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

CD2665 showed a pan-genotypic anti-HBV effect

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

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

Discussion

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

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

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

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

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Abbreviations

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

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Figure Legends

Fig. 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 dotted 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 U/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, 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, J) and HBe antigen in the culture supernatant (I) were quantified. The data show the means of three independent experiments. SDs are also shown as error bars. Statistical significance was determined using Student's t-test (*P<0.05, **P<0.01).

Fig. 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. 1A. Secreted HBs was detected by ELISA (left). Cell viability was also determined by ELISA (right). (B) Left, 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, HepG2 cells transfected with the reporter plasmids carrying HBV Enhancer I+II, HBV Enh 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, 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. 1A. (D) Upper scheme shows the experimental procedure for examining cell surface bound HBV. The cells were pretreated with compounds (50 U/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 U/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 inoculated for 16 h in the absence of the compounds.

Fig. 3. Ro41-5253 reduced NTCP expression. (A) HepaRG cells were treated or untreated with 10 and 20 μM Ro41-5253 or 50 U/ml heparin for 12 h and 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 RARα (si-RARα) plus that against RXRα (si-RXRα), that against NTCP (si-NTCP), and a randomized siRNA (si-control) for three days, and then were re-treated with siRNAs for three 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.

Fig. 4. RAR could regulate human NTCP (hNTCP) promoter activity. (A) Left, HuS-E/2 cells were transfected for 6 h with a hNTCP reporter construct with -1143/+108 of the hNTCP promoter region cloned upstream of the Gluc gene (upper, phNTCP-Gluc), together with an internal control plasmid expressing secreted alkaline phosphatase (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, 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 RAR-binding elements (RARE) (upper, pRARE-Fluc) and Rluc-encoding reporter plasmid driven from herpes simplex virus (HSV) thymidine kinase (TK) 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 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), RARα (middle), and actin (lower, as an internal control) were determined by western blot analysis of differentiated HepaRG, undifferentiated HepaRG, and HepG2 cells.

Fig. 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 a 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 in Experimental Procedures with Huh7-25 cells transfected with or without an expression plasmid for FLAG-tagged RARα plus that for RXRα in the presence or absence of ATRA stimulation. (C) Left, a 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 (gray regions), and -112 to -96 (black regions: GAATCCAGCAGAGGTCA)] in nt -1143 to +108 of hNTCP. 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, relative luciferase activities upon overexpression with or without RARα plus RXRα in the presence or absence of Ro41-5253. (D) A 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.

Fig. 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 20 mM for 48 h. Relative Fluc values normalized by Rluc are shown. (B, 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).

Fig. 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 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) and actin proteins as an internal control (lower) were examined by western blot analysis of HepaRG cells treated with or without the indicated compounds at 20 μM.

Fig. 8. CD2665 showed a pan-genotypic anti-HBV activity. (A-E) Primary human hepatocytes were pretreated with or without compounds (50 U/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. Red and blue signals in the left panels show the detection of HBc protein and nucleus, respectively.

Fig. 9. Schematic representation of the mechanism for RAR involvement in the regulation of NTCP expression and HBV infection. Left, 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, 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.

