Supporting information

Supporting Experimental Procedures

Cell culture

HepaRG cells (Biopredic) were cultured with Williams' medium E (Invitrogen) supplemented with 2 mM L-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 5 μg/ml insulin (Wako), 20 ng/ml EGF (Peprotech), 50 μM hydrocortisone (Sigma) and 2% DMSO (Sigma). HepAD38 cells (kindly provided by Dr. Christoph Seeger at Fox Chase Cancer Center) (33) 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 in the presence of 400 μg/ml G418 (Nacalai) and 0.3 μg/ml tetracycline. Tetracycline was removed to induce HBV production. Huh-7.5.1 cells (kindly provided by Dr. Francis Chisari at Scripps Research Institute) were cultured as described previously (51). The isolation of and the culture medium for primary human hepatocytes (PHH) (Phoenixbio) were described previously (52).

HBV preparation and infection

Most experiments in this study utilized HBV derived from HepAD38 cells (33). To induce virus production, HepAD38 cells were cultured in the absence of tetracycline. Culture medium, collected every three days over the period 7-31 days post-induction, was passed through a 0.45 μm filter. Virus was precipitated using 10% PEG8000 and 2.3% NaCl. The precipitates were washed and resuspended with medium at approximately 200-fold concentration. HBV DNA was quantified by real time PCR. For Fig. 3A, we recovered the media of HepG2 cells transfected with an expression plasmid for HBV genotype A, B, or D (HBV/Aeus, HBV/C-AT, or HBV/D-IND60) (53) at 3 and 6 days posttransfection. Virus was concentrated and quantified as described for HepAD38 cells.

HepaRG cells and PHH were infected with HBV at 2000-20000 (normally 6000) genome equivalents (GEq)/cell in the presence of 4% PEG8000 at 37˚C for 16 h as previously described (23). Under these conditions, efficient infection of HepaRG cells using virus derived from HepAD38 or HepG2.2.15 cells requires an inoculum of $>10^4$ HBV GEq/cell (i.e. 1.25 - 40 x 10^4 GEq/cell) (54).

Indirect immunofluorescence analysis

Indirect immunofluorescence analysis was performed essentially as described previously (16). Briefly, after fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton X-100, an anti-HBc antibody (DAKO, #B0586) was used as the primary antibody.

ELISA

To quantify HBs protein by ELISA, microwell antigen capture plates (Maxisorp nunc-immuno plate, Nunc #439454) were prepared by overnight incubation at 4˚C with a sheep anti-HBs antibody at 1:5000 dilution, followed by coating with 0.2% BSA/0.02% NaN_3/1 x PBS at 4˚C until use. For HBs detection, samples were incubated in individual wells of the capture plates for 2 h. After washing, horseradish peroxidase-labeled rabbit anti-HBs antibody was added for an additional 2 h incubation. The substrate solution (from the HCV core ELISA kit: Ortho) was reacted for 15-60 min before the OD_{450} values were measured.

Real time PCR and RT-PCR

HBV DNA was extracted from cells using a QIAamp mini kit (QIAGEN) according to the manufacturer's protocol. HBV DNA was quantified by real time PCR analysis using the primer set 5'-ACTCACCAACCTCCTGTCCT-3' and 5'-GACAAACGGGCAACATACCT-3' and probe 5'FAM-TATCGCTGGATGTGTCTGCGGCGT-TAMRA3'. Detection of cccDNA was achieved using 5'-CGTCTGTGCCTTCTCATCTGC-3' and 5'-GCACAGCTTGGAGGCTTGAA-3' as primers and 5'-CTGTAGGCATAAATTGGT (MGB)-3' as a probe. mRNAs for NTCP, CyPA, CyPB, and GAPDH were detected using a one step RNA PCR kit (Takara) following the manufacturer's protocol. Primers are 5'-CCGGCTGAAGAACATTGAGGCACTGG-3' and 5'-AGGGAGGAGGTGGCAATCAAGAGTGG-3' for NTCP, 5'-CTCCTTTGAGCTGTTTGCAGACAAGGTCCC -3' and 5'-CATTTGCCATGGACAAGATGCCAGGACCCG -3' for CyPA, 5'-AGACTGTTCCAAAAACAGTGGATAA-3' and 5'-AGTGCTTCAGTTTGAAGTTCTCATC-3' for CyPB, and 5'-CCATGGAGAAGGCTGGGG-3' and 5'-CAAAGTTGTCATGGATGACC-3' for GAPDH,

respectively.

