Kondo Y, Iwao T, Yoshihashi S, Mimori K, Ogihara R, Nagata K, Kurose K, Saito M, Niwa T, Suzuki T, Miyata N, Ohmori S, Katsunori Nakamura K, **Matsunaga T**	Histone deacetylase inhibitor valproic acid promotes the differentiation of human induced pluripotent stem cells into hepatocyte-like cells.	PLoS One.	9(8)	e104010	2014
Katsuda T, Kurata H, Tamai R, Banas A, Ishii T, Ishikawa S, **Ochiya T**.	The in vivo evaluation of the therapeutic potential of human adipose tissue-derived mesenchymal stem cells for acute liver disease.	Methods Mol Biol.	1213	57-67	2014
Ogawa M, **Fukasawa M**, Satoh M, Hanada K, Saijo M, Uchiyama T, Ando S.	The intracellular pathogen Orientia tsutsugamushi responsible for scrub typhus induces lipid droplet formation in mouse fibroblasts.	Microbe Infect.	16(11)	962-966	2014
Ohtaki H, Ito H, Ando K, **Ishikawa T**, Hoshi M, Ando T, Takamatsu M, Hara A, Moriwaki H, Saito K, Seishima M.	Kynurenine production mediated by indoleamine 2,3-dioxygenase aggravates liver injury in HBV-specific CTL-induced fulminant hepatitis.	Biochim Biophys Acta.	1842(9)	1464-1471	2014
*Maeda S, Wada H, Naito Y, Nagano H, Simmons S, Kagawa Y, Naito A, Kikuta J, Ishii T, Tomimaru Y, Hama N, Kawamoto K, Kobayashi S, Eguchi H, Umeshita K, Ishii H, Doki Y, Mori M, **Ishii M**.	Interferon-α Acts on the S/G2/M Phases to Induce Apoptosis in the G1 Phase of an IFNAR2-Expressing Hepatocellular Carcinoma Cell Line.	J Biol Chem.	289(34)	23786-795	2014

Ninomiya M, **Kondo Y**, Niihori T, Nagashima T, Kogure T, Kakazu E, Kimura O, Aoki Y, Matsubara Y, Shimosegawa T.	Sequential analysis of amino acid substitutions with hepatitis B virus in association with nucleoside/nucleotide analog treatment detected by deep sequencing.	Hepatol Res.	44(6)	678-684	2014
***Kondo Y** and Shimosegawa T.	Significant roles of regulatory T cells and myeloid derived suppressor cells in hepatitis B virus persistent infection and hepatitis B virus-related HCCs.	Int J Mol Sci.	16(2)	3307-3322	2015
*Chuma M, Terashita K, **Sakamoto N**.	New molecularly targeted therapies against advanced hepatocellular carcinoma: From molecular pathogenesis to clinical trials and future directions.	Hepatol Res.	in press		2014
*Tsunematsu S, Chuma M, Kamiyama T, Miyamoto N, Yabusaki S, Hatanaka K, Mitsuhashi T, Kamachi H, Yokoo H, Kakisaka T, Tsuruga Y, Orimo T, Wakayama K, Ito J, Sato F, Terashita K, Nakai M, Tsukuda Y, Sho T, Suda G, Morikawa K, Natsuizaka M, Nakanishi M, Ogawa K, Taketomi A, Matsuno Y, **Sakamoto N**.	Intratumoral artery on contrast-enhanced computed tomography imaging: differentiating intrahepatic cholangiocarcinoma from poorly differentiated hepatocellular carcinoma.	Abdom Imaging.	in press		2015

*Tsukuda S, **Watashi K**, Iwamoto M, Suzuki R, Aizaki H, Okada M, Sugiyama M, Kojima S, **Tanaka Y**, Mizokami M, Li J, Tong S, Wakita T.	Dysregulation of retinoic acid receptor diminishes hepatocyte permissiveness to hepatitis B virus infection through modulation of sodium taurocholate cotransporting polypeptide (NTCP) expression.	J Biol Chem.	290(9)	5673-5684	2015
*Ogura N, **Watashi K**, Noguchi T, Wakita T.	Formation of covalently closed circular DNA in Hep38.7-Tet cells, a tetracycline inducible hepatitis B virus expression cell line.	Biochem Biophys Res Commun.	452(3)	315-321	2014

Ⅳ. 研究成果の刊行物・別冊

Cyclosporin A and Its Analogs Inhibit Hepatitis B Virus Entry Into Cultured Hepatocytes Through Targeting a Membrane Transporter, Sodium Taurocholate Cotransporting Polypeptide (NTCP)

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Chronic hepatitis B virus (HBV) infection is a major public health problem worldwide. Although nucleos(t)ide analogs inhibiting viral reverse transcriptase are clinically available as anti-HBV agents, emergence of drug-resistant viruses highlights the need for new anti-HBV agents interfering with other targets. Here we report that cyclosporin A (CsA) can inhibit HBV entry into cultured hepatocytes. The anti-HBV effect of CsA was independent of binding to cyclophilin and calcineurin. Rather, blockade of HBV infection correlated with the ability to inhibit the transporter activity of sodium taurocholate cotransporting polypeptide (NTCP). We also found that HBV infection-susceptible cells, differentiated HepaRG cells and primary human hepatocytes expressed NTCP, while nonsusceptible cell lines did not. A series of compounds targeting NTCP could inhibit HBV infection. CsA inhibited the binding between NTCP and large envelope protein *in vitro*. Evaluation of CsA analogs identified a compound with higher anti-HBV potency, having a median inhibitory concentration <0.2 μM. *Conclusion*: This study provides a proof of concept for the novel strategy to identify anti-HBV agents by targeting the candidate HBV receptor, NTCP, using CsA as a structural platform. (HEPATOLOGY 2014;59:1726-1737)

Hepatitis B virus (HBV) infection is a substantial public health problem, affecting ~350 million people worldwide.[1-3] HBV-infected patients have an elevated risk for developing liver cirrhosis and hepatocellular carcinoma. Currently, clinical treatment for HBV infection includes interferon alpha (IFN-α) and nucleos(t)ide analogs. IFN-α therapy yields long-term clinical benefit in only less than 40% of patients and can cause significant side effects. Nucleos(t)ide analog treatment can suppress HBV replication and is accompanied by substantial biochemical and histological improvement; however, it may select for drug-resistant viruses, which limit the efficacy of long-term treatment. To overcome these problems, the development of new anti-HBV agents targeting a different step of the HBV life cycle is urgently needed.

As HBV has only one viral gene encoding an enzymatic activity, the polymerase, there is no apparent strategy to develop a new class of antiviral agents other than polymerase inhibitors. Hence, it is important to define alternative molecular targets for anti-HBV agents as well as to identify potential anti-HBV

Abbreviations: CN, calcineurin; CsA, cyclosporin A; CyPs, cyclophilins; HBs, viral envelope protein; HBV, hepatitis B virus; HCV, hepatitis C virus; HCVpp, HCV pseudoparticles; IFN, interferon; LHBs, large envelope protein; MDR, multidrug resistance; MHBs, middle envelope protein; MRP, MDR-related protein; NTCP, sodium taurocholate cotransporting polypeptide; PHH, primary human hepatocytes; PPIase, peptidyl prolyl cis/trans-isomerase; SHBs, small envelope protein; TCA, taurocholic acid; TUDCA, tauroursodeoxycholic acid.

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compounds.[3,4] Myrcludex-B is a peptide mimicking pre-S1, which is crucial for the virus-cell membrane interaction. Pretreatment with this peptide has been shown to prevent virus entry and spread of virus infection.[5,6] Phenylpropenamide derivatives and heteroarylpyrimidines (HAP) suppressed HBV replication through capsid disassembly.[7-10] Although the development of the former was discontinued because of significant toxicity,[3] HAP exhibited anti-HBV efficacy in the absence of robust toxicity.[8,10] Deoxynojirimycin derivatives are iminosugars that inhibit alpha-glucosidases. Although treatment with these compounds suppressed HBV secretion in both cell culture and mouse models,[11,12] further investigation will be required to assess their anti-HBV efficacy and the specificity to HBV. Thus, it is an attractive strategy to identify a cellular factor that is specifically involved in HBV infection and relevant for the development of anti-HBV agents.

Cyclosporin A (CsA) is an immunosuppressant clinically used for suppression of the immunological failure of xenograft tissues. CsA primarily targets cellular peptidyl prolyl cis/trans-isomerase (PPIase) cyclophilins (CyPs).[13] The resultant CsA/CyP complex subsequently binds to and inhibits calcineurin (CN), a phosphatase that dephosphorylates nuclear factor of activated T cell (NF-AT) to allow nuclear translocation and transactivation of downstream genes. This CN inhibition contributes to the suppression of immune responses. In addition, CsA is known to inhibit the transporter activity of membrane transporters, including the multidrug resistance (MDR) and MDR-related protein (MRP) families.[14] Previously, we demonstrated that CsA and its nonimmunosuppressive derivatives suppress hepatitis C virus (HCV) replication,[15,16] with the anti-HCV activity being mediated by the inhibition of CyPs.[17-19] Currently, a series of drugs classified as CyP inhibitors are in clinical development for treatment of HCV-infected patients.[20,21]

In this study we report that CsA and its analogs inhibited HBV entry through a CyP-independent mechanism. We established a screening system that can identify small molecules inhibiting HBV entry. Screening in this system revealed that CsA blocked HBV entry. The anti-HBV activity of CsA was not correlated with binding to CyPs and CN. CsA inhibited the transporter activity of sodium taurocholate cotransporting polypeptide (NTCP), a recently reported candidate for the HBV entry receptor,[22] and interrupted the binding between NTCP and large envelope protein *in vitro*. Other NTCP inhibitors also blocked HBV infection. Analog testing identified CsA-related compounds with higher anti-HBV potency than CsA. Thus, CsA and NTCP inhibitors can be used as a platform to develop a novel class of anti-HBV agents.

Materials and Methods

Cell Culture. HepaRG (Biopredic), HepAD38 (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center), and primary human hepatocytes (PHHs) (Phoenixbio) were cultured as described previously.[23]

HBV Preparation and Infection. The HBV used in this study was mainly derived from the culture supernatant of HepAD38 cells. HBV infection was performed as described previously.[23] More detailed procedures are given in the Supporting Information.

