Figure 1

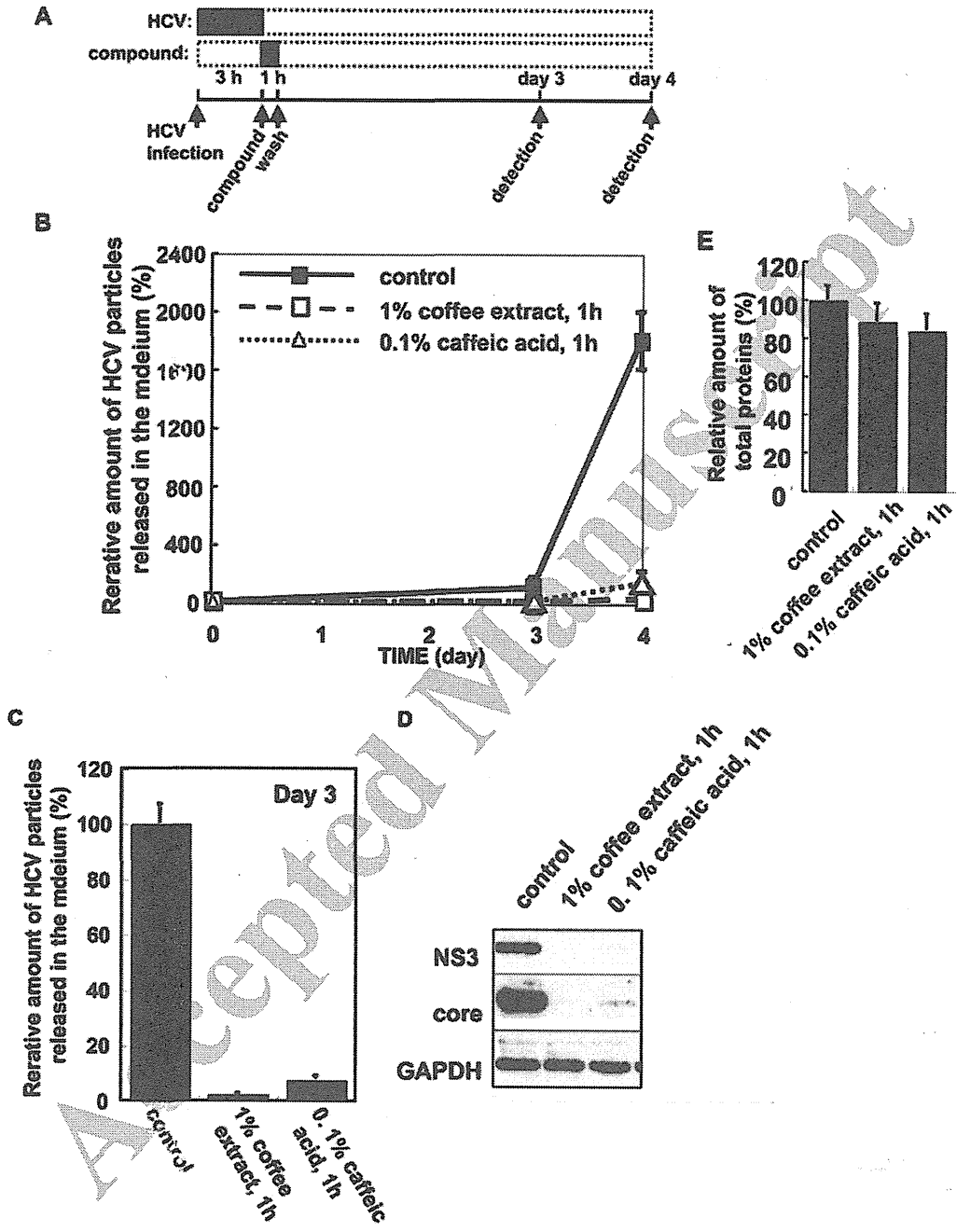


Figure 2

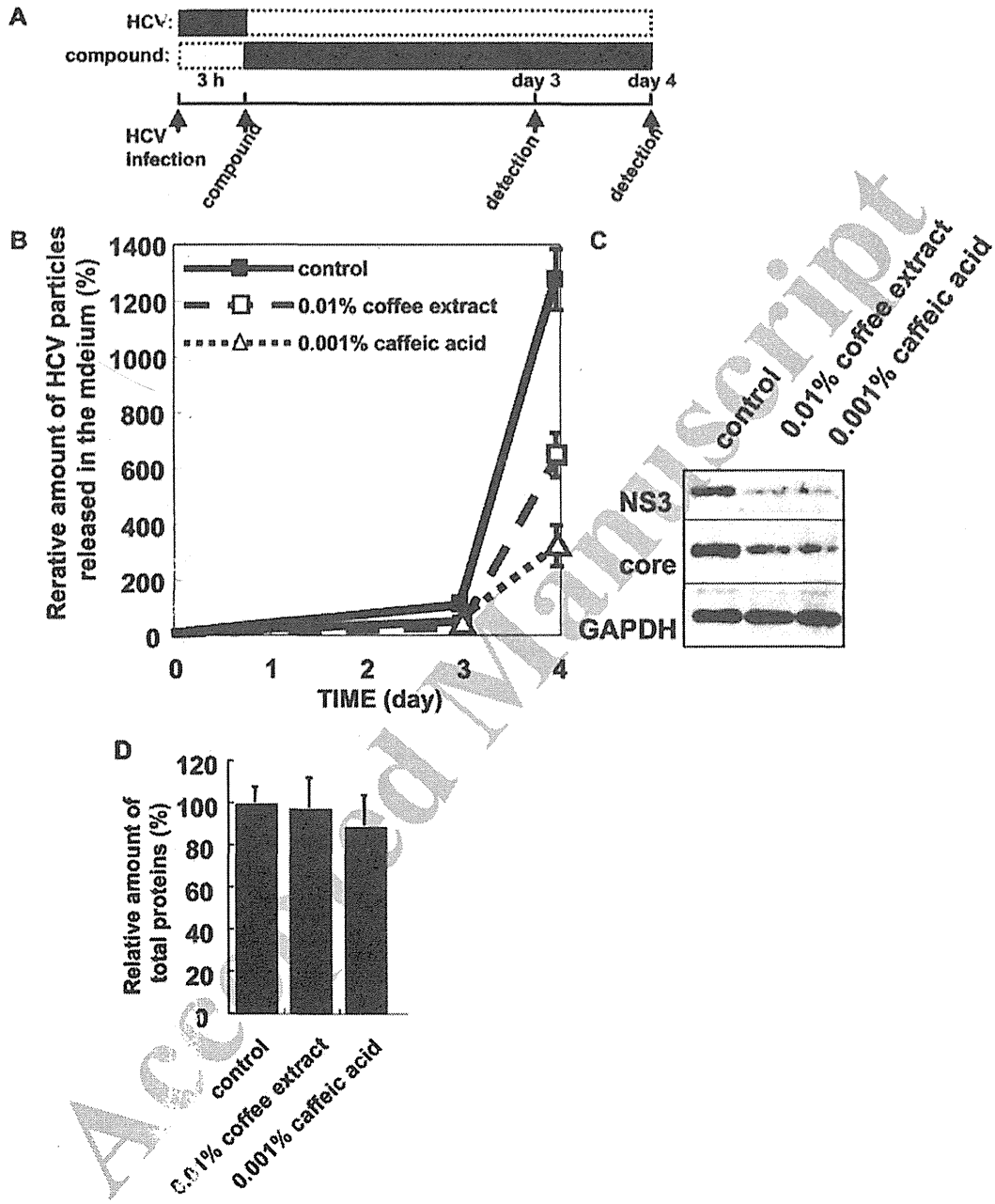


Figure 3

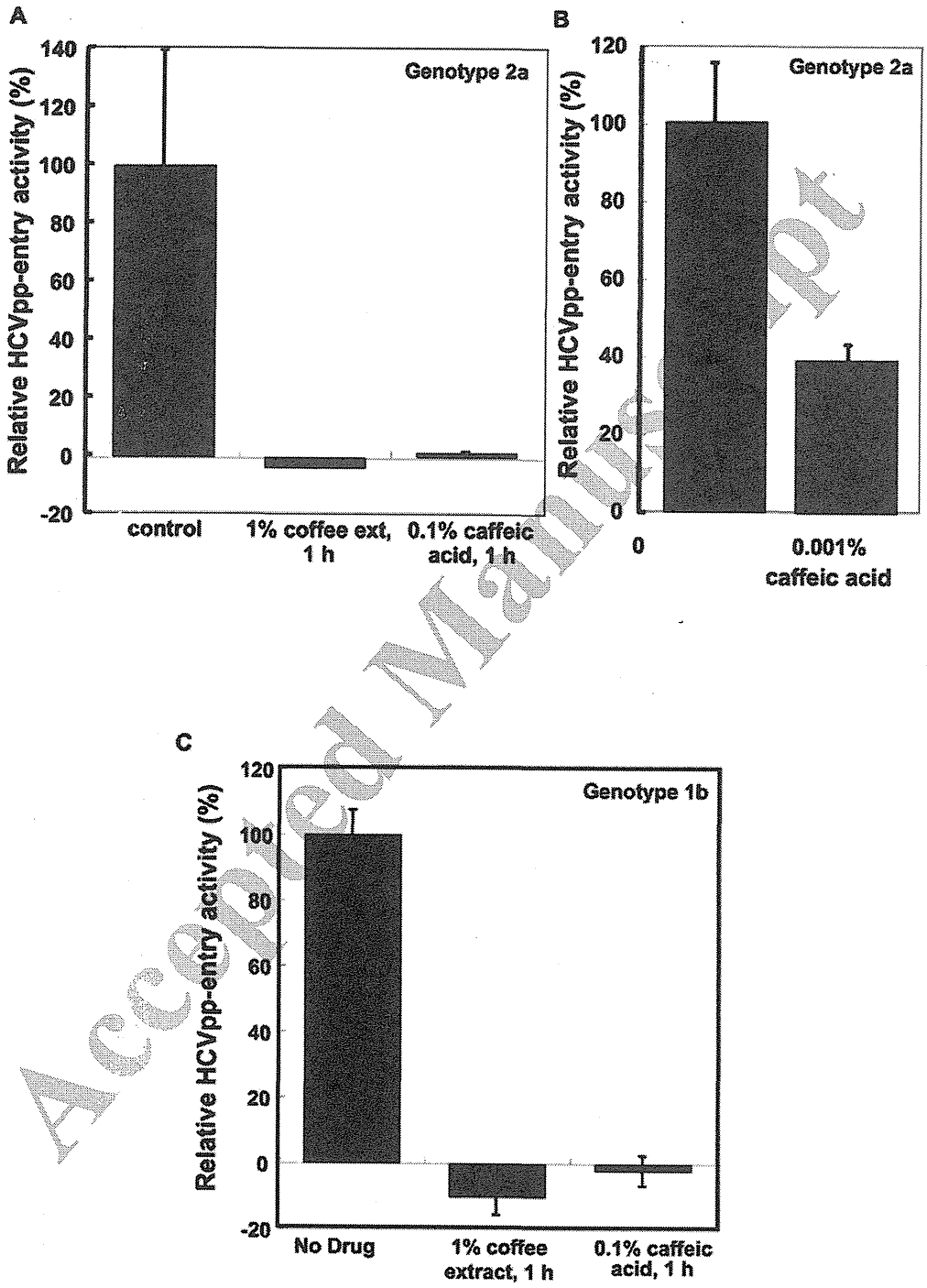
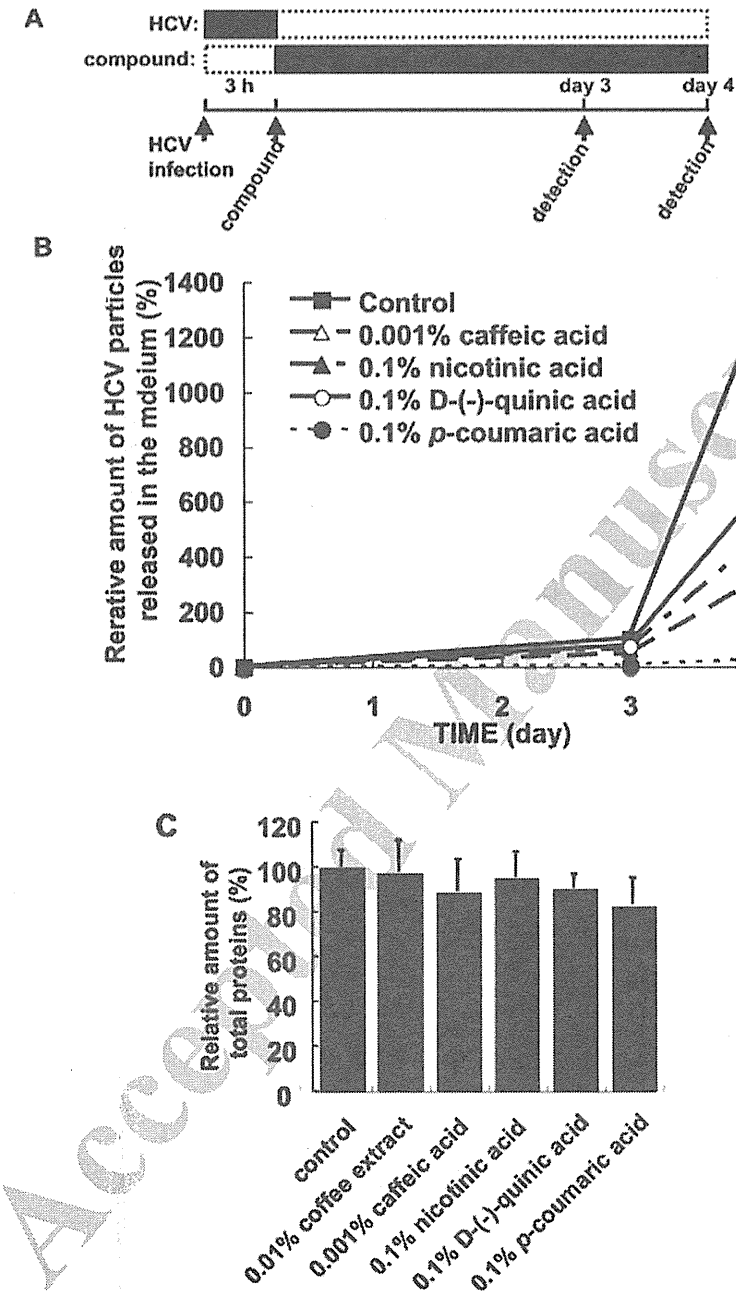


Figure 4



JBC Papers in Press. Published on December 30, 2014 as Manuscript M114.602540
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M114.602540

Dysregulation of retinoic acid receptor diminishes hepatocyte permissiveness to hepatitis B virus infection through modulation of NTCP expression

Senko Tsukuda[1,2], Koichi Watashi[1]*, Masashi Iwamoto[1], Ryosuke Suzuki[1], Hideki Aizaki[1], Maiko Okada[3], Masaya Sugiyama[4], Soichi Kojima[2], Yasuhito Tanaka[5], Masashi Mizokami[4], Jisu Li[6], Shuping Tong[6], Takaji Wakita[1]

[1]Department of Virology II, National Institute of Infectious Diseases, Tokyo, 162-8640, Japan, [2]Micro-signaling Regulation Technology Unit, RIKEN Center for Life Science Technologies, Wako, 351-0198, Japan. [3]Department of Translational Oncology, St. Marianna University School of Medicine, Kawasaki, 216-8511, Japan, [4]The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, 272-8516, Japan, [5]Department of Virology and Liver Unit, Nagoya City University Graduate School of Medicinal Sciences, Nagoya, 467-8601, Japan, [6]Liver Research Center Rhode Island Hospital, Warren Alpert School of Medicine, Brown University, Province, Rhode Island, 02912, USA.