Fig. 1

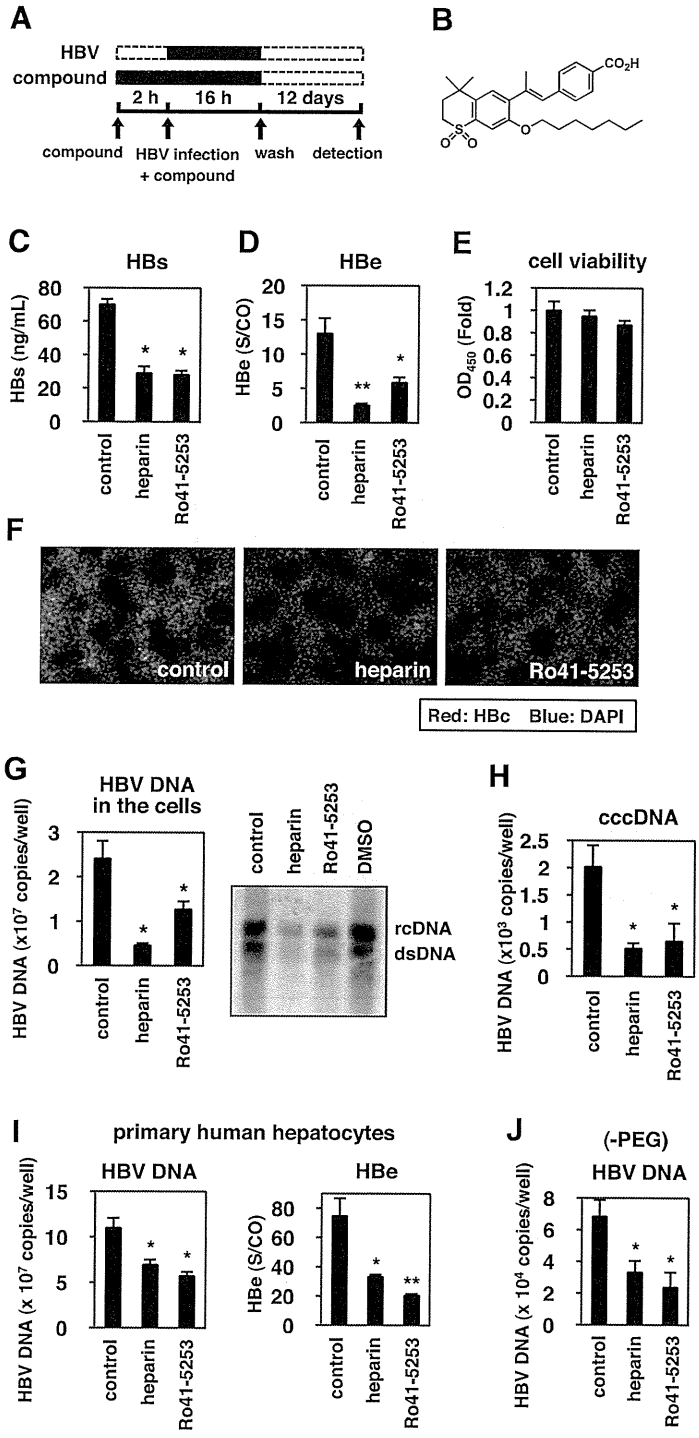


