Dysregulation of Retinoic Acid Receptor Diminishes Hepatocyte Permissiveness to Hepatitis B Virus Infection through Modulation of Sodium Taurocholate Cotransporting Polypeptide (NTCP) Expression*

Senko Tsukuda†, Koichi Watashi†, Masashi Iwamoto†, Ryosuke Suzuki†, Hideki Aizaki‡, Maiko Okada§, Masaya Sugiyama†, Soichi Kojima†, Yasuhito Tanaka**, Masashi Mizokami†, Jisu Li**, Shoping Tong‡‡, and Takaji Wakita‡

From the †Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, the ‡Department of Virology and Liver Unit, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, the §Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, the ¶Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, the **Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, the ‡‡Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan, and the ‡‡‡Research Center for Hepatitis and Liver Disease, Tokyo 162-8640, Japan.

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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.

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 nucleotides −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 the 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 that was resistant to nucleoside analogs. Thus, RAR is crucial for regulating NTCP expression that determines permissiveness to HBV infection. This is the first demonstration showing host regulation of NTCP to support HBV infection.

Hepatitis B virus (HBV) infection is a major public health problem, as the virus chronically infects ~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 analogs, 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 analogs 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 of the reverse transcriptase of HBV, while resistance to other nucleos(t)ide analogs is restricted to less than 40%. Thus, there is a need for new antiviral agents that target novel host factors as potential therapeutic targets.

The abbreviations used are: HBV, hepatitis B virus; NTCP, sodium taurocholate cotransporting polypeptide; RAR, retinoic acid receptor; LMV, lamivudine; ETV, entecavir; HB, 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; FXR, farnesoid X receptor; MIT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nt, nucleotide; cccDNA, covalently closed circular DNA.

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† To whom correspondence should be addressed: Dept. 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.
polymerase L180M and M204V plus either one of Thr-184, Ser-202, or Met-250 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 for 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 it also suggests a novel concept whereby manipulation of this regulation machinery can be useful for preventing HBV infection.

**EXPERIMENTAL PROCEDURES**

**Reagents**—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-1β was purchased from PeproTech. CD2665, BMS195614, BMS493, and MM11253 were purchased from Tocris Bioscience.

**Cell Culture**—HepaRG cells (BiOPREDIC) and primary human hepatocytes (Phoebos) 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 GeneCopeia 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 the following primer sets: F1, 5’-CACATCTTGGAAATTTCCCAAAATC-3’ and 5’-GGAGGGATGTTGTCATGAAATTTGTTGAGGCTGAGAGATCCGAGATTGCATCTCCTCCT-3’ and primer sets 5’-CTCTACGCTTCATATACATTTCAATGGAGGACATCACCCTCTCTCAGGACAT-3’ and R6, 5’-CTCGGTACCAAGCTTTCCTTGTG-3’.

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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. 8, A–E, we used concentrated (~200-fold) media of HepG2 cells transfected with an expression plasmid for either HBV genotypes A, B, C, D or genotype C carrying mutations at L180M, S202G, and C230M, and M204V (HBV/Aeus, HBV/Bj35s, HBV/C-AT, HBV/D-IN560, 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 described previously (19). HBV for Fig. 8F (genotype C) was purchased from Phoenixbiox.

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’-AGGAGGA-GTGTTCT-3’ and 5’-CCGGCTGAAGA-GTTGGGTC-3’ for NTCP, 5’-GTGCGCTTG-ACATGGTTCT-3’ and 5’-GACCCAATAGGCCAAGATA-3’ for ASBT, 5’-CAGCTATGTGCACCTCATCG-3’ and 5’-CCAAGGCTCCAGACACG-3’ for SHP, and 5’-CCATGGAGA-AGCTGGG-3’ and 5’-AAAGTTGCATGGAGTACC-3’ for GAPDH, respectively.

Immunofluorescence Analysis—Immunofluorescence was conducted essentially as described (25) using an anti-Hbc antibody (DAKO, catalog no. B0586) at a dilution of 1:1000.

