genomes by PCRs with a set of primers; 5′-ACTGCAAACAAG TTTCCGTTGT-3′ and 5′-AGTGTTACTGACTTGAGATTACTA -3′. Amplified sequence fragments were directly sequenced using an ABI3130xl sequencer. Genomic DNA was extracted from sperm cells of the F4–F9 animals. The genomes of F0–F3 and F10 were accidentally lost and were not analyzed.

We also amplified a genomic fragment of the scaffold KHL81 from the F9 genome with the following primers. 5′-AACGAATTGTAAAGTACGCTCACGA-3′, and 5′-AGAAGCT CTCAGCCAATGAGCGT-3′.

The *Themis-B* locus of the genomes of the F9 animal and wild-caught animals was amplified with the following primers: 5′-CTGGAAAGTTATCCAACCAGTT-3′ and 5′-TTGTTGGGA TATTT(A/G)TTGATTTCT-3′. Amplified fragments were cloned and sequenced. For each genome, we sequenced at least 18 clones.

The *Themis-A* locus of the F9 genome was amplified with the following primers: 5′-ATTTCGATCTCAATAGACACCAA-3′ and 5′-TGTTACATTCAATGTGCCAAGT-3′.

Measurement of Copy Number Variations

DNA extracted from sperm cells of the F9 animal and wild-caught animals were used to determine copy number variation. We performed two distinct multiplex-quantitative-PCRs for each genomic DNA. We amplified a constant region of the *s-Themis-B* and *Macho-1* genes in the first reaction and *FoxA-a* and *Macho-1* genes in the second reaction. We adopted a TaqMan method using a TaqMan Universal PCR Master Mix (Invitrogen). Probes for *s-Themis-B*, *FoxA-a*, and *Macho-1* are as follows: *s-Themis-B*, 5′-(FAM)-CAGCGCTATCATTAG AT-(MGB)-3′; *FoxA-a*, 5′-(FAM)-TCTGCCGTTGAAGTTAGTT CGCCATCC-(TAMARA)-3′; *Macho-1*, 5′-(VIC)-ACGGTCACT TTAGCACCTCCACCA-(TAMARA)-3′. As calibrators, we made two DNA constructs, which contained amplicon sequences of *s-Themis-B* and *Macho-1*, and of *FoxA-a* and *Macho-1*. Sequences of these calibrators are shown in supplementary fig. S6, Supplementary Material online. After calibrating the different efficiencies between probe/primer sets with these calibrators, we calculated expected numbers of *s-Themis-B* and *FoxA-a* per diploid, assuming that *Macho-1* exists in a single copy gene per haploid (two copies per diploid). Standard errors were calculated among triplicates. PCR primers were as follows: *s-Themis-B*, 5′-TGATGAATGTAAATTGGTTCAAGTCAA-3′ and 5′-TGAG GAACGGTTTCAAACACTTG-3′; *FoxA-a*, 5′-TTCAACACCAC CACACTCAACAG-3′ and 5′-CGTGTTCAATGCCATGTTC-3′; *Macho-1*, 5′-CCCAGTATGCACCAAATTCAGA-3′, and 5′-TG GTGAGAAAACGGGTGAAAC-3′. The fertilization ratio was determined as previously described (Harada et al. 2008).

Supplementary Material

Supplementary table S1 and figures S1–S6 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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References

Bachtrog D. 2013. Y-chromosome evolution: emerging insights into processes of Y-chromosome degeneration. *Nat Rev Genet.* 14:113–124.
Boyes DC, Nasrallah ME, Vrebalov J, Nasrallah JB. 1997. The self-incompatibility (S) haplotypes of *Brassica* contain highly divergent and rearranged sequences of ancient origin. *Plant Cell* 9:237–247.
Charlesworth D, Charlesworth B, Marais G. 2005. Steps in the evolution of heteromorphic sex chromosomes. *Heredity* 95:118–128.
Charlesworth D, Willis JH. 2009. The genetics of inbreeding depression. *Nat Rev Genet.* 10:783–796.
Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein DM, et al. 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* 298:2157–2167.
Delsuc F, Brinkmann H, Chourrout D, Philippe H. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature* 439:965–968.
Eichler EE, Sankoff D. 2003. Structural dynamics of eukaryotic chromosome evolution. *Science* 301:793–797.
Ferris PJ, Armbrust EV, Goodenough UW. 2002. Genetic structure of the mating-type locus of *Chlamydomonas reinhardtii*. *Genetics* 160: 181–200.
Fraser JA, Heitman J. 2004. Evolution of fungal sex chromosomes. *Mol Microbiol.* 51:299–306.
Harada Y, Takagaki Y, Sunagawa M, Saito T, Yamada L, Taniguchi H, Shoguchi E, Sawada H. 2008. Mechanism of self-sterility in a hermaphroditic chordate. *Science* 320:548–550.
Harismendy O, Ng PC, Strausberg RL, Wang X, Stockwell TB, Beeson KY, Schork NJ, Murray SS, Topol EJ, Levy S, et al. 2009. Evaluation of next generation sequencing platforms for population targeted sequencing studies. *Genome Biol.* 10:R32.
Kamau E, Charlesworth D. 2005. Balancing selection and low recombination affect diversity near the self-incompatibility loci of the plant *Arabidopsis lyrata*. *Curr Biol.* 15:1773–1778.
Kano S, Satoh N, Sordino P. 2006. Primary genetic linkage maps of the ascidian, *Ciona intestinalis*. *Zoolog Sci.* 23:31–39.
Kent WJ. 2002. BLAT–the BLAST-like alignment tool. *Genome Res.* 12: 656–664.
Kimura M, Crow JF. 1964. The number of alleles that can be maintained in a finite population. *Genetics* 49:725–738.
Lahn BT, Page DC. 1999. Four evolutionary strata on the human X chromosome. *Science* 286:964–967.
Lengeler KB, Fox DS, Fraser JA, Allen A, Forrester K, Dietrich FS, Heitman J. 2002. Mating-type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosomes. *Eukaryot Cell.* 1:704–718.
Liu Z, Moore PH, Ma H, Ackerman CM, Ragiba M, Yu Q, Pearl HM, Kim MS, Charlton JW, Stiles JI, et al. 2004. A primitive Y chromosome in papaya marks incipient sex chromosome evolution. *Nature* 427: 348–352.

Llaurens V, Gonthier L, Billiard S. 2009. The sheltered genetic load linked to the s locus in plants: new insights from theoretical and empirical approaches in sporophytic self-incompatibility. *Genetics* 183: 1105–1118.

Morgan TH. 1944. Some further data on self fertilization in *Ciona*. *J Exp Zool.* 97:231–248.

Nielsen R, Paul JS, Albrechtsen A, Song YS. 2011. Genotype and SNP calling from next-generation sequencing data. *Nat Rev Genet.* 12: 443–451.

Ning Z, Cox AJ, Mullikin JC. 2001. SSAHA: a fast search method for large DNA databases. *Genome Res.* 11:1725–1729.

Patel RK, Jain M. 2012. NGS QC Toolkit: a toolkit for quality control of next generation sequencing data. *PLoS One* 7:e30619.

Saito T, Shiba K, Inaba K, Yamada L, Sawada H. 2012. Self-incompatibility response induced by calcium increase in sperm of the ascidian *Ciona intestinalis*. *Proc Natl Acad Sci U S A.* 109:4158–4162.

Satou Y, Mineta K, Ogasawara M, Sasakura Y, Shoguchi E, Ueno K, Yamada L, Matsumoto J, Wasserscheid J, Dewar K, et al. 2008. Improved genome assembly and evidence-based global gene model set for the chordate *Ciona intestinalis*: new insight into intron and operon populations. *Genome Biol.* 9: R152.

Satou Y, Shin-i T, Kohara Y, Satoh N, Chiba S. 2012. A genomic overview of short genetic variations in a basal chordate, *Ciona intestinalis*. *BMC Genomics* 13:208.

Uyenoyama MK. 1997. Genealogical structure among alleles regulating self-incompatibility in natural populations of flowering plants. *Genetics* 147:1389–1400.

Uyenoyama MK. 2005. Evolution under tight linkage to mating type. *New Phytol.* 165:63–70.

Wright S. 1939. The distribution of self-sterility alleles in populations. *Genetics* 24:538–552.

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Original Article

Use of nucleoside analogs in patients with chronic hepatitis B in Nepal: A prospective cohort study in a single hospital

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Aim: There still remain many concerns about the present status of antiviral therapy for chronic hepatitis B in developing countries in Asia, where the monitoring systems of virological markers have not been well established, despite the high prevalence of hepatitis B virus (HBV) infection. To investigate it in Nepal, this prospective cohort study was conducted at the Teaching Hospital of Tribhuvan University in Kathmandu.

Methods: From 2007 to 2012, 65 patients were consecutively enrolled, 44 of whom received nucleoside analogs (NA), such as lamivudine (LMV), adefovir or tenofovir (TDF), on the decision of the local hepatologist. Virological determinations were performed in Japan, by using the serially collected serum samples at the Teaching Hospital. Statistical analysis was performed, using Mann–Whitney *U*-test or Fisher's exact test.