Fig. 2

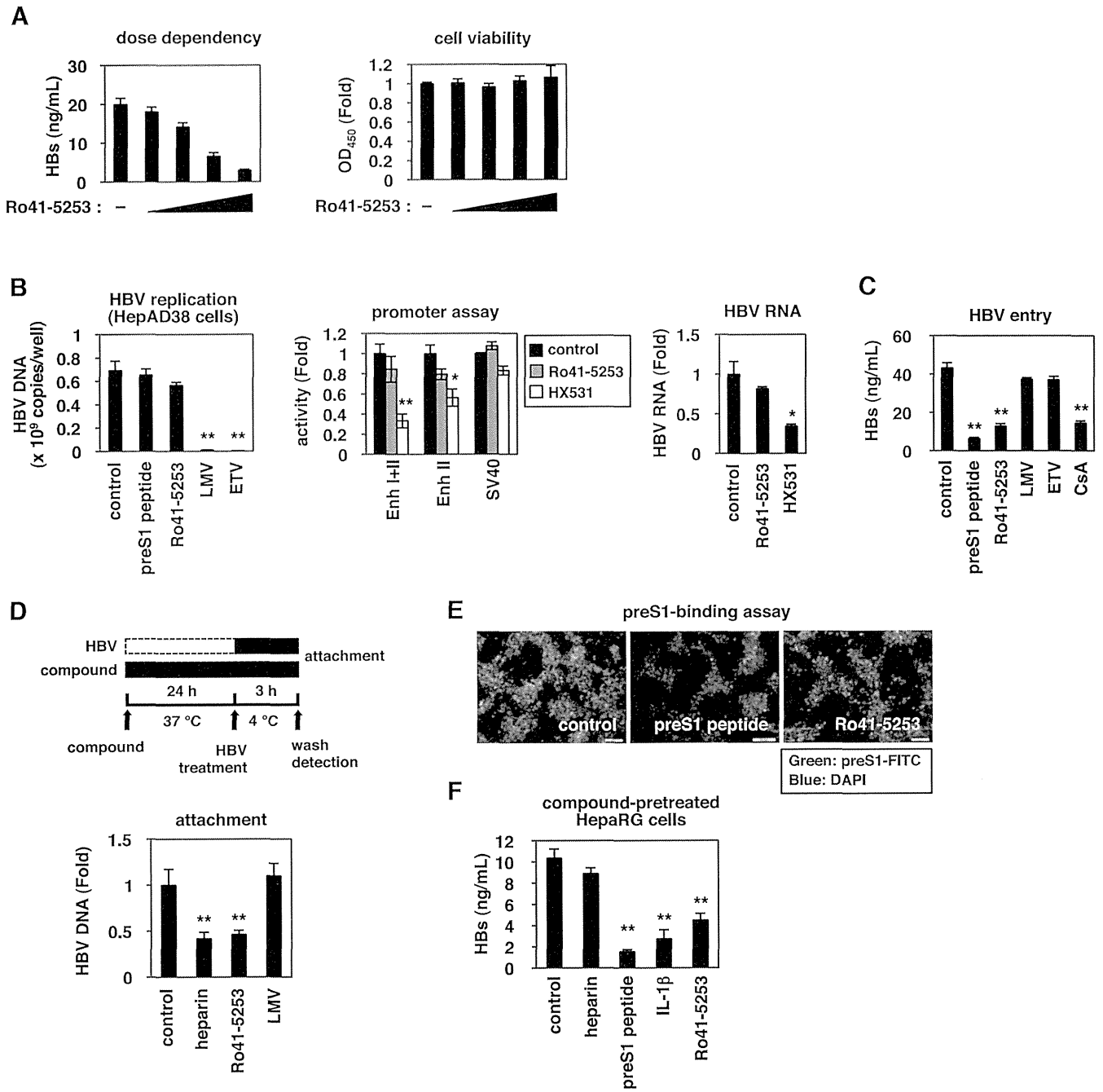