Detection of HBs and HBe Antigens—HBs and HBe antigens were detected by ELISA and chemiluminescence immunoblot assay, 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 × 10⁶ primary human hepatocytes were incubated for 30 min with a 1:50 dilution of anti-NTCP antibody (Abcam) and then washed and incubated with a dye-labeled secondary antibody (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-buffered 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 post-transfection, cells were stimulated with the indicated compounds for a 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 using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’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") and that carrying enhancer II and core promoter (nt 1413–1788) ("Enh II"). These were constructed by inserting the corresponding sequences derived from a genotype D HBV in HepG2.2.15 cells into pGL4.28 vector (Promega), pGL3 promoter vector (Promega), which carries SV40 promoter ("SV40") was used 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 mg/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 lysed, digested with micrococcal nuclease, and 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’-CCCAGGGCCACCTGAATTCT-3’ and 5’-TAGATTCAGGTGCGCTTGGGG-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 for which bioactivity was 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...
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FIGURE 1. Ro41-5253 decreased susceptibility to HBV infection. A, schematic representation of the schedule for treatment of HepaRG cells with compounds and infection with HBV. HepaRG cells were pretreated with compounds for 2 h and then inoculated with HBV in the presence of compounds for 16 h. After washing out the free HBV and compounds, cells were cultured in the absence of compounds for an additional 12 days followed by quantification of secreted HBs protein. Black and dashed bars indicate the interval for treatment and without treatment, respectively. B, chemical structure of Ro41-5253. C–E, HepaRG cells were treated with or without 10 μM Ro41-5253 or 50 units/ml heparin according to the protocol shown in A, and HBs (C) and HBc (D) antigens in the culture supernatant were measured. Cell viability was also examined by MTT assay (E). F–H, HBc protein (F), HBV DNAs (G), and cccDNA (H) in the cells according to the protocol shown in A were detected by immunofluorescence, real time PCR, and Southern blot analysis. Red and blue in F show the detection of HBc protein and nuclear staining, respectively. I and J, primary human hepatocytes were treated with the indicated compounds and infected with HBV in the presence (I) or absence (J) of PEG8000 according to the protocol shown in A. The levels of HBV DNA in the cells (I and J) and HBc (I) or absence (J) were quantified. The data show the means of three independent experiments. Standard deviations are also indicated by error bars. Statistical significance was determined using Student’s t test (*, p < 0.05; **, p < 0.01).
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 compounds (200 nM preS1 peptide, 20 μM Ro41-5253, 1 μM lamivudine, or 1 μM entecavi) for 6 days without tetracycline quantified by real time PCR. Middle panel, HepG2 cells transfected with the reporter plasmids carrying HBV Enhancer (Enh) I and II, HBV Enhancer II, or SV40 promoter (“Experimental Procedures”) were treated with or without Ro41-5253 or HX531 as a positive control to measure the luciferase activity. Right panel, HepG2.2.15 cells were treated with or without Ro41-5253 or HX531 for 6 days, and intracellular HBV RNA was quantified by real time RT-PCR. C, HepaRG cells were treated with or without indicated compounds (200 nM preS1 peptide, 20 μM Ro41-5253, 1 μM lamivudine, 1 μM entecavi, or 4 μM CsA) followed by HBV infection according to the protocol shown in Fig. 1A. D, upper scheme shows the experimental procedure for examining cell surface-bound HBV. The cells were pretreated with compounds (50 units/ml heparin, 20 μM Ro41-5253, or 1 μM lamivudine) at 37 °C for 24 h and then treated with HBV at 4 °C for 3 h to allow HBV attachment but not internalization into the cells. After removing free virus, cell surface HBV DNA was extracted and quantified by real time PCR. E, HepaRG cells pretreated with the indicated compounds (1 μM unconjugated preS1 peptide, 20 μM Ro41-5253) for 24 h were treated with 40 nM FITC-conjugated pre-S1 peptide (FITC-preS1) in the presence of compounds at 37 °C for 30 min. Green and blue signals show FITC-preS1 and nuclear staining, respectively. F, HepaRG cells pretreated with the indicated compounds (50 units/ml heparin, 200 nM preS1 peptide, 100 ng/ml IL-1β, or 20 μM Ro41-5253) for 24 h were used for the HBV infection assay, where HBV was inoculated for 16 h in the absence of the compounds. Statistical significance was determined using Student’s t test (*, p < 0.05, and **, p < 0.01).