Results: The younger, especially female patients of reproductive age were more frequently prescribed with these NA, and an increased preference for the use of TDF was observed over time. However, there was insufficient follow up of the NA-treated patients in this cohort, and not a few patients developed emergence of NA-resistant HBV: known resistance to LMV in 3 patients and incidental resistance to non-administrated NA in four patients.

Conclusion: The results of the present study indicate that education for physicians as well as for infected patients regarding the proper use of NA, together with establishment of appropriate monitoring systems for virological markers, is warranted to prevent an increase in NA-resistant HBV infections in Nepal.

Key words: antiviral therapy, chronic hepatitis B, hepatitis B virus, nucleoside analogs, resistance

INTRODUCTION

ACCORDING TO ESTIMATES by the World Health Organization, almost 240 million people are infected by hepatitis B virus (HBV) worldwide, and up to 780 000 deaths related to liver cirrhosis or hepatocellular carcinoma caused by chronic HBV infection occur annually.[1] HBV infection is endemic, particularly in Asian and African countries, and the prevalence is reported to be as high as 20% of the general population in some countries.[2] In the rural areas of Nepal, one of the poorest countries in the world, the prevalence of HBV and hepatitis C virus (HCV) among blood donors was reported to be 0.86% and 0.52%, respectively, in 2001–2002.[3] Our epidemiological study conducted between 1997 and 2002 also demonstrated that HBV and HCV carrier rates among 540 blood donors in the urban area of Nepal were 2.4% and 1.1%, respectively (e.g. N. Masaki, unpubl. data, 2003).

The recent introduction of nucleoside analogs (NA) such as lamivudine (LMV), adefovir (ADF), entecavir (ETV) and tenofovir (TDF) into clinical settings has significantly attenuated the progression of liver fibrosis and

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**Author contribution: N. M. designed the research protocol, obtained funding and wrote the first draft of the manuscript. P. K. S. was the counterpart of this research in Nepal and contributed to the collection of serum samples as well as clinical information. S. N. and K. I. contributed to the acquisition of data and the analysis. M. S. carried out the molecular analyses of hepatitis B virus. M. M. supervised the entire research project. All authors read and approved the final manuscript.*

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hepatocarcinogenesis,[4] leading to improvements in the prognosis of HBV-related liver diseases. However, because the pharmacological nature of these NA is chiefly confined to competitive inhibition of HBV DNA polymerase, semi-permanent use of NA is principally necessary to prevent the relapse of hepatitis accompanying HBV proliferation. However, the long-term use of NA may result in emergence of NA-resistant HBV,[5] therefore, it is necessary to regularly monitor the serum viral load during treatment.

Although the HBV and HCV carrier rates in Nepal appear to be relatively lower than in other developing countries in Asia, it is important to understand the current use of therapeutic strategies. However, little information about the present status of antiviral therapy for chronic HBV is available in this country, especially in terms of NA use. Furthermore, there are several serious socioeconomic problems to be addressed in Nepal. In relation to chronic HBV, domestic laboratories in Nepal are not able to perform virological determinations of HBV genotypes or HBV viral load, and sending serum samples to overseas commercial labs is costly. For example, the measurement of HBV viral load in India costs approximately 8000 Nepalese rupees (i.e. $US 127). In contrast, NA are relatively inexpensive in Nepal, because of an influx of generic drugs produced by foreign pharmaceutical companies. One tablet of LMV, ADF, ETV or TDF costs 18 ($US 0.28), 30 ($US 0.48), 80 ($US 1.26) and 43 Nepalese rupees ($US 0.68), respectively. As a result, NA may be disorderly administrated in this country. This study aimed to document NA use for HBV and discuss the socioeconomic problems that need to be addressed in most of the developing countries in Asia.

METHODS

CONSECUTIVE NEPALESE PATIENTS with chronic HBV infection in the outpatient clinic of the Tribhuvan University Teaching Hospital in Kathmandu were enrolled between August 2007 and August 2012, and the observation period extended from the first day of enrollment to February 2013. The Teaching Hospital, which was established in 1983 with the support of the Japanese Government as an integral part of the Institute of Medicine of Tribhuvan University, provides academic training programs (basic, graduate and postgraduate) for the Institute and, as a national hospital, renders medical care and services to the Nepalese people.

Nucleoside analog administration was based on the decision of the local hepatologist (P. K. S.), and the clinical course was prospectively analyzed. NA treatment was categorized into three groups: LMV-, ADF- and TDF-based. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees of the National Center for Global Health and Medicine, Japan, on human experimentation and with the Declaration of Helsinki of 1975, as revised in 2008. At that time, when we had started this project, the ethics committee of Tribhuvan University Teaching Hospital was not organized yet; hence, oral or written informed consent was obtained by the local hepatologist (P. K. S.), and this study was conducted as part of routine clinical practice.

Determination of HBV markers

Only a qualitative determination of hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (HBsAb), hepatitis B e-antigen (HBeAg) and hepatitis B e antibody (HBeAb) was available in Nepal, therefore, quantitative determination of the virological parameters was performed in Japan using serum samples collected at the Teaching Hospital. In addition to the samples collected at enrollment, the local hepatologist was advised to collect serial serum samples at least every 6 months in NA-treated patients.

Hepatitis B surface antigen, HBeAg and HBeAb were determined by chemiluminescent microparticle immunoassay (ARCHITECT; Abbot Japan, Tokyo, Japan). The HBV viral load was determined by transcription-mediated amplification (Chugai Diagnostics Science, Tokyo, Japan) or AccuGene m-HBV (Abbott RealTime HBV; Abbot Japan; lower limits of detection, 1.7 log copies/mL), according to the manufacturers' instructions. HBV genotype was determined by restriction fragment length polymorphism, as described previously.[6] NA-resistant HBV was determined by INNO-LiPA HBV DR version 2, according to the manufacturer's instructions (INNOGENETICS, Ghent, Belgium). This line-probe assay can simultaneously detect HBV mutations resistant to LMV, ADF, ETV and TDF.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test for comparison of the means of continuous variables or Fisher's exact test for categorical variables. A two-tailed *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Demographic features and laboratory data at enrollment

TABLE 1 PROVIDES the characteristics of the 65 patients (72% male) who were enrolled during the study period. The average ± standard deviation age was

Table 1 Demographic features and laboratory data at the enrollment

	n (%)[†]
Sex (men/women)	47 (72%)/18 (28%)
Age (years)	33 ± 13[‡] (range, 16–71)
Serum ALT (IU/mL)	34 ± 32[‡] (range, 2–176)
HBeAg/HBeAb	
+/−	29 (46.0%)
+/+	1 (1.6%)
−/−	1 (1.6%)
−/+	32 (49.2%)
HBV viral load (log copies/mL)	
≤3.9	24 (36.9%)
4.0–6.9	16 (24.6%)
≥7.0	25 (38.5%)
HBV genotype	
Aa (A)	12 (18.5%)
C	14 (21.5%)
C/D recombinant	1 (1.5%)
D	33 (50.8%)
Not detected	5 (7.7%)

[†]Unless otherwise indicated.
[‡]Mean ± standard deviation.
ALT, alanine aminotransferase; HBeAb, hepatitis B e antibody; HBeAg, hepatitis B e-antigen; HBV, hepatitis B virus.

Table 2 Profiles of the NA-treated 44 patients enrolled in this prospective study

	No. of patients[†]
Sex (male/female)	28/16
Age (years)	31 ± 12[‡] (range: 16–71)
Treatment history of NA at enrollment	
Naive cases	22
for LMV/for ADF/for TDF	7/3/12
On NA treatment	19
of LMV/of ADF/of LMV + ADF/of TDF	8/3/2/6
Previous history of NAs	3
of LMV/of LMV + ADF	2/1
Treatment profiles of NA during observation period[§]	
LMV-based	12
on LMV alone/LMV alone→stop	9/3
ADF-based	6
on ADF alone/on LMV + ADF/LMV + ADF→stop	2/3/1
TDF-based	23
LMV→TDF/ LMV→ADF→TDF/on TDF alone	4/2/17

[†]Unless otherwise indicated,
[‡]Mean ± standard deviation.
[§]After excluding three patients with only previous history of NA, the treatment profiles were evaluated in 41 patients, as of February 2013.
ADF, adefovir; LMV, lamivudine; NA, nucleoside analogs; TDF, tenofovir.

33 ± 13 years, and 32% and 28% were aged in their 20s and 30s, respectively. The extent of liver inflammation was relatively mild. The proportions of HBeAg positive and HBeAb positive patients were similar, and 38.5% of the patients had a viral load of 7.0 log copies/mL or more. The grades of viral load were quite consistent with the HBeAg/HBeAb status, namely, 21 (72%) out of 29 patients positive for HBeAg and negative for HBeAb had a higher viral load (≥7.0 log copies/mL), while 20 (63%) out of 32 patients negative for HBeAg and positive for HBeAb had a lower viral load (≤3.9 log copies/mL, $P < 0.001$). In this cohort, HBV genotype D was the most prevalent, affecting as many as 50% of the patients.

