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Note

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

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ABSTRACT

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.

1. Introduction

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 10–40% 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

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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.

[^{3}H]-ETV (American Radiolabeled Chemicals, St. Louis, MO, USA) or [^{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

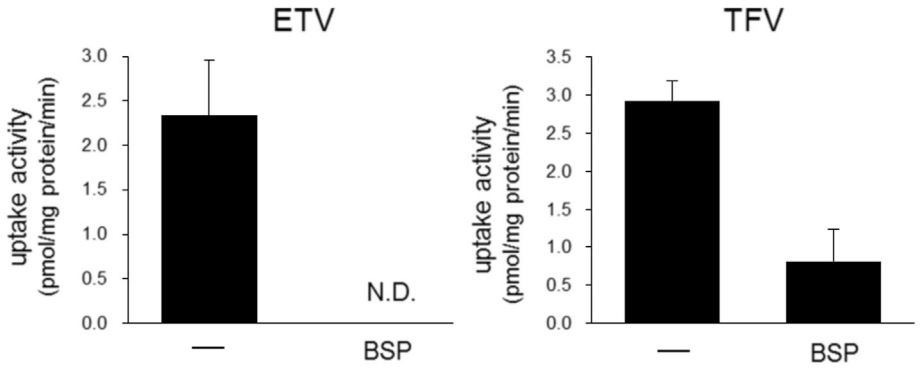

Fig. 1. ETV and TFV uptake by primary human hepatocytes. ETV (1 μM, left) and TFV (1 μM, right) uptake by pooled (5-donor) primary human hepatocytes were examined using suspension transport assays. BSP (200 μM) was used as an inhibitor of drug transporters. The assays were also performed at 4 °C, and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