Ro41-5253 Down-regulated 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 down-regulated 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 infec-


Retinoic Acid Receptor Regulated NTCP Promoter Activity—

To determine the mechanism for Ro41-5253-induced down-regulation of NTCP, we used a reporter construct inserting nucleotides (nt) −1143 to +108 of the human NTCP (hNTCP) promoter upstream of the Gluc gene (Fig. 4A, upper panel). Ro41-5253 dose-dependently decreased the luciferase activity driven from this promoter, although the effect was modest and showed up to ~40% reduction (Fig. 4A, left panel). Ro41-5253 had little effect on the herpes simplex virus thymidine kinase promoter (Fig. 4A, right panel), suggesting that Ro41-5253 specifically repressed hNTCP promoter activity. As reported previously (38), Ro41-5253 specifically inhibited RAR-mediated transcription (Fig. 4, B and 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 the RAR-responsive element (RARE) predominantly in the form of a heterodimer with 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, RARβ, 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 (Fig. 4G) (29). This expression pattern was consistent with the expression of RAR and with HBV susceptibility, suggesting the significance of RAR in regulating NTCP expression.

Promoter Analysis of hNTCP—We next examined whether RAR regulation of the hNTCP promoter is direct or indirect. From the analyses so far using the 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 the rNtcp promoter to repress the promoter activity (39). Then we examined whether RAR affected the expression of human SHP. As shown in Fig. 5A, although an FXR agonist GW4064 remarkably induced SHP expression as reported (39), RAR did not have a remarkable effect on the SHP level in HepaRG cells (Fig. 5A). To assess the direct involvement of RAR in hNTCP regulation, the ChIP assay showed that RAR was associated with the 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 the hNTCP promoter possesses five putative RAREs in nt −1143 to +108 (Fig. 5C). Introduction of mutations in all of these five elements lost the promoter activation by RAR/RXR overexpression (Fig. 5C, 5-Mut). Although the promoters mutated in the motif nt −491 to −479, −368 to −356, −274 to −258, or −179 to −167 were activated by ectopic expression of RAR/RXR and this activation was cancelled by Ro41-5253 treatment, the hNTCP promoter with
FIGURE 4. RAR could regulate hNTCP promoter activity. A, left panel, HuS-E/2 cells were transfected for 6 h with an hNTCP reporter construct with −1143/+108 of the hNTCP promoter region cloned upstream of the Gluc gene (upper panel, phNTCP-Gluc), together with an internal control plasmid expressing SEAP (pSEAP). Cells were treated or untreated with various concentrations of Ro41-5253 (5–40 μM) for 48 h. The Gluc and SEAP activities were determined, and the Gluc values normalized by SEAP are shown. Right panel, HuS-E/2 cells transfected with a reporter construct carrying the herpes simplex virus thymidine kinase promoter (pTK-Rluc) were examined for luciferase activity in the presence or absence of Ro41-5253 (10–40 μM). B, HuS-E/2 cells transfected with a Fluc-encoding reporter plasmid carrying three tandem repeats of RARE (upper panel, pRARE-Fluc), and Rluc-encoding reporter plasmid driven from herpes simplex virus thymidine kinase promoter (pTK-Rluc) were treated with or without 20 μM Ro41-5253 in the presence or absence of an RAR agonist, ATRA, 1 μM for 24 h. Relative values for Fluc normalized by Rluc are shown. C, HuS-E/2 cells transfected with pRARE-Fluc and pTK-Rluc with or without expression plasmids for RARs (RARα, RARβ, or RARγ) and RXRα were treated with (black) or without (white) Ro41-5253 for 48 h. Relative values for Fluc/Rluc are shown. D, HuS-E/2 cells were cotransfected with phNTCP-Gluc and pSEAP with or without the expression plasmids for RARs (RARα, RARβ, or RARγ), followed by 24 h of treatment with or no treatment with 20 μM Ro41-5253. Relative Gluc/SEAP values are shown. E, phNTCP-Gluc and pSEAP were transfected into HuS-E/2 cells together with siRNAs against RARα (si-RARα), RXRα (si-RXRα), si-RARα plus si-RXRα, or randomized siRNA (si-control) for 48 h. Relative Gluc/SEAP values are indicated. Endogenous RARα, RXRα, and actin proteins were detected by Western blot analysis (lower panels). F, mRNA levels for NTCP and GAPDH were detected in differentiated HepaRG cells treated with or without ATRA (0.5 and 1 μM) for 24 h. G, protein levels for endogenous NTCP (upper panel), RARα (middle panel), and actin (lower panel, as an internal control) were determined by Western blot analysis of differentiated HepaRG, undifferentiated HepaRG, and HepG2 cells. Statistical significance was determined using Student’s t test (*, p < 0.05).
mutations in nt −112 to −96 had no significant response by RAR/RXR (Fig. 5C). Promoter activity of hNTCP that lacked the region nt −112 to −96 (nt −53 to +108) was not affected by Ro41-5253 (Fig. 5D). 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 down-regulation of NTCP protein (Fig. 7D).