Profiles of the NAs-treated patients

Of the 65 enrolled patients, 44 (68%) received NA during the observation period or before enrollment. NA were prescribed in 16 of the 18 female patients (88.9%) and 28 of the 47 male patients (59.6%, $P < 0.05$). The average age of the NA-treated patients was significantly younger than that of the NA-untreated patients (31 ± 12 years vs 39 ± 15 years, $P < 0.05$).

In Table 2, the profiles of the NA-treated patients are detailed. Among 44 patients enrolled in this study, 22 patients (50%) were naive cases for NA, and LMV, ADF and TDF were administrated in seven, three and 12 patients, respectively. Nineteen patients had already been of NA treatment at enrollment: LMV, ADF, LMV + ADF and TDF were administrated in eight, three, two and six patients, respectively. Three patients had only previous history of NA, and received no further treatment. As for the treatment profiles of the 41 patients treated with NA during the observation period until February 2013, LMV-, ADF- and TDF-based therapies were carried out in 12, six and 23 patients, respectively. Four patients stopped NA: one receiving LMV alone and one receiving LMV + ADF because of HBsAg loss during the observation period, while the remaining two stopped LMV due to the decision of the patients. The preference for TDF as the first or second line of treatment was clearly demonstrated in this prospective study (56.1%).

Follow up of the NA-treated patients and emergence of NA-resistant HBV

The number of patients who returned to the hospital for follow-up visits decreased over time. As detailed in

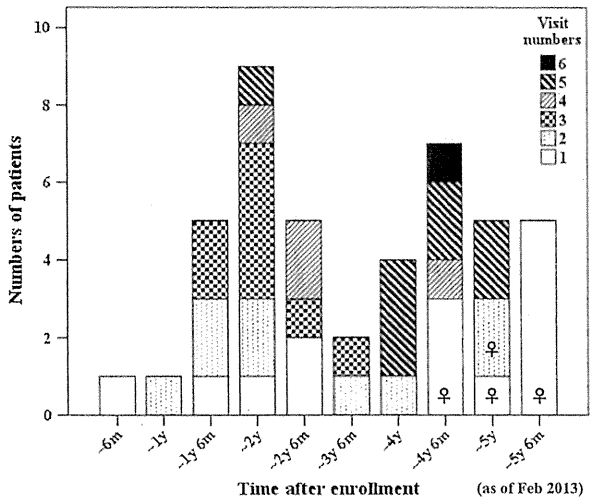

Figure 1 Subsequent hospital visits decreased in this prospective cohort of patients treated with nucleoside analogs. The local hepatologist was advised to collect serial serum samples approximately every 6 months in patients with chronic hepatitis B treated by nucleoside analogs. The numbers of hospital visits during the observation period were evaluated as of February 2013. For the patients who were enrolled more than 3 years from the end of observation period, the numbers of follow-up visits to the Teaching Hospital were significantly less than expected. ♀ denotes the four female patients who were lost to follow up before February 2013.

Figure 1, 44 NA-treated patients were classified according to both the time after enrollment (≈every 6 months) and the numbers of follow-up visits to the Teaching Hospital (range, 1–6). Especially for the patients who were enrolled more than 3 years prior from the end of the observation period (i.e. February 2013), the numbers of follow-up visits were significantly less than expected. Under these circumstances, serial determinations of serum HBV DNA levels and the emerging rate of NA-resistant HBV were difficult to achieve in clinical settings.

Of those patients who were followed, seven developed NA-resistant HBV during the observation period (Fig. 2); as the first line treatment, five of these patients were treated with LMV and two patients were treated with TDF. In three patients (patients CHB-16, CHB-22, CHB-23) with LMV-resistant HBV, the clinical course varied. A change to TDF dramatically decreased the HBV viral load in patient CHB-16; however, serum levels of HBV DNA rose up again thereafter, probably due to poor adherence to TDF, considering absence of NA-resistant HBV; the M204I mutation spontaneously disappeared in patient CHB-22 despite the continuation of LMV; and in patient CHB-23, the M204I mutation disappeared immediately after replacement with TDF, without viral breakthrough. Interestingly, in the remaining four patients (i.e. CHB-25, CHB-28, CHB-38 and CHB-49), incidental and unrelated resistance to NA that had not been administrated (i.e. ETV or ADF) was detected; however, except in one patient in whom LMV was withdrawn (i.e. CHB-28), the HBV DNA levels were fully suppressed, despite the emergence of incidental resistance. In addition, in TDF-treated patients (i.e. CHB-38 and CHB-49), such incidental resistance (i.e. ADF resistance and ETV resistance, respectively) was detected just transiently and spontaneously disappeared.

To evaluate the effects of TDF, serial changes of serum HBV DNA were examined in 16 patients naive to TDF treatment, for whom multiple serum samples were available. As shown in Figure 3, 14 out of 16 patients (88%) achieved a fair response to TDF, except for two patients whose HBV DNA levels were unchanged, likely as a result of poor adherence. In 13 patients (81%), HBV DNA levels decreased to less than 3.0 log copies/mL within 12 months of the start of TDF. As detailed above, even though two patients (i.e. CHB-38 and CHB-49 in Fig. 2) developed incidental resistance to either ADF or ETV that was not administered, the virological response to TDF was satisfactorily maintained (Fig. 3).

DISCUSSION

IN THIS PROSPECTIVE study, we found that NA were prescribed preferably in younger female patients with chronic hepatitis B and that the monitoring systems for HBV markers were extremely insufficient in this country. As a consequence, not a few patients developed emergence of NA-resistant HBV: known resistance to LMV in three patients and incidental resistance to non-administrated NA in four patients.

In developing countries, where health insurance systems are not available, patients can obtain medications without a doctor's prescription after the first visit to the pharmacy. Moreover, socioeconomic problems such as persistent poverty and insufficient infrastructure may prevent regular medical checkups,[7] even with chronic liver disease, as was confirmed in the present study.

One concern in younger patients and, in particular, female patients of reproductive age, is the use of NA without appropriate checkups. All of the approved NA have prominent warnings regarding the potential risk of fetal anomaly with their use just before and during pregnancy. Telbivudine and TDF are considered Pregnancy Category B drugs (i.e. no known teratogenicity or embryotoxicity in animal studies, but inadequate human studies) by the

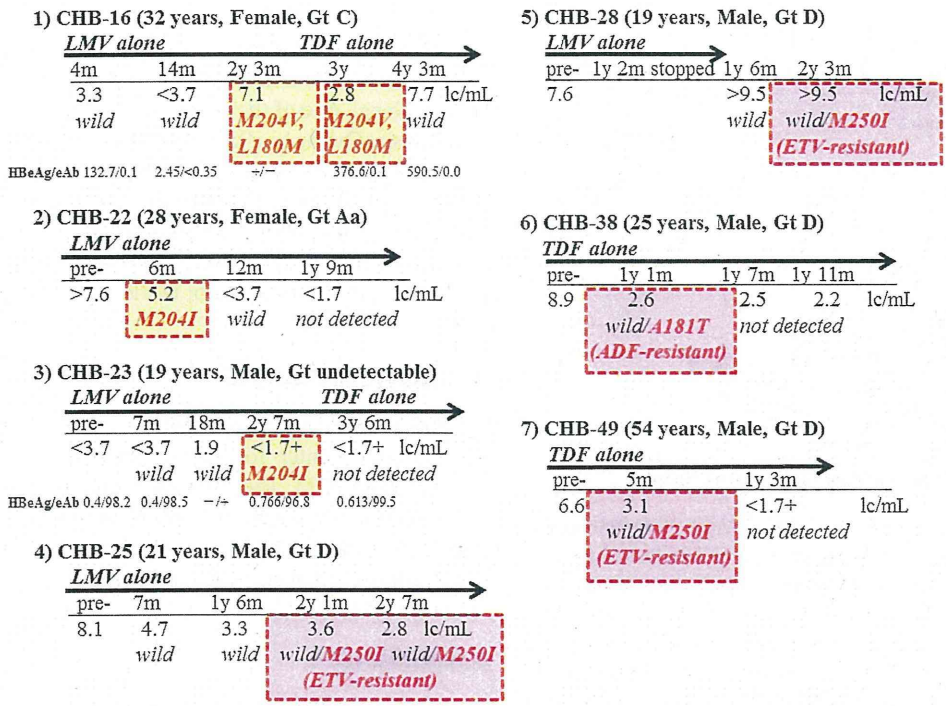

Figure 2 Emergence of NA-resistant hepatitis B virus. Seven patients developed NA-resistant hepatitis B virus in this prospective cohort. Patients 1–5 received LMV-based treatment, and patients 6 and 7 received TDF-based treatment. In two patients (i.e. CHB-25 and CHB-28) treated with LMV and two patients (i.e. CHB-38 and CHB-49) treated with TDF, incidental resistance to unused NA such as ETV or ADF was also detected. ADF, adefovir; ETV, entecavir; Gt, genotype; lc, log copies; LMV, lamivudine; NA, nucleoside analog; TDF, tenofovir.

