Supplementary Data

Supporting Experimental Procedures

Sample harvest from tupaias.

Blood samples from 10 HBV-inoculated tupaias and 10 control tupaias were taken from the tail venous plexus for serological analysis and ALT detection on day 4 prior to inoculation and on days 9, 15, 21, and 42 post-inoculation (p.i.). Liver biopsy samples were obtained by surgery from the other 4 HBV-inoculated tupaias at the intervals mentioned above for immunohistochemistry, pathological assessments, and cccDNA detection. A liver biopsy sample from the 1 remaining PBS-inoculated tupaia was obtained as a control.

Infection inhibition by an inhibitory peptide for viral entry

The inhibitory peptide contained 47 amino acids based on the Pre-S113–59 of HBV genotype C with a myristoylation at the N-terminus. The complete sequence is: myr-GTNLSVPNPLGFFPDHQLDPAFGANSNNPDWDFNPNKDHWPEANQVG-NH2.

This peptide was chemically synthesized with more than 98% purity (Chinese Peptide Company, Hangzhou, China) and could inhibit HBV infection of primary tupaia hepatocytes *in vitro* with a 50% inhibitory concentration (IC50) of 1.6 nM. To block HBV infection *in vivo*, the peptide was subcutaneously administered at a dose of 2 mg/kg to another group of 10 tupaias simultaneously with HBV inoculation and then again on days 1, 2, 3, 5, 7, 9, and 13 p.i.

Protein-free DNA extraction.

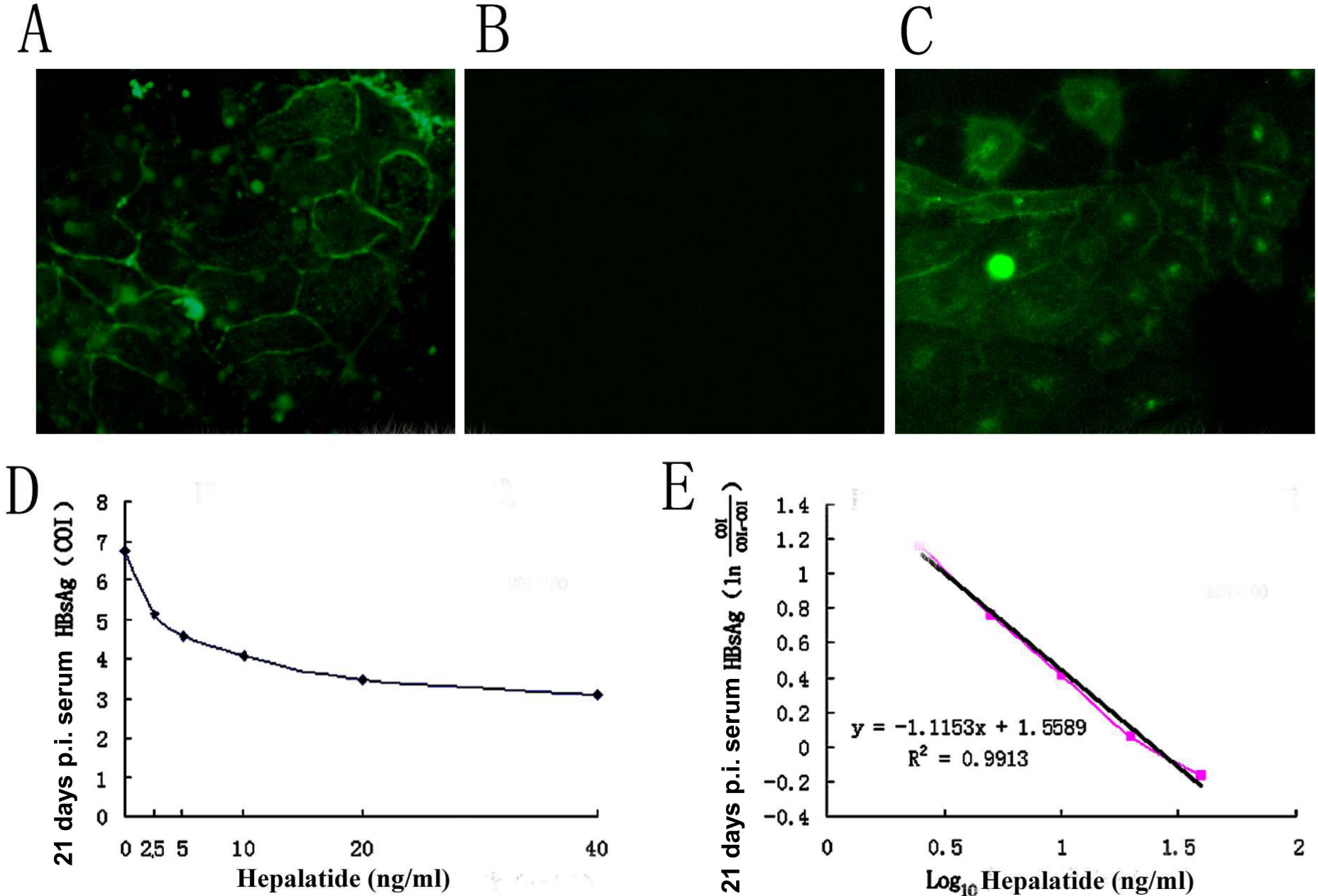
Liver samples were homogenized with 200 μL of homogenization buffer (50 mM Tris·HCl [pH 8], 1 mM EDTA, 0.2% Nonidet P-40, 0.15 M NaCl) in a Potter-Elvehjem tissue grinder. The cell nuclei in the homogenate was lysed in 200 μL lysis buffer (6% [vol/vol] SDS/0.1 M NaOH) followed by incubation at 37°C for 30 min and occasional mixing. The alkaline lysate was neutralized by 100 μL neutralization buffer (3 M KAc [pH 4.8]), followed by centrifugation at 12,000 × *g* for 15 min at 4°C. The nucleic acids in the supernatant were extracted with 600 μL water-saturated phenol (pH 4.5) and chloroform, and then precipitated by isopropanol in the presence of 10 μg glycogen. The precipitated protein-free nucleic acids were dissolved in 50 μL TE (10 mM Tris·HCl [pH 7.5]/1 mM EDTA) and stored at -70 °C for HBV cccDNA analysis.