Fig. 3

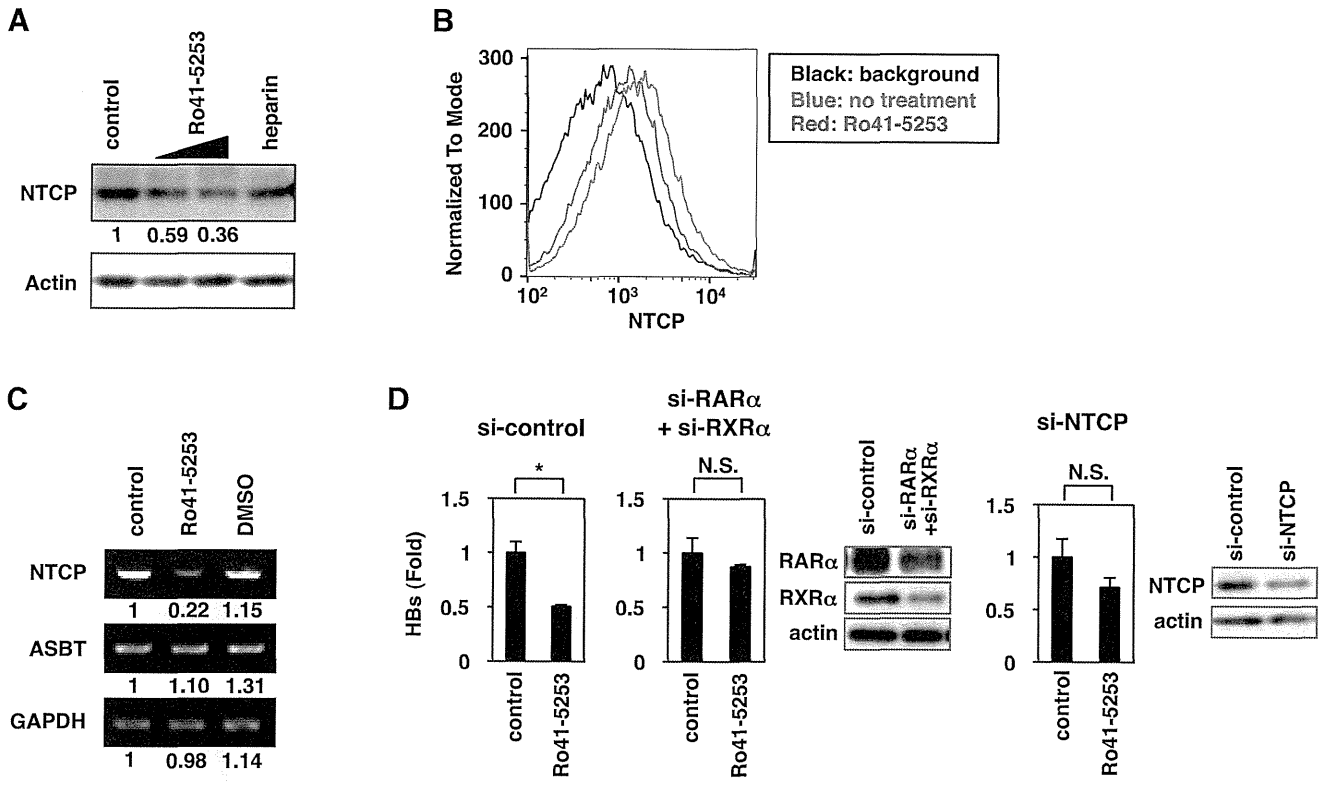