US Federal Drug Administration, while LMV, ADF and ETV are Category C (i.e. embryotoxic or teratogenic in animal studies, but inadequate human studies).[8] Accordingly, their use in men and women of child-bearing age should be limited.

Also of concern is that long-term NA use may result in NA-resistant HBV. The first approved NA for chronic HBV was LMV, and the cumulative incidence of LMV resistance, including M204V/I or L180M, has been reported up to 70% at treatment year 5,[4] leading to hepatitis flare-up as well as viral breakthrough. ADF add-on therapy is effective for the prevention of these adverse events; however, additional use of medication may increase patients' mental and financial burden. In contrast, ETV and TDF are potent HBV inhibitors with a high barrier to resistance.[9–13] Of the limited number of patients with follow-up data in the current study, seven developed NA-resistant HBV; five were treated with LMV and two with TDF. The classic YMDD mutation was detected in three patients treated with LMV, and, in the remaining four patients, incidental resistance to other non-administrated NA (ETV and ADF) was found. This information is relevant to future consideration of drug choice in these patients. Because, in CHB-38 and CHB-49 treated with TDF, we did not check the NA-resistant HBV at pretreatment, the possibility of pre-existing ADF-resistant or ETV-resistant HBV could not be excluded. According to the recent systematic review and meta-analysis,[14] the incidence of naturally occurring resistance rates in naive chronic hepatitis B was reported to be 5.39% globally (including China, Japan, Turkey, Korea, Iran, India, Pakistan, Thailand, Australia, USA, Brazil, Spain, Italy, France and South Africa; 95% confidence interval, 4.54–6.24%); the highest incidence was 7.83% (6.48–9.18%) in China, while 1.39% (0.67–2.10%) in other countries. In subgroup analysis, it was shown that genotype C HBV infection, male and HBeAg negative patients had a slightly higher natural mutation rate, although the precise mechanisms were not elucidated. In contrast, in CHB-25 and CHB-28, ETV-resistant HBV first emerged during the observation period. In CHB-25, the virological response to LMV seemed to be relatively slow, and in CHB-28, LMV was stopped owing to the patient's will at 1 year and 1 month before. Although these two cases could not yet be classified as multidrug resistance evolution in patients failing NA,[15] further follow ups

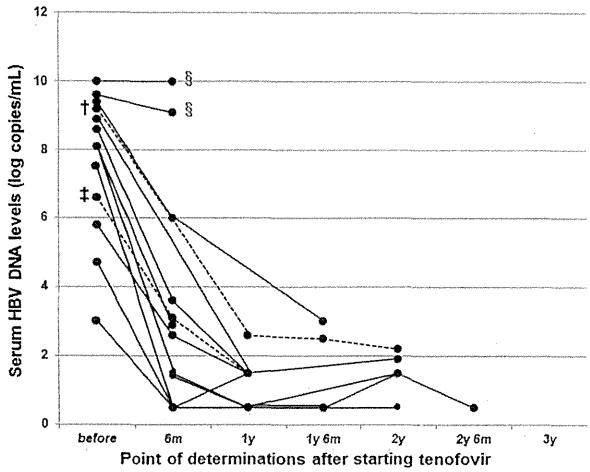

Figure 3 Serial changes in serum HBV DNA levels in patients naive to tenofovir treatment. In 16 patients naive to TDF treatment, multiple serum samples were available for determination of serum HBV DNA levels. Fourteen of 16 patients (88%) achieved a fair response to TDF, except for two patients (§) who likely had poor adherence, considering no emergence of NA-resistant HBV. In 13 patients (81%), HBV DNA levels decreased to less than 3.0 log copies/mL within 12 months after the start of TDF. Two patients (i.e. CHB-38 [†] and CHB-49 [‡] in Fig. 2) developed incidental resistance to either ADF or ETV, that was not administrated, the virological response to TDF was satisfactorily maintained (dashed line). ADF, adefovir; ETV, entecavir; HBV, hepatitis B virus; LMV, lamivudine; NA, nucleoside analog; TDF, tenofovir.

should be mandatory to prevent progression of chronic liver diseases.

A recent study reported that no resistance to TDF was detected after 6 years of treatment in patients with HBeAg positive and HBeAg negative chronic HBV, and regular use of TDF could satisfactorily suppress HBV proliferation,[16] which was also demonstrated in the present cohort. These lines of evidences may explain the recent preference for TDF in Nepal, even though the monitoring systems for virological markers have not been well established.

There are several limitations in this study. First, owing to the small sample size, we must be prudent in drawing conclusions. However, we believe that the use of antiviral medications documented in this study are representative of general practice at this hospital, the affiliated hospital of the only national university of Nepal. Second, it is difficult to determine the specific reasons other than poor drug adherence in patients with poor response to NA, because we could did not have enough data regarding the quality or storage conditions of generic drugs from neighboring countries.

In conclusion, this prospective study documented the present status of antiviral therapy in patients with chronic HBV in Nepal, one of the representative developing countries in Asia. Owing to a lack of monitoring systems, there is a risk of an increase in NA-resistant HBV infections. Given the prevalence of HBV in these countries, this could represent a global public health problem. In addition, education for physicians as well as for infected patients regarding the appropriate use of NA is urgently required.

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REFERENCES

1 World Health Organization. Hepatitis B Fact sheet N°204 Updated July 2014. Available at: http://www.who.int/mediacentre/factsheets/fs204/en/. Accessed August 14, 2014.
2 Chen CJ, Wang LY, Yu MW. Epidemiology of hepatitis B virus infection in the Asia-Pacific region. *J Gastroenterol Hepatol* 2000; 15(Suppl): E3–E6.
3 Karki S, Ghimire P, Tiwari BR, Maharjan A, Rajkarnikar M. Trends in hepatitis B and hepatitis C seroprevalence among Nepalese blood donors. *Jpn J Infect Dis* 2008; 61: 324–6.
4 Chang TT, Liaw YF, Wu SS *et al.* Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B. *Hepatology* 2010; 52: 886–93.
5 European Association for the Study of the Liver. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol* 2012; 57: 167–85.
6 Mizokami M, Nakano T, Orito E *et al.* Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* 1999; 450: 66–71.
7 Liaw YF. Antiviral therapy of chronic hepatitis B: opportunities and challenges in Asia. *J Hepatol* 2009; 51: 403–10.
8 Fontana RJ. Side effects of long-term oral antiviral therapy for hepatitis B. *Hepatology* 2009; 49(5 Suppl): S185–S195.
9 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; 354: 1001–10.
10 Marcellin P, Heathcote EJ, Buti M *et al.* Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 2008; 359: 2442–55.
11 Chang TT, Lai CL, Kew Yoon S *et al.* Entecavir treatment for up to 5 years in patients with hepatitis B e antigen-positive chronic hepatitis B. *Hepatology* 2010; 1: 422–30.

12 Heathcote EJ, Marcellin P, Buti M *et al.* Three-year efficacy and safety of tenofovir disoproxil fumarate treatment for chronic hepatitis B. *Gastroenterology* 2011; **140**: 132–43.
13 Lai CL, Shouval D, Lok AS *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; **354**: 1011–20.
14 Zhang Q, Liao Y, Cai B *et al.* Incidence of natural resistance mutations in naïve chronic hepatitis B patients: a systematic review and meta-analysis. *J Gastroenterol Hepatol* 2014. DOI: 10.1111/jgh.12831
15 Locarnini S Primary resistance, multidrug resistance, and cross-resistance pathways in HBV as a consequence of treatment failure. *Hepatol Int* 2008; **2**: 147–51.
16 Kitrinos KM, Corsa A, Liu Y *et al.* No detectable resistance to tenofovir disoproxil fumarate after 6 years of therapy in patients with chronic hepatitis B. *Hepatology* 2014; **59**: 434–42.

Microbiology:

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

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

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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* (h*NTCP*) gene and that Ro41-5253 repressed the hNTCP promoter by antagonizing RAR. RAR recruited to the h*NTCP* promoter region, and nucleotides −112 to −96 of the h*NTCP* 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)[2] 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

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[2] 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; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; nt, nucleotide; cccDNA, covalently closed circular DNA.