Indirect Immunofluorescence Analysis, Real-Time Polymerase Chain Reaction (PCR), Southern Blot Analysis, 3-(4,5-Dimethylthiazol-2-yl)−2,5-diphenyltetrazolium Bromide (MTT) Assays, and Reporter Assays. Indirect immunofluorescence analysis, real-time PCR, southern blot analysis, MTT assays, and reporter assays were performed essentially as described.[23] More detailed procedures are given in the Supporting Information.

Detection of HBs and HBe Antigens. HBs antigen was quantified by enzyme-linked immunosorbent assay (ELISA) as described previously.[23] HBe antigen was detected by a Chemiluminescent Immuno-Assay (Mitsubishi Chemical Medience).

HCV Pseudoparticle Assay. The HCV pseudoparticles (HCVpp), which reproduce HCV envelope-mediated entry, were generated by transfecting the

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Additional Supporting Information may be found in the online version of this article.

expression plasmids for MLV Gag-Pol, HCV E1E2, and a luciferase that can be packaged into the virion (kindly provided by Dr. Francois-Loic Cosset at the University of Lyon) into 293T cells. HCVpp recovered from the culture supernatant of transfected cells were used in a HCV entry assay as described previously.[24]

Transporter Assay. The transporter activity of NTCP was assayed essentially as described[25] using 293 (Sekisui Medical) and HepG2 cells permanently overexpressing human NTCP. Briefly, the cells were preincubated with compounds at 37°C for 15 minutes and then incubated with radiolabeled substrate, [^{3}H]taurocholic acid (TCA), at 37°C for 5 minutes to allow substrate uptake into the cells. The cells were then washed and lysed to measure the accumulated radioactivity. In this assay, we did not observe cytotoxic effects of compounds at any of the concentrations tested. More detailed procedures are given in the Supporting Information.

AlphaScreen Assay. Recombinant NTCP and HBs proteins, which were tagged with 6xHis and biotin, respectively, were synthesized using a wheat cell-free protein system as described previously.[26] Protein-protein interactions were detected using the AlphaScreen IgG (ProteinA) detection kit (PerkinElmer) according to the manufacturer's instruction. Briefly, the recombinant tagged proteins were incubated with streptavidin-coated donor beads and anti-6xHis antibody-conjugated acceptor beads that generate a luminescence signal when brought into proximity by binding to interacting proteins. Luminescence was analyzed with the AlphaScreen detection program of an Envision spectrophotometer (PerkinElmer). More detailed procedures for the AlphaScreen assay are described in the Supporting Information.

Additional experimental procedures are included in the Supporting Information.

Results

Cyclosporin A Blocked HBV Infection. We focused on HBV entry and established a cell culture system to evaluate this step in HBV infection. To identify small molecules inhibiting HBV entry, we pretreated HepaRG cells[27] with compounds for 2 hours, then added a HBV inoculum and continued incubation with compounds for 16 hours (Fig. 1A). After washing out free HBV and compounds, the cells were cultured for an additional 12 days in the absence of compounds (Fig. 1A). For robust chemical screening, HBV infection was monitored by the viral envelope protein (HBs) level secreted from the infected cells at 12 days postinfection by ELISA. This assay could identify heparin, an HBV attachment inhibitor,[28,29] and bafilomycin A1, a v-type H^+ ATPase inhibitor that blocks acidification of vesicles and HBV entry,[30] but not lamivudine, a reverse transcriptase inhibitor,[31] as compounds reducing HBs protein level in the medium (Fig. 1B). In addition, use of an anti-HBs antibody to neutralize viral entry, but not use of an anti-FLAG antibody, reduced viral protein secreted from the HBV-infected cells (Fig. 1B). Thus, this system is likely to evaluate the effect of compounds on the early phase of the HBV life cycle, including attachment and entry, but not effects on HBV replication. A chemical screen with this system revealed that CsA reduced HBs secretion from HBV-infected cells (Fig. 1B). Treatment with CsA significantly decreased HBc protein expression (Fig. 1C) and HBV DNA as well as cccDNA (Fig. 1D) in the cells and HBe in the medium (Fig. 1E), without causing cytotoxicity (Supporting Fig. S1A). This effect of CsA was not limited to infection of HepaRG cells, as we observed a similar anti-HBV effect of CsA for PHHs (Fig. 1F). The anti-HBV effect of CsA was also observed on HBV infection of PHHs in the absence of PEG8000 (Fig. S1B), indicating that the effect of CsA did not depend on PEG8000, which was normally included in the HBV infection experiments. These data suggest that CsA blocked HBV infection.

Effect of Cyclosporin A on HBV Entry. CsA decreased HBs and HBe secreted from the infected cells in a dose-dependent manner (Fig. 2A). We next investigated which step in the HBV life cycle was blocked by CsA. The HBV life cycle can be divided into two phases: the early phase of infection including attachment, entry, nuclear import, and cccDNA formation, and the following late phase representing HBV replication that includes transcription, assembly, reverse transcription, and viral release.[32] Lamivudine drastically decreased HBV DNAs in HepAD38 cells,[33] which reproduce HBV replication but not the early phase of infection (Fig. 2B). In addition, continuous treatment with lamivudine as well as entecavir and interferon-α for 4 days after HBV infection could decrease HBV DNA levels in HBV-infected HepaRG cells, which suggests an inhibition of HBV replication (Fig. 2C). Nevertheless, lamivudine did not show an anti-HBV effect when applied only prior to and during HBV infection (Fig. 1A,B), suggesting that the anti-HBV compounds identified in Fig. 1A interrupted the early phase of the HBV life cycle.

We then examined whether CsA inhibited attachment or entry. For evaluating HBV attachment,[34] cell surface HBV DNA was extracted and quantified from HepaRG cells exposed to HBV at 4°C for 3 hours and

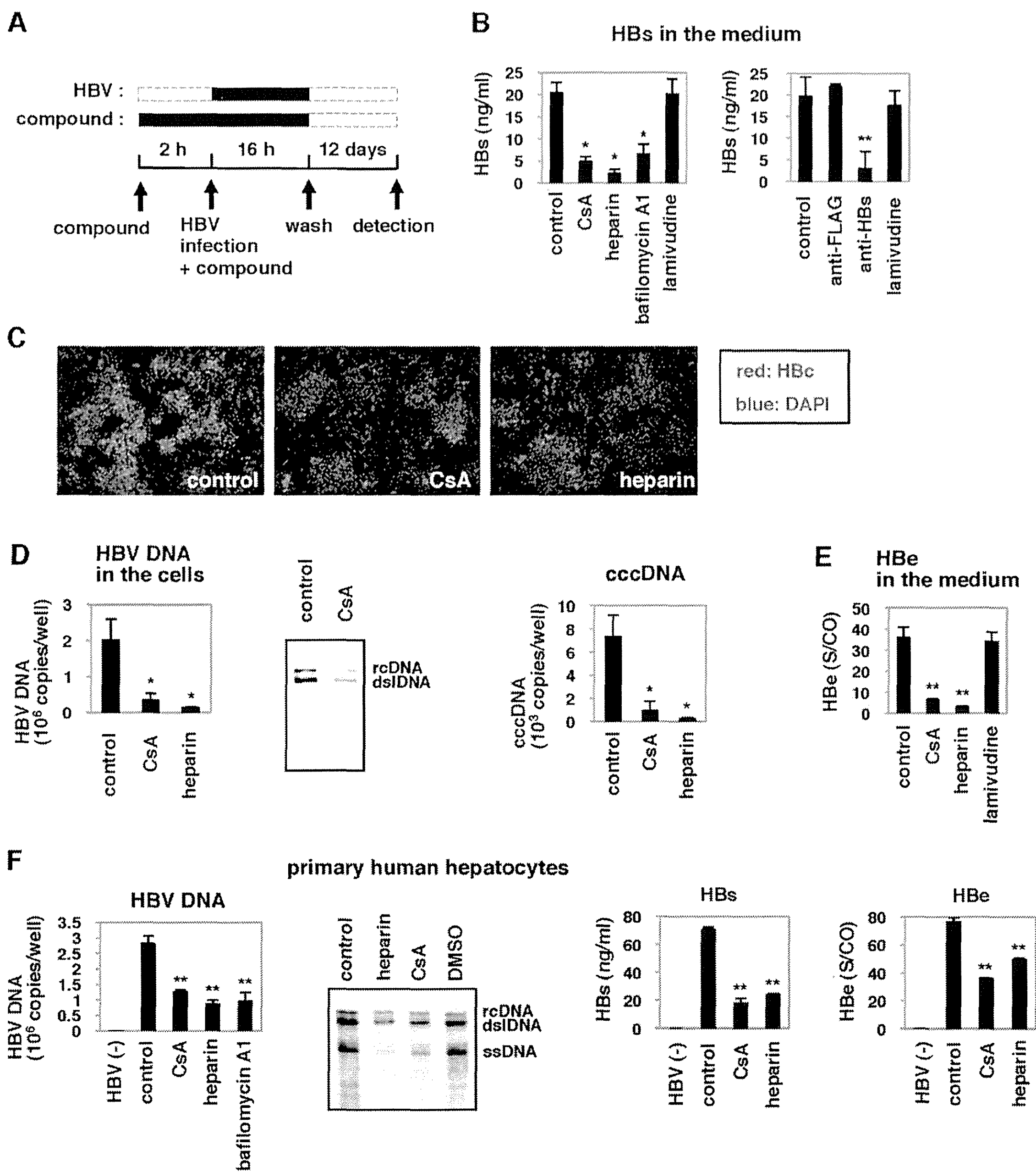


Fig. 1. Cyclosporin A (CsA) blocked HBV infection. (A) Schematic representation of the schedule for exposing HepaRG cells to compounds and HBV. HepaRG cells were pretreated with compounds for 2 hours and then inoculated with HBV for 16 hours. After washing out the free HBV and compounds, the cells were cultured with the medium in the absence of compounds for an additional 12 days to quantify HBs protein secreted from the infected cells into the medium. Black and dotted bars indicate the interval for treatment and without treatment, respectively. (B) CsA 4 μM, heparin 25 U/mL, bafilomycin A1 200 nM, lamivudine 1 μM, anti-FLAG 10 μg/mL, and anti-HBs antibody 10 μg/mL, were tested for effect on HBV infection according to the protocol shown in (A). (C-E) HBc protein (C), HBV DNAs, and cccDNA (D) in the cells as well as HBe antigen in the medium (E) at 12 days postinfection according to the protocol shown in (A) were detected by immunofluorescence, real-time PCR analysis, southern blot, and ELISA. Red and blue in (C) show the detection of HBc protein and nuclear staining, respectively. (F) PHHs were treated with the indicated compounds and infected with HBV using the protocol shown in (A). The levels of HBV DNAs in the cells, as well as of HBs and HBe antigens in the medium, were determined. Statistical significance was determined using the Student *t* test (*$P < 0.05$, **$P < 0.01$).