Fig. 4

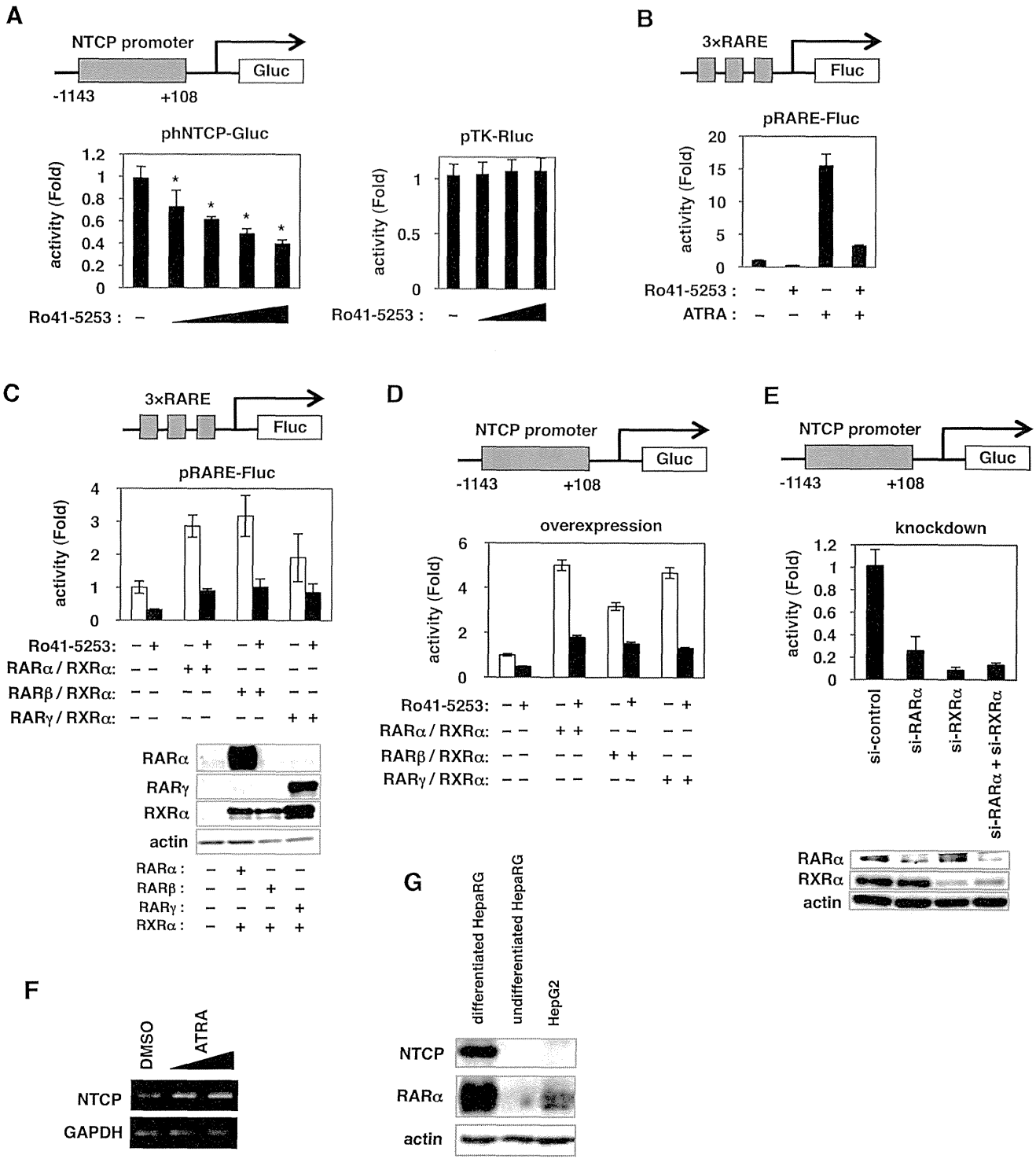