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* (h*NTCP*) gene. Retinoic acid receptor (RAR) played a crucial role in regulating the promoter activity of h*NTCP*, 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 (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 the following primer sets: F1, 5′-CAGATCTTGGAATTCCCAAAATC-3′ and 5′-GAGGGGATGTGTCCATTGAAATGTTAATGGGAGCTGAGAGGATGCCAGTATCCTCCCT-3′ and 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 to −479)-Gluc. Other promoter mutants were prepared by the same method using the following primer sets: F1, 5′-GTGGGTTATCATTTGTTTCCCGAAAACATTAGAGTGAAAGGAGCTGGGTGTTGCCTTTGG-3′ and 5′-TCCTTTCACTCTAATGTTTTCGGGAAACAAATGATAACCCACTGGACATGGGGAGGGCAC-3′; R6 for −368 to −356; F1 and 5′-AATCTAGGTCCAGCCTATTTAAGTCCCTAAATTTCCTTTTCCCAGCTCCGCTCTTGATTCCTT-3′, 5′-CTGGGAAAAGGAAATTTAGGGACTTAAATAGGCTGGACCTAGATTCAGGTGGGCCCTGGGCAG-3′, and R6 for −274 to −258; F1 and 5′-TTCTGGGCTTATTTCTATATTTTGCAATCCACTGAGTGTGCCTCATGGGCATTCATTC-3′, 5′-CACACTCAGTGGATTGCAAAATATAGAAATAAGCCCAGAAGCAGCAAAGTGACAAGGG-3′, and R6 for −179 to −167; F1 and 5′-AGCTCTCCCAAGCTCAAAGATAAATGCTAGTTTCCTGGGTGCTACTTGTACTCCTCCCTTGTC-3′, 5′-GTAGCACCCAGGAAACTAGCATTTATCTTTGAGCTTGGGAGAGCTAGGGCAGGCAGATAAGGT-3′, and R6 for −112 to −96, respectively. For constructing the hNTCP promoter carrying these five mutations (5-Mut), five DNA segments were amplified using the primers as follows: segment 1, F1 and 5′-GAGGGGATGTGTCCATGACC-3′; segment 2, 5′-AGCTCCTTTCACTCTCATGGGT-3′ and 5′-TCCTTTTCCCAGCTCCGC-3′; segment 3, 5′-GAGCTGGGAAAAGGAGCTGC-3′ and 5′-CCACTGAGTGTGCCTCATGG-3′; segment 4, 5′-AGGCACACTCAGTGGAGGG-3′ and 5′-CTGGGTGCTACTTGTACTCCTCC-3′; and segment 5, 5′-CAAGTAGCACCCAGGAATCCA-3′ and R6. For producing a deletion construct for the h*NTCP* promoter, phNTCP (−53 to +108)-Gluc, DNA fragment was amplified using the primer sets 5′-GGTGAATTCTGTTCCTCTTTGGGGCGACAGC-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. 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 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 described previously (19). HBV for Fig. 8*F* (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, 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 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—1×10^6 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 with 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. 2*B*, 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 RARa and for RXRa were treated with 5 mg/ml actinomycin D for 2 h. The cells were then washed and treated with or without 2 mM 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′-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 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. 1*A*). 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

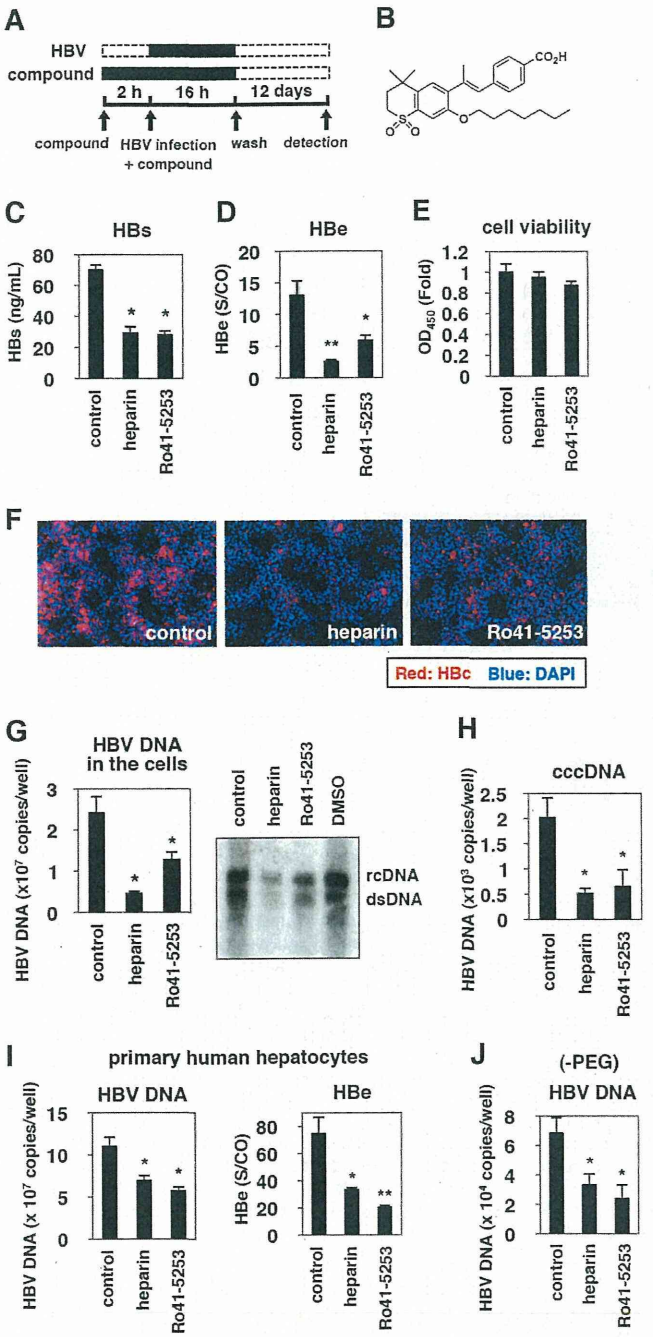

FIGURE 1. **Ro41-5253 decreased susceptibility to HBV infection.** *A*, schematic representation of the schedule for treatment of HepaRG cells with compounds and infection with HBV. HepaRG cells were pretreated with compounds for 2 h and then inoculated with HBV in the presence of compounds for 16 h. 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 HBe (*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 HBe antigen in the culture supernatant (*I*) were quantified. The data show the means of three independent experiments. Standard deviations are also shown as *error bars*. Statistical significance was determined using Student's *t* test (*, $p < 0.05$; **, $p < 0.01$).

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. 1*B*) as well as heparin, a competitive viral attachment inhibitor that served as a positive control (Fig. 1*C*) (14). HBe in the medium (Fig. 1*D*) as well as intracellular HBc protein (Fig. 1*F*), HBV replicative (Fig. 1*G*), and cccDNA (Fig. 1*H*) were consistently decreased by treatment with Ro41-5253, without serious cytotoxicity (Fig. 1*E*). 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. 1*I*). The anti-HBV effect of Ro41-5253 on HBV infection of primary human hepatocytes was also observed in the absence of PEG8000 (Fig. 1*J*), 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. 2*A*). 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 as follows: 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. 2*B*, *left panel*), 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. 1*A*), in contrast to the anti-HBV effect of CsA, an HBV entry inhibitor (Fig. 2*C*) (19, 35), 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. 2*C* but not in Fig. 2*B*. 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. 2*B*, *middle panel*), and by the HBV RNA level in HepG2.2.15 cells, persistently producing HBV (Fig. 2*B*, *right panel*) (36). We then examined whether Ro41-5253 pretreatment affected viral attachment to host cells. To this end, HepaRG cells were exposed to HBV at 4 °C for 3 h, which allowed HBV attachment but not subsequent internalization (19) (Fig. 2*D*). After washing out free viruses, cell surface HBV DNA was extracted and quantified to evaluate HBV cell attachment (Fig. 2*D*). Pretreatment with Ro41-5253 significantly reduced HBV DNA attached to the cell surface, as did heparin (Fig. 2*D*). In a preS1 binding assay, where FITC-labeled preS1 lipopeptide was used as a marker for HBV attachment to the cell surface, Ro41-5253-