Running Title: Retinoids reduced HBV susceptibility by downregulating NTCP

***Address correspondence to:** Koichi Watashi, Ph.D.
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: kwatashi@nih.go.jp

Keywords: hepatitis virus, transporter, retinoid, transcription, chemical biology, HBV, infection, RAR, permissive, NTCP

Background: Host factors regulating hepatitis B virus (HBV) entry receptors are not well defined.
Results: Chemical screening identified that retinoic acid receptor (RAR) regulates sodium taurocholate cotransporting polypeptide (NTCP) expression and supports HBV infection.
Conclusion: RAR regulates NTCP expression, and thereby supports HBV infection.
Significance: RAR regulation of NTCP can be a target for preventing HBV infection.

Abstract

Sodium taurocholate cotransporting polypeptide (NTCP) is an entry receptor for hepatitis B virus (HBV) and is regarded as one of the determinants that confer HBV permissiveness to host cells. However, how host factors regulate the ability of NTCP to support HBV infection is largely unknown. We aimed to identify the host signaling that regulated NTCP expression and, thereby, permissiveness to HBV. Here, a cell-based chemical screening method identified that Ro41-5253 decreased host susceptibility to HBV infection. Pretreatment with Ro41-5253 inhibited the viral entry process without affecting HBV replication. Intriguingly, Ro41-5253 reduced expression of both NTCP mRNA and protein. We found that retinoic acid receptor (RAR) regulated the promoter activity of the human NTCP (hNTCP) gene, and that Ro41-5253 repressed the hNTCP promoter by antagonizing RAR. RAR recruited to the hNTCP promoter region, and nt -112 to -96 of the hNTCP was suggested to be critical for RAR-mediated transcriptional activation. HBV susceptibility was decreased in pharmacologically RAR-inactivated cells. CD2665 showed a stronger anti-HBV potential and disrupted the spread of HBV infection that was achieved by continuous reproduction of whole HBV life cycle. In addition, this mechanism was significant for drug development, as antagonization of RAR blocked infection of multiple HBV genotypes and also a clinically relevant HBV mutant which was resistant to nucleoside analogs. Thus, RAR is crucial for regulating NTCP expression which determines permissiveness to HBV infection. This is the first demonstration showing host regulation of NTCP to support HBV infection.

Introduction

Hepatitis B virus (HBV) infection is a major public health problem, as the virus chronically infects approximately 240 million people worldwide (1-3). Chronic HBV infection elevates the risk for developing liver cirrhosis and hepatocellular carcinoma (4-6). Currently, two classes of antiviral agents are available to combat chronic HBV infection. First, interferon (IFN)-based drugs including IFNα and pegylated-IFNα modulate host immune function and/or directly inhibit HBV replication in hepatocytes (7,8). However, the antiviral efficacy of IFN-based drugs is restricted to less than 40% (9,10). Second, nucleos(t)ide analogues including lamivudine (LMV), adefovir, entecavir (ETV), tenofovir and telbivudine suppress HBV by inhibiting the viral reverse transcriptase (11,12). Although they can provide significant clinical improvement, long-term therapy with nucleos(t)ide analogues often results in the selection of drug resistant mutations in the target gene, which limits the treatment outcome. For example, in patients treated with ETV, at least three mutations can arise in the reverse transcriptase sequence at of the polymerase L180M, M204V plus either one of T184, S202 or M250 codon changes to acquire drug resistance (13). Therefore, development of new anti-HBV agents targeting other molecules requires elucidation of the molecular mechanisms underlying the HBV life cycle.

HBV infection of hepatocytes involves multiple steps. The initial viral attachment to the host cell surface starts with a low affinity binding involving heparan sulfate proteoglycans, and the following viral entry is mediated by a specific interaction between HBV and its host receptor(s) (14). Recently, sodium taurocholate cotransporting polypeptide (NTCP) was reported as a functional receptor for HBV (15). NTCP interacts with HBV large surface protein (HBs) to mediate viral attachment and the subsequent entry step. NTCP, also known as solute carrier protein 10A1 (SLC10A1), is physiologically a sodium-dependent transporter for bile salts located on the basolateral membrane of hepatocytes (16). In the liver, hepatocytes take up bile salts from the portal blood and secrete them into bile for enterohepatic

circulation, and NTCP-mediated uptake of bile salts into hepatocytes occurs largely in a sodium-dependent manner. Although NTCP is abundant in freshly isolated primary hepatocytes, it is weakly or no longer expressed in most cell lines such as HepG2 and Huh-7, and these cells rarely support HBV infection (17,18). In contrast, primary human hepatocytes, primary tupaia hepatocyte and differentiated HepaRG cells, which are susceptible to HBV infection, express significant levels of NTCP (19). Thus, elucidation of the regulatory mechanisms for NTCP gene expression is important for understanding the HBV susceptibility of host cells as well as for developing a new anti-HBV strategy. HBV entry inhibitors are expected to be useful for preventing *de novo* infection after liver transplantation, for post-exposure prophylaxis, or vertical transmission by short-term treatment (20,21).

In this study, we used a HepaRG-based HBV infection system to screen for small molecules capable of decreasing HBV infection. We found that pretreatment of host cells with Ro41-5253 reduced HBV infection. Ro41-5253 reduced NTCP expression by repressing the promoter activity of the human NTCP (hNTCP) gene. Retinoic acid receptor (RAR) played a crucial role in regulating the promoter activity of hNTCP, and Ro41-5253 antagonized RAR to reduce NTCP transcription and consequently HBV infection. This and other RAR inhibitors showed anti-HBV activity against different genotypes and an HBV nucleoside analog-resistant mutant, and moreover inhibited the spread of HBV. This study clarified one of the mechanisms for gene regulation of NTCP to support HBV permissiveness, and also suggests a novel concept whereby manipulation of this regulation machinery can be useful for preventing HBV infection.

Experimental Procedures

Regents

Heparin was obtained from Mochida Pharmaceutical. Lamivudine, cyclosporin A, all-trans retinoic acid (ATRA), and TO901317 were obtained from Sigma. Entecavir was obtained from Santa Cruz Biotechnology. Ro41-5253 was obtained from Enzo life sciences. PreS1-lipopeptide and FITC-labeled preS1 were synthesized by CS bio. IL-1b was purchased from Peprotech. CD2665, BMS195614, BMS493, and MM11253 were purchased from Tocris Bioscience.

Cell culture

HepaRG cells (BIOPREDIC) and primary human hepatocytes (Phoenixbio) were cultured as described previously (19). HepG2 and HepAD38 cells (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center) (22), were cultured with DMEM/F-12+GlutaMax (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, and 5 μg/ml insulin. HuS-E/2 cells (kindly provided by Dr. Kunitada Shimotohno at National Center for Global Health and Medicine) were cultured as described previously (23).

Plasmid construction

phNTCP-Gluc, pTK-Rluc was purchased from Genecopoeia and Promega, respectively. pRARE-Fluc was generated as described (25). For constructing phNTCP-Gluc carrying a mutation in a putative RARE (nt -491 to -479), the DNA fragments were amplified by PCR using phNTCP-Gluc as a template with primer sets F1; 5'-CAGATCTTGGAATTCCCAAAATC-3' and 5'-GAGGGGATGTGTCCATTGAAATGTTAATGGGAGCTGAGAGGATGCCAGTATCCTCCCT-3', and with primer sets 5'-CTCTCAGCTCCCATTAACATTTCAATGGACACATCCCCTCCTGGAGGCCAGTGACATT-3' and R6: 5'-CTCGGTACCAAGCTTTCCTTGTT-3'. The resultant products were further amplified by PCR with F1 and R6, and then inserted into the EcoRI/HindIII sites of phNTCP-Gluc to generate phNTCP Mut(-491~-479)-Gluc. Other promoter mutants were prepared by the same method using the primer sets, F1 and 5'-GTGGGTTATCATTTGTTTCCCGAAAACATTAGAGTGAAAGGAGCTGGGTGTTGCCTTTGG-3', 5'-TCCTTTCACTCTAATGTTTTCGGGAAACAAATGATAACCCACTGGACATGGGGAGGGCAC-3' and R6 for -368~-356; F1 and 5'-AATCTAGGTCCAGCCTATTTAAGTCCCTAAATTTCCTTTTCCCAGCTCCGCTCTTGATTCCTT-3', 5'-CTGGGAAAAGGAAATTTAGGGACTTAAAT