cccDNA detection

cccDNA was amplified with RCA using a phi29 phage DNA polymerase (New England BioLabs, Ipswich, MA); this method was specific for cccDNA, as the virion-derived relaxed circular HBV DNA could not be amplified by this method. After digestion with the SpeI restriction enzyme (New England BioLabs), the RCA products were probed with a digoxin-labeled HBV-specific probe as previously described (28). Furthermore, the cccDNA content was determined using a commercially available real-time PCR kit (Shanghai Fosun Biology High Technology Ltd.). The selective primers for cccDNA amplification (sense: nucleotides 1540–1570; antisense: nucleotides 1910–1870) and FAM-labeled probe (nucleotides 1540–1580) were positioned at the Pre-C region, which spanned the

single-stranded gap of relaxed circular HBV DNA. To enhance the specificity, DNA samples were digested with plasmid-safe DNase to remove HBV relaxed genomic DNA from the cccDNA. In this real-time PCR system, the cccDNA could be specifically amplified while avoiding false negative reactions in the presence of HBV DNA at no more than 10^7 copies/mL. The detection limit was 20 copies/mg protein-free DNA.

Supplementary Fig. 1. The Pre-S1-derived HBV entry inhibitor, hepalatide, bound to the HBV receptor NTCP and blocked HBV infection of primary tupaia hepatocytes *in vitro*. (A) FITC-labeled hepalatide was inoculated with cultured primary tupaia hepatocytes for 10 min at a concentration of 100 μg/mL, followed by washing 3 times with PBS. The cells were then observed under a fluorescence microscope at 490 nm. To confirm whether hepalatide could bind human NTCP, the functional receptor for HBV infection, FITC-labeled hepalatide was inoculated with the LO2 liver cell line stably transfected with vector plasmid (B) or a human NTCP-encoding plasmid (C). To test the inhibitory effect on HBV infection by hepalatide, cultured primary tupaia hepatocytes were inoculated with HBV purified from CHB patients at 100 copies of genomic HBV DNA per cell; simultaneously, hepalatide was administered at final concentrations of 0, 2.5, 5, 10, 20, and 40 ng/mL. Inoculum-containing supernatant was removed 12 h after inoculation, and the cells were washed 3 times with PBS. The culture was continued with medium refreshed every 3 days. HBsAg secreted into the culture medium on day 9 p.i. was quantitatively analyzed by ECLIA (D). A COI greater than 1 was considered positive. Because of the competitive relationship between HBV and the entry inhibitor in terms of binding to the same HBV receptor, NTCP, the association between the HBV infection parameter of HBsAg and inhibitor concentration was regressed into a linear equation after logit-log transformation (E). The calculated 50% inhibitory concentration (IC50) was 8.5 ng/mL (equivalent to 1.6 nM).