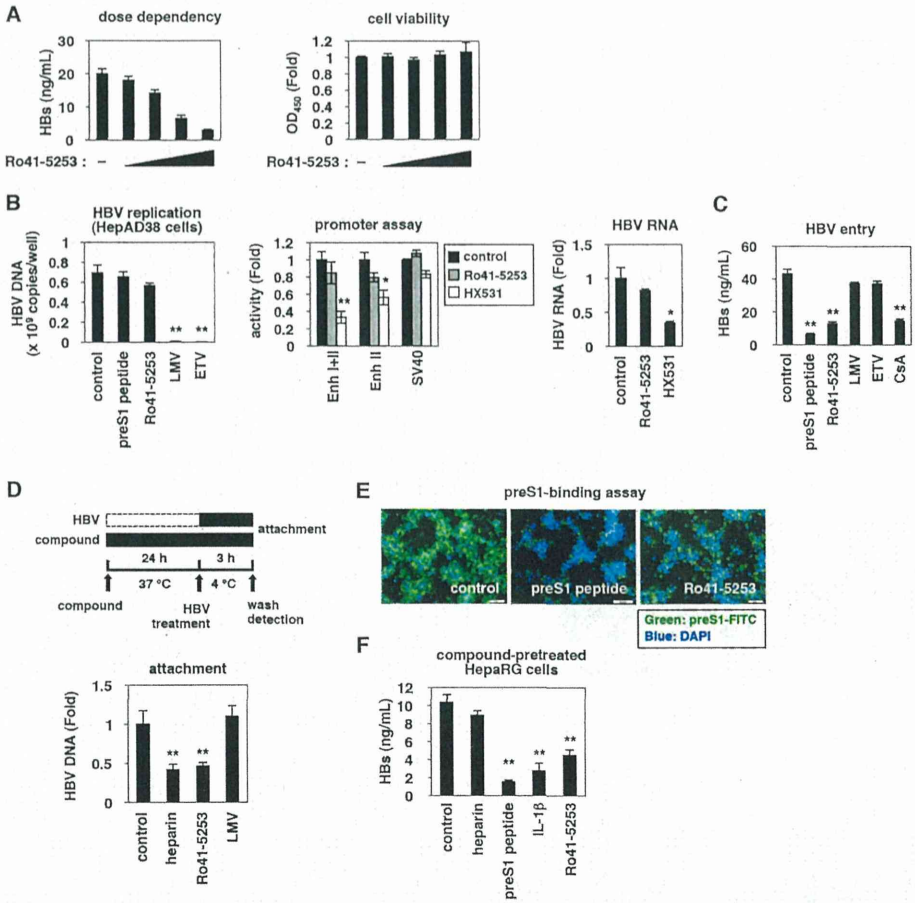

FIGURE 2. **Ro41-5253 decreased HBV entry.** *A,* HepaRG cells were treated with or without various concentrations (2.5, 5, 10, and 20 μM) of Ro41-5253 followed by HBV infection according to the protocol shown in Fig. 1*A*. Secreted HBs was detected by ELISA (*left panel*). Cell viability was also determined by ELISA (*right panel*). *B, left panel,* nucleocapsid-associated HBV DNA in HepAD38 cells treated with the indicated compounds (200 nM preS1 peptide, 20 μM Ro41-5253, 1 μM lamivudine, or 1 μM entecavir) for 6 days without tetracycline was quantified by real time PCR. *Middle panel,* HepG2 cells transfected with the reporter plasmids carrying HBV Enhancer (*Enh*) I + 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 entecavir, or 4 μM CsA) followed by HBV infection according to the protocol shown in Fig. 1*A*. *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 **, $p < 0.01$).

treated cells showed a reduced FITC fluorescence measuring viral attachment (Fig. 2*E*). 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. 2*F*). 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 (Fig. 2*F*, *2nd* and *3rd lanes*) (14). HBV infection was also diminished in HepaRG cells pretreated with IL-1β, which induced an innate immune response (Fig. 2*F*, *4th lane*) (37). In this experiment, Ro41-5253-pretreated HepaRG cells were less susceptible to HBV infection (Fig. 2*F*, *5th lane*), suggesting that the activity of Ro41-5253 in host cells contributed to the inhibition of HBV entry.

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. 3*A*). Flow cytometry showed that NTCP protein on the cell surface was consistently down-regulated following treatment with Ro41-5253 (Fig. 3*B*, 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. 3*C*). 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. 3*D*), suggesting that the inhibitory activity of Ro41-5253 to HBV infec-

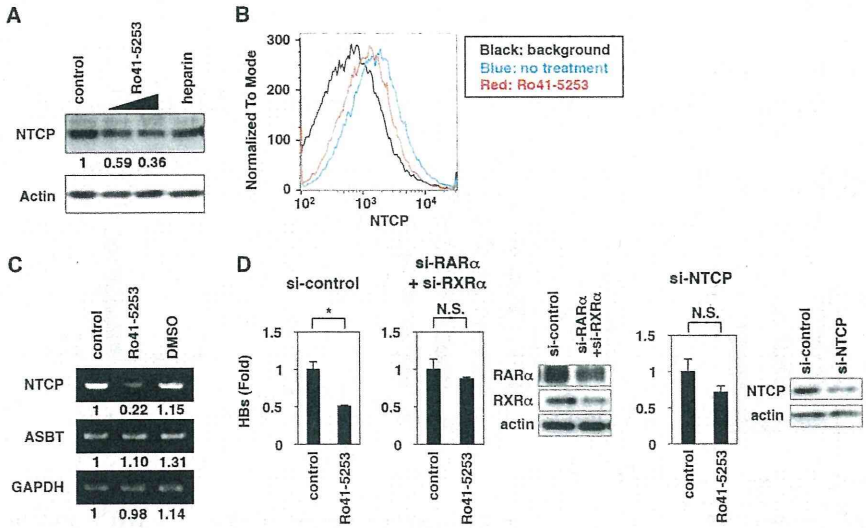

FIGURE 3. **Ro41-5253 reduced NTCP expression.** *A,* HepaRG cells were treated or untreated with 10 and 20 μM Ro41-5253 or 50 units/ml heparin for 12 h, and the levels of NTCP (*upper panel*) and actin (*lower panel*) were examined by Western blot analysis. The relative intensities for the bands of NTCP measured by densitometry are shown below the *upper panel*. *B,* flow cytometric determination of NTCP protein level on the cell surface of primary human hepatocytes treated with 20 μM Ro41-5253 (*red*) for 24 h or left untreated (*blue*). The *black line* indicates the background signal corresponding to the cells untreated with the primary antibody. *C,* RT-PCR determination of the mRNA levels for *NTCP* (*upper panel*), *ASBT* (*middle panel*), and *GAPDH* (*lower panel*) in cells treated with 20 μM Ro41-5253 or 0.1% DMSO for 12 h or left untreated. The relative intensities for the bands measured by densitometry are shown *below the panels*. *D,* HepaRG cells were treated with siRNA against RARa (*si-RARa*) plus that against RXRa (*si-RXRa*), that against NTCP (*si-NTCP*), and a randomized siRNA (*si-control*) for 3 days and then were re-treated with siRNAs for 3 days. The cells were pretreated with or without Ro41-5253 for 24 h and then infected with HBV for 16 h. HBs antigen produced from the infected cells were measured at 12 days postinfection. Statistical significance was determined using Student's *t* test (*, $p < 0.05$; *NS*, not significant).

tion was, at least in part, mediated by targeting NTCP. These data suggest that Ro41-5253 down-regulated 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 down-regulation of NTCP, we used a reporter construct inserting nucleotides (nt) −1143 to +108 of the human *NTCP* (h*NTCP*) promoter upstream of the Gluc gene (Fig. 4*A*, *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. 4*A*, *left panel*). Ro41-5253 had little effect on the herpes simplex virus thymidine kinase promoter (Fig. 4*A*, *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 h*NTCP* promoter. As shown in Fig. 4*D*, 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. 4*D*). Knockdown of endogenous RARα, RXRα, or both dramatically impaired the activity of the h*NTCP* promoter (Fig. 4*E*). These results suggest that RAR/RXR is involved in the transcriptional regulation of the h*NTCP* gene. Consistently, an RAR agonist, ATRA, induced *NTCP* mRNA expression (Fig. 4*F*).

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. 4*G*) (29). 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 the h*NTCP* promoter is direct or indirect. From the analyses so far using the rat *Ntcp* (r*Ntcp*) 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 r*Ntcp* expression; FXR induces its downstream small heterodimer partner (Shp), another nuclear receptor, and Shp recruits to the r*Ntcp* promoter to repress the promoter activity (39). Then we examined whether RAR affected the expression of human SHP. As shown in Fig. 5*A*, 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. 5*A*). To assess the direct involvement of RAR in h*NTCP* regulation, the ChIP assay showed that RAR was associated with the h*NTCP* promoter both in the presence and absence of ATRA (Fig. 5*B*), consistent with the characteristic that RAR/RXR binds to RARE regardless of ligand stimulation (40). The Genomatix software predicts that the h*NTCP* promoter possesses five putative RAREs in nt −1143 to +108 (Fig. 5*C*). Introduction of mutations in all of these five elements lost the promoter activation by RAR/RXR overexpression (Fig. 5*C*, *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 h*NTCP* promoter with