AGGCTGGACCTAGATTCAGGTGGGCCCTG GGCAG-3' and R6 for -274 ~ -258; F1 and 5'-TTCTGGGCTTATTTCTATATTTTGCAATCCA CTGAGTGTGCCTCATGGGCATTCATTC-3', 5'-CACACTCAGTGGATTGCAAAATATAGAAA TAAGCCCAGAAGCAGCAAAGTGACAAGGG -3' and R6 for -179 ~ -167; F1 and 5'-AGCTCTCCCAAGCTCAAAGATAAATGCTA GTTTCCTGGGTGCTACTTGTACTCCTCCCTT GTC-3', 5'-GTAGCACCCAGGAAACTAGCATTTATCTTT GAGCTTGGGAGAGCTAGGGCAGGCAGATA AGGT-3' and R6 for -112 ~ -96, respectively. For constructing hNTCP promoter carrying these five mutations (5-Mut), five DNA segments were amplified using the primers as follows: for segment 1, F1 and 5'- GAGGGGATGTGTCCATGACC-3'; for segment 2, 5'-AGCTCCTTTCACTCTCATGGGT-3' and 5'-TCCTTTTCCCAGCTCCGC-3'; for segment 3, 5'-GAGCTGGGAAAAGGAGCTGC-3' and 5'-CCACTGAGTGTGCCTCATGG-3'; for segment 4, 5'- AGGCACACTCAGTGGAGGG-3' and 5'-CTGGGTGCTACTTGTACTCCTCC-3'; for segment 5, 5'-CAAGTAGCACCCAGGAATCCA-3' and R6. For producing a deletion construct for hNTCP promoter, phNTCP (-53~+108)-Gluc, DNA fragment was amplified using the primer sets 5'-GGTGAATTCTGTTCCTCTTTGGGGCGACAG C-3' and 5'-GGTGGTAAGCTTTCCTTGTTC TCCGGCTGACTCC-3' and then inserted into the EcoRI and HindIII sites of phNTCP-Gluc.

HBV preparation and infection

HBV was prepared and infected as described (19). HBV used in this study was mainly derived from HepAD38 cells (22). For Fig. 6A-E, we used concentrated (approximately 200-fold) media of HepG2 cells transfected with an expression plasmid for either HBV genotype A, B, C, D, or genotype C carrying mutations at L180M, S202G, and M204V [HBV/Aeus, HBV/Bj35s, HBV/C-AT, HBV/D-IND60, or HBV/C-AT(L180M/S202G/M204V)] (24), and infected into the cells at 2000 GEq/cell in the presence of 4% PEG8000 at 37 °C for 16 h as previously described (19). HBV for Fig. 6F (genotype C) was purchased from Phoenixbio.

Real time PCR and RT-PCR

Real time PCR for detecting HBV DNAs and cccDNA was performed as described (19). RT-PCR detection of mRNAs for NTCP, ASBT, SHP, and GAPDH was performed with one step RNA PCR kit (TaKaRa) following the manufacturer's protocol with primer set 5'-AGGGAGGAGGTGGCAATCAAGAGTGG-3' and 5'-CCGGCTGAAGAACATTGAGGCACTGG-3' for NTCP, 5'- GTTGGCCTTGGTGATGTTCT-3' and 5'- CGACCCAATAGGCCAAGATA-3' for ASBT, 5'- CAGCTATGTGCACCTCATCG-3' and 5'- CCAGAAGGACTCCAGACAGC-3' for SHP, and 5' -CCATGGAGAAGGCTGGGG-3' and 5'-CAAAGTTGTCATGGATGACC-3' for GAPDH, respectively.

Immunofluorescence analysis

Immunofluorescence was conducted essentially as described (25) using an anti-HBc antibody (DAKO, #B0586) at a dilution of 1:1,000.

Detection of HBs and HBe antigens

HBs and HBe antigens were detected by ELISA and chemiluminescence immunoassay, respectively, as described (19).

MTT assay

The MTT cell viability assay was performed as described previously (19).

Southern blot analysis

Isolation of cellular DNA and southern blot analysis to detect HBV DNAs were performed as described previously (19).

Immunoblot Analysis

Immunoblot analysis was performed as described previously (26,27). Anti-NTCP (Abcam) (1:2000 dilution), anti-RARα (Santa Cruz Biotechnology) (1:6000 dilution), anti-RARβ (sigma) (1:6000 dilution), anti-RARγ (abcam) (1:2000 dilution), anti-RXRα (Santa Cruz Biotechnology) (1:8000 dilution), and anti-actin (Sigma) (1:5000 dilution) antibodies were used for primary antibodies.

Flow cytometry

1 x 10^6 primary human hepatocytes were incubated for 30 min with a 1:50 dilution of anti-NTCP Ab (Abcam), then washed and incubated with a dye-labeled secondary Ab (Alexa Fluor 488, Invitrogen) at 1:500 dilution in the dark. Staining and washing were carried out at 4 °C in PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide. The signals were analyzed with Cell Sorter SH8000 (SONY).

FITC-preS1 peptide-binding assay

Attachment of preS1 peptide with host cells was examined by preS1-binding assay essentially as described previously (28). HepaRG cells treated with or without Ro41-5253 (28) for 24 h or unlabeled preS1 peptide for 30 min were incubated with 40 nM FITC-labeled preS1 peptide (FITC-preS1) at 37°C for 30 min. After washing the cells twice with culture medium and once with phosphate-buffer saline (PBS), the cells were fixed with 4% paraformaldehyde. Then, the cells were treated with 4% Block Ace (DS Pharma Biomedical) containing DAPI for 30 min.

Reporter assay

HuS-E/2 cells were transfected with phNTCP-Gluc (GeneCopoeia), a reporter plasmid carrying the NTCP promoter sequence upstream of the Gaussia luciferase (Gluc) gene, and pSEAP (GeneCopoeia), expressing the secreted alkaline phosphatase (SEAP) gene, together with or without expression plasmids for RARα, RARβ, RARγ with RXRα using lipofectamine 2000 (Invitrogen). At 24 h posttransfection, cells were stimulated with the indicated compounds for further 24 h. The activities for Gluc as well as for SEAP were measured using a Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia) according to the manufacturer's protocol, and Gluc values normalized by SEAP are shown.

pRARE-Fluc, carrying three tandem repeats of RAR binding elements upstream of Firefly luciferase (Fluc) and pTK-Rluc (Promega), which carries herpes simplex virus thymidine kinase promoter expressing Renilla luciferase (Rluc) (25), were used in dual luciferase assays for detecting Fluc and Rluc. Fluc and Rluc were measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacture's protocol, and Fluc activities normalized by Rluc are shown.

For evaluating HBV transcription in Fig. 2B, we used a reporter construct carrying HBV enhancer I, II, and core promoter (nt 1039-1788) ("Enh I+II"), that carrying enhancer II and core promoter (nt 1413-1788) ("Enh II") that are derived from a genotype D HBV in HepG2.2.15 cells, which was inserted into pGL4.28 vector (Promega), and pGL3 promoter vector (Promega), which carries SV40 promoter ("SV40") as a control.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a Pierce Agarose ChIP Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Huh7-25 cells transfected with phNTCP-Gluc together with or without expression plasmids for FLAG-tagged RARα and for RXRα were treated with 5 μg/ml actinomycin D for 2 h. The cells were then washed and treated with or without 2 μM ATRA for 60 min. Formaldehyde cross-linked cells were lyzed, digested with micrococcal nuclease, immunoprecipitated with anti-FLAG antibody (Sigma) or normal IgG. Input samples were also recovered without immunoprecipitation. DNA recovered from the immunoprecipitated or the input samples was amplified with primers 5'-CCCAGGGCCCACCTGAATCTA-3' and 5'-TAGATTCAGGTGGGCCCTGGG-3' for detection of NTCP.