then washed (Fig. 2D-a). For the internalization assay,[34] the above cells, after washing, were further cultured at 37°C for 16 hours to allow HBV to internalize into the cells, and then trypsinized to digest HBV remaining on the cell surface to allow quantification of internalized HBV DNA (Fig. 2D-b). CsA slightly reduced the amount of attached HBV DNA, although the effect was not statistically significant

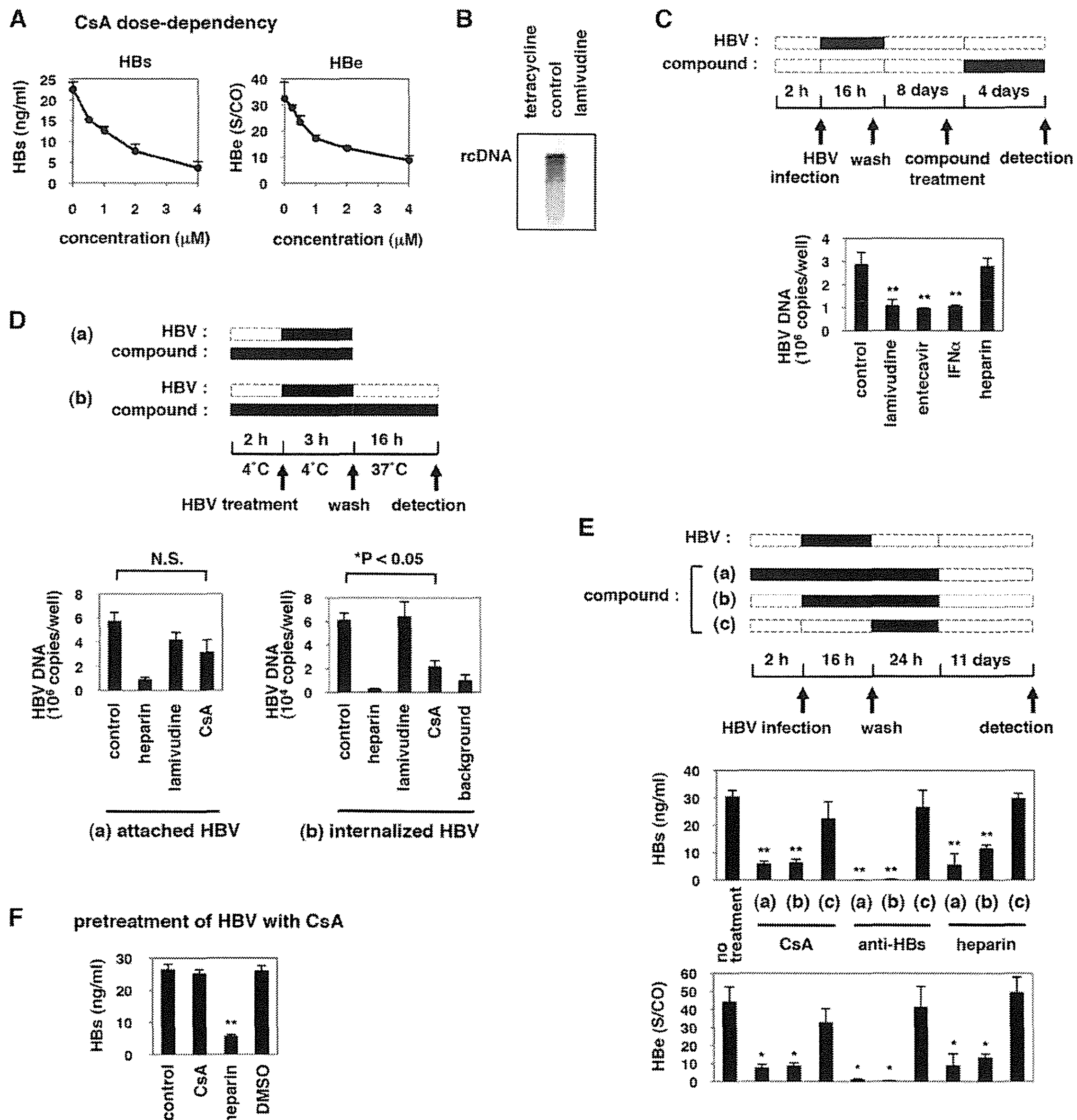


Fig. 2. CsA reduced internalized HBV. (A) HepaRG cells were treated with or without various concentrations of CsA (0.5, 1, 2, and 4 μM) as shown in Fig. 1A. HBV infection was monitored by HBs and HBe secretion. (B) HBV DNA in core particles was detected by southern blot analysis of DNA extracts from HepAD38 cells treated for 6 days with or without tetracycline 0.5 μg/mL and lamivudine 1 μM. (C) Upper scheme indicates the treatment schedule of HepaRG cells with compounds and HBV. HepaRG cells were infected with HBV for 16 hours. After washing out the input virus, cells were cultured in the absence of compounds for 8 days. The cells were then cultured with compounds (lamivudine 1 μM, entecavir 1 μM, IFN-α 100 IU/mL, or heparin 25 U/mL) for 4 days and recovered for detection of HBV DNA. Black and dotted boxes indicate the periods with and without treatment, respectively. Lower graph shows the quantified relative HBV DNA level in cells treated according to the above scheme. (D) Upper scheme shows the experimental procedure for examining the attached and internalized HBV. (a) The cells were pretreated with compounds (heparin 25 U/mL, lamivudine 1 μM, or CsA 4 μM) at 4°C for 2 hours and then treated together with HBV at 4°C for 3 hours to allow HBV attachment to the cells. After washing out the free virus, cell surface HBV DNA was extracted and quantified by real-time PCR. (b) After attachment of HBV at 4°C for 3 hours and the following wash, the cells were cultured in the presence or absence of compounds at 37°C for 16 hours to allow the cells to internalize bound HBV. The cells were then trypsinized and extensively washed prior to quantifying the cellular HBV DNA. The lower graphs show the level of HBV DNA attached to the cells (a) and internalized inside the cells (b). "Background" in (b) indicates the signal from cells incubated at 4°C, instead of 37°C, for 16 hours after washing out the virus in (b), which shows the background signal level of the assay. (E) The upper scheme shows the procedure for the time of addition experiment. Compounds (CsA 4 μM, anti-HBs antibody 10 μg/mL, or heparin 25 U/mL) were applied beginning 2 hours prior to HBV infection (a), beginning during HBV infection (b), or beginning immediately after HBV infection (c) until 24 hours postinfection. HBs and HBe protein secretion were measured at 12 days postinfection. Middle and lower graphs indicate HBs and HBe secretion, respectively, from the cells treated according to the above scheme. (F) Preincubation of HBV with compounds. HBV was preincubated with the indicated compounds for 30 minutes at 37°C. Compounds were then removed by ultrafiltration. The recovered compound-treated HBV was used to infect HepaRG cells (16 hours incubation), and HBV infection was monitored with HBs antigen secreted into the medium at 12 days postinfection. *$P < 0.05$, **$P < 0.01$, N.S., not significant.

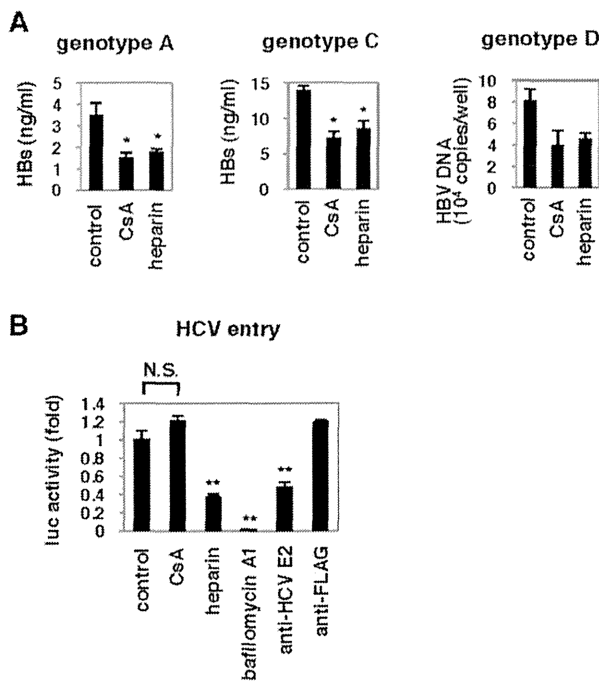
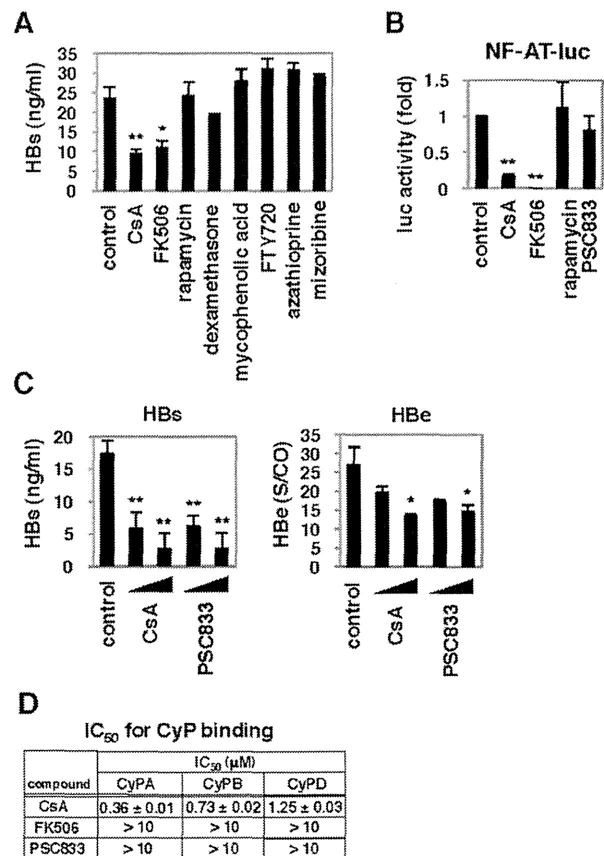


Fig. 3. CsA showed a pan-genotypic anti-HBV effect. (A) PHHs were treated with compounds (CsA 4 μM or heparin 25 U/mL) according to the scheme in Fig. 1A with different genotypes of HBV inoculum, and either HBs protein in the medium or HBV DNA in the cells at 12 days postinfection was quantified. (B) CsA did not affect the entry of HCV. Huh-7.5.1 cells were pretreated with the indicated compounds for 1 hour and then infected with HCVpp for 4 hours. At 72 hours postinfection, intracellular luciferase activity was measured. $*P < 0.05$, $**P < 0.01$, N.S., not significant.