A
B
C
D
21 days p.i. serum HBsAg (COI)
8
7
6
5
4
3
2
1
0
0 2.5 5 10 20 40
Hepalatide (ng/ml)
E
21 days p.i. serum HBsAg (ln COI/(COI-COI))
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
y = -1.1153x + 1.5589
R² = 0.9913
0 0.5 1 1.5 2
Log10 Hepalatide (ng/ml)

Supplementary Table 1. HBsAg, HBeAg, and HBV DNA concentration in the serum of each of the 10 HBV-inoculated tupaias before (b.i) and after (p.i) inoculation

Animal	HBsAg (cut-off index)					HBeAg (cut-off index)					HBV DNA (copies/ml)				
(No.)	day -4	day 9	day 15	day 21	day 42	day -4	day 9	day 15	day 21	day 42	day -4	day 9	day 15	day 21	day 42
1	0.933	0.781	0.593	0.834	0.327	0.109	0.124	0.128	0.138	0.149	ND	ND	ND	ND	ND
2	0.93	8.89	1.1	0.968	0.476	0.115	9.06	1.6	0.171	0.629	ND	438	466	ND	ND
3	0.879	21.98	1.68	0.902	0.465	0.221	22.15	11.65	0.204	0.184	ND	15000	6730	ND	ND
4	0.969	25.39	1.3	0.412	0.786	0.114	5.9	0.583	0.232	0.391	ND	436	460	ND	ND
5	0.783	1.7	0.661	0.923	0.526	0.116	22.42	0.508	0.135	0.425	ND	570	345	97	38
6	0.729	6.96	1.48	0.625	0.59	0.122	43.68	2.76	0.567	0.143	ND	444	278	1020	ND
7	0.562	213.5	21.49	11.87	0.573	0.102	26.18	21.76	16.92	0.204	ND	14000	18400	1420	ND
8	0.414	3.66	1.27	1.92	0.247	0.122	9.02	1.82	0.416	0.264	ND	1600	1400	300	ND
9	0.547	1.88	0.951	0.574	0.217	0.119	4.93	0.769	0.283	0.443	ND	1520	1900	ND	ND
10	0.628	1.24	3.9	0.472	0.21	0.109	12.99	4.98	0.338	0.401	ND	ND	ND	52	ND

ND: non-detectable; □No infection; □Super infection; □Routine infection

Supplementary Table 2. Intrahepatic cccDNA levels detected in liver biopsy from a single tupaia by real-time PCR (copies/mg protein-free DNA).

Test	Days b.i.	Days p.i.			
	4	9	15	21	42
1	0	2,268	739	68	5
2	0	2,357	774	59	3
3	0	2,410	759	59	4
Mean	0	2,345	757	62	4

Supplementary Table 3. ALT activity in serum from each of the 10 HBV-inoculated tupaias before (b.i.) and after (p.i.) inoculation.

Animal	Day 4 b.i.		Day 21 p.i.	
(No.)	IU/mL	×NL*	IU/mL	×NL*
1	65	1.1	311	5.5
2	48	0.8	60	1.1
3	11	0.2	31	0.5
4	88	1.5	360	6.3
5	49	0.9	72	1.3
6	49	0.9	95	1.7
7	28	0.5	170	3.0
8	121	2.1	214	3.8
9	68	1.2	106	1.9
10	101	1.8	194	3.4
Mean ± SD	62.8 ± 33.3	1.1 ± 0.6	161.3 ± 109.8	2.9 ± 1.9
P^{**}	0.009			

*Normal Level (NL) = 56.8 IU/ml; **By paired-sample *t*-test using SPSS 16.0 software.