Results

Anti-HBV activity of Ro41-5253

We searched for small molecules capable of decreasing HBV infection in a cell-based chemical screening method using HBV-susceptible HepaRG cells (29). As a chemical library, we used a set of compounds of which bioactivity is already characterized (19). HepaRG cells were pretreated with compounds and then further incubated with HBV inoculum in the presence of compounds for 16 h (Fig. 1A). After removing free HBV and compounds by washing, the cells were cultured for an additional 12 days without compounds. For robust screening, HBV infection was monitored by ELISA quantification of HBs antigen secreted from the infected cells at 12 days postinfection. This screening revealed that HBs was significantly reduced by treatment with Ro41-5253 (Fig. 1B) as well as heparin, a competitive viral attachment

inhibitor which served as a positive control (14) (Fig. 1C). HBe in the medium (Fig. 1D) as well as intracellular HBc protein (Fig. 1F), HBV replicative (Fig. 1G), and cccDNA (Fig. 1H) were consistently decreased by treatment with Ro41-5253, without serious cytotoxicity (Fig. 1E). This effect of Ro41-5253 was not limited to infection of HepaRG cells because we observed a similar anti-HBV effect in primary human hepatocytes (Fig. 1I). The anti-HBV effect of Ro41-5253 on HBV infection of primary human hepatocytes was also observed in the absence of PEG8000 (Fig. 1J), which is frequently used to enhance HBV infectivity *in vitro* (14,29). These data suggest that Ro41-5253 treatment decreases hepatocyte susceptibility to HBV infection.

Reduced HBV entry in Ro41-5253-treated cells

Ro41-5253 decreased HBs secretion from infected cells in a dose-dependent manner without significant cytotoxicity (Fig. 2A). We next investigated which step in the HBV life cycle was blocked by Ro41-5253. The HBV life cycle can be divided into two phases: 1) the early phase of infection including attachment, internalization, nuclear import, and cccDNA formation, and 2) the following late phase representing HBV replication that includes transcription, pregenomic RNA encapsidation, reverse transcription, envelopment, and virus release (19,20,30-34). LMV and ETV, inhibitors of reverse transcriptase, dramatically decreased HBV DNA in HepAD38 cells (Fig. 2B, left), which can replicate HBV DNA but are resistant to infection (22). However, LMV and ETV did not show a significant effect in HepaRG-based infection (Fig. 1A), in contrast to the anti-HBV effect of CsA, an HBV entry inhibitor (19,35) (Fig. 2C), suggesting that this infection assay could be used to evaluate the early phase of infection without the replication process including the reverse transcription. Ro41-5253 was suggested to inhibit the early phase of infection prior to genome replication as an anti-HBV activity was evident in Fig. 2C but not in Fig. 2B. Moreover, Ro41-5253 had little effect on HBV transcription, which was monitored by a luciferase activity driven from the HBV enhancer I, II, and the core promoter (Fig. 2B, middle), and by the HBV RNA level in HepG2.2.15 cells, persistently producing HBV (36) (Fig. 2B, right). We then examined whether Ro41-5253 pretreatment affected viral attachment to host cells. To this end, HepaRG cells were exposed to HBV at 4℃ for 3 h, which allowed HBV attachment but not subsequent internalization (19) (Fig. 2D). After washing out free viruses, cell surface HBV DNA was extracted and quantified to evaluate HBV-cell attachment (Fig. 2D). Pretreatment with Ro41-5253 significantly reduced HBV DNA attached to the cell surface, as did heparin (Fig. 2D). In a preS1-binding assay, where FITC-labeled preS1 lipopeptide was used as a marker for HBV attachment to the cell surface, Ro41-5253-treated cells showed a reduced FITC fluorescence measuring viral attachment (Fig. 2E). Thus, Ro41-5253 primarily decreased the entry step, especially viral attachment. Next, to examine whether Ro41-5253 targeted HBV particles or host cells, HepaRG cells pretreated with compounds were examined for susceptibility to HBV infection in the absence of compounds (Fig. 2F). As a positive control, HBV infection was blocked by pretreatment of cells with an NTCP-binding lipopeptide, preS1(2-48)^{myr} (preS1 peptide) (15), but not by heparin, which binds HBV particles instead (14) (Fig. 2F, lanes 2 and 3). HBV infection was also diminished in HepaRG cells pretreated with IL-1β, which induced an innate immune response (37) (Fig. 2F, lane 4). In this experiment, Ro41-5253-pretreated HepaRG cells were less susceptible to HBV infection (Fig. 2F, lane 5), suggesting that the activity of Ro41-5253 in host cells contributed to the inhibition of HBV entry.

Ro41-5253 downregulated NTCP

Next, we examined how treatment of hepatocytes with Ro41-5253 decreased HBV susceptibility. Recently, NTCP was reported to be essential for HBV entry (15). Intriguingly, we found that Ro41-5253 decreased the level of NTCP protein in HepaRG cells (Fig. 3A). Flow cytometry showed that NTCP protein on the cell surface was consistently downregulated following treatment with Ro41-5253 (Fig. 3B, compare red and blue). Semi-quantitative RT-PCR revealed that mRNA levels for NTCP, but not apical sodium-dependent bile salt transporter (ASBT, also known as NTCP2 or SLC10A2), another SLC10 family transporter, were reduced by Ro41-5253 in HepaRG cells (Fig. 3C). Thus, Ro41-5253 could reduce NTCP expression. When endogenous NTCP and RAR

was knocked down by siRNA, the anti-HBV effect of Ro41-5253 was significantly diminished (Fig. 3D), suggesting that the inhibitory activity of Ro41-5253 to HBV infection was, at least in part, mediated by targeting NTCP. These data suggest that Ro41-5253 downregulated NTCP, which probably contributed to the anti-HBV activity of Ro41-5253.

Retinoic acid receptor regulated NTCP promoter activity

To determine the mechanism for Ro41-5253-induced downregulation of NTCP, we used a reporter construct inserting nucleotides (nt) -1143 ~ +108 of the human NTCP (hNTCP) promoter upstream of the Gluc gene (Fig. 4A, upper). Ro41-5253 dose-dependently decreased the luciferase activity driven from this promoter, although the effect was modest that showed up to approximately 40% reduction (Fig. 4A, left). Ro41-5253 had little effect on the herpes simplex virus thymidine kinase promoter (Fig. 4A, right), suggesting that Ro41-5253 specifically repressed hNTCP promoter activity. As reported previously (38), Ro41-5253 specifically inhibited retinoic acid receptor (RAR)-mediated transcription (Fig. 4B, C). RARα, RARβ, and RARγ are members of the nuclear hormone receptor superfamily, which are ligand-activated transcription factors that regulate the transcription of specific downstream genes by binding to RAR responsive element (RARE) predominantly in the form of heterodimer with retinoid X receptor (RXR). We therefore asked whether RAR could regulate the hNTCP promoter. As shown in Fig. 4D, hNTCP promoter activity was stimulated by overexpression of either RARα, RARβ, or RARγ together with RXRα, and transcription augmented by RAR could be repressed by Ro41-5253 (Fig. 4D). Knockdown of endogenous RARα, RXRα, or both dramatically impaired the activity of the hNTCP promoter (Fig. 4E). These results suggest that RAR/RXR is involved in the transcriptional regulation of the hNTCP gene. Consistently, an RAR agonist, ATRA, induced NTCP mRNA expression (Fig. 4F).

Importantly, endogenous expression of RARα was more abundant in differentiated HepaRG cells which are susceptible to HBV infection, than that in undifferentiated HepaRG and HepG2 cells, which are not susceptible (29) (Fig. 4G). This expression pattern was consistent with the expression of NTCP and with HBV susceptibility, suggesting the significance of RAR in regulating NTCP expression.

