Supplementary Material

Supplementary materials and methods

These methods have been described previously by Berke et al. (1), and are described briefly below.

Size exclusion chromatography and electron microscopy studies. Test compound was incubated with a mixture of Cp149 protein, storage buffer and NaCl. Aliquots were injected onto a size exclusion column for separation. Compound-induced capsid formation was assessed by calculating the ratio of the area under the curve (280 nm) of the dimer and capsid fractions. Aliquots were also negatively stained, and hepatitis B virus (HBV) capsid particles were analyzed by transmission electron microscopy according to Mast & Demeestere (2).

Antiviral assay with HepG2.117 and HepG2.2.15 cells and cytotoxicity assay (HepG2). JNJ-6739, at several concentrations, was added to cultured HepG2.117 cells (kindly provided by Dr M. Nassal, University Hospital Freiburg, Germany), and HepG2.2.15 cells (kindly provided by Dr. B. Korba, Georgetown University, USA). On day 6, HBV DNA was extracted from the cell culture supernatant and quantified by a quantitative polymerase chain reaction (qPCR) assay as described by Berke et al. (1). Percent inhibition (50% effective concentration [EC_{50}] and 90% effective concentration [EC_{90}] values) was calculated using the difference in threshold cycle number between treated and control wells. Cultured HepG2 cells were incubated for 4 days with several concentrations of JNJ-6379. Cytotoxicity was evaluated by resazurin read-out, and 50% cytotoxic concentration CC_{50} and 90% cytotoxic concentration (CC_{90}) were calculated. Selectivity index was defined as the ratio of the mean CC_{50} or CC_{90} value of JNJ-6379 in HepG2 cells to the mean EC_{50} value of JNJ-6379 assessed in HepG2.117 cells.

Antiviral studies in HBV-infected primary human hepatocytes (PHHs). Freshly thawed cryopreserved PHHs (Life Technologies or In vitro ADMET Laboratories) were

infected with cell-derived HBV (1). Stable HBV replicating HepG2.2.15 cells (provided by Dr Brent Korba, Georgetown University, Washington, DC, USA) were cultured until confluency in collagen-coated flasks. The medium was then replaced by virus production medium and the supernatant was replaced every 7–10 days for virus harvest. The virus was concentrated from the supernatants (Centricon Pus 70 Filter device, as per manufacturer's instructions), and genome equivalents/mL were determined using quantitative polymerase chain reaction (qPCR). Briefly, PHHs were seeded (80,000 cells/well) in culture medium for 24 h, then infected overnight with 5,000 genome equivalents of the cell-derived HBV per cell in PHH culture medium (1).

JNJ-6379, tenofovir disoproxil (TDF), or entecavir (ETV), (in dimethyl sulphoxide [DMSO]) were added to the PHHs either at the same time as the viral inoculum (condition 1) or on day 4 or 5 post infection (condition 2). Culture medium (which contained 4% polyethylene glycol 8000 as well as other additives as described in [1]) was replenished every 3–4 days. On day 11 (condition 1) or day 15 (condition 2) the supernatants were collected and stored frozen. Cells were removed from the culture wells and lysed with RLT buffer (Qiagen RNeasy 96 kit) with 1% β-mercapto-ethanol.

Extracellular HBV DNA levels were assessed by qPCR. HBV DNA was extracted from the cell culture supernatant of PHHs using the MagNA Pure 96 DNA and Viral NA Small Volume Kit according to the manufacturer's instructions. The primers used are described by Berke et al. (1). For the PCR, samples were denatured by heat, followed by 40 cycles of amplification. HBV DNA levels were quantified using a standard curve of a plasmid containing full genome HBV. Differences in HBV DNA levels between compound treated and DMSO controls were calculated. Results were expressed as EC_{50} and EC_{90} values based on the relative HBV DNA quantification.

Total intracellular HBV RNA in PHH cell cultures was quantified by extracting RNA from cell lysates of treated PHHs and complementary DNA was prepared and amplified. A standard curve of in-vitro transcribed HBV RNA in a background of HepG2 RNA was constructed, and used to calculate the number of copies of HBV RNA, which was then normalized by the glyceraldehyde 3-phosphate dehydrogenase concentration in the sample.

Quantification of extracellular hepatitis B e antigen and hepatitis B core-related antigens (HBe/c Ag) and hepatitis B surface antigen (HBsAg) in PHH cell culture supernatant was achieved with an in-house modified Amplified Luminescent Proximity Homogeneous Assay (AlphaLISA, Perkin Elmer). Biotinylated anti-HBe/cAg (Acris) and anti-HBsAg (in house), bound to streptavidin donor beads. A mixture of biotinylated antibodies and acceptor beads conjugated with the relevant antibody were added to supernatants from treated PHHs, followed by streptavidin donor beads. Plates were evaluated (Envision, Perkin Elmer); excitation: 650 nm; emission: 615 nm. Standard curves were constructed using recombinant HBeAg (Jena Bioscience) and HBsAg (in house).

Human albumin was quantified using an ELISA quantitation set (Bethyl Laboratories) as per the manufacturer's instructions (excluding the blocking step).

Immunofluorescence analysis of HBsAg was conducted in PHHs. Fixed and permeabilized PHHs were incubated with the primary anti-HBsAg antibody. Washed cells were then incubated with Alexa Fluor 568 Goat Anti-Human IgG (H+L) Antibody (Invitrogen). Total cell numbers were assessed using counterstains for nucleic acid (Hoechst 33258) and cells (CellMask) (Life Technologies). Washed plates were analyzed on an Opera Phenix platform (Perkin Elmer). Percentage of infected PHHs were determined with automated image analysis.

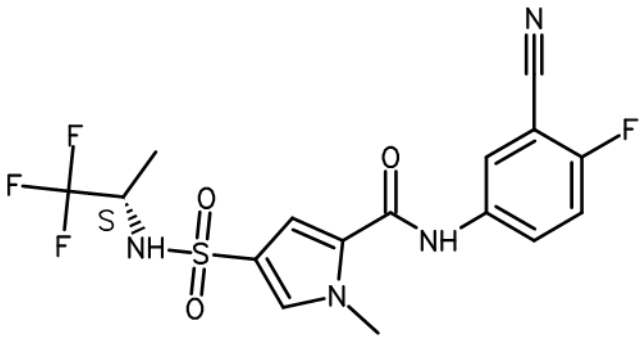
Hirt-extracted DNA from HBV-infected PHHs and Southern Blotting. Extraction of covalently closed circular DNA was conducted by the Hirt method (3, 4). In brief, covalently

closed circular (cccDNA) extracts were resolved on an agarose gel (5). DNA was transferred from the gel to a nylon membrane overnight, after UV crosslinking, hybridization was carried out (EasyHyb ready-to-use buffer with a DIG-labeled HBV probe). The DIG-labeled probe was detected using a DIG luminescent detection kit (Roche) according to the manufacturer's instructions. Visualization of hybridization signals was achieved with a chemiluminescent substrate, and the light signal produced was captured with an ImageQuant LAS 4000.

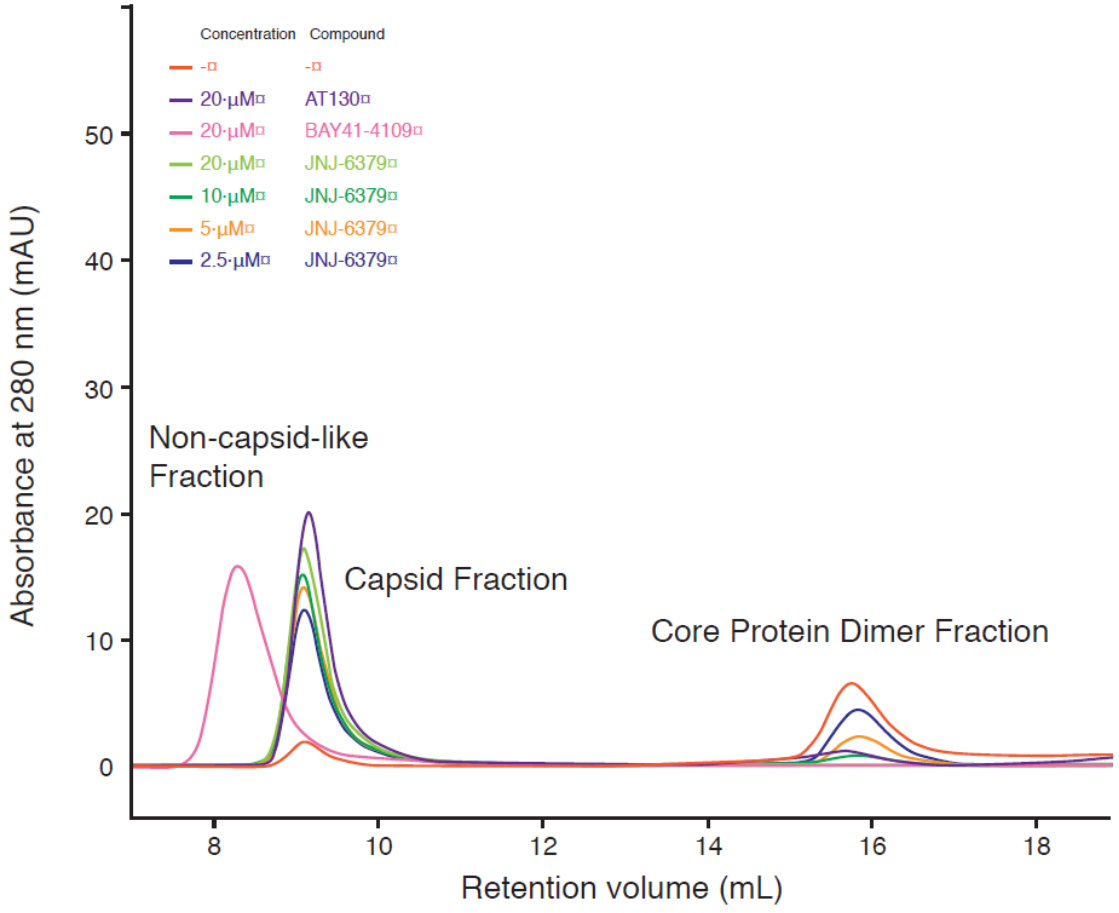
REFERENCES

1. Berke JM, Dehertogh P, Vergauwen K, Van Damme E, Mostmans W, Vandyck K, Pauwels F. 2017. Capsid assembly modulators have a dual mechanism of action in primary human hepatocytes infected with hepatitis B virus. Antimicrob Agents Chemother 61. pii:e00560–17.
2. Mast J, Demeestere L. 2009. Electron tomography of negatively stained complex viruses: application in their diagnosis. Diagnostic Pathology 4:5.
3. Hirt B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J Mol Biol 26:365–369.
4. Zhou T, Guo H, Guo JT, Cuconati A, Mehta A, Block TM. 2006. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. Antiviral Res 72:116–124.
5. Cai D, Nie H, Yan R, Guo JT, Block TM, Guo H. 2013. A southern blot assay for detection of hepatitis B virus covalently closed circular DNA from cell cultures. Methods Mol Biol 1030:151–161.