Southern blot analysis

Southern blotting was performed as described previously (23).

HBV attachment and internalization assay

To allow HBV attachment to the cells without subsequent internalization, HBV was added to HepaRG cells at 4˚C for 3 h. Free HBV was removed by washing the cells with PBS. Attached HBV was quantified by real time PCR analysis of DNA extracted from the washed cells. To allow virus internalization, cells treated with HBV as above were transferred to 37˚C and incubated for an additional 16 h. The cells were then trypsinized to digest the cell surface HBV and extensively washed with PBS. Internalized HBV from the washed cells was quantified by real time PCR analysis of HBV DNA.

Pretreatment of HBV particles with compounds

HBV inoculum, prepared as described above, was preincubated with the indicated compounds (Fig. 2F) for 30 min at 37˚C. Each compound-treated HBV inoculum was concentrated from 450 ml to 15 ul via ultrafiltration, followed by dilution to 450 ml with PBS and re-concentration to recover 15 ul of HBV-containing medium. This procedure is expected to result in a 900-fold reduction in compound concentration. The infectivity of the resultant HBV-containing concentrate (10 ml) was evaluated by inoculating HepaRG cells as described above.

MTT assay

The MTT cell viability assay was performed as described previously (23).

Reporter assay

Gene expression reporter assays were performed essentially as described (18). Briefly, the reporter plasmids pNF-AT-luc (promoter binding elements for NF-AT upstream of the firefly luciferase gene) and pRL-TK (herpes simplex virus thymidine kinase promoter upstream of the renilla luciferase gene) were transfected into Jurkat cells using lipofectamine 2000. At 4

h posttransfection, cells were stimulated with the indicated compounds. After an additional 28 h incubation, the cells were stimulated with 1 mM PMA and 1 mM ionomycin for 16 h and the luciferase activities were measured.

HCV pseudoparticle assay

The HCV pseudoparticle (HCVpp) assay was essentially performed as previously described (55, 56). Plasmids used to produce HCVpp, were kindly provided by Dr. Francois-Loic Cosset at University of Lyon.

Cyclophilin binding assay

Binding to human cyclophilins A, B, and D by CsA, CsA analogs, and other compounds was measured in a fluorescence polarization ligand competition assay. The binding probe for this assay (FP-CsA ligand) was the carboxyfluorescein (CF)-labeled [Ser]8-CsA derivative [O-(CF-NH(CH2)2NHC(O) CH2-)D-Ser]8CsA, synthesized at Scynexis as described in (57). Compound stock solutions were serially diluted in DMSO, and added to assay buffer (20 mM HEPES pH 8.0, 0.01% Triton-X100) containing 20 nM FP-CsA ligand. Competitive ligand mixtures were then added to assay buffer containing a cyclophilin protein (1500 nM for CypA, 500 nM for CypB and CypD) in black 96-well microtiter plates. Final compound concentrations in the assay ranged from 10 to 0.15625 μM. Plates were incubated at room temperature on an orbital shaker for 30 min prior to reading fluorescence polarization. Fluorescence polarization measurements were performed on a PheraStar fluorescence reader (BMG Labtech, Cary, NC) equipped with a filter set for fluorescein polarization measurement (FP480/FP535). The anisotropy values (r) were calculated from the fluorescence intensity parallel (F_1) and perpendicular (F_2) to the excitation plane: $r = (F_1-F_2)/(F_1+2F_2)$. The anisotropy values obtained were plotted and IC_{50} values calculated using Excel Fit Software.

Isolation of HepG2 cells stably overexpressing NTCP (HepG2-NTCP cells)

HepG2 cells were transfected with an expression plasmid for NTCP (25) using TransIT LT1 transfection reagent (Mirus). The cells were plated into a 10 cm dish at 4 h posttransfection, and cultured in the presence of G418 1 mg/ml to select plasmid-bearing cells beginning on day 2 posttransfection. After 20 days of growth, individual cell colonies were isolated and

expanded. Each cell clone and parental HepG2 cells as a negative control were infected with HBV as described above for HepaRG cells, and cell clones highly susceptible to HBV infection were selected for use in experiments.