Promoter analysis of hNTCP

We next examined whether RAR regulation of hNTCP promoter is direct or indirect. From the analyses so far using rat Ntcp (rNtcp) promoter, one of the major regulators for rNtcp expression is farnesoid X receptor (FXR), which is a nuclear receptor recognizing bile acids (39). FXR, which is activated upon intracellular bile acids, indirectly regulates rNtcp expression: FXR induces its downstream small heterodimer partner (Shp), another nuclear receptor, and Shp recruits to rNtcp promoter to repress the promoter activity (39). Then, we examined whether RAR affected the expression of human SHP. As shown in Fig. 5A, while a FXR agonist GW4064 remarkably induced SHP expression as reported (39), RAR did not have a remarkable effect on SHP level in HepaRG cells (Fig. 5A). To assess the direct involvement of RAR in hNTCP regulation, ChIP assay showed that RAR was associated with hNTCP promoter both in the presence and absence of ATRA (Fig. 5B), consistent with the characteristic that RAR/RXR binds to RARE regardless of ligand stimulation (40). The genomatix software predicts that hNTCP promoter possesses five putative RAREs in nt -1143 ~ +108 (Fig. 5C). Introduction of mutations in all of these five elements lost the promoter activation by RAR/RXR overexpression (Fig. 5C, "5-Mut"). While the promoters mutated in the motif nt -491~-479, -368~-356, -274~-258, or -179~-167 was activated by ectopic expression of RAR/RXR and this activation was cancelled by Ro41-5253 treatment, the hNTCP promoter with mutations in nt -112~-96 had no significant response by RAR/RXR (Fig. 5C). These data suggest that the nt -112 to -96 region is responsible for RAR-mediated transcriptional activation of hNTCP.

HBV susceptibility was decreased in RAR-inactivated cells

We further investigated the impact of RAR antagonization on HBV infectivity. BMS195614, BMS493, and MM11253, which repressed RAR-mediated transcription (Fig. 6A), all decreased the susceptibility of HepaRG cells to HBV infection

(Fig. 6B) without significant cytotoxicity (Fig. 6C). These data confirmed that HBV infection was restricted in RAR-inactivated cells. Among these, CD2665, a synthetic retinoid that is known to inhibit RAR-mediated transcription (Fig. 7A), had more potent anti-HBV activity than Ro41-5253 (Fig. 7B), which was accompanied by the inhibition of the hNTCP promoter (Fig. 7C) and downregulation of NTCP protein (Fig. 7D).

CD2665 showed a pan-genotypic anti-HBV effect

We then examined the effect of CD2665 on the infection of primary human hepatocytes with different HBV genotypes. CD2665 significantly reduced the infection of HBV genotypes A, B, C, and D, as revealed by quantification of HBs and HBe antigens in the culture supernatant of infected cells (Fig. 8A-D). Additionally, this RAR inhibitor decreased the infection of ETV- and LMV-resistant HBV genotype C clone carrying mutations in L180M, S202G, and M204V (Fig. 8E and F). Thus, CD2665 showed pan-genotypic anti-HBV effects and was also effective on an HBV isolate with resistance to nucleoside analogs.

We further investigated whether RAR inhibitors could prevent HBV spread. It was recently reported that HBV infection in freshly isolated primary human hepatocytes could spread during long term culture through production of infectious virions and reinfection of surrounding cells (41). As shown in Fig. 8G, the percentage of HBV-positive cells increased up to 30 days postinfection without compound treatment (Fig. 8G, panels a-d). However, such HBV spread was clearly interrupted by treatment with Ro41-5263 and CD2665 as well as preS1 peptide (Fig. 8G, panels e-p). The rise of HBs antigen in the culture supernatant along with the culture time up to 30 days was remarkably inhibited by continuous treatment with Ro41-5253 and CD2665 as well as preS1 peptide without serious cytotoxicity (Fig. 8G, right graph). Thus, continuous RAR inactivation could inhibit the spread of HBV by interrupting *de novo* infection.

Discussion

In this study, we screened a chemical library using a HepaRG-based HBV infection system and found that pretreatment with Ro41-5253 decreased HBV infection by blocking viral entry. HBV entry follows multiple steps starting with low affinity viral attachment to the cell surface followed by specific binding to entry receptor(s) including NTCP. NTCP is reported to be essential for HBV entry (42). So far, we and other groups have reported that NTCP-binding agents including cyclosporin A and its derivatives, as well as bile acids including ursodeoxycholic acid and taurocholic acid inhibited HBV entry by interrupting the interaction between NTCP and HBV large surface protein (19,35). Ro41-5253 was distinct from these agents and was found to decrease host susceptibility to HBV infection by modulating the expression levels of NTCP. These results suggest that the regulatory circuit for NTCP expression is one of the determinants for susceptibility to HBV infection. We previously showed that the cell surface NTCP protein expression correlated with susceptibility to HBV infection (43). We therefore screened for compounds inhibiting hNTCP promoter activity to identify HBV entry inhibitors (data not shown) (44). Intriguingly, all of the compounds identified as repressors of the hNTCP promoter were inhibitors of RAR-mediated transcription. This strongly suggests that RAR plays a crucial role in regulating the activity of the hNTCP promoter (Fig. 9). We consistently found that RAR was abundantly expressed in differentiated HepaRG cells susceptible to HBV infection, in contrast to the low expression of RAR in undifferentiated HepaRG and HepG2 cells, which were not susceptible to HBV (Fig. 4F). RARE is also found in the HBV enhancer I region (45). RAR is likely to have multiple roles in regulating the HBV life cycle.

So far, only transcriptional regulation of rat Ntcp has been extensively analyzed (39,46,47). However, the transcription of hNTCP was shown to be differently regulated mainly because of sequence divergence in the promoter region (48), and transcriptional regulation of hNTCP remains poorly understood. Hepatocyte nuclear factor (HNF)1α and HNF4α, which positively regulated the rat Ntcp promoter, had little effect on hNTCP promoter activity (48). HNF3β bound to the promoter region and inhibited promoter activities of both hNTCP and rat Ntcp. CCAAT/enhancer-binding protein (C/EBP) also bound and regulated the hNTCP promoter (44,48). A previous study, which was mainly based on reporter assays using a construct of the region from -188 to +83 of hNTCP promoter, concluded that RAR did not affect hNTCP transcription

(48). By using a reporter carrying a longer promoter region, our study is the first to implicate RARs in the regulation of hNTCP gene expression (Fig. 9). The turnover of NTCP protein was reported to be rapid, with a half-life of much less than 24 h (49). Consequently, reduction in NTCP transcription by RAR inhibition could rapidly decrease the NTCP protein level and affect HBV susceptibility.

NTCP plays a major role in the hepatic influx of conjugated bile salts from portal circulation. Because NTCP-knockout mice are so far unavailable, it is not known whether loss of NTCP function can cause any physiological defect *in vivo*. However, no serious diseases are reported in individuals carrying single nucleotide polymorphisms (SNPs) that significantly decrease the transporter activity of NTCP (50,51), suggesting that NTCP function may be redundant with other proteins. Organic anion transporting polypeptides (OATPs) are also known to be involved in bile acid transport. Moreover, an inhibition assay using Myrcludex-B showed that the IC_{50} for HBV infection was approximately 0.1 nM (52) while that for NTCP transporter function was 4 nM (28), suggesting that HBV infection could be inhibited without fully inactivating the NTCP transporter (53). HBV entry inhibitors are expected to be useful for preventing *de novo* infection upon post-exposure prophylaxis or vertical transmission where serious toxicity might be avoided with a short term treatment (54). For drug development studies against HIV, downregulation of an HIV co-receptor CCR5 by ribozymes could inhibit HIV infection both *in vitro* and *in vivo* (55). Disruption of CCR5 by zinc-finger nucleases could reduce permissiveness to HIV infection and was effective in decreasing viral load *in vivo* (56). Thus, interventions to regulate viral permissiveness could become a method for eliminating viral infection (55). Our findings suggest that the regulatory mechanisms of NTCP expression could serve as targets for the development of anti-HBV agents. High throughput screening with a reporter assay using an NTCP promoter-driven reporter, as exemplified by this study, will be useful for identifying more anti-HBV drugs.