(Fig. 2D-a). In contrast, CsA caused a significant reduction of HBV DNA in the internalization assay (Fig. 2D-b). In the time of addition assay as shown in Fig. 2E, treatment with CsA during HBV infection decreased HBs and HBe production (Fig. 2E-b), while CsA did not have an anti-HBV effect when delivered after HBV infection (Fig. 2E-c). Thus, CsA appears to primarily block the entry step including internalization. To examine whether CsA targeted HBV particles or host cells, we preincubated HBV with CsA and then purified the CsA from the HBV inoculum, followed by measurement of the HBV infectivity using HepaRG cells (Fig. 2F). Preincubation with CsA did not affect HBV infectivity, in contrast to the antagonizing effect of heparin to HBV particles (Fig. 2F), suggesting that CsA did not affect HBV particles but rather targeted host cells.

Cyclosporin A Showed a Pan-Genotypic Anti-HBV Effect. We examined the anti-HBV effect of CsA on the infection of different genotypes of HBV into PHHs. As shown in Fig. 3A, CsA reduced the infection of HBV genotype A, C, or D, which differ in sequences from the virus strain used in all of the other figures. However, CsA did not affect the entry of HCV, in contrast to the inhibition of HCV entry by heparin, bafilomycin A1, or an anti-HCV E2 antibody (Fig. 3B).

Effect of Immunosuppressants on HBV Infection. CsA is used clinically as an immunosuppressant, such as in patients following liver transplantation.[13] We therefore investigated the activity of other immunosuppressants on HBV infection. Among the additional immunosuppressive drugs examined, only FK506 was able to suppress HBV infection (Fig. 4A). CsA is known to have three major cellular targets: cellular cyclophilins (CyPs), calcineurin (CN), and transporters including MDRs and MRPs.[18] Although both CsA and FK506 can inhibit CN (Fig. 4B), this activity



compound	IC_{50} (μM)		
	CyPA	CyPB	CyPD
CsA	0.36 ± 0.01	0.73 ± 0.02	1.25 ± 0.03
FK506	> 10	> 10	> 10
PSC833	> 10	> 10	> 10

Fig. 4. Effect of immunosuppressants on HBV infection. (A,C) HepaRG cells were treated with or without the indicated compounds at 2 μM (FK506 4 μM) in (A), and CsA (2 and 4 μM) and PSC833 (2 and 4 μM) in (C), according to the scheme in Fig. 1A. HBs (A,C) and HBe (C) secretion was determined. (B) Effect of compounds on the activity of the calcineurin/NF-AT pathway. Jurkat cells transfected with pNF-AT-luc and pRL-TK were stimulated with PMA and ionomycin in the presence or absence of CsA, FK506, and PSC833 for 24 hours to measure the luciferase activity. (D) Cyclophilin binding activity of CsA, FK506, and PSC833 was determined in a competitive binding assay as described in the Materials and Methods using a CsA-derived fluorescent probe. IC_{50}s (μM) for the inhibition of probe binding to CyPA, CyPB, and CyPD are shown. $*P < 0.05$, $**P < 0.01$.

was dispensable for the anti-HBV effect, as PSC833, a CsA derivative inactive for CN inhibition (Fig. 4B),[18] could still inhibit HBV infection (Fig. 4C). As PSC833 and FK506 did not bind to the active site of CyPs (Fig. 4D), CyP inhibition is not likely to be responsible for the anti-HBV activity.

CsA Blocked HBV Infection Through Targeting NTCP. Recently, NTCP was reported as a candidate entry receptor for HBV.[22] A transporter activity assay showed that CsA inhibited the activity of NTCP both in 293 (Fig. 5A) and HepG2 cells (Fig. 5B) engineered to stably overexpress NTCP, as previously reported.[35] CsA was also suggested to bind to NTCP on the membrane in a ligand binding assay using HepG2-NTCP cells (Fig. S2).

NTCP messenger RNA (mRNA) was expressed in HepaRG cells and PHH, which are HBV-susceptible, while little to no expression was detected in HBV-nonsusceptible cell lines including HepG2, Huh-7, FLC4, and nonhepatocyte HeLa cells (Fig. 5C). In contrast, CyPA and CyPB were expressed in all of these cell lines, irrespective of infection susceptibility. Intriguingly, we found that the inhibition of NTCP transporter activity correlated with anti-HBV entry activity (Figs. 5A, 4A,B). These results suggest the possibility that compounds targeting NTCP have the potential to block HBV infection. To test this prediction, we treated HepaRG cells with compounds known to inhibit NTCP, including ursodeoxycholate, cholic acid, propranolol, progesterone, and bosentan[35,36] to investigate the effect on HBV entry using the protocol in Fig. 1A. As shown in Fig. 5D, these compounds inhibited HBV infection. Thus, inhibition of NTCP blocked HBV infection. We also showed that HepG2 cells overexpressing NTCP were susceptible to HBV infection (Fig. 5E), as reported recently.[22] Treatment with CsA also reduced HBs and HBe secretion when these cells were infected with HBV (Fig. 5E), suggesting that CsA inhibited NTCP-mediated HBV infection.

The binding of the HBV large envelope protein (LHBs) to NTCP was reported to be important for HBV entry.[22] Thus, one mechanism by which compounds that directly inhibit NTCP activity may block HBV entry is interruption of the binding between NTCP and LHBs. To test this possibility, we established an AlphaScreen assay to evaluate LHBs-NTCP binding *in vitro* as described in the Materials and Methods. *In vitro* synthesized NTCP and LHBs were at least partially functional, as NTCP bound to its substrate TCA (Fig. S3A) and LHBs could neutralize HBV infection into HepaRG cells (Fig. S3B). As shown in Fig. 5F, incubation of recombinant NTCP with LHBs but not middle (MHBs) and small envelope protein (SHBs) produced a significant AlphaScreen signal (Fig. 5F-a, left) indicative of a direct protein-protein interaction. In contrast to NTCP, recombinant GST or other nonrelevant proteins, LCK and FYN,[37] did not produce a binding signal when incubated with LHBs (Fig. 5F-a), suggesting that our AlphaScreen assay produced a specific signal for the interaction of NTCP with LHBs. Consistent with the report that the pre-S1 region of LHBs was important for the binding to NTCP,[22] the signal was decreased in a dose-dependent manner by the addition of pre-S1 lipopeptide HBVpreS/2-48^{myr},[5] (Fig. 5F-b) but not of an inactive mutant of pre-S1 (Fig. S3C), indicating a competition of pre-S1 with LHBs for NTCP binding. In this assay, CsA as well as FK506 and a CsA derivative, SCYX1454139 (see the next section), were shown to reduce the signal for LHBs-NTCP binding in a dose-dependent manner (Fig. 5F-c,d,e). These results suggest that CsA targets NTCP and thereby inhibits the interaction between LHBs and NTCP.

Identification of CsA Analogs Possessing a Higher Anti-HBV Potential. Considering CsA as a lead compound, we tested CsA analogs for anti-HBV activity. As shown in Fig. 6A, SCYX618806 reduced HBs secretion after HBV infection, while a related analog SCYX1774198 did not have a significant anti-HBV effect (Fig. 6A,C). Additional analogs, SCYX827830 and SCYX1454139, had significant anti-HBV activities (Fig. 6A,C). Alisporivir (Debio 025), an anti-HCV drug candidate,[38] also decreased HBV infection to the equivalent level to CsA (Fig. 6B). Figure 6D shows a dose-dependent reduction of HBs secretion by treatment with SCYX618806, SCYX827830, and SCYX1454139, all of which had more potent anti-HBV activities than CsA (compare Fig. 6D with Fig. 2A). These results indicate that anti-HBV activity is not disrupted by at least some changes to the 3-glycine, 4-leucine, and 8-alanine residues of CsA, although additional analogs will need to be evaluated for a full understanding of the structure-activity relationships. Notably, SCYX618806 and alisporivir bear modifications on the 4-leucine residue of the CsA backbone that prevent CN binding and immunosuppressive activity (Table S1), further confirming that anti-HBV activity does not require immunosuppressive activity. Notably, SCYX1454139 showed the strongest anti-HBV entry activity among 50 CsA derivatives examined (data not shown and Fig. 6E). The median inhibitory concentrations (IC_{50}s) for anti-HBV activity as well as CC_{50}s determined by an MTT-based cell viability assay are shown in Fig. 6E. The IC_{50} and CC_{50} of SCYX1454139 were 0.17 ± 0.02 and >10