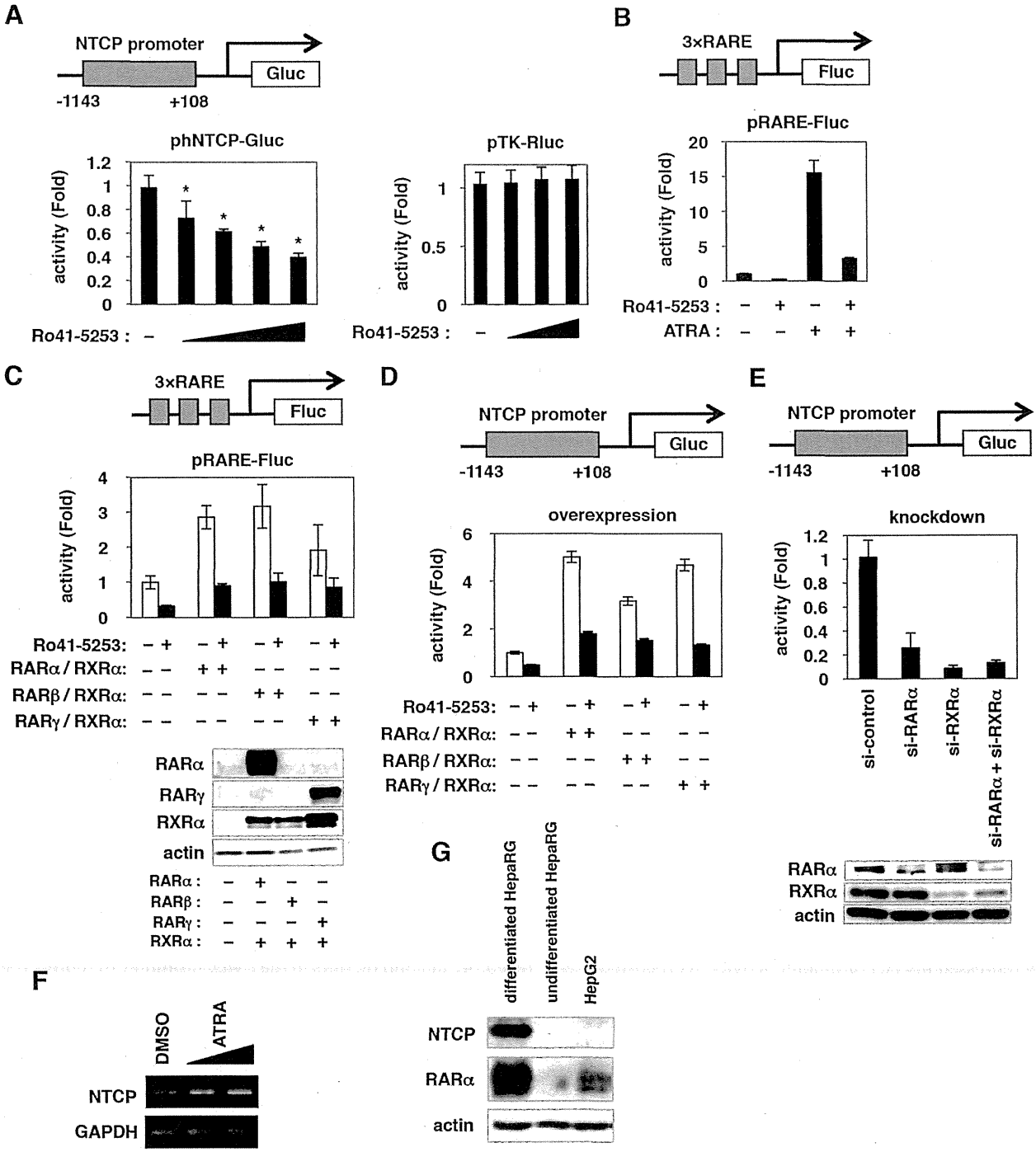

FIGURE 4. **RAR could regulate h*NTCP* promoter activity.** *A, left panel,* HuS-E/2 cells were transfected for 6 h with an hNTCP reporter construct with −1143/+108 of the h*NTCP* promoter region cloned upstream of the Gluc gene (*upper panel, phNTCP-Gluc*), together with an internal control plasmid 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) and RXRα, followed by 24 h of treatment 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 (*, $p < 0.05$).

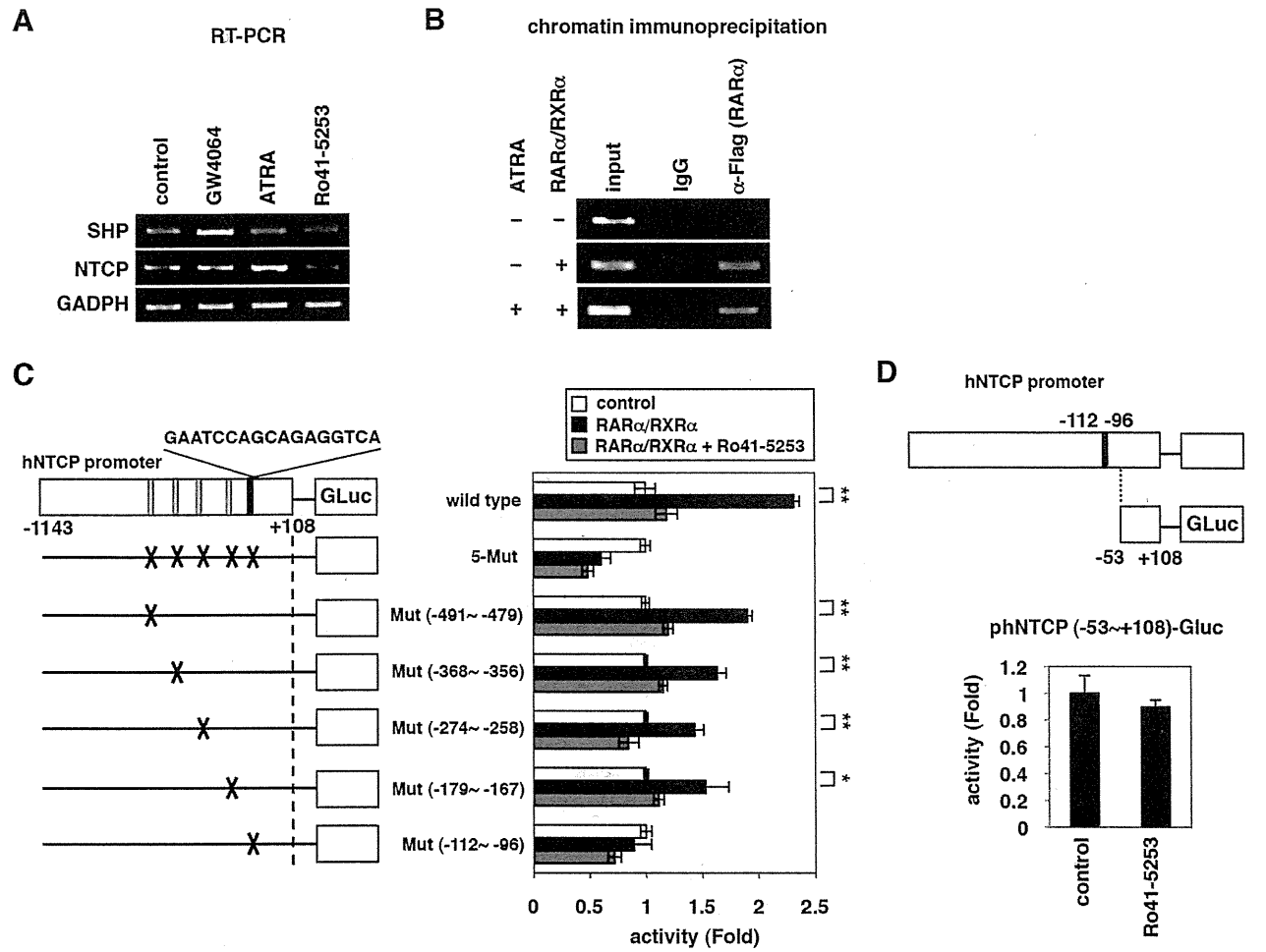

FIGURE 5. **RAR directly regulated the activity of hNTCP promoter.** *A*, HepaRG cells were treated with or without ATRA, Ro41-5253, or a positive control GW4064, which is an FXR agonist, for 24 h. mRNAs for SHP as well as *NTCP* and *GAPDH* were detected by RT-PCR. *B*, ChIP assay was performed as described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

mutations in nt −112 to −96 had no significant response by RAR/RXR (Fig. 5*C*). Promoter activity of h*NTCP* that lacked the region nt −112 to −96 (nt −53 to +108) was not affected by Ro41-5253 (Fig. 5*D*). These data suggest that the nt −112 to −96 region is responsible for RAR-mediated transcriptional activation of h*NTCP*.

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. 6*A*), all decreased the susceptibility of HepaRG cells to HBV infection (Fig. 6*B*) without significant cytotoxicity (Fig. 6*C*). 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. 7*A*), had more potent anti-HBV activity than Ro41-5253 (Fig. 7*B*), which was accompanied by the inhibition of the h*NTCP* promoter (Fig. 7*C*) and down-regulation of NTCP protein (Fig. 7*D*).

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. 8*G*, the percentage of HBV-positive cells increased up to 30 days postinfection without compound treatment (Fig. 8*G*,

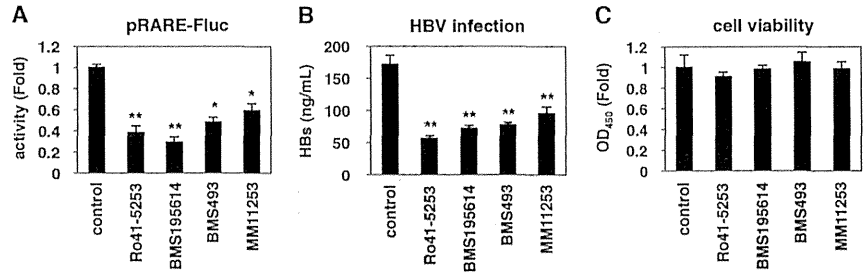

FIGURE 6. **HBV susceptibility was decreased in RAR-inactivated cells.** *A*, HuS-E/2 cells were transfected with the pRARE-Fluc and pTK-Rluc for 6 h followed by treatment with or without the indicated compounds at 20 μM for 48 h. Relative Fluc values normalized by Rluc are shown. *B* and *C*, HepaRG cells treated with or without the indicated compounds 20 μM were subjected to the HBV infection assay according to the scheme in Fig. 1*A*. 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 **, $p < 0.01$).