References

1. Liang, T. J. (2009) Hepatitis B: the virus and disease. *Hepatology* **49**, S13-21
2. Ott, J. J., Stevens, G. A., Groeger, J., and Wiersma, S. T. (2012) Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* **30**, 2212-2219
3. Zoulim, F., and Locarnini, S. (2013) Optimal management of chronic hepatitis B patients with treatment failure and antiviral drug resistance. *Liver international : official journal of the International Association for the Study of the Liver* **33 Suppl 1**, 116-124
4. Arbuthnot, P., and Kew, M. (2001) Hepatitis B virus and hepatocellular carcinoma. *International journal of experimental pathology* **82**, 77-100
5. Kao, J. H., Chen, P. J., and Chen, D. S. (2010) Recent advances in the research of hepatitis B virus-related hepatocellular carcinoma: epidemiologic and molecular biological aspects. *Advances in cancer research* **108**, 21-72
6. Lok, A. S. (2002) Chronic hepatitis B. *The New England journal of medicine* **346**, 1682-1683
7. Pagliaccetti, N. E., Chu, E. N., Bolen, C. R., Kleinstein, S. H., and Robek, M. D. (2010) Lambda and alpha interferons inhibit hepatitis B virus replication through a common molecular mechanism but with different in vivo activities. *Virology* **401**, 197-206
8. Robek, M. D., Boyd, B. S., and Chisari, F. V. (2005) Lambda interferon inhibits hepatitis B and C virus replication. *Journal of virology* **79**, 3851-3854
9. Dusheiko, G. (2013) Treatment of HBeAg positive chronic hepatitis B: interferon or nucleoside analogues. *Liver international : official journal of the International Association for the Study of the Liver* **33 Suppl 1**, 137-150
10. Lau, G. K., Piratvisuth, T., Luo, K. X., Marcellin, P., Thongsawat, S., Cooksley, G., Gane, E., Fried, M. W., Chow, W. C., Paik, S. W., Chang, W. Y., Berg, T., Flisiak, R., McCloud, P., Pluck, N., and Peginterferon Alfa-2a, H.-P. C. H. B. S. G. (2005) Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *The New England journal of medicine* **352**, 2682-2695
11. Chen, L. P., Zhao, J., Du, Y., Han, Y. F., Su, T., Zhang, H. W., and Cao, G. W. (2012) Antiviral treatment to prevent chronic hepatitis B or C-related hepatocellular carcinoma. *World journal of virology* **1**, 174-183

12. Ohishi, W., and Chayama, K. (2012) Treatment of chronic hepatitis B with nucleos(t)ide analogues. *Hepatology research : the official journal of the Japan Society of Hepatology* **42**, 219-225

13. Liu, F., Wang, X., Wei, F., Hu, H., Zhang, D., Hu, P., and Ren, H. (2014) Efficacy and resistance in de novo combination lamivudine and adefovir dipivoxil therapy versus entecavir monotherapy for the treatment-naive patients with chronic hepatitis B: a meta-analysis. *Virology journal* **11**, 59

14. Schulze, A., Gripon, P., and Urban, S. (2007) Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology* **46**, 1759-1768

15. Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H., Fu, L., Song, M., Chen, P., Gao, W., Ren, B., Sun, Y., Cai, T., Feng, X., Sui, J., and Li, W. (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife* **1**, e00049

16. Stieger, B. (2011) The role of the sodium-taurocholate cotransporting polypeptide (NTCP) and of the bile salt export pump (BSEP) in physiology and pathophysiology of bile formation. *Handbook of experimental pharmacology*, 205-259

17. Kotani, N., Maeda, K., Debori, Y., Camus, S., Li, R., Chesne, C., and Sugiyama, Y. (2012) Expression and transport function of drug uptake transporters in differentiated HepaRG cells. *Molecular pharmaceutics* **9**, 3434-3441

18. Kullak-Ublick, G. A., Beuers, U., and Paumgartner, G. (1996) Molecular and functional characterization of bile acid transport in human hepatoblastoma HepG2 cells. *Hepatology* **23**, 1053-1060

19. Watashi, K., Sluder, A., Daito, T., Matsunaga, S., Ryo, A., Nagamori, S., Iwamoto, M., Nakajima, S., Tsukuda, S., Borroto-Esoda, K., Sugiyama, M., Tanaka, Y., Kanai, Y., Kusuhara, H., Mizokami, M., and Wakita, T. (2014) Cyclosporin A and its analogs inhibit hepatitis B virus entry into cultured hepatocytes through targeting a membrane transporter, sodium taurocholate cotransporting polypeptide (NTCP). *Hepatology* **59**, 1726-1737

20. Gripon, P., Cannie, I., and Urban, S. (2005) Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. *Journal of virology* **79**, 1613-1622

21. Petersen, J., Dandri, M., Mier, W., Lutgehetmann, M., Volz, T., von Weizsacker, F., Haberkorn, U., Fischer, L., Pollok, J. M., Erbes, B., Seitz, S., and Urban, S. (2008) Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. *Nature biotechnology* **26**, 335-341

22. Ladner, S. K., Otto, M. J., Barker, C. S., Zaifert, K., Wang, G. H., Guo, J. T., Seeger, C., and King, R. W. (1997) Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. *Antimicrobial agents and chemotherapy* **41**, 1715-1720

23. Aly, H. H., Watashi, K., Hijikata, M., Kaneko, H., Takada, Y., Egawa, H., Uemoto, S., and Shimotohno, K. (2007) Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes. *Journal of hepatology* **46**, 26-36

24. Sugiyama, M., Tanaka, Y., Kato, T., Orito, E., Ito, K., Acharya, S. K., Gish, R. G., Kramvis, A., Shimada, T., Izumi, N., Kaito, M., Miyakawa, Y., and Mizokami, M. (2006) Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* **44**, 915-924

25. Watashi, K., Hijikata, M., Tagawa, A., Doi, T., Marusawa, H., and Shimotohno, K. (2003) Modulation of retinoid signaling by a cytoplasmic viral protein via sequestration of Sp110b, a potent transcriptional corepressor of retinoic acid receptor, from the nucleus. *Molecular and cellular biology* **23**, 7498-7509

26. Marusawa, H., Hijikata, M., Watashi, K., Chiba, T., and Shimotohno, K. (2001) Regulation of Fas-mediated apoptosis by NF-kappaB activity in human hepatocyte derived cell lines. *Microbiology and immunology* **45**, 483-489

27. Watashi, K., Khan, M., Yedavalli, V. R., Yeung, M. L., Strebel, K., and Jeang, K. T. (2008) Human immunodeficiency virus type 1 replication and regulation of APOBEC3G by peptidyl prolyl isomerase Pin1. *Journal of virology* **82**, 9928-9936

28. Ni, Y., Lempp, F. A., Mehrle, S., Nkongolo, S., Kaufman, C., Falth, M., Stindt, J., Koniger, C., Nassal, M., Kubitz, R., Sultmann, H., and Urban, S. (2014) Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. *Gastroenterology* **146**, 1070-1083

29. Gripon, P., Rumin, S., Urban, S., Le Seyec, J., Glaise, D., Cannie, I., Guyomard, C., Lucas, J., Trepo, C., and Guguen-Guillouzo, C. (2002) Infection of a human hepatoma

cell line by hepatitis B virus. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15655-15660

30. Cattaneo, R., Will, H., and Schaller, H. (1984) Hepatitis B virus transcription in the infected liver. *The EMBO journal* **3**, 2191-2196

31. Hirsch, R. C., Lavine, J. E., Chang, L. J., Varmus, H. E., and Ganem, D. (1990) Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as wel as for reverse transcription. *Nature* **344**, 552-555

32. Huan, B., and Siddiqui, A. (1993) Regulation of hepatitis B virus gene expression. *Journal of hepatology* **17 Suppl 3**, S20-23

33. Newman, M., Suk, F. M., Cajimat, M., Chua, P. K., and Shih, C. (2003) Stability and morphology comparisons of self-assembled virus-like particles from wild-type and mutant human hepatitis B virus capsid proteins. *Journal of virology* **77**, 12950-12960

