qPCR</td>
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<td>5'-TGC ACC ACC AAC TGC TTA-3'</td>
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<tr>
<td></td>
<td>Anti-sense</td>
<td>5'-GGA TGC AGG GAT GAT GTT C-3'</td>
</tr>
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</table>

* The EcoRI site and the BamHI site are included in the sense and the antisense primer, respectively.

The transporter activity level of each expression system was determined based essentially on methods described previously [2-4]. Substrates and inhibitors are summarized in Table S2. The vehicle was sterile water or dimethylsulfoxide. The ETV and TFV uptake incubation times for each transporter were set at the same values employed for classical substrate uptake, which are: 3 min for OATP1B1, 5 min for OATP1B3, 3 min for OATP2B1, 1.5
min for OAT2, 2 min for OAT7, 1.5 min for OCT1, and 1.5 min for NTCP.

In kinetic parameter analysis, transporter activity levels were calculated by subtracting the value obtained from mock-cells from the value obtained from transporter-expressed cells. ETV concentrations used in this analysis were 0.5, 1, 10, 50, 100, and 200 μM. Transport kinetics were first evaluated using an Eadie-Hofstee plot, and then apparent $K_m$ and $V_{max}$ values were estimated by a computer program (DeltaGraph Ver 4.5, SPSS Inc., Chicago, IL), which is designed for non-linear regression analysis.

Using the same experimental procedures, equilibrative nucleoside transporter-mediated ETV or TFV uptake was examined using HepG2 cells. Adenosine (10 μM) and NBMPR (100 μM) were used as the model substrate and inhibitor, respectively. Incubation time was set at 1 min. HepG2 cells were cultured using the same method as that used for the HEK293 cells.

OAT1-mediated TFV uptake was examined using the transient expression assay. The OAT1 expression plasmid was transfected with HEK293 cells. The procedures used for the TFV transport assay was the same as those employed for other transporters.

Table S2. Summary of transporter substrates and inhibitors used in this study

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate (concentration)</th>
<th>Inhibitor (concentration)</th>
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<tr>
<td>OATP1B1</td>
<td>Estradiol-17β-D-glucuronide (100 nM)</td>
<td>Rifampicin (10 μM)</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>Cholecystokinin Octapeptide, sulfated (10 nM)</td>
<td>Bromosulfophthalein (100 μM)</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>Estrone-3-sulfate (50 μM)</td>
<td>Bromosulfophthalein (100 μM)</td>
</tr>
<tr>
<td>OAT1</td>
<td>Tenofovir (1 μM)</td>
<td>Not used</td>
</tr>
<tr>
<td>OAT2</td>
<td>cyclic GMP (2 μM)</td>
<td>Indomethacin (100 μM)</td>
</tr>
<tr>
<td>OAT7</td>
<td>Estrone-3-sulfate (1 μM)</td>
<td>Bromosulfophthalein (100 μM)</td>
</tr>
<tr>
<td>OCT1</td>
<td>1-methyl-4-phenylpyridinium (10 μM)</td>
<td>Tetraethylammonium (20 mM)</td>
</tr>
<tr>
<td>NTCP</td>
<td>Taurocholic acid (1 μM)</td>
<td>Cholic acid (100 μM)</td>
</tr>
<tr>
<td>All</td>
<td>Entecavir (1 μM)</td>
<td>Either one of the above</td>
</tr>
<tr>
<td>All</td>
<td>Tenofovir (1 μM)</td>
<td>Either one of the above</td>
</tr>
</tbody>
</table>

References cited in the supplemental materials


Fig. s1. Examination of ENT-mediated ETV or TFV uptake in HepG2 cells.

The ETV or TFV uptake activities of ENTs were examined using HepG2 cells. ENTs functions were confirmed using adenosine (10 μM) as a typical substrate. NBMPR (100 μM) is an ENTs inhibitor. Similarly, ENT-mediated ETV and TFV uptake by HepG2 cells was tested. Each value is the mean ± S.D. of transport activity for three independent experiments, each performed in duplicate.
**Fig. s2. Additional characterization of OAT2-mediated ETV uptake in OAT2/HEK.**

**A**, ETV uptake by OAT2/HEK was inhibited by BSP (100 μM). Each value is the mean ± S.D. of transport activity for three independent experiments, each performed in duplicate.

**B**, Kinetic parameters of OAT2-mediated ETV uptake were estimated using OAT2/HEK. Substrate concentrations were 0.5, 1, 10, 50, 100, and 200 μM. The assays were also performed using Mock/HEK, and each data was calculated by subtracting the activity obtained by Mock/HEK from that of OAT2/HEK. Each value is the mean ± S.D. of transport activity for three independent experiments, each performed in duplicate. Transport kinetics were first evaluated using an Eadie-Hofstee plot \[y = -193.17x + 1775.8, R^2 = 0.902\], and then apparent \(K_m\) and \(V_{max}\) values were estimated by a computer program (DeltaGraph Ver 4.5).
Fig. s3. Inhibitory effects of indomethacin on ETV uptake by primary human hepatocytes.

ETV (1 μM) uptake by pooled (5-donor) primary human hepatocytes were examined using suspension transport assays in the presence or absence of indomethacin (IDM, 50 μM), which is an OAT2 substrate/inhibitor. The assays were also performed at 4° C, and each data was calculated by subtracting the 4° C activity from that at 37° C. The value are expressed as relative uptake levels and represents the mean ± S.D. The experiments were repeated four times, each performed in duplicate.
TFV uptake activity was examined using HEK293 cells transiently expressing OAT1 (OAT1/HEK) and those transfected with empty vector (Mock/HEK). Each value is the mean ± S.D. of transport activity for three independent experiments, each performed in duplicate.

Fig. s4. Validation of TFV uptake by OAT1.
Fig. s5. Confirmation of transporter mRNA expression in HEK293 expression systems developed in this study.

**A**, Total RNA were isolated from OAT2/HEK, OCT1/HEK, NTCP/HEK and Mock/HEK, and their cDNA were synthesized. RT-PCR was performed to detect OAT2 mRNA, OCT1 mRNA or NTCP mRNA. GAPDH mRNA was used as an internal control. NTC, non-template control. The representative results from three independent experiments are shown.

**B**, The OAT2 mRNA expression level in OAT2/HEK was compared with that of pooled (5-donor) primary human hepatocytes by quantitative real-time PCR. GAPDH mRNA was used as an internal control. The value are expressed as relative mRNA levels and represents the mean ± S.D. The experiments were repeated three times, each performed in duplicate.