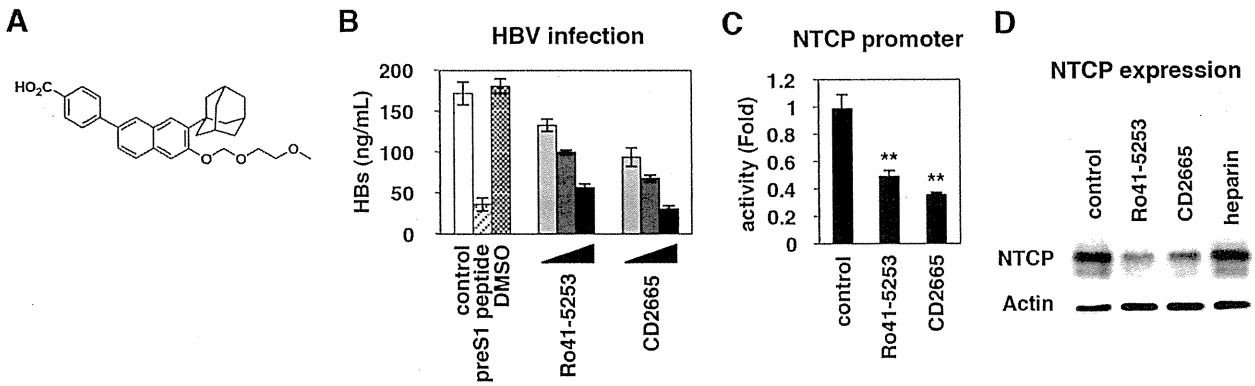

FIGURE 7. **CD2665 had a stronger anti-HBV activity than Ro41-5253.** *A*, chemical structure of CD2665. *B*, HepaRG cells treated with or without 1 μM preS1 peptide, 0.1% DMSO, or various concentrations of Ro41-5253 or CD2665 (5, 10, and 20 μM) were subjected to HBV infection according to the protocol shown in Fig. 1*A*. 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 phNTCP-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 (**, $p < 0.01$).

panels a–d). However, such HBV spread was clearly interrupted by treatment with Ro41-5253 and CD2665 as well as preS1 peptide (Fig. 8*G*, *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. 8*G*, *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 h*NTCP* promoter activity to identify HBV entry inhibitors (data not shown) (44). Intriguingly, all of the compounds identified as repressors of the h*NTCP* promoter were inhibitors of RAR-mediated transcription. This strongly suggests that RAR plays a crucial role in regulating the activity of the h*NTCP* 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. 4*G*). 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 h*NTCP* was shown to be differently regulated mainly because of sequence divergence in the promoter region (48), and transcriptional regulation of h*NTCP* remains poorly understood. Hepatocyte nuclear factor (HNF)1α and HNF4α, which positively regulated the rat *Ntcp* promoter, had little effect on h*NTCP* promoter activity (48). HNF3β bound to the promoter region and inhibited promoter activities of both h*NTCP* and rat

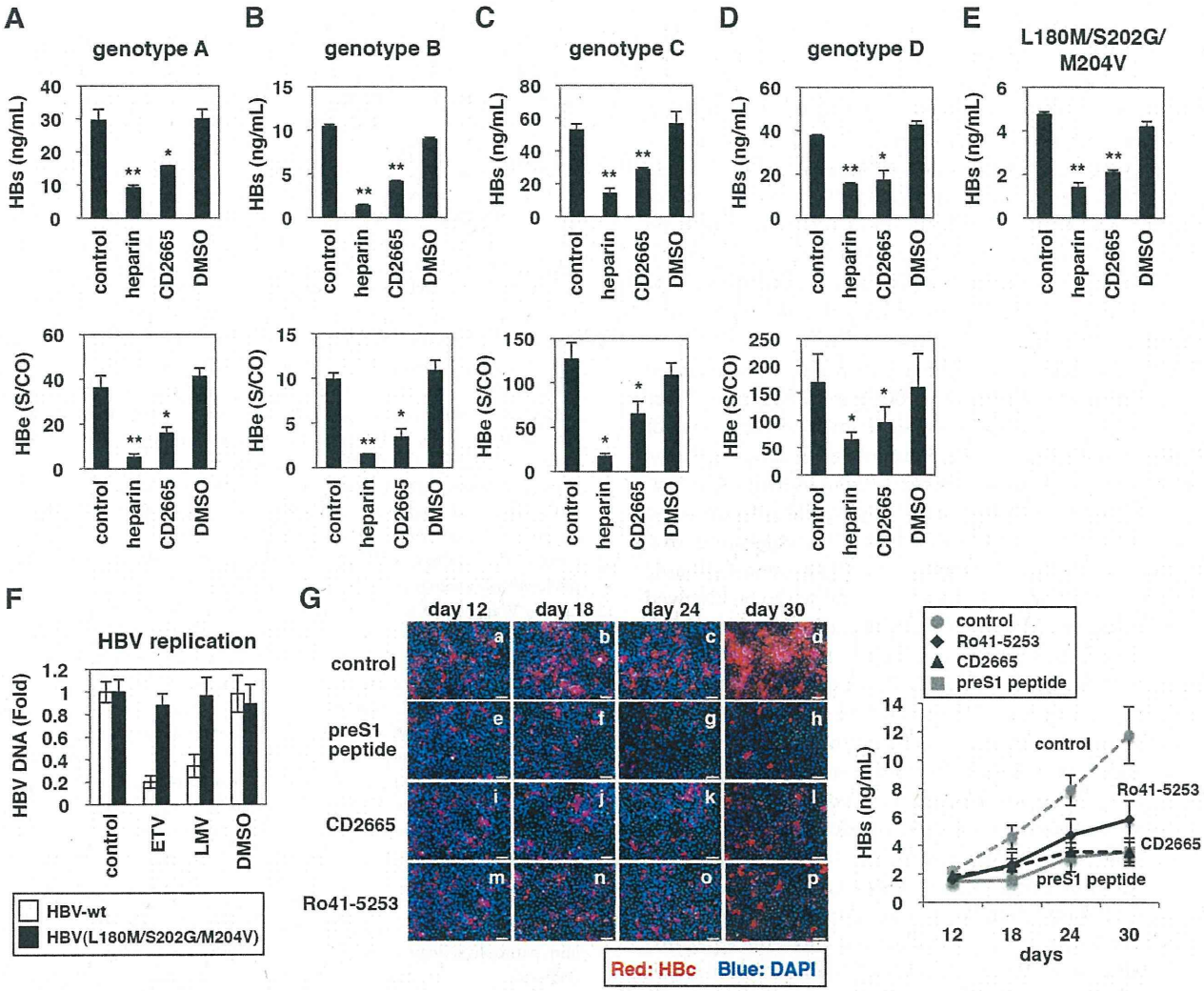

FIGURE 8. **CD2665 showed a pan-genotypic anti-HBV activity.** *A–E,* primary human hepatocytes were pretreated with or without compounds (50 units/ml heparin, 20 μM CD2665, or 0.1% DMSO) and inoculated with different genotypes of HBV according to the scheme show in Fig. 1*A*. HBs (*A–E*) and HBe (*A–D*) 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. *Red* and *blue* signals in the *left panels* show the detection of HBc protein and nucleus, respectively. Statistical significance was determined using Student's *t* test (*, $p < 0.05$, and **, $p < 0.01$).

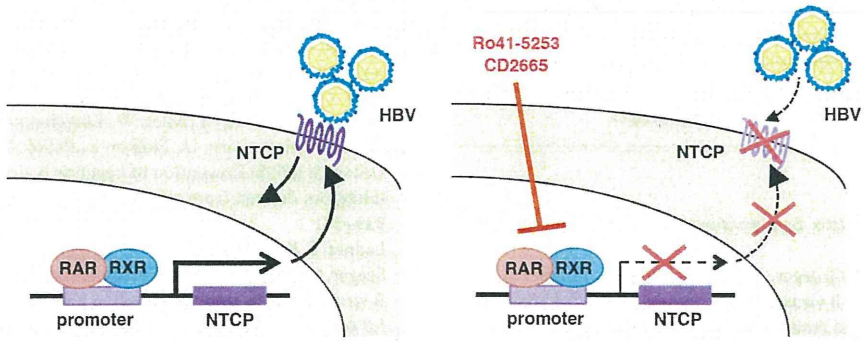

FIGURE 9. **Schematic representation of the mechanism for RAR involvement in the regulation of NTCP expression and HBV infection.** *Left panel,* RAR/RXR recruits to the promoter region of *NTCP* and regulates the transcription. The expression of NTCP in the plasma membrane supports HBV infection. *Right 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.