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Cyclosporin derivatives inhibit hepatitis B virus entry without interfering with NTCP transporter activity

Satomi Shimura1,2, Koichi Watashi1,3,4,⇑, Kento Fukano1,5, Michael Peel2, Ann Sluder2, Fumihiro Kawai6, Masashi Iwamoto1,3, Senko Tsukuda1,7, Junko S. Takeuchi1, Takeshi Miyake6, Masaya Sugiyama9, Yuki Ogasawara5, Sam-Yong Park6, Yasuhiro Tanaka10, Hiroyuki Kusuhara8, Masashi Mizokami10, Camille Sureau11, Takaji Wakita1

1Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan; 2SCYNEXIS, Inc., Durham, NC 27713, USA; 3Department of Applied Biological Science, Tokyo University of Sciences, Noda 278-8510, Japan; 4Department of Analytical Biochemistry, Meiji Pharmaceutical University, Kiyose 204-8588, Japan; 5Drug Design Laboratory, Graduate School of Medical Life Science, Yokohama City University, Yokohama 230-0045, Japan; 6Micro-signaling Regulation Technology Unit, RIKEN Center for Life Science Technologies, Wako 351-0198, Japan; 7The University of Tokyo, Graduate School of Pharmaceutical Sciences, Tokyo 113-0033, Japan; 8The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa 272-8516, Japan; 9Department of Virology and Liver Unit, Nagoya City University Graduate School of Medalic Sciences, Nagoya 467-8601, Japan; 10Laboratoire de Virologie Moléculaire, Institut National de la Transfusion Sanguine (INTS), Paris, France

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Corresponding author. Address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel.: +81 3 5285 1111; fax: +81 3 5285 1161. E-mail address: kwatashi@nih.go.jp (K. Watashi).

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Background & Aims: The sodium taurocholate co-transporting polypeptide (NTCP) is the main target of most hepatitis B virus (HBV) specific entry inhibitors. Unfortunately, these agents also block NTCP transport of bile acids into hepatocytes, and thus have the potential to cause adverse effects. We aimed to identify small molecules that inhibit HBV entry while maintaining NTCP transporter activity.

Methods: We characterized a series of cyclosporine (CsA) derivatives for their anti-HBV activity and NTCP binding specificity using HepG2 cells overexpressing NTCP and primary human hepatocytes. The four most potent derivatives were tested for their capacity to prevent HBV entry, but maintain NTCP transporter function. Their antiviral activity against different HBV genotypes was analysed.

Results: We identified several CsA derivatives that inhibited HBV infection with a sub-micromolar IC50. Among them, SCY446 and SCY450 showed low activity against calcineurin (CN) and cyclophilins (CyPs), two major CsA cellular targets. This suggested that instead, these compounds interacted directly with NTCP to inhibit viral attachment to host cells, and have no immunosuppressive function.

Importantly, we found that SCY450 and SCY995 did not impair the NTCP-dependent uptake of bile acids, and inhibited multiple HBV genotypes including a clinically relevant nucleoside analog-resistant HBV isolate.

Conclusions: This is the first example of small molecule selective inhibition of HBV entry with no decrease in NTCP transporter activity. It suggests that the anti-HBV activity can be functionally separated from bile acid transport. These broadly active anti-HBV molecules are potential candidates for developing new drugs with fewer adverse effects.

Lay summary: In this study, we identified new compounds that selectively inhibited hepatitis B virus (HBV) entry, and did not impair bile acid uptake. Our evidence offers a new strategy for developing anti-HBV drugs with fewer side effects.