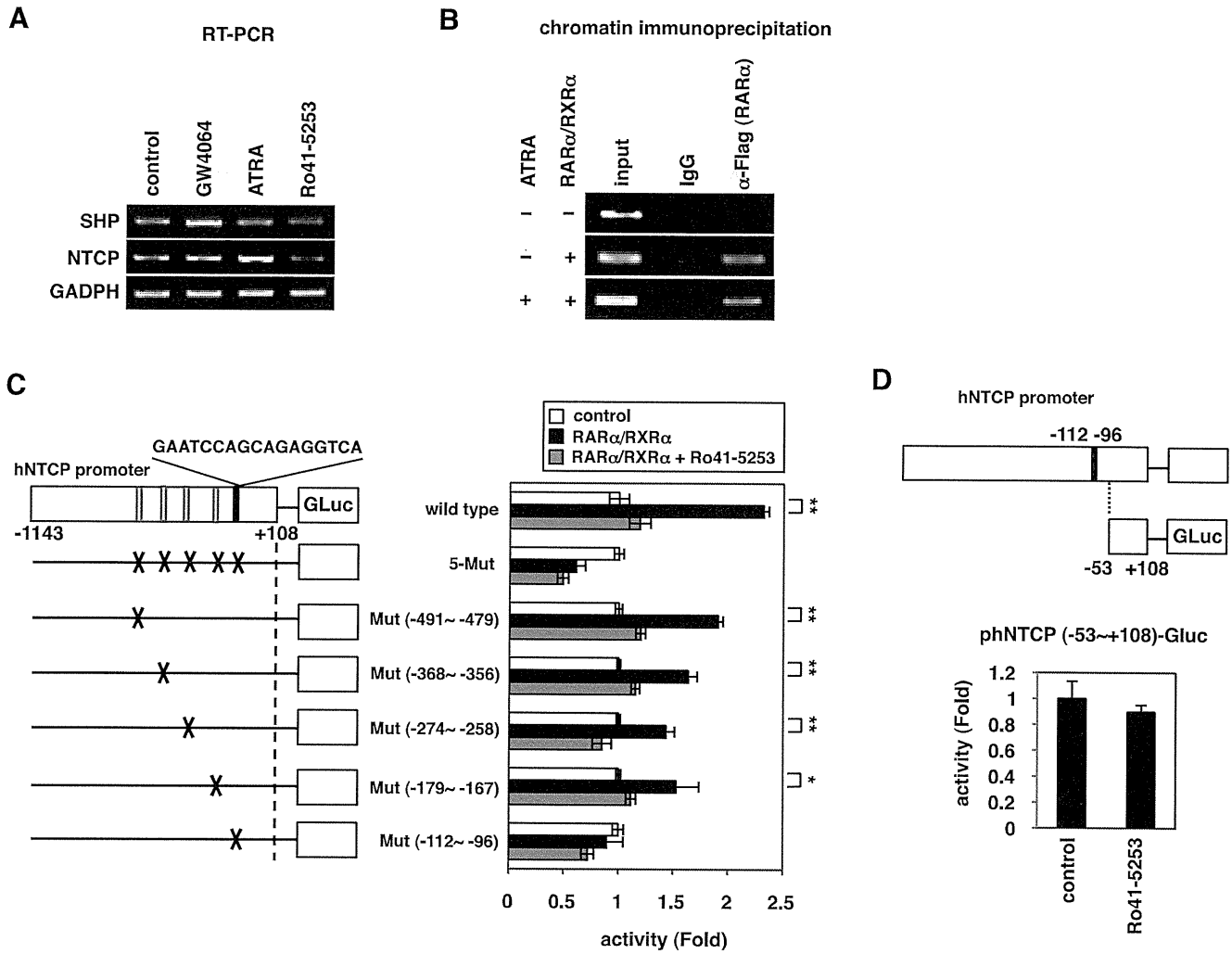