**FIGURE 5. RAR directly regulated the activity of hNTCP promoter.** A, HepaRG cells were treated with or without ATRA, Ro41-5253, or a positive control GW4064, which is an FXR agonist, for 24 h. mRNAs for SHP as well as NTCP and GAPDH were detected by RT-PCR. B, ChIP assay was performed as described under “Experimental Procedures” with Huh7-25 cells transfected with or without an expression plasmid for FLAG-tagged RARα plus that for RXRa in the presence or absence of ATRA stimulation. C, left panel, schematic representation of hNTCP promoter and the reporter constructs used in this study. hNTCP promoter has five putative RAREs (nt −491 to −479, −368 to −356, −274 to −258, −179 to −167, and −112 to −96) in nt −1143 to +108 of hNTCP. The mutant constructs possessing mutations within each putative RARE and in all of five elements (5-Mut) as well as the wild type construct are shown. Right panel, relative luciferase activities upon overexpression with or without RARα plus RXRa in the presence or absence of Ro41-5253. D, deletion reporter construct carrying the region nt −53 to +108 of the hNTCP upstream of the Gluc gene was used for the reporter assay in the presence or absence of Ro41-5253.

**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. 8, A–D). Additionally, this RAR inhibitor decreased the infection of the ETV- and LMV-resistant HBV genotype C clone carrying mutations in L180M, S202G, and M204V (Fig. 8, E 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,
These results suggest that the regulatory circuit for NTCP HBV infection by modulating the expression levels of NTCP.

These agents and was found to decrease host susceptibility to large surface protein (19, 35). Ro41-5253 was distinct from entry by interrupting the interaction between NTCP and HBV ursodeoxycholic acid and taurocholic acid, inhibited HBV cyclosporin A and its derivatives, as well as bile acids, including groups have reported that NTCP-binding agents, including reported to be essential for HBV entry (42). So far, we and other binding to entry receptor(s), including NTCP. NTCP is affinity viral attachment to the cell surface followed by specific viral entry. HBV entry follows multiple steps starting with low ment with Ro41-5253 decreased HBV infection by blocking HepaRG-based HBV infection system and found that pretreat-

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 undifferen-

panels a–d). However, such HBV spread was clearly interrupted by treatment with Ro41-5253 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.

FIGURE 6. HBV susceptibility was decreased in RAR-inactivated cells. A, HuS-E/2 cells were transfected with the pRARE-Fluc and pTK-Rluc for 6 h followed by treatment with or without the indicated compounds at 20 μM for 48 h. Relative Fluc values normalized by Rluc are shown. B and C, HepaRG cells treated with or without the indicated compounds 20 μM were subjected to the HBV infection assay according to the scheme in Fig. 1A. HBs antigen in the culture supernatant was determined by ELISA (B). Cell viability was also quantified by MTT assay (C). Statistical significance was determined using Student’s t test (**, *p < 0.05, and ***, *p < 0.01).