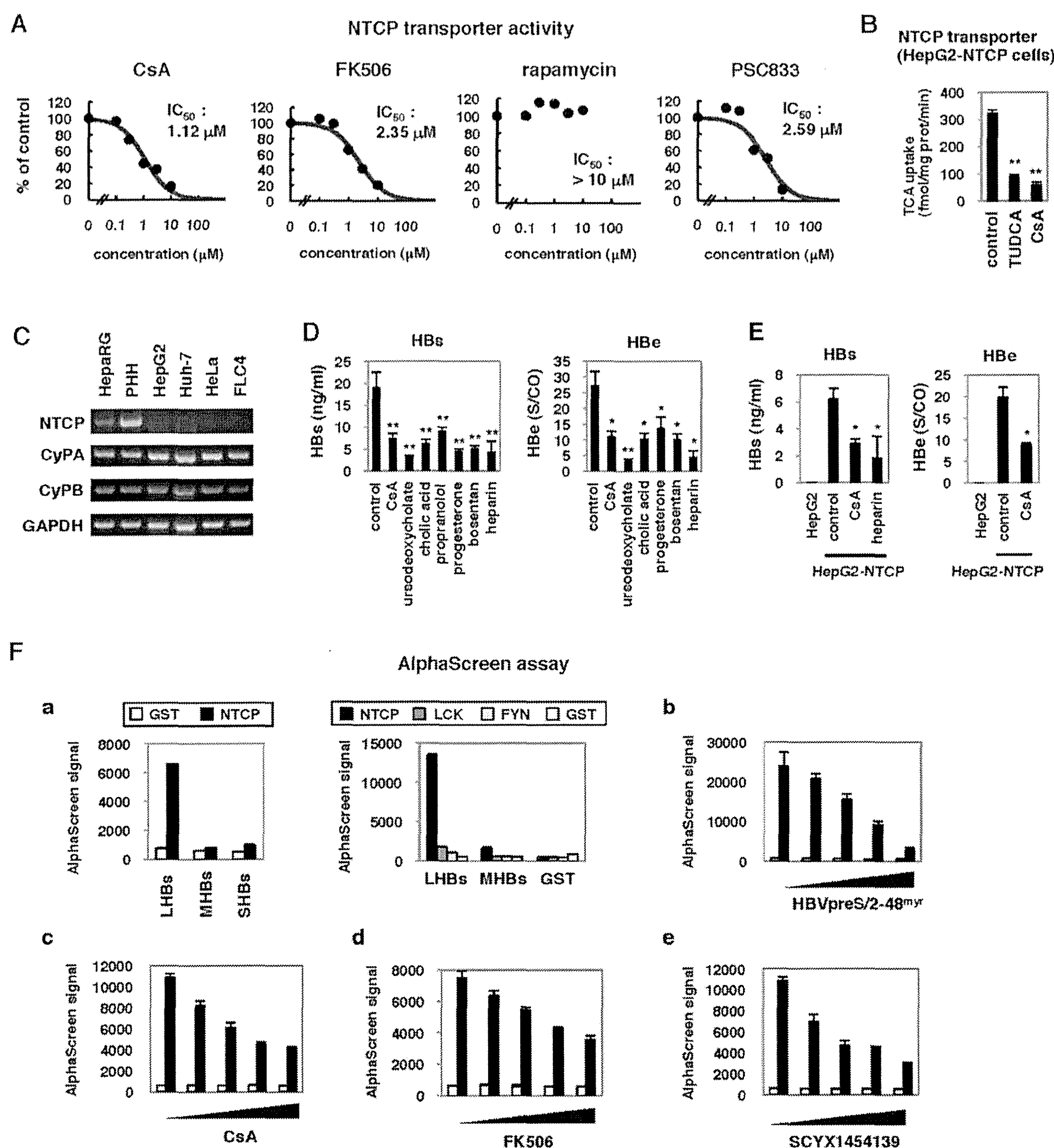
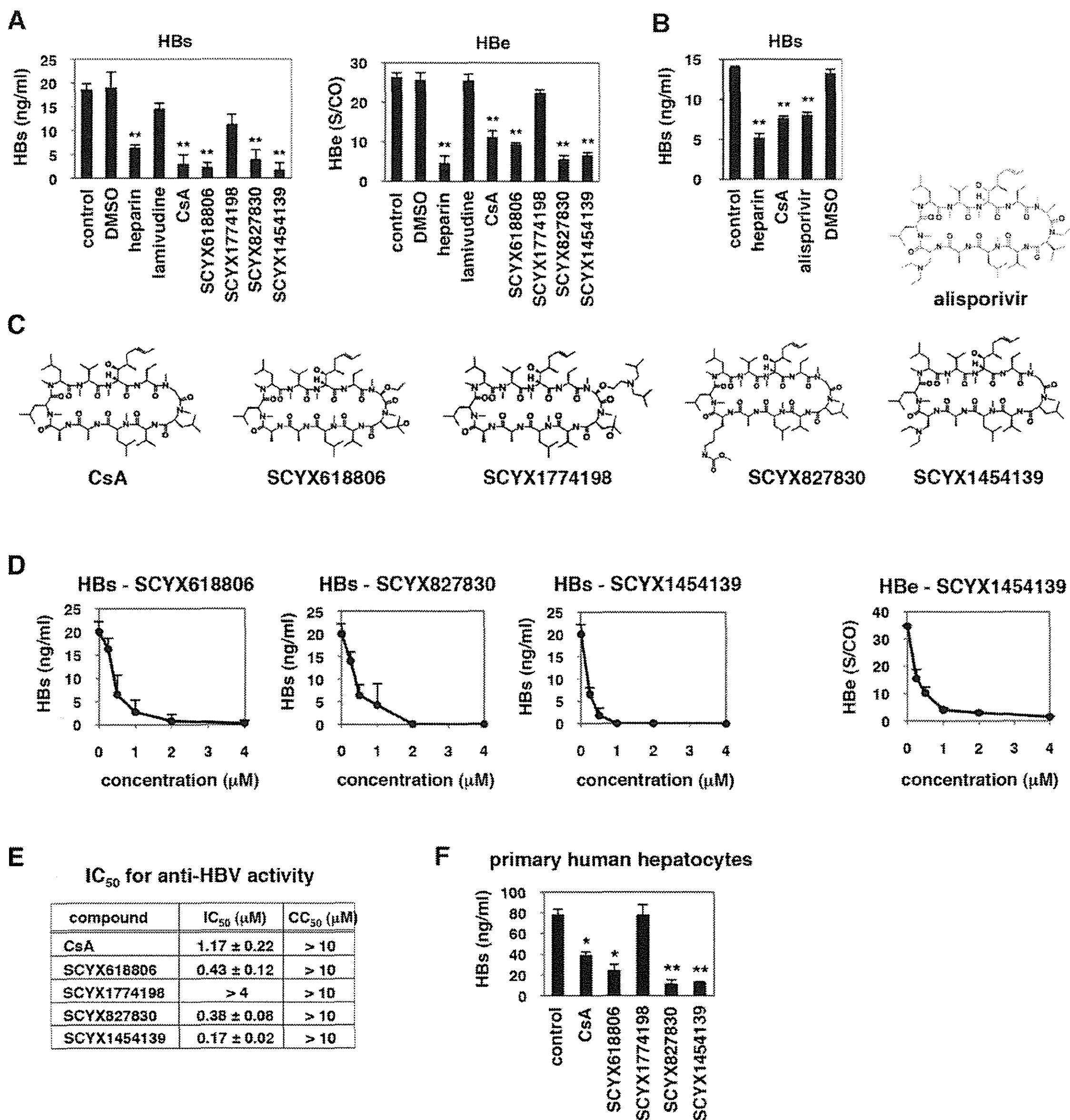


Fig. 5. NTCP inhibitors blocked HBV infection. (A) NTCP transporter activity was examined following CsA, FK506, rapamycin, and PSC833 treatment of 293 cells overexpressing NTCP, as described in the Materials and Methods. Dose-response curves and IC_{50}s for inhibition of NTCP transporter activity are shown. (B) NTCP transporter activity was measured in HepG2-NTCP cells treated with or without CsA 10 μM or tauroursodeoxycholic acid (TUDCA) 10 μM as a positive control. (C) Expression of mRNAs for NTCP, CyPA, CyPB, and GAPDH in HepaRG, PHHs, HepG2, Huh-7, HeLa, and FLC4 cells was determined by RT-PCR. (D) HepaRG cells were treated with or without CsA 4 μM, ursodeoxycholate 100 μM, cholic acid 100 μM, propranolol 100 μM, progesterone 40 μM, bosentan 100 μM, and heparin 25 U/mL according to the scheme in Fig. 1A. Secretion of HBs and HBe was quantified. (E) HepG2 cells overexpressing NTCP (HepG2-NTCP) and the parental HepG2 cells were pretreated with or without CsA or heparin for 2 hours, then treated with HBV for 16 hours. HBV infection was monitored with HBs and HBe secreted from the cells. (F) AlphaScreen assay to evaluate the binding between NTCP and large envelope protein (LHBs) as described in the Materials and Methods. (a) Left, His-tagged GST (white bars) or NTCP (black bars) are incubated with large (LHBs), middle (MHBs), or small envelope protein (SHBs). Right, His-tagged NTCP and other nonrelevant proteins, LCK and FYN, and GST were incubated with LHBs, MHBs, and GST. (b-e) His-tagged GST (white bars) or NTCP (black bars) were incubated with LHBs in the presence of varying amounts of pre-S1 lipopeptide HBVpreS/2-48^{myr} (b; 0, 7.7, 15.3, 30.7, and 61.3 μM), CsA (c; 0, 37.5, 75, 150, and 300 μM), FK506 (d; 31, 63, 125, 250, and 500 μM), and SCYX1454139 (e; 0, 37.5, 75, 150, and 300 μM), respectively. $^{*}P < 0.05$, $^{**}P < 0.01$.

μM, respectively, a profile superior to that of CsA (IC_{50} and CC_{50} of 1.17 ± 0.22 and >10 μM, respectively). Inhibition of HBV infection by treatment with SCYX1454139 was also observed in PHHs, in which also the anti-HBV effect of SCYX1454139 was more remarkable than that of CsA (Fig. 6F). These results



compound	IC_{50} (μM)	CC_{50} (μM)
CsA	1.17 ± 0.22	> 10
SCYX618806	0.43 ± 0.12	> 10
SCYX1774198	> 4	> 10
SCYX827830	0.38 ± 0.08	> 10
SCYX1454139	0.17 ± 0.02	> 10

Fig. 6. Analysis of CsA analogs. (A,B) Anti-HBV activity of CsA analogs. HepaRG cells were treated with or without dimethyl sulfoxide (DMSO), heparin 10 U/mL, lamivudine 1 μM, CsA 4 μM, or its analogs, SCYX618806, SCYX1774198, SCYX827830, and SCYX1454139 (A) or alisporivir (B) at 4 μM, as shown in Fig. 1A to measure HBs and HBe secretion level. (C) Chemical structures of CsA and its derivatives. (D) Dose-response curves for CsA analogs. HepaRG cells were treated with or without various concentrations of SCYX618806, SCYX827830, or SCYX1454139 (0.25, 0.5, 1, 2, and 4 μM) as shown in Fig. 1A. (E) IC_{50}s (μM) for CsA and its analogs in blocking HBV infection are shown. CC_{50}s (μM) determined by the MTT cell viability assay are also shown. (F) PHHs were treated with CsA and its derivatives at 4 μM or left untreated according to the protocol in Fig. 1A, and HBV infection was monitored by HBs protein secretion. *$P < 0.05$, **$P < 0.01$.

clearly indicate that analogs of CsA may include compounds with greater anti-HBV potency than that of CsA itself.