NTCP transporter assay

NTCP transporter activity (Fig. 5A) was assayed essentially as described (25, 58) using 293 cells permanently overexpressing human NTCP (Sekisui Medical). In this assay, we observed no cytotoxic effects at any of the compound concentrations tested. Cells were pre-incubated at 37°C for 15 min in Hanks' Balanced Salt Solution buffer (HBSS buffer, 142 mM NaCl, 23.8 mM $NaHCO_3$, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$, adjusted to pH 7.4) with or without CsA, FK506, rapamycin, or PSC833. After pre-incubation, the HBSS was removed completely, and the transport assay was initiated by adding [^{3}H]taurocholic acid (TCA) solution with or without compounds at 37°C for 5 min. The transport assay was terminated by adding ice-cold PBS containing 0.2% BSA (Sigma-Aldrich). Cells were washed twice with 1 mL of ice-cold PBS, then pelleted cells were solubilized by pipetting in 0.5mL of 0.1 N NaOH. Cell lysate (300 µL) was combined with 10 mL of scintillator (Hionic-Fluor) in a glass scintillation vial, and the radioactivity was measured using a liquid scintillation counter (TRI-CARB 2500TR, PerkinElmer). Protein concentration was determined with 20 µL of the remaining cell lysate using a BCA-protein assay kit (Thermo Fisher Scientific, Waltham, MA). Cleared volume (ml/mg protein) was calculated from the uptake amount (disintegrations/well), protein amount (mg protein/well) and initial concentration (disintegrations/ml). The percent of control (%) was calculated from the ratio of cleared volume in the presence of the compound to that in the absence of the compound. The IC_{50} value was calculated from the relationship between the concentration of the compounds and the percent of the control using eq.1: Percent of control (%) = IC_{50} /(IC_{50}+I) x 100, where I is concentration of the compound (mM).

For the HepG2-based transporter assay in Fig. 5B, the uptake experiments were performed as described (59, 60) by using HepG2 and HepG2-NTCP with modification. The uptake of [^{3}H]TCA was measured at 37^{o}C for 5 min in HBSS containing 125 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 5.6 mM D-glucose, 25 mM HEPES (pH 7.4) and 50 nM [^{3}H]TCA (370 GBq/mmol; American Radiolabeled Chemicals, Inc; St. Louis, MO, USA) in the presence or absence of 10 µM inhibitors as indicated in the figure. NTCP

specific TCA uptake values were obtained by subtracting background (HepG2 uptake value) from HepG2-NTCP uptake value.

Binding assay for CsA to NTCP-containing membranes

Crude membrane fractions of HepG2 and HepG2-NTCP cells were prepared as described (60). For the binding assay, 42.5 mg of crude membrane fraction was mixed with 100 ml of 30 nM [^{3}H]cyclosporin A (CsA) (1.23 GBq/mmol; Perkin Elmer) in buffer containing 20 mM KPi pH 7.0 and 150 mM NaCl. The reaction was incubated at 25^{o}C for 5 min. Membrane–bound [^{3}H]CsA was captured using rapid filtration and quantified using a liquid scintillation counter (61).

Scintillation proximity assay for binding of [^{3}H]TCA to crude recombinant NTCP

A scintillation proximity assay (SPA) (62) was utilized to test substrate binding by in vitro synthesized NTCP. Three mg/ml of crude recombinant NTCP-mycHis or GST-mycHis was mixed with 100 nM [^{3}H]TCA (0.6 GBq/mmol), followed by 250 mg of YSi Copper HIS TAG SPA bead (Perkin Elmer) in binding buffer containing 50 mM KPi pH 7.0, 100 mM NaCl, 0.2% w/v bovine serum albumin (Cohn fraction V) and 0.05% n-dodecyl b-D-maltopyroside. The reaction was incubated at 4^{o}C for 1 h. The radioactivity of bound [^{3}H]TCA was measured using a 1450 MicroBeta TriLux scintillation counter (Perkin Elmer), and corrected for the assay background that was measured following addition of 200 mM imidazole to each well. The total radioactivity (ccpm) in each well was obtained in the presence of OptiPhase Supermix scintillation cocktail, and used for calculation of ccpm-to-mol of substrate/well.

AlphaScreen assay for NTCP-HBs binding

Recombinant NTCP and HBs proteins were synthesized using a wheat cell-free protein system as described previously (63-65). The template for transcription was produced by split-primer PCR using the following primers: a target protein specific primer (5’-5'-CCACCCACCACCACCAatgnnnnnnnnnnnnnnnn-3'; lowercase indicates the 5'-coding region of the target gene) and the SV40_5-23 (5’- AAAACGATTCCGAAGCCC) or the pcDNA_Neo472 (5’- CTGATGCTCTTCGTCCAG). The second round of PCR was performed with a 1/100 volume of the first PCR product using 100 nM of SPu primer (5’-GCGTAGCATTTAGGTGACACT-3’), 100 nM of the SV40_2-21 (5’-AAACGATTCCGAAGCCCAA) or the pcDNA_Neo469 (5’- ATGCTCTTCGTCCAGATC)