Fig. 5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ATRA
Ro41-5253
SHP
NTCP
GADPH
B
chromatin immunoprecipitation
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input
IgG
α-Flag (RARα)
C
GAATCCAGCAGAGGTCA
hNTCP promoter
GLuc
-1143
+108
control
RARα/RXRα
RARα/RXRα + Ro41-5253
wild type
5-Mut
Mut (-491~ -479)
Mut (-368~ -356)
Mut (-274~ -258)
Mut (-179~ -167)
Mut (-112~ -96)
**
**
**
*
0
0.5
1
1.5
2
2.5
activity (Fold)
D
hNTCP promoter
-112 -96
GLuc
-53 +108
phNTCP (-53~+108)-Gluc
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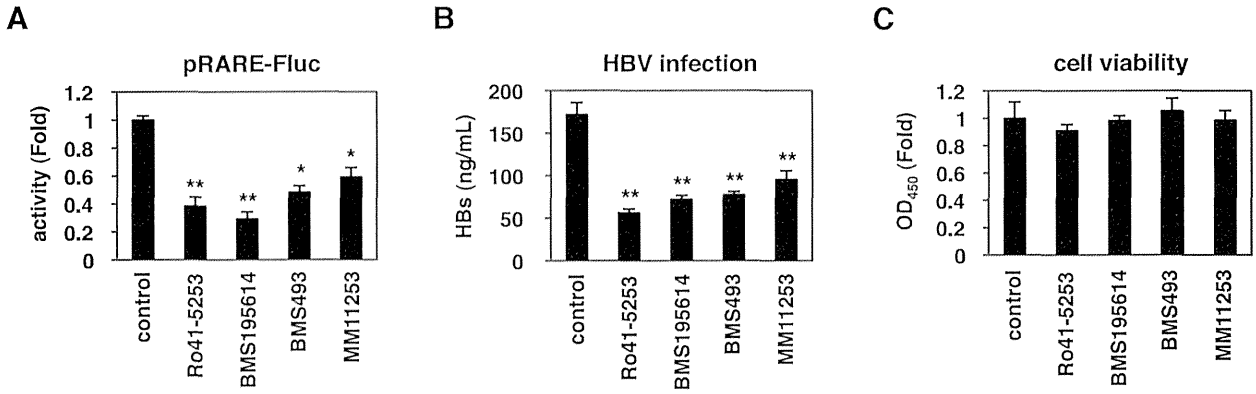
0.2
0
control
Ro41-5253