Discussion

Previous reports have demonstrated that CsA suppresses the replication of a variety of viruses including human immunodeficiency virus, HCV, influenza virus, severe acute respiratory syndrome coronavirus, human papillomavirus, flaviviruses, vesicular stomatitis virus, and vaccinia virus.[16,39-46] Virological analyses using CsA further demonstrate that CyPs are involved in the replication of these viruses. In this study, we showed that CsA inhibited the entry of HBV but in an apparent CyP-independent manner. It was previously reported that CsA suppressed HBV replication in a cell culture system carrying an HBV transgene.[47] However,

this antireplication effect is not likely to be responsible for the anti-HBV activity observed in this study, based on several observations. First, the experimental system mainly used in this study (Fig. 1A) is likely to evaluate the early phase of HBV infection but not HBV replication. Second, the suppression of HBV replication by CsA reported previously was mediated by blocking the mitochondrial permeability transition pore possibly through binding to mitochondrial CyPD.[47,48] The anti-HBV activity shown in this study, however, had no correlation with binding to CyPs, suggesting that the inhibition of HBV infection in HepaRG cells and PHHs is not from the result of suppression of HBV replication. Rather, CsA inhibited NTCP transporter activity and disrupted the binding between NTCP and LHBs *in vitro*. Moreover, inhibition of HBV infection could be observed by treatment with other compounds having the capacity to inhibit NTCP. These results suggest that targeting NTCP blocks HBV infection.

The current anti-HBV agents are mainly comprised of nucleos(t)ide analogs and IFNs. Development of anti-HBV agents targeting different molecules is greatly needed for achieving improved treatment of HBV infection, especially to combat drug-resistant virus. HBV cell entry mechanisms have been poorly defined. At the initial stage, HBV attaches to target cells with low affinity through binding involving cellular factors including heparan sulfate proteoglycans.[28,29] For the subsequent entry mechanism, it has recently been reported that NTCP is essential for HBV-specific entry.[22] Although the precise mechanism for entry and internalization is as yet incompletely understood, interference with this step has emerged as an attractive approach for development of novel therapeutics. For example, Gripon et al.[5] demonstrated that a peptide mimicking the pre-S1 region of large envelope protein prevented HBV infection in a mouse model. These results suggest that inhibition of virus cell entry could be an effective strategy for preventing HBV infection to achieve clinical outcomes such as for postexposure prophylaxis, blockage of vertical transmission, and prevention of HBV recurrence after liver transplantation. Given that HBV reactivation generally occurs under immunosuppressive conditions,[49,50] it is uncertain whether clinically relevant doses of CsA or FK506 could be helpful in preventing HBV reactivation after liver transplantation. It remains also unknown in general whether entry inhibitors could be effective in eliminating chronic HBV infection. Future studies should evaluate whether inhibition of HBV entry by CsA or its derivatives can reduce persistent HBV infection, especially in combination with nucleos(t)ide analogs or interferons. In this study, we obtained nonimmunosuppressive CsA derivatives that could inhibit HBV entry (Fig. 6). Moreover, a small-scale analog analysis identified a CsA derivative exhibiting more potent inhibition of HBV infection, with an IC_{50} of 0.1-0.2 μM (Fig. 6). This IC_{50} is equivalent to the anti-HCV replication activities of alisporivir or SCY-635 (0.22 μM and 0.08 μM, respectively), drugs which have been shown to reduce HCV viral load in infected patients during clinical trials.[38] Further analog analysis using CsA as a platform may identify more potent anti-HBV compounds.

In general, antiviral drugs targeting a cellular factor select drug-resistant viruses at a lower frequency than do direct-acting antiviral agents. Cellular targets relevant for anti-HBV drug development have been poorly defined to date. This study has demonstrated that small molecules targeting NTCP can inhibit HBV infection. Further study of NTCP inhibitors and CsA derivatives may provide a new anti-HBV strategy targeting a cellular factor, which is less likely to foster emergence of drug-resistant viruses.

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References

1. Pawlotsky JM, Dusheiko G, Hatzakis A, Lau D, Lau G, Liang TJ, et al. Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach. Gastroenterology 2008;134:405-415.
2. Rapicetta M, Ferrari C, Levrero M. Viral determinants and host immune responses in the pathogenesis of HBV infection. J Med Virol 2002;67:454-457.
3. Zoulim F. Hepatitis B virus resistance to antiviral drugs: where are we going? Liver Int 2011;31(Suppl 1):111-116.
4. Grimm D, Thimme R, Blum HE. HBV life cycle and novel drug targets. Hepatol Int 2011;5:644-653.
5. Gripon P, Cannie I, Urban S. Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. J Virol 2005;79:1613-1622.
6. Petersen J, Dandri M, Mier W, Lutgehetmann M, Volz T, von Weizsacker F, et al. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nat Biotechnol 2008;26:335-341.

7. Delaney WEt, Edwards R, Colledge D, Shaw T, Furman P, Painter G, et al. Phenylpropenamide derivatives AT-61 and AT-130 inhibit replication of wild-type and lamivudine-resistant strains of hepatitis B virus in vitro. Antimicrob Agents Chemother 2002;46:3057-3060.
8. Deres K, Schroder CH, Paessens A, Goldmann S, Hacker HJ, Weber O, et al. Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. Science 2003;299:893-896.
9. King RW, Ladner SK, Miller TJ, Zaifert K, Perni RB, Conway SC, et al. Inhibition of human hepatitis B virus replication by AT-61, a phenylpropenamide derivative, alone and in combination with (-)beta-L-2',3'-dideoxy-3'-thiacytidine. Antimicrob Agents Chemother 1998; 42:3179-3186.
10. Weber O, Schlemmer KH, Hartmann E, Hagelschuer I, Paessens A, Graef E, et al. Inhibition of human hepatitis B virus (HBV) by a novel non-nucleosidic compound in a transgenic mouse model. Antiviral Res 2002;54:69-78.
11. Block TM, Lu X, Mehta AS, Blumberg BS, Tennant B, Ebling M, et al. Treatment of chronic hepadnavirus infection in a woodchuck animal model with an inhibitor of protein folding and trafficking. Nat Med 1998;4:610-614.
12. Block TM, Lu X, Platt FM, Foster GR, Gerlich WH, Blumberg BS, et al. Secretion of human hepatitis B virus is inhibited by the imino sugar N-butyldeoxynojirimycin. Proc Natl Acad Sci U S A 1994;91: 2235-2239.
13. Watashi K, Shimotohno K. Cyclophilin and viruses: cyclophilin as a cofactor for viral infection and possible anti-viral target. Drug Target Insights 2007;2:9-18.
14. Loor F, Tiberghien F, Wenandy T, Didier A, Traber R. Cyclosporins: structure-activity relationships for the inhibition of the human MDR1 P-glycoprotein ABC transporter. J Med Chem 2002;45:4598-4612.
15. El-Farrash MA, Aly HH, Watashi K, Hijikata M, Egawa H, Shimotohno K. In vitro infection of immortalized primary hepatocytes by HCV genotype 4a and inhibition of virus replication by cyclosporin. Microbiol Immunol 2007;51:127-133.
16. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. HEPATOLOGY 2003;38:1282-1288.
17. Nakagawa M, Sakamoto N, Tanabe Y, Koyama T, Itsui Y, Takeda Y, et al. Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. Gastroenterology 2005;129: 1031-1041.
18. Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, et al. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol Cell 2005;19:111-122.
19. Yang F, Robotham JM, Nelson HB, Irsigler A, Kenworthy R, Tang H. Cyclophilin A is an essential cofactor for hepatitis C virus infection and the principal mediator of cyclosporine resistance in vitro. J Virol 2008;82:5269-5278.
20. Schlutter J. Therapeutics: new drugs hit the target. Nature 2011;474: S5-S7.
21. Watashi K. Alisporivir, a cyclosporin derivative that selectively inhibits cyclophilin, for the treatment of HCV infection. Curr Opin Investig Drugs 2010;11:213-224.
22. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 2012;1:e00049.
23. Watashi K, Liang G, Iwamoto M, Marusawa H, Uchida N, Daito T, et al. Interleukin-1 and tumor necrosis factor-alpha trigger restriction of hepatitis b virus infection via a cytidine deaminase activation-induced cytidine deaminase (AID). J Biol Chem 2013;288:31715-31727.
24. Nakajima S, Watashi K, Kamisuki S, Tsukuda S, Takemoto K, Matsuda M, et al. Specific inhibition of hepatitis C virus entry into host hepatocytes by fungi-derived sulochrin and its derivatives. Biochem Biophys Res Commun 2013;440:515-520.
25. Mita S, Suzuki H, Akita H, Hayashi H, Onuki R, Hofmann AF, et al. Inhibition of bile acid transport across Na+/taurocholate cotransporting polypeptide (SLC10A1) and bile salt export pump (ABCB 11)-coexpressing LLC-PK1 cells by cholestasis-inducing drugs. Drug Metab Dispos 2006;34:1575-1581.
26. Takai K, Sawasaki T, Endo Y. Practical cell-free protein synthesis system using purified wheat embryos. Nat Protoc 2010;5:227-238.
27. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci U S A 2002;99:15655-15660.
28. Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. Cell Microbiol 2008;10: 122-133.
29. Schulze A, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. HEPATOLOGY 2007;46:1759-1768.
30. Funk A, Mhamdi M, Hohenberg H, Will H, Sirma H. pH-independent entry and sequential endosomal sorting are major determinants of hepadnaviral infection in primary hepatocytes. HEPATOLOGY 2006;44:685-693.
31. De Clercq E, Ferir G, Kaptein S, Neyts J. Antiviral treatment of chronic hepatitis B virus (HBV) infections. Viruses 2010;2:1279-1305.
32. Locarnini S, Zoulim F. Molecular genetics of HBV infection. Antivir Ther 2010;15(Suppl 3):3-14.
33. Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, et al. Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother 1997;41: 1715-1720.
34. Aizaki H, Morikawa K, Fukasawa M, Hara H, Inoue Y, Tani H, et al. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 2008;82:5715-5724.
35. Kim RB, Leake B, Cvetkovic M, Roden MM, Nadeau J, Walubo A, et al. Modulation by drugs of human hepatic sodium-dependent bile acid transporter (sodium taurocholate cotransporting polypeptide) activity. J Pharmacol Exp Ther 1999;291:1204-1209.
36. Leslie EM, Watkins PB, Kim RB, Brouwer KL. Differential inhibition of rat and human Na+-dependent taurocholate cotransporting polypeptide (NTCP/SLC10A1)by bosentan: a mechanism for species differences in hepatotoxicity. J Pharmacol Exp Ther 2007;321:1170-1178.
37. Palacios EH, Weiss A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. Oncogene 2004;23:7990-8000.
38. Paeshuyse J, Kaul A, De Clercq E, Rosenwirth B, Dumont JM, Scalfaro P, et al. The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro. HEPATOLOGY 2006;43:761-770.
39. Bienkowska-Haba M, Patel HD, Sapp M. Target cell cyclophilins facilitate human papillomavirus type 16 infection. PLoS Pathog 2009;5: e1000524.
40. Bose S, Mathur M, Bates P, Joshi N, Banerjee AK. Requirement for cyclophilin A for the replication of vesicular stomatitis virus New Jersey serotype. J Gen Virol 2003;84:1687-1699.
41. Damaso CR, Moussatche N. Inhibition of vaccinia virus replication by cyclosporin A analogues correlates with their affinity for cellular cyclophilins. J Gen Virol 1998;79(Pt 2):339-346.
42. Liu X, Zhao Z, Li Z, Xu C, Sun L, Chen J, et al. Cyclosporin A inhibits the influenza virus replication through cyclophilin A-dependent and -independent pathways. PLoS One 2012;7:e37277.
43. Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff SP. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell 1993;73:1067-1078.
44. Pfefferle S, Schopf J, Kogl M, Friedel CC, Muller MA, Carbajo-Lozoya J, et al. The SARS-coronavirus-host interactome: identification of cyclophilins as target for pan-coronavirus inhibitors. PLoS Pathog 2011;7:e1002331.
45. Qing M, Yang F, Zhang B, Zou G, Robida JM, Yuan Z, et al. Cyclosporine inhibits flavivirus replication through blocking the interaction between host cyclophilins and viral NS5 protein. Antimicrob Agents Chemother 2009;53:3226-3235.