and 1 nM of deSP6E01 primer (5'-GGTGACACTATAGAACTCACCTATCTCCCCAACACCTAATAACATTCAATCACTCTTTCCACTAACCACCTCCACCCACCACCACCAATG-3') or deSP6E02-bls-S1 primer (5'-GGTGACACTATAGAACTCACCTATCTCTCTACACAAAACATTTCCCTACATACAACTTTCAACTTCCTATTATGGGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGAACTCCACCCACCACCACCAATG). In vitro transcription and wheat cell-free protein synthesis were performed in accordance with the manufacturer's instructions (CellFree Sciences, Yokohama, Japan).

The binding assay was carried out in a total volume of 15 µl consisting of 100 mM Tris-HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 0.1 µl crude recombinant NTCP-His and 0.1 µl crude recombinant Biotin-HBs at 26°C for 1 hr in a 384-well Optiplate (PerkinElmer, Boston, MA, USA). For the binding inhibitor assay, 0.1 µl recombinant NTCP-His was incubated with serially diluted inhibitors at 26°C for 10 min followed by addition of biotinylated HBs protein. Mixtures were then incubated at 26°C for 1 hr in a 384-well Alphaplate. In accordance with the AlphaScreen IgG (ProteinA) detection kit instruction manual (PerkinElmer), 10 µl of detection mixture containing 100 mM Tris-HCl pH 8.0, 0.01% Tween-20, 1 mg/ml BSA, 5 µg/ml Anti-6xHis antibody (GeneTex Inc., Irvine, CA, USA), 0.1 µl streptavidin-coated donor beads and 0.1 µl anti-IgG (ProteinA) acceptor beads were added to each well of the 384 Alphaplate followed by incubation at 26°C for 1 hr. Luminescence was analyzed using the AlphaScreen detection program of an Envision spectrophotometer (PerkinElmer). The pre-S1 lipopeptide HBVpreS/2-48^{myr} was kindly provided by Dr. Stephan Urban at University Hospital Heidelberg. The mutant peptide for HBVpreS/2-48^{myr} (myr-SLNTGVQNAEPLPNPVWHDKNDPFFGPNFDWPDLQHDPNNSNAGFA), which was shown to be deficient for inhibiting HBV infection, was synthesized (CS Bio, Co.).

Statistical analyses

Statistical significance was determined using the Student's t-test.

Supporting References

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Supporting Table S1

Activity of CsA analogs for immunosuppression and CyP binding

		Binding IC_{50} (nM)		
Compound	IL-2 IC_{50} (nM) *	CyPA	CyPB	CyPD
CsA	0.065	0.36	0.73	1.25
SCYX618806	2366	0.76	2.6	1.7
SCYX1774198	5085	>10	>10	>10
SCYX827830	4	2.0	6.1	5.0
SCYX1454139	166	4.7	>10	>10

* *In vitro* immunosuppression assay measured inhibition of IL-2 secretion from activated Jurkat cells as described in (66).

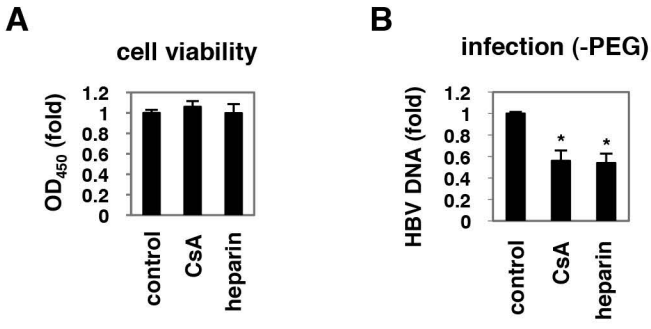
Supporting Figure Legends

Fig. S1. Cyclosporin A (CsA) blocked HBV infection. (A) Cells were treated with 4 mM CsA and 25 U/ml heparin according to the protocol shown in Fig. 1A. Cell viability was quantified using the MTT assay. (B) Primary human hepatocytes were pretreated with or without 4 mM CsA and 25 U/ml heparin and then inoculated with HBV in the absence of 4% PEG8000 using the protocol shown in Fig. 1A.