FIGURE 7. CD2665 had a stronger anti-HBV activity than Ro41-5253. A, chemical structure of CD2665. B, HepaRG cells treated with or without 1 μM preS1 peptide, 0.1% DMSO, or various concentrations of Ro41-5253 or CD2665 (5, 10, and 20 μM) were subjected to HBV infection according to the protocol shown in Fig. 1A. HBV infection was detected by quantifying the HBs secretion into the culture supernatant by ELISA. The efficiency of HBV infection was monitored by ELISA detection of secreted HBs. C, HuS-E/2 cells transfected with pNTCP-Gluc and pSEAP were treated with the indicated compounds at 20 μM for 24 h. Relative Gluc/SEAP values are shown. D, NTCP (upper panel) and actin proteins as an internal control (lower panel) were examined by Western blot analysis of HepaRG cells treated with or without the indicated compounds at 20 μM. Statistical significance was determined using Student’s t test (**, *p < 0.01).
Retinoids Reduced HBV Susceptibility by Down-regulating NTCP

FIGURE 8. CD2665 showed a pan-genotypic anti-HBV activity. A–E, primary human hepatocytes were pretreated with or without compounds (50 units/ml heparin, 20 μM CD2665, or 0.1% DMSO) and inoculated with different genotypes of HBV according to the scheme show in Fig. 1A. HBs (A–E) and HBe (A–D) antigen secreted into the culture supernatant was quantified by ELISA. Genotypes A (A), B (B), C (C), D (D), and an HBV carrying mutations (L180M/S202G/M204V) (E) were used as inoculum. F, HBV(L180M/S202G/M204V) was resistant to nucleoside analogs. HepG2 cells transfected with the expression plasmid for HBV/C-AT (white) or HBV/C-AT(L180M/S202G/M204V) (black) were treated with or without 1 μM ETV, 1 μM LMV, or 0.1% DMSO for 72 h. The cells were lysed, and the nucleocapsid-associated HBV DNAs were recovered. Relative values for HBV DNAs are indicated. G, continuous RAR inactivation could inhibit HBV spread. Freshly isolated primary human hepatocytes were pretreated with or without indicated compounds (1 μM preS1 peptide, 10 μM Ro41-5253, or 10 μM CD2665) and inoculated with HBV at day 0. After removing free viruses, primary human hepatocytes were cultured in the medium supplemented with the indicated compounds for up to 30 days postinfection. At 12, 18, 24, and 30 days postinfection, Hbc protein in the cells (left panels, red) and HBs antigen secreted into the culture supernatant (right graph) were detected by immunofluorescence and ELISA, respectively. Statistical significance was determined using Student's t test (*, p < 0.05, and **, p < 0.01).

FIGURE 9. Schematic representation of the mechanism for RAR involvement in the regulation of NTCP expression and HBV infection. Left panel, RAR/RXR recruits to the promoter region of NTCP and regulates the transcription. The expression of NTCP in the plasma membrane supports HBV infection. Right panel, RAR antagonists, including Ro41-5253 and CD2665, repress the transcription of NTCP via RAR antagonization, which decreases the expression level of NTCP in the plasma membrane and abolishes the entry of HBV into host cells.
Retinoids Reduced HBV Susceptibility by Down-regulating NTCP

Ntcp. CCAAT/enhancer-binding protein 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 the 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 the 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 knock-out 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 that significantly decrease the transporter activity of NTCP (50, 51), suggesting that NTCP function may be redundant with other proteins. Organic anion transporting polypeptides are also known to be involved in bile acid transport. Moreover, an inhibition assay using Myrcludex-B showed that the IC_{50} value for HBV infection was −0.1 nM (52), although 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, down-regulation of the HIV coreceptor 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.

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