46. Towers GJ, Hatziioannou T, Cowan S, Goff SP, Luban J, Bieniasz PD. Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. Nat Med 2003;9:1138-1143.
47. Bouchard MJ, Puro RJ, Wang L, Schneider RJ. Activation and inhibition of cellular calcium and tyrosine kinase signaling pathways identify targets of the HBx protein involved in hepatitis B virus replication. J Virol 2003;77:7713-7719.
48. Xia WL, Shen Y, Zheng SS. Inhibitory effect of cyclosporine A on hepatitis B virus replication in vitro and its possible mechanisms. Hepatobil Pancreat Dis Int 2005;4:18-22.
49. Coffin CS, Terrault NA. Management of hepatitis B in liver transplant recipients. J Viral Hepat 2007;14(Suppl 1):37-44.
50. Fox AN, Terrault NA. The option of HBIG-free prophylaxis against recurrent HBV. J Hepatol 2012;56:1189-1197.

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MAJOR ARTICLE

Effect of Hepatitis B Virus Reverse Transcriptase Variations on Entecavir Treatment Response

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Background. Entecavir therapy often reduces hepatitis B virus (HBV) DNA to an undetectable level, but HBV DNA remain detectable in some patients. We investigated whether baseline HBV reverse transcriptase (rt) polymorphism and quasispecies complexity and diversity were associated with treatment response.

Methods. Pretreatment HBV DNA levels, HBV rt sequence, serology, and quasispecies complexity and diversity from 305 entecavir-treated patients were determined. These data were tested for their association with year 1 virological outcome, defined by optimal response (undetectable HBV DNA; lower limit of detection, ≤12 IU/mL) or partial response (detectable HBV DNA).

Results. Four rt variants were more frequently detected in the 64 partial responders than in the 241 optimal responders (all $P < .05$). Multivariate analysis revealed that high baseline HBV DNA level ($P < .0001$; odds ratio [OR], 2.32), HBV e antigen (HBeAg) positivity ($P < .001$; OR, 3.70), and rt124N ($P = .002$; OR, 3.06) were associated with a partial entecavir response. Compared with the optimal responders, the partial responders had a lower quasispecies complexity and diversity.

Conclusions. Apart from the known factors (high baseline HBV DNA level and HBeAg positivity), a novel single nucleotide polymorphism (rt124N) and lower quasispecies complexity and diversity were associated with partial entecavir response at year 1.

Keywords. antiviral therapy; hepatitis B; chronic viral hepatitis; drug response.

Nucleos(t)ide analogue (NA) therapy is currently the mainstay of treatment for chronic hepatitis B virus (HBV) infection worldwide. One of the problems of administering NAs for the treatment of chronic HBV infection is the development of drug-resistant mutations after prolonged treatment. Although the incidence of resistance to the current 2 first-line NAs, entecavir and tenofovir, is low, there are situations, such as severe acute exacerbation of chronic hepatitis B, in which more-rapid lowering of the HBV DNA level is preferred. Moreover, it has been found that early viral suppression to an undetectable level during the first year of therapy is important in reducing the chance of development of drug resistance [1–4].

According to 2 multicenter pivotal phase III entecavir clinical trials [5, 6], around 10%–35% of patients receiving entecavir still have detectable HBV DNA after 1 year of treatment. A rational hypothesis is that there may be some differences within the natural polymorphism in the HBV "wild-type" reverse transcriptase (rt) sequences that confer primary hypo-responsiveness. This approach has been used to predict antiviral response in human immunodeficiency virus (HIV)–infected patients [7].

So far, only a limited number of small cohort or case report studies have investigated the association between pretreatment HBV rt natural variants and NA treatment response [8–16]. Large-scale studies with statistically significant findings are lacking, and studies

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on the predictive HBV rt sequences for entecavir treatment are scarce.

In this study, our primary aim was to investigate whether there are some pretreatment HBV rt sequence variations that can predict response to entecavir. Identifiable sequence variations would be subjected to a molecular docking stimulation analysis to determine the possible underlying mechanisms [17]. It is not known whether viral quasispecies heterogeneity will affect entecavir response. The secondary aim was to examine whether there was an association between baseline quasispecies complexity and diversity with response to entecavir.

METHODS

Study Subjects

We recruited 370 patients with chronic hepatitis B who started entecavir therapy between January 2002 and September 2009 in the Liver Clinic at the Queen Mary Hospital, Hong Kong. None of these 370 patients had received prior NA therapy or interferon/pegylated-interferon therapy or had other chronic liver diseases, including chronic hepatitis C virus and hepatitis D virus coinfection, autoimmune diseases, and alcoholic liver diseases. All 370 patients had taken 0.5 mg of entecavir continuously, had been followed-up in our clinic for >1 year, and had baseline, year 1, and year 3 (if applicable) HBV DNA levels measured. Liver cirrhosis was defined by the presence of cirrhosis-related complications, such as ascites, and esophageal/gastric varices, with or without ultrasonographic evidence of small-sized or nodular-surfaced liver. All patients consented to have baseline and subsequent serum samples stored for analysis. This study was approved by the Institutional Review Board of The University of Hong Kong and Hospital Authority Hong Kong West Cluster, Hong Kong.

HBV DNA Levels and Definition of Treatment Response

HBV DNA levels were measured by the Cobas TaqMan HBV Test (Roche Diagnostics, Branchburg, NJ), which has a lower limit of detection of 12 IU/mL (60 copies/mL) and a linear range of up to 1.1×10^8 IU/mL (6.4×10^8 copies/mL). For statistical and numerical analysis, samples with a viral load of $>1.1 \times 10^8$ IU/mL were regarded to have a viral load of 1.1×10^8 IU/mL. Optimal and partial virological responses were defined as undetectable HBV DNA by the Cobas TaqMan assay (≤12 IU/mL) and detectable HBV DNA (>12 IU/mL), respectively, at the end of 1 year of treatment [18].

Direct Polymerase Chain Reaction (PCR) Sequencing of the Gene Encoding HBV rt

HBV DNA was isolated from 200 µL of serum samples, using the QIAamp DNA blood mini kit (Qiagen, Hilden, Germany). The HBV polymerase rt domain was PCR amplified using the Platinum Taq High Fidelity DNA Polymerase, the forward primer HBV56s (5′-CCTGCTGGTGGCTCCAGTTC-3′), and the reverse primer HBV1234a (5′-GACACAAAGGTCCCACGCAT-3′). The 1.1-kb amplicon was sequenced bidirectionally, using the BigDye Terminator v3.1 Cycle Sequencing Kit and the Applied Biosystems Prism 3730*xl* Genetic Analyzer (Life Technologies, Carlsbad, CA).

Clonal Sequencing

PCR amplicons were cloned into the TA cloning sequencing vector system (Life Technologies) and transformed into competent *Escherichia coli* JM109 cells. For each isolate, 10–20 white colonies (median, 18 colonies) were picked and sequenced, as described above.

DNA Sequence Analysis

HBV DNA sequences were assembled and aligned using the CLC Main Workbench 6.6.2 (CLC Bio, Katrinebjerg, Denmark). HBV quasispecies heterogeneity was evaluated by 2 parameters: quasispecies complexity and quasispecies diversity. Quasispecies complexity is a measure of heterogeneity within a sample, while quasispecies diversity refers to the relatedness (genetic distance) of individual genomes within the population. Complexity is measured by normalized Shannon entropy (Sn) at both nucleotide and amino acid levels, using the following formula: $Sn = -\Sigma_i [p_i \times \ln p_i]/\ln N$, where p_i is the observed proportion of each different sequence of the mutant spectrum, and N the total number of clones compared [19]. Normalized Sn ranges from 0 (when all clones are conserved) to 1 (when all clones are different). Quasispecies diversity was assessed by the mean genetic distance (d) at both nucleotide and amino acid levels, the number of synonymous substitutions per synonymous site (dS), and the number of nonsynonymous substitutions per nonsynonymous site (dN), using the MEGA software [20, 21].

Molecular Docking Simulation

As there are no data on the crystal structures of the HBV rt, the crystal structures of the HIV type 1 (HIV-1) rt heterodimer (Protein Data Bank accession number 3KLI) were used as a template for molecular modeling [22]. Protein-compound docking simulation was performed by the Sievegene module in the myPresto Program and an in-house program OPPIH (Option Program for myPresto In HTML tools) [23, 24].

Statistical Analysis

Statistical analyses were performed using SPSS 18.0 (SPSS, Chicago, IL). Continuous variables with normal and skewed distributions were compared using the Student *t* test and the Mann-Whitney test, respectively. Categorical variables were tested using the χ^2 test or the Fisher exact test as appropriate. Bonferroni correction was used to control for type I error in multiple comparisons. Stepwise logistic regression was performed to test the factors associated with complete or

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partial virological response. Statistical significance was denoted by $P < .05$.