Fig. 6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0.6
0.4
0.2
0
**
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*
*
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Ro41-5253
BMS195614
BMS493
MM11253
B
HBV infection
HBs (ng/mL)
200
150
100
50
0
**
**
**
**
control
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BMS195614
BMS493
MM11253
C
cell viability
OD450 (Fold)
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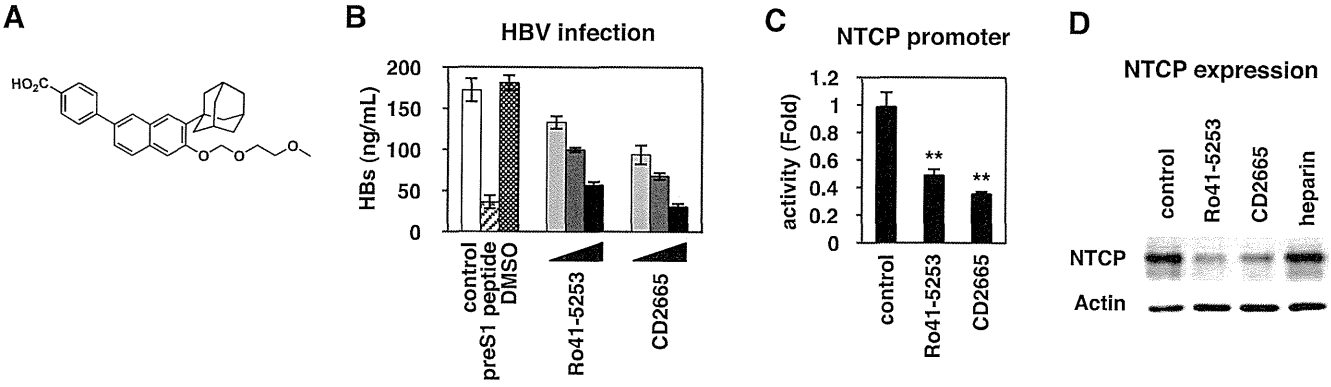
Fig. 7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B
HBV infection
HBs (ng/mL)
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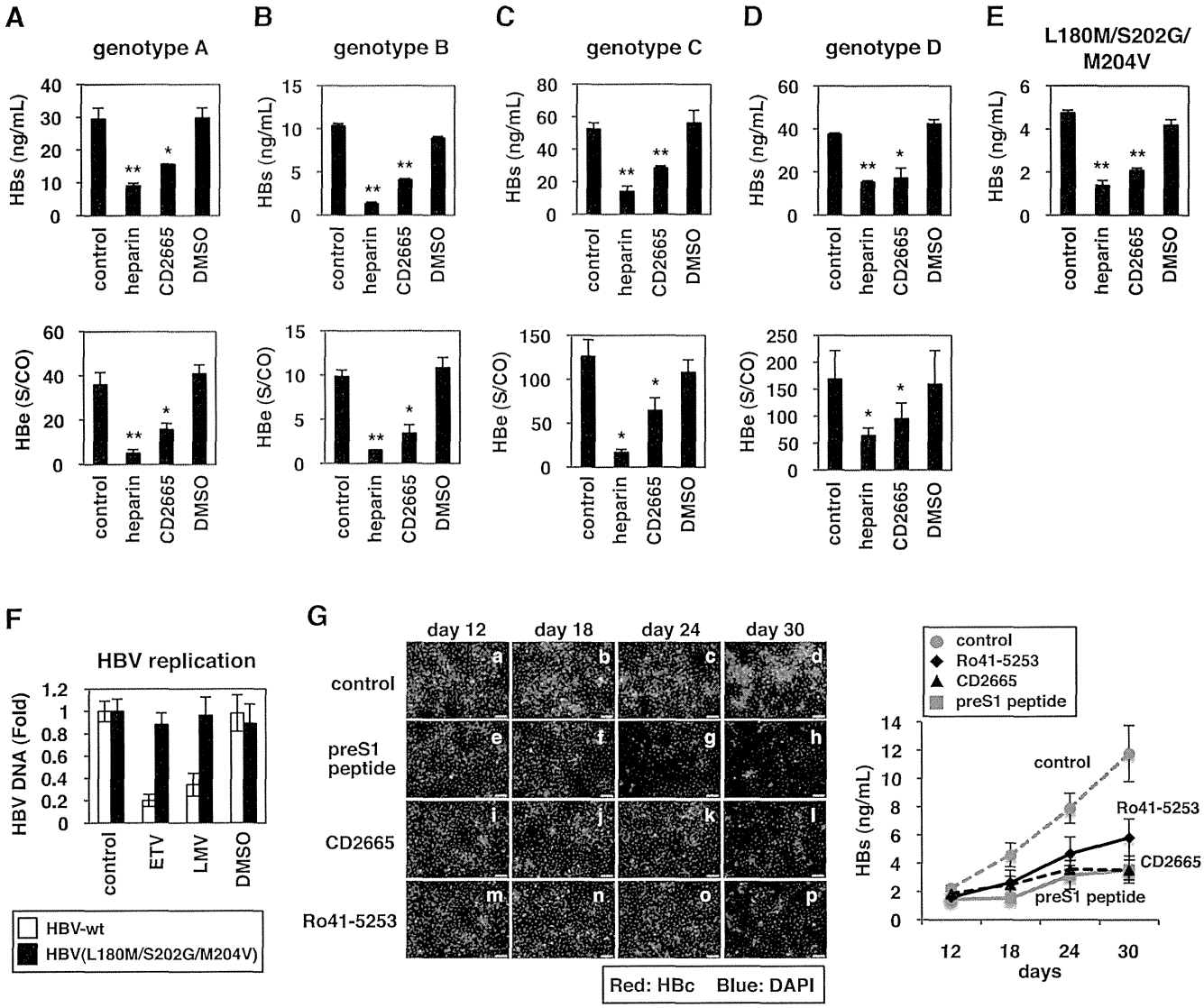
150
100
50
0
control
preS1 peptide
DMSO
Ro41-5253
CD2665
C
NTCP promoter
activity (Fold)
1.2
1
0.8
0.6
0.4
0.2
0
**
**
control
Ro41-5253
CD2665
D
NTCP expression
control
Ro41-5253
CD2665
heparin
NTCP
Actin

Fig. 8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B
genotype B
C
genotype C
D
genotype D
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M204V
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HBe (S/CO)
control
heparin
CD2665
DMSO
F
HBV replication
HBV DNA (Fold)
control
ETV
LMV
DMSO
HBV-wt
HBV(L180M/S202G/M204V)
G
day 12
day 18
day 24
day 30
control
preS1 peptide
CD2665
Ro41-5253
Red: HBc Blue: DAPI
control
Ro41-5253
CD2665
preS1 peptide
HBs (ng/mL)
days