Fig. S2. Binding of [^{3}H]CsA to native membrane-bound NTCP protein. The binding of [^{3}H]CsA was assayed as described in the Supplemental Experimental Procedures using the membrane fraction of HepG2 and HepG2-NTCP cells. Membranes containing NTCP protein bound increased levels of CsA.

Fig. S3. Characterization of recombinant NTCP and LHBs proteins. (A) Binding capacity of recombinant NTCP protein for [^{3}H]TCA. Scintillation proximity assay was performed using in vitro synthesized NTCP protein as described in Experimental Procedures. Recombinant NTCP protein bound to TCA. (B) Neutralizing activity of recombinant LHBs in HBV infection. HepaRG cells were pretreated with or without BSA or recombinant 300 ng LHBs for 1 h, and then infected with HBV for 16 h. HBV infection was evaluated by quantifying HBs antigen in the medium at 12 days postinfection. Pretreatment with recombinant LHBs significantly reduced HBV infection. (C) An AlphaScreen protein-interaction assay was performed by incubating LHBs and GST-His or NTCP-His in the presence or absence of wild-type or mutant HBVpreS/2-48myr peptide. The assay results are shown normalized to the AlphaScreen signal from the interaction of LHBs and NTCP-His in the absence of peptide.

Fig. S1.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cell viability
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infection (-PEG)
HBV DNA (fold)
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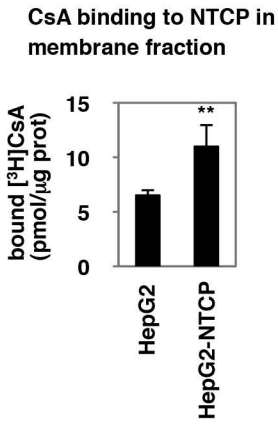
control
CsA
heparin

Fig. S2.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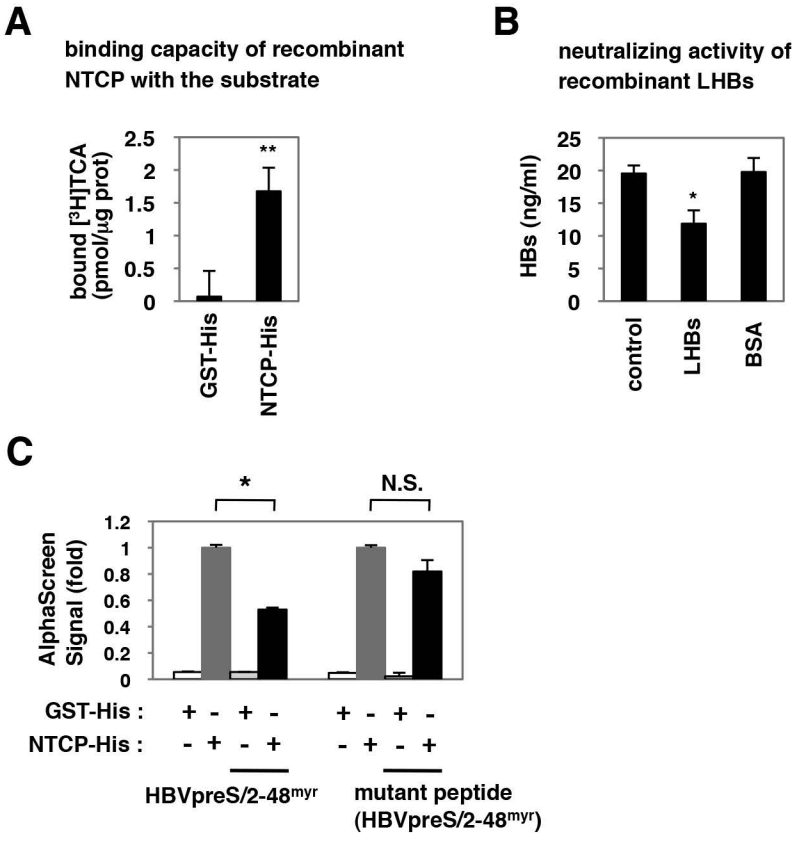
10
5
0
**
HepG2
HepG2-NTCP

Fig. S3.


A
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bound [3H]TCA (pmol/μg prot)
2.5
2
1.5
1
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GST-His
NTCP-His
B
neutralizing activity of recombinant LHBs
HBs (ng/ml)
25
20
15
10
5
0
*
control
LHBs
BSA
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N.S.
AlphaScreen Signal (fold)
1.2
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GST-His : + - + - + - + -
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HBVpreS/2-48myr
mutant peptide (HBVpreS/2-48myr)