RESULTS

Baseline Characteristics

Full-length HBV rt DNA was successfully amplified by PCR in 305 patients. Of these 305 patients, 114 (37%) were HBV e antigen (HBeAg) positive and 191 (63%) were HBeAg negative at baseline. At baseline, 87 patients (29%) had clinical evidence of liver cirrhosis. At year 1, 64 patients (21%) had partial virological response to entecavir (HBV DNA level, >2 IU/mL). The median HBV DNA level at year 1 for the partial responders was 110 IU/mL (range, $12.4 \times 10^6 - 5.12 \times 10^6$ IU/mL). The baseline characteristics of the 305 patients are listed in Table 1. The sex ratios, baseline albumin levels, and bilirubin levels were comparable between the partial and optimal responders. Compared with the optimal responders, the partial responders were younger, had greater proportions of HBeAg-positive patients, and had higher baseline HBV DNA and alanine aminotransferase (ALT) levels. Compared with the optimal responders, the partial responders also had a greater proportion of patients with HBV genotype B and a smaller proportion of patients with cirrhosis.

HBV rt Sequence Analysis

The amino acid sequence for the whole HBV rt region (344 amino acids) was studied. Major known drug resistance mutations (rtL80I/V, I169T, V173L, L180M, A181V/T, T184G, A194T, S202I, M204I/V, N236T, and M250V) were not detected in these patients, except that, in one patient, concomitant rtL80I and rtL180M mutations were detected. Of the 344 rt amino acid positions, 217 amino acid residues were conserved among these 305 patients. Twenty rt variations were found only in partial responders (distributed among 17 partial responders) but not in any of the optimal responders (Supplementary Table 1). Because of the rarity of these cases, association between these polymorphisms and partial/slow response was not statistically different.

Association analysis revealed that 17 amino acid variants were detected more frequently in the 64 partial responders than in the 241 optimal responders (Table 2). Since multiple comparisons of 127 nonconserved amino acid positions were performed, Bonferroni correction was used to control for type I error. After Bonferroni adjustment, 4 rt variants, namely rt53N, rt118N, rt124N, and rt332S, occurred significantly more frequently in the partial responders than in the optimal responders (Table 2). Of these 305 patients, 52 harbored HBV with all 4 variants (rt53N, rt118N, rt124N, and rt332S). The proportion of patients harboring all of these 4 variants was higher in the partial responders (25/64 [39%]) than in the optimal responders (27/241 [11%]; $P < .0001$).

We investigated the HBV rt sequence at year 1 in the partial responders. We successfully amplified the HBV rt region in 18 partial responders, all with an HBV DNA level of >500 IU/mL at year 1. HBV entecavir resistance mutations were not detected.

Table 1. Baseline Characteristics of Patients With an Optimal or Partial Response to Entecavir

Characteristic	Optimal Responders (n = 241)	Partial Responders (n = 64)	P
Age, y, mean ± SD	48.3 ± 13.1	41.5 ± 10.7	<.001
Sex, male:female	167:74	42:22	.574
HBeAg positivity, patients, no. (%)	67 (28)	47 (73)	<.001
HBV DNA level, $\log_{10}$ IU/mL	6.2 (2.3–8.0)	8.0 (4.4–8.0)	<.001
HBV genotype, B:C	65:176	30:33[a]	.002
Cirrhosis, patients, no. (%)	78 (32)	9 (14)	.004
ALT level, U/L	77 (11–3000)	110 (20–2144)	.004
Albumin level, g/L	42 (22–51)	42 (25–48)	.459
Total bilirubin level, μmol/L	12 (4–261)	13 (2–216)	.256

Continuous parameters are expressed as median value (range), unless otherwise indicated.

Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B virus e antigen; HBV, hepatitis B virus.

[a] One patient had HBV genotype A.

Table 2. Reverse Transcriptase (rt) Variants With Significantly Different Distribution Among Patients With an Optimal or Partial Response to Entecavir

rt Variant	Percentage of Variants Found in Partial Responders	Percentage of Variants Found in Optimal Responders	P	
			Unadjusted	After Bonferroni Correction
rt9H	68.8	53.0	.025	1
rt16T	50.0	29.6	.002	.254
rt53N	51.6	27.4	.00024	**.031**
rt109S	54.7	35.3	.005	.635
rt118N	48.4	25.3	.00034	**.043**
rt121I	51.6	29.5	.001	.127
rt124N	50.0	23.7	.000038	**.0048**
rt127R	46.9	29.0	.007	.889
rt131N	50.0	28.2	.001	.127
rt134N	35.9	16.2	.00048	.061
rt151Y	51.6	30.3	.001	.127
rt221Y	53.1	37.8	.026	1
rt222A	40.6	21.6	.002	.254
rt238H	50.0	31.1	.005	.635
rt271M	48.4	26.1	.001	.127
rt278V	70.3	54.8	.025	1
rt332S	43.8	20.1	.00010	**.013**

rt variants with significant different distribution after Bonferroni correction are shown in bold.

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Compared with baseline, no change in the variants at year 1 at positions rt53, rt118, rt124, and rt332 was observed, indicating that these variants persisted over time in these patients.

The relationship between these 4 rt variants and other baseline parameters were studied. The 4 rt variants were distributed equally in patients of different sexes and HBeAg statuses (all $P > .05$). However, these 4 variants were found more frequently among patients without cirrhosis, those with HBV genotype B, and those with a high HBV DNA level (all $P < .05$).

Multivariable logistic regression analysis was performed to determine the independent effects of the baseline factors of age, HBeAg status, liver cirrhosis, ALT level, HBV DNA level, HBV genotype, and rt variants on partial entecavir response at year 1. We found that high baseline HBV DNA levels ($P < .0001$; odds ratio [OR], 2.32; 95% confidence interval [CI], 1.61–3.36), HBeAg positivity ($P < .001$; OR, 3.70; 95% CI, 1.79–7.65), and rt variant rt124N ($P = .002$; OR, 3.06; 95% CI, 1.53–6.11) were associated with partial entecavir response at year 1.

We also studied whether these HBV variants were associated with suboptimal entecavir response if other cutoff HBV DNA levels were used. Of these 305 patients, 13 (4.3%) had an HBV DNA level of >2000 IU/mL at year 1. A higher proportion of patients with an HBV DNA level of >2000 IU/mL had the rt124N variant, compared with patients with an HBV DNA level of ≤2000 IU/mL (7/13 [53.8%] vs 82/292 [28.1%]; $P = .046$). However, no significant difference in the variant frequencies of rt53N, rt118N, and rt332S was found ($P = .281$, .057, and .076, respectively). When other cutoff HBV DNA levels, such as 1000 IU/mL and 200 IU/mL, were used, no significant differences in the HBV variant frequencies were found (data not shown).

Molecular Docking Simulation

We used a molecular docking simulation model to assess whether rt124N causes a steric hindrance to the binding of entecavir to HBV rt [17]. On the basis of the crystal structure of the HIV-1 rt, we constructed a molecular model of the catalytic binding pocket of the HBV rt heterodimer (Figure 1). In this model, residue rt124 was positioned behind the helix, which consists of rt180L and rt184T. As shown in Figure 1*A*, rt124Y, the prevailing rt sequence for HBV genotype C, did not interfere with binding of entecavir to the rt catalytic pocket. In contrast, rt124N, the prevailing sequence of HBV genotype B, was in close proximity to entecavir and might cause a slight interference to the binding of entecavir to rt (Figure 1*B*).

HBV Quasispecies Complexity and Diversity

Quasispecies complexity and diversity were compared between the optimal and partial responders. As shown in Table 3, the optimal responders had a significantly higher quasispecies complexity than the partial responders at the nucleotide level

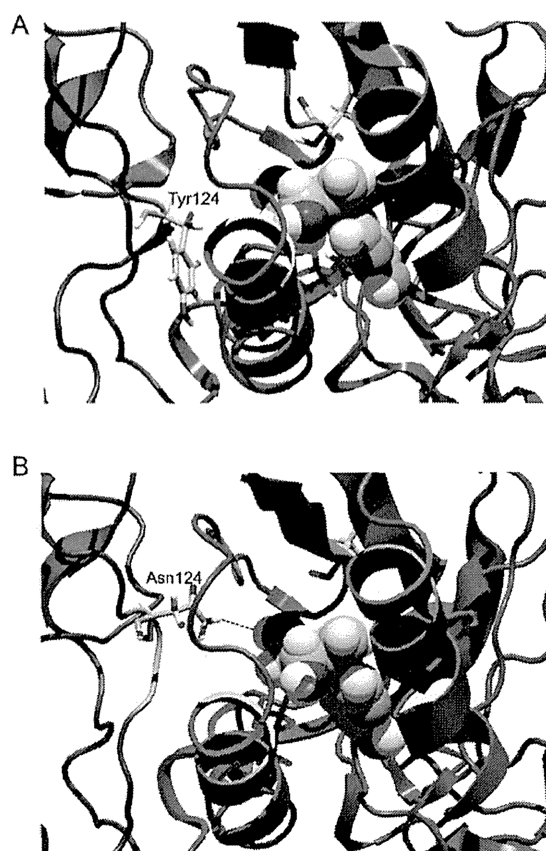

Figure 1. Molecular docking simulation model of the hepatitis B virus (HBV) reverse transcriptase (rt) catalytic domain. The HBV subunits corresponding to the human immunodeficiency virus type 1 rt p66 and p51 subunits are shown as blue and green ribbons, respectively. The entecavir molecule is shown in the middle, with a 3D space-filling representation. The figures were created using MolFeat (FiatLux, Tokyo, Japan). *A*, Catalytic pocket of rt with rt124Y (Tyr124). *B*, Catalytic pocket of rt with rt124N (Asn124).

($P = .036$), and the quasispecies complexity at the amino acid level also tended to be higher in the optimal responders ($P = .087$). Similarly, the optimal responders had a greater quasispecies diversity than the partial responders in terms of all 4 parameters (mean genetic distance at both the nucleotide and amino acid levels, number of synonymous substitutions per synonymous site, and number of nonsynonymous substitutions per nonsynonymous site; all $P < .05$; Table 3). In other words, lower baseline quasispecies complexity and diversity were associated with partial response to entecavir at year 1.

Longer-Term Entecavir Treatment Response

We also determined whether pretreatment rt variations were associated with differences in longer term treatment response. Among the 64 suboptimal responders, 55 patients had continuous entecavir treatment with year 3 HBV DNA data available. At year 3, 14 of 55 patients (25%) still had detectable HBV

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