34. Yeh, C. T., and Ou, J. H. (1991) Phosphorylation of hepatitis B virus precore and core proteins. *Journal of virology* **65**, 2327-2331

35. Nkongolo, S., Ni, Y., Lempp, F. A., Kaufman, C., Lindner, T., Esser-Nobis, K., Lohmann, V., Mier, W., Mehrle, S., and Urban, S. (2013) Cyclosporin A inhibits hepatitis B and hepatitis D virus entry by cyclophilin-independent interference with the NTCP receptor. *Journal of hepatology*

36. Sells, M. A., Zelent, A. Z., Shvartsman, M., and Acs, G. (1988) Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. *Journal of virology* **62**, 2836-2844

37. Watashi, K., Liang, G., Iwamoto, M., Marusawa, H., Uchida, N., Daito, T., Kitamura, K., Muramatsu, M., Ohashi, H., Kiyohara, T., Suzuki, R., Li, J., Tong, S., Tanaka, Y., Murata, K., Aizaki, H., and Wakita, T. (2013) Interleukin-1 and tumor necrosis factor-alpha trigger restriction of hepatitis B virus infection via a cytidine deaminase activation-induced cytidine deaminase (AID). *The Journal of biological chemistry* **288**, 31715-31727

38. Apfel, C., Bauer, F., Crettaz, M., Forni, L., Kamber, M., Kaufmann, F., LeMotte, P., Pirson, W., and Klaus, M. (1992) A retinoic acid receptor alpha antagonist selectively counteracts retinoic acid effects. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 7129-7133

39. Denson, L. A., Sturm, E., Echevarria, W., Zimmerman, T. L., Makishima, M.,

Mangelsdorf, D. J., and Karpen, S. J. (2001) The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. *Gastroenterology* **121**, 140-147

40. Bastien, J., and Rochette-Egly, C. (2004) Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1-16

41. Ishida, Y., Yamasaki, C., Yanagi, A., Yoshizane, Y., Chayama, K., and Tateno, C. (2013) Development of novel in vitro HBV infection model by using fresh human hepatocytes isolated from humanized mouse liver. *International Meeting on Molecular Biology of Hepatitis B Virus* **P13**

42. Yan, H., Peng, B., Liu, Y., Xu, G., He, W., Ren, B., Jing, Z., Sui, J., and Li, W. (2014) Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide. *Journal of virology* **88** 3273-3284

43. Iwamoto, M., Watashi, K., Tsukuda, S., Aly, H. H., Fukasawa, M., Fujimoto, A., Suzuki, R., Aizaki, H., Ito, T., Koiwai, O., Kusuhara, H., and Wakita, T. (2014) Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP. *Biochemical and biophysical research communications* **443**, 808-813

44. Shiao, T., Iwahashi, M., Fortune, J., Quattrochi, L., Bowman, S., Wick, M., Qadri, I., and Simon, F. R. (2000) Structural and functional characterization of liver cell-specific activity of the human sodium/taurocholate cotransporter. *Genomics* **69**, 203-213

45. Huan, B., and Siddiqui, A. (1992) Retinoid X receptor RXR alpha binds to and trans-activates the hepatitis B virus enhancer. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 9059-9063

46. Geier, A., Martin, I. V., Dietrich, C. G., Balasubramaniyan, N., Strauch, S., Suchy, F. J., Gartung, C., Trautwein, C., and Ananthanarayanan, M. (2008) Hepatocyte nuclear factor-4alpha is a central transactivator of the mouse Ntcp gene. *American journal of physiology. Gastrointestinal and liver physiology* **295**, G226-233

47. Zollner, G., Wagner, M., Fickert, P., Geier, A., Fuchsbichler, A., Silbert, D., Gumhold, J., Zatloukal, K., Kaser, A., Tilg, H., Denk, H., and Trauner, M. (2005) Role of nuclear receptors and hepatocyte-enriched transcription factors for Ntcp repression in biliary obstruction in mouse liver. *American journal of physiology. Gastrointestinal and liver*

physiology **289**, G798-805

48. Jung, D., Hagenbuch, B., Fried, M., Meier, P. J., and Kullak-Ublick, G. A. (2004) Role of liver-enriched transcription factors and nuclear receptors in regulating the human, mouse, and rat NTCP gene. *American journal of physiology. Gastrointestinal and liver physiology* **286**, G752-761

49. Rippin, S. J., Hagenbuch, B., Meier, P. J., and Stieger, B. (2001) Cholestatic expression pattern of sinusoidal and canalicular organic anion transport systems in primary cultured rat hepatocytes. *Hepatology* **33**, 776-782

50. Ho, R. H., Leake, B. F., Roberts, R. L., Lee, W., and Kim, R. B. (2004) Ethnicity-dependent polymorphism in Na+-taurocholate cotransporting polypeptide (SLC10A1) reveals a domain critical for bile acid substrate recognition. *The Journal of biological chemistry* **279**, 7213-7222

51. Pan, W., Song, I. S., Shin, H. J., Kim, M. H., Choi, Y. L., Lim, S. J., Kim, W. Y., Lee, S. S., and Shin, J. G. (2011) Genetic polymorphisms in Na+-taurocholate co-transporting polypeptide (NTCP) and ileal apical sodium-dependent bile acid transporter (ASBT) and ethnic comparisons of functional variants of NTCP among Asian populations. *Xenobiotica : the fate of foreign compounds in biological systems* **41**, 501-510

52. Schulze, A., Schieck, A., Ni, Y., Mier, W., and Urban, S. (2010) Fine mapping of pre-S sequence requirements for hepatitis B virus large envelope protein-mediated receptor interaction. *Journal of virology* **84**, 1989-2000

53. Watashi, K., Urban, S., Li, W., and Wakita, T. (2014) NTCP and beyond: opening the door to unveil hepatitis B virus entry. *International journal of molecular sciences* **15**, 2892-2905

54. Deuffic-Burban, S., Delarocque-Astagneau, E., Abiteboul, D., Bouvet, E., and Yazdanpanah, Y. (2011) Blood-borne viruses in health care workers: prevention and management. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* **52**, 4-10

55. Bai, J., Gorantla, S., Banda, N., Cagnon, L., Rossi, J., and Akkina, R. (2000) Characterization of anti-CCR5 ribozyme-transduced CD34+ hematopoietic progenitor cells in vitro and in a SCID-hu mouse model in vivo. *Molecular therapy : the journal of the American Society of Gene Therapy* **1**, 244-254

56. Perez, E. E., Wang, J., Miller, J. C., Jouvenot, Y., Kim, K. A., Liu, O., Wang, N., Lee, G.,

Bartsevich, V. V., Lee, Y. L., Guschin, D. Y., Rupniewski, I., Waite, A. J., Carpenito, C., Carroll, R. G., Orange, J. S., Urnov, F. D., Rebar, E. J., Ando, D., Gregory, P. D., Riley, J. L., Holmes, M. C., and June, C. H. (2008) Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nature biotechnology* **26**, 808-816

Abbreviations

NTCP, sodium taurocholate cotransporting polypeptide; HBV, hepatitis B virus; RAR, retinoic acid receptor; IFN, interferon; LMV, lamivudine; ETV, entecavir; HBs, HBV surface protein; SLC10A1, solute carrier protein 10A1; hNTCP, human NTCP; ATRA, all-trans retinoic acid; SHP, small heterodimer partner; ASBT, apical sodium-dependent bile salt transporter; RARE, RAR responsive element; RXR, retinoid X receptor; SEAP, secreted alkaline phosphatase; ChIP, chromatin immunoprecipitation; FXR, farnesoid X receptor

Acknowledgments

HepAD38 and HuS-E/2 cells were kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center and Dr. Kunitada Shimotohno at National Center for Global Health and Medicine. We are also grateful to all of the members of Department of Virology II, National Institute of Infectious Diseases. This study was partly supported by grants-in-aid from the Ministry of Health, Labor, and Welfare, Japan, from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, and from Japan Society for the Promotion of Science, and by the incentive support from Liver Forum in Kyoto.