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Introduction

Hepatitis B virus (HBV) infection is a worldwide public health problem, which is estimated to chronically infect approximately 240 million individuals [1,2]. Chronic HBV infection elevates the risk of developing liver cirrhosis and hepatocellular carcinoma [3–5]. Current clinical treatments for HBV infection include interferons (IFN)s and nucleos(t)ide analogs (NAs) [6–8]. IFNxs and its pegylated form (PegIFNx) modulate host immune response to viral infection and directly inhibit HBV replication in hepatocytes. NAs, including adefovir, entecavir (ETV), lamivudine, telbivudine, and tenofovir, suppress HBV replication by inhibiting reverse transcription. These agents significantly improve the progression of HBV-associated pathogenesis; however, they rarely lead to complete elimination of HBV from infected cells. Treatment studies for human immunodeficiency virus (HIV) and hepatitis C virus
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(HCV) have shown that multidrug cocktails can greatly improve the clinical outcome [9,10]. To achieve better anti-HBV treatment in the future, the development of new anti-HBV agents targeting different steps of the HBV life cycle, and their application to multidrug treatment are needed.

Cyclosporin A (CsA), a well-known immunosuppressive agent classified as a calcineurin (CN) inhibitor, is clinically used for prevention of graft rejection after organ transplantation [11,12]. CsA primarily targets cellular peptidyl prolyl cis/trans-isomerase cyclophilins (CyPs), and suppresses their enzymatic activity. The resulting CsA/CyP complex subsequently binds CN to inhibit its phosphatase activity, which in turn inactivates NF-AT, an essential transcription factor for immune responses. In addition to this primary activity of CyPs- and CN inhibition, CsA is also reported to inhibit the transporter activity of membrane transporter families [13]. Thus far, CsA has been reported to inhibit the replication of numerous viruses, including HIV, HCV, HBV, herpesviruses, dengue virus, West Nile virus, human papillomavirus, and coronaviruses [14,15]. In most of these cases, CyP-inhibition is the principal mechanism of the antiviral activity, as CyPs play a critical role in the viral life cycles. We, and other groups, have recently reported that CsA inhibits HBV entry through targeting a membrane transporter, sodium taurocholate co-transporting polypeptide (NTCP) [16–18].

HBV enters into hepatocytes via specific interaction of the preS1 region in the HBV large surface protein with NTCP, a recently identified HBV entry receptor [19]. Thus far, NTCP has been specifically expressed on the hepatic basolateral membranes and functions for co-transporting bile acids with sodium ions into hepatocytes [20–25]. Identification of NTCP as an HBV receptor raises the entry process as an attractive target for drug development [26]. By inhibiting the entry step, subsequent formation of covalently closed circular DNA (cccDNA), a persistent viral reservoir that is difficult to eliminate by the current treatment, will be prevented. To date, several compounds have been reported to inhibit HBV infection by targeting NTCP, including myrcludex-B, iberitasarin, ezetimibe, ritonavir, vanitarcin A, and bile acids in addition to CsA [17,18,27–31]. However, all of these drugs can also inhibit the transporter function of NTCP and impair the sodium-dependent bile acid uptake, which may induce significant adverse effects: NTCP-deficient mice, and a patient carrying a defective polymorphism mutation in NTCP, exhibit an elevated level of serum bile acids, and develop the related pathologies including growth retardation and hypercholanemia [32,33]. Thus, it is a significant challenge to identify a compound that specifically inhibits HBV infection by targeting NTCP, without any effect on bile acid uptake.

In this study, we describe the identification of new CsA derivatives that inhibit HBV infection without affecting the transporter function of NTCP. Moreover, the anti-HBV activity can be achieved by compounds having minimal inhibition activity against CyPs and CN. Importantly, these compounds are effective against entry of multiple HBV genotypes and a clinically relevant NA-resistant HBV isolate. Non-immunosuppressive CsA derivatives, such as alisporivir, NIM811, and SCY-635, have been developed as anti-HCV candidates in clinical trial to phase II and III [14,34,35]. These findings suggest that CsA derivatives constitute a useful platform for the discovery of novel anti-HBV agents with specific activity but less adverse effects.

Materials and methods

Cell culture

HepG2-hNTCP-C4, Hep38.7-Tet, and HepG2.2.15.7 cells, and primary human hepatocytes (PhoenixBio) were cultured as described previously [16,36,37]. The parental HepG2 cells were purchased from ATCC. These cells were all confirmed to be mycoplasma-negative.

HBV preparation and infection

The HBV (genotype D) used in this study was mainly derived from the culture supernatant of Hep38.7-Tet cells as described previously [38]. HBV (genotype A, C, or A carrying the mutations L180M/S202I/M204V) was prepared from the culture supernatant of HepG2 cells transfected with the corresponding expression plasmid [39]. These inoculants contained both virions and nucleocapsids without envelopes, with ratio of 53:47 in Hep38.7-Tet-derived HBV (Supplementary Fig. 1). HBV infection was performed at 6000 (Fig. 1), 2000 (Fig. 6D and ‘B-D), or 1000 (Fig. 6F) genome equivalents (GEq)/cell (in which HBV GEq contains HBV DNA from both virions and nucleocapsids) in the presence of 4% PEG8000 as described previously [38].

Transporter assay

Bile acid uptake activity was measured in the presence or absence of sodium using HepG2-hNTCP-C4 cells and primary human hepatocytes, essentially as described [31]. Cells were preincubated with compounds at 37 °C for 15 min and then incubated with [3H]-taurocholic acid (TCA) in the presence of compounds at 37 °C for 15 min to allow substrate uptake into the cells. Radioactivity inside the cells was measured with a liquid scintillator.

Statistics

Statistical significance was determined using Student’s t test (*p <0.05, **p <0.01).

Detailed materials and methods are described in the Supplementary materials.

Results

Characterization of cyclosporin derivatives

We investigated the anti-HBV activity of synthesized CsA derivatives, designated as SCY series. HepG2-hNTCP-C4 cells, overexpressing the human NTCP gene in HepG2 cells and susceptible to HBV infection, were exposed to HBV in the presence or absence of these compounds for 16 h. After washing out free HBV inoculum and compounds, the cells were cultured for an additional 12 days in the absence of compounds, and HBV proteins were detected as an indicator for HBV infection. Hepatitis B surface (HBs) antigens secreted into the culture supernatant (Fig. 1A) and hepatitis B core (HBc) protein in the cells (Fig. 1C and D) at day 13 post infection were greatly reduced by preS1 peptide, a N-myristoylated peptide consisting of 2–48 aa of the preS1 region of the HBV large surface protein, as a positive control. Among the CsA derivatives, SCY806, SCY446, SCY450, SCY453, and SCY995 showed significant reduction of HBs and HBc, without causing cytotoxicity (Fig. 1A–D). In contrast, other CsA derivatives including SCYS52, SCY651, SCY660, SCY198, SCY506, and SCY640 did not show significant effect on HBV infection (Fig. 1A–D).

CsA is known to primarily bind to cellular cyclophilins (CyPs), in addition to the interaction with a phosphatase, calcineurin (CN), which is essential for its immunosuppressive function [15]. To characterize the activity of these new CsA derivatives,
we evaluated the effect of each derivative on the inhibitory activity against CN and CyPA. As shown in Table 1, some of the compounds including SCY806, SCY651, SCY446, SCY198, SCY450, SCY453, SCY995, SCY506, and SCY640 lost the ability for CN inhibition, indicating the elimination of immunosuppressive activity (Table 1). Further, derivatives such as SCY446, SCY198, SCY450, and SCY453 showed very weak or no binding to CyPA (Table 1). Drugs that cause immunosuppression may promote virus infection/replication in patients [40], so it is desirable that immunosuppression should be eliminated from new agents. Based on these results, we then focused on four CsA derivatives in the following study, including SCY806, SCY446, SCY450, and SCY995, which showed high anti-HBV activity but with no immunosuppressive ability.

**Four CsA derivatives are potent inhibitors against HBV infection**

To more precisely evaluate the anti-HBV activity of the new CsA derivatives, an HBV infection assay was performed with primary cultures of human hepatocytes, a more physiologically relevant model [36]. CsA and its derivatives (Fig. 2A) significantly decreased HBV infection in these cells, as monitored by HBV DNA and cccDNA in the cells at day 13 post infection (Fig. 2B and C). Hepatitis Be (HBe) antigens secreted into the culture supernatant were consistently reduced by treatment with the compounds in a dose-dependent manner (Fig. 2D). The half-maximal inhibitory concentrations (IC50) were calculated to be 0.5–2.0 μM, with SCY446, SCY450, and SCY806 were, by this order, more potent than CsA itself (Fig. 2D). All of these compounds did not show any significant cytotoxic effects in primary human hepatocytes, with >80 μM as the half-maximal cytotoxic concentrations (CC50) (Supplementary Fig. 2).

**CsA derivatives inhibit HBV entry by targeting NTCP**

The HBV life cycle is composed of multiple steps, including the early phase (attachment/entry, trafficking to the nucleus, and cccDNA formation) and the replication phase (transcription, nucleocapsid assembly, reverse transcription, envelopment, and release) [4,18]. To identify the step at which CsA derivatives are active within the HBV life cycle, we assessed the effect of these four CsA derivatives on both HBV entry and replication processes. HBV replication was evaluated using HepG2.2.15.7 cells, which
human hepatocytes were infected with HBV with or without various concentrations of indicated compounds (0.25, 0.5, 1, 2, 4, and 8 µM) for 16 h. HBV DNA (B) and cccDNA (C) in the cells were detected by real-time PCR analysis at day 16 post infection. (D) Dose-response curves for anti-HBV activity. Primary human hepatocytes were exposed to HBV with or without indicated compounds at 40 µM.(B, C) Primary human hepatocytes were exposed to HBV with or without compounds [DMSO 0.16%, preS1 peptide 100 nM, CsA and its derivatives (SCYs)]8. are permissive to HBV replication, but not the entry step[37], by treating the cultures with compounds for six days (Fig. 3A). HBV DNA released into the culture supernatant was significantly reduced by a nucleoside analog, entecavir, as a positive control, CsA and its derivatives inhibited the early phase of infection, as shown in Fig. 3B and C. It has been reported that CsA inhibits HBV entry by preventing attachment of preS1 to NTCP [17,18]. Therefore, we performed the preS1 binding assay to evaluate the effect of CsA and its derivatives on the preS1 attachment to host cells. In this assay, fluorescent-labeled preS1 peptide attached onto host cells was reduced by the treatment with CsA and its derivatives, SCY806, SCY446, SCY450, SCY995, but not by SCY582, an inactive CsA derivative on HBV infection (Fig. 3B; quantified data and C; picture). These results indicate that CsA and its derivatives inhibit preS1-mediated HBV attachment onto host cells.

We have reported that CsA interacted with NTCP on the cell surface of host hepatocytes [18]. We thus investigated whether CsA derivatives could bind to NTCP using surface plasmon resonance (SPR) analysis. As a control experiment, we confirmed that the recombinant NTCP protein used in this study (Supplementary Fig. 3; Materials and methods) could specifically interact with preS1 (2–48 aa) peptide in SPR analysis (Supplementary Fig. 4A) and in pull down assay (Supplementary Fig. 4B), and competitively inhibited the preS1 binding to host cells (Supplementary Fig. 4C), showing that this recombinant NTCP protein, at least in part of them, was functional. As shown in Fig. 4, each CsA derivative was shown to bind to recombinant NTCP protein in a dose-dependent manner (Fig. 4, left), while no significant interaction was observed using bovine serum albumin (BSA) as a negative control (Fig. 4, right). Thus, these CsA derivatives can interact with NTCP, similar to CsA.

Effects of CsA derivatives on NTCP transporter activity

To date, several anti-HBV entry inhibitors that interact with NTCP have been reported, including myrcludex-B, ezetimibe, iberasartan, 901T3, vanitarcin A, and bile acids in addition to CsA [1

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was evaluated with HepG2-hNTCP-C4 cells (Fig. 5A) and primary human hepatocytes (Fig. 5B) in the presence or absence of an excess amount of CsA or its derivatives (10µM) using [3H]-labeled taurocholic acid (TCA) as a substrate. As shown in Fig. 5A, a preS1 peptide and CsA decreased the sodium-dependent TCA uptake, which indicated NTCP transporter inhibitory activity, in HepG2-hNTCP-C4 cells, as reported previously [41,42,45] (Fig. 5A, dark blue bars). Among CsA derivatives, SCY446 showed an apparent reduction of the TCA uptake, while SCY806, SCY450, and SCY995 did not significantly affect NTCP activity (Fig. 5A, dark blue bars). In primary human hepatocytes, SCY806, in addition to SCY446, within the SCY series significantly reduced the bile acid uptake (Fig. 5B). The dose-response curves for inhibition of NTCP transporter activity indicate that SCY446 dramatically impaired the bile acid uptake as the case with CsA, while SCY995 did not show significant effect on the transport (Fig. 5C and Supplementary Fig. 5). Although there is still possibility that SCY995 can inhibit NTCP transporter at higher concentrations, it was clearly shown that the activity of SCY995 to NTCP transporter, if any, was much lower than CsA or SCY446 (IC50: 1.49, 1.92, and >25µM for CsA, SCY446, and SCY995, respectively). The above results reveal two types of anti-HBV entry inhibitors: those impairing bile acid uptake and those minimally affecting transporter function. This effect was not likely to be due to the enhancement of NTCP endocytosis, as these CsA derivatives did not apparently change the cell surface localization of NTCP (Supplementary Fig. 6C–F).

The bile acid transport activity of NTCP can be separated from its HBV entry function

Our data suggest that certain compounds can inhibit HBV entry without significantly affecting the transporter function of NTCP. So far it was suggested that molecular determinants of NTCP critical for HBV entry overlap with those required for bile acid transport. To further confirm whether the function for bile acid transport and the capacity to support HBV entry can be distinguishable, we searched for compounds that bind NTCP and inhibit its function as a bile acid transporter but cannot inhibit HBV infection. Sulfobromophthalein is known to function as a substrate of NTCP that interacts with the bile acid pocket of NTCP [46]. As shown in Fig. 6A and Supplementary Fig. 7, sulfobromophthalein

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**Fig. 4. Interaction of CsA derivatives with recombinant NTCP protein by surface plasmon resonance (SPR) binding analysis.** CsA derivatives were injected over a sensor chip with immobilized recombinant NTCP (left) or BSA (right), and real-time binding profiles for increasing concentrations of compounds (red: 500µM, pink: 100µM, light blue: 50µM, dark blue: 5µM, purple: 1µM) are analyzed as shown in the materials and methods. Compound-free buffer was injected after 120 s.

**Fig. 5. Anti-HBV CsA derivatives, SCY450 and SCY995, had no significant effect on NTCP transporter activity.** (A) Taurocholic acid (TCA) uptake activity of HepG2-hNTCP-C4 cells was measured with or without indicated compounds (DMSO 0.2%, preS1 peptide 100 nM, CsA and SCY compounds 10 µM) either in a sodium-free (light blue bars) or containing buffer (dark blue bars). (B) Sodium-dependent TCA uptake in primary human hepatocytes was assessed with or without the indicated compounds in a sodium-containing buffer. (C) Dose-response curves for CsA and its derivatives on NTCP transporter activity. NTCP transporter activity of HepG2-hNTCP-C4 cells was quantified as shown in (B) at various concentrations of compounds (0, 0.625, 1.25, 2.5, 5 and 10 µM). IC50 values calculated for each compound are also shown above the graphs.
mophthalein impaired the bile acid uptake in sodium-containing but not sodium-free condition in HepG2-hNTCP-C4 cells, and it did not affect bile acid uptake of NTCP-deficient HepG2 cells. This suggests that sulfobromophthalein inhibited the NTCP-mediated bile acid transport, as reported previously [47] (Fig. 6A and Supplementary Fig. 7). However, this compound did not inhibit HBV infection under the same concentration conditions (Fig. 6B). Fur-

Discussion

The finding that NTCP functions as an HBV entry receptor has enabled discovery efforts to identify agents that specifically inhibit HBV entry. To date, myrcludex-B, CsA, irbesartan, ezetimibe, ritonavir, and vanitaracin A have been reported to inhibit HBV infection by directly targeting NTCP [17,18,27–29,31,41–45]. However, all of these agents have the potential to inhibit the function of NTCP for uptake of bile acids into hepatocytes. Although the IC50 of myrcludex-B for inhibiting the NTCP transporter is less than that for HBV entry inhibition, and it is thus possible to seek the window to inhibit HBV infection without significant loss of transporter function [49], other small molecules shown above have similar IC50 for inhibiting the transporter to those for HBV inhibition. Therefore, seeking small molecules that inhibit HBV infection without affecting the NTCP transporter activity is one of the major challenges of exploiting entry inhibition as a safe and effective therapy for HBV.

Previously, point mutation analyses within the NTCP gene suggested a close relationship between its transporter activity and the viral receptor function. Any substitution of amino acid residues related to the transporter function, including sodium binding (Q68, S105, N106, E257, and Q261), or bile acid binding (N262, Q293, and L294), were found to reduce or completely abolish the capacity of NTCP to bind preS1 and support HBV entry [45]. These results suggested that the residues essential for the transporter function are also utilized by HBV for entry into host cells. In addition, previously reported inhibitors of NTCP transport activity were found to reduce the capacity of NTCP to support HBV infection. Until now, there is no report clearly demonstrating that the two functionalities of NTCP can be distinguished. The possible mode of mechanism for cyclosporins–NTCP interaction is discussed in the Supplementary note of the Supplementary materials. In the present study, we identified CsA derivatives (SCy450 and SCy995) that can inhibit HBV entry without affecting the NTCP transport activity, with equivalent, or even higher, anti-entry activity than the parental CsA. Thus, by minimizing the side-effect on NTCP function in bile acid transport, it is possible to develop compounds with acceptable side-effect profiles. Strong support for the concept of separating the antiviral activity of a compound from its inhibitory effects on the original function of the receptor is found among antiviral drugs against HIV. Maraviroc and TAK-220 both target an HIV coreceptor CCR5 and inhibit HIV entry with comparable potencies. While Maraviroc also inhibits the binding of CCR5 to its cognate ligands (RANTES, MIP-1α, and MIP-1β) [50], TAK-220 does not affect binding of CCR5 to MIP-1β [51]. CsA is also reported to have a potential but weak to inhibit the activity of apical sodium-dependent bile acid transporter (ASBT), also known as NTCP2, another family transporter working for bile acid uptake [52]. Con-

do in the experiments shown above (Fig. 1–6), we used a genotype D isolate. Here, in addition to the effect on HBV genotype D, we show that the new CsA derivatives inhibited infection by HBV genotypes A, B, and C (Fig. 7B–D). Importantly, these compounds also inhibited the infection of a clinically relevant entecavir-resistant HBV, carrying mutations rtL180M/S202I/ M204V [31] (Fig. 7E). Thus, CsA derivatives demonstrate considerable potential for the development of pan-genotypic anti-HBV agents.

CsA derivatives show pan-genotypic anti-HBV effects

We further examined the effect of CsA derivatives on hepatitis D virus (HDV), which enters host hepatocytes through NTCP [19,48]. As shown in Fig. 7A, HDV infection of HepG2-hNTCP-C4 cells was significantly reduced in the presence of CsA or its derivatives (Fig. 7A), consistent with the essential role of NTCP in HDV entry.

To assess the antiviral potential of CsA derivatives, we investigated the anti-HBV activity against different genotypes of HBV, and against an entecavir-resistant HBV in primary human hepato-

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Fig. 6. Two functions of NTCP, transporting bile acids and supporting HBV infection, are separable. (A, B) An NTCP-targeting agent, sulfobromophthalein inhibited NTCP transporter activity but had no effect on HBV infection. (A) NTCP transporter activity of HepG2-hNTCP-C4 cells was measured as shown in Fig. 5 with or without compounds (preS1 peptide 100 nM, sulfobromophthalein 40 μM (light blue) and 100 μM (dark blue)). (B) HBV infection assay was performed as shown in Fig. 1 with or without compounds. (C, D) NTCP transporter activity depends on sodium concentration. TCA uptake of HepG2-hNTCP-C4 cells was significantly reduced in the presence of CsA or its derivatives. The possible mode of mechanism for cyclosporins–NTCP interaction is discussed in the Supplementary note of the Supplementary materials. In the present study, we identified CsA derivatives (SCy450 and SCy995) that can inhibit HBV entry without affecting the NTCP transport activity, with equivalent, or even higher, anti-entry activity than the parental CsA. Thus, by minimizing the side-effect on NTCP function in bile acid transport, it is possible to develop compounds with acceptable side-effect profiles. Strong support for the concept of separating the antiviral activity of a compound from its inhibitory effects on the original function of the receptor is found among antiviral drugs against HIV. Maraviroc and TAK-220 both target an HIV coreceptor CCR5 and inhibit HIV entry with comparable potencies. While Maraviroc also inhibits the binding of CCR5 to its cognate ligands (RANTES, MIP-1α, and MIP-1β) [50], TAK-220 does not affect binding of CCR5 to MIP-1β [51]. CsA is also reported to have a potential but weak to inhibit the activity of apical sodium-dependent bile acid transporter (ASBT), also known as NTCP2, another family transporter working for bile acid uptake [52]. Con-
sistentlly, SCY450 and SCY995, as well as CsA, did not show significant reductions in ABST transporter by 10 μM (Supplementary Fig. 8), excluding the possibilities that these two derivatives are more potent ASBT inhibitors than CsA.

As immunosuppression induced by compounds is likely to facilitate viral infection/replication in vivo [40], it is desirable that the immunosuppressive activity of CsA be removed. This was achieved during the development of alisporivir, SCY-635, and NIM811, as non-immunosuppressive cyclophilin inhibitors as anti-HCV drugs [34,35,53]. Most of the CsA derivatives examined in this present study were deficient in calcineurin inhibition, which is essential for the immunosuppressive activity, but still exerted anti-HBV activity. Interestingly, some of these compounds were also found to have weak inhibition on cyclophilins, including SCY446 and SCY450. This modification may be preferable from the aspect of further improving the specificity of the compounds activity.

Importantly, these compounds are effective against different HBV genotypes and clinically relevant entecavir-resistant HBV isolate. Moreover, these agents are expected to raise less frequent drug resistant viral species as they target a cellular factor. Thus, our study showed the advantage of CsA derivatives for developing new antiviral agents that are potent, well-tolerated, and broadly active inhibitors of HBV and HDV infection. Further in depth derivative analyses, including the antiviral activity, toxicity, and pharmacokinetic and dynamic properties in vivo, are expected.

Authors’ contributions

S.S. performed research, analyzed data, and wrote the paper; K. W. designed research, performed research, analyzed data, and wrote the paper; K.F., M.P, A.S. performed research, analyzed data; F.K., M.S., S.Y.P., Y.T., H.K., M.M., C.S. contributed reagents or analytic tools; M.L., S.T., J.S.T., T.M. performed research; Y.O., T.W., analyzed data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2016.11.009.

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Conflict of interest

S.S., M.P., and A.S. are employees of SCYNEXIS, Inc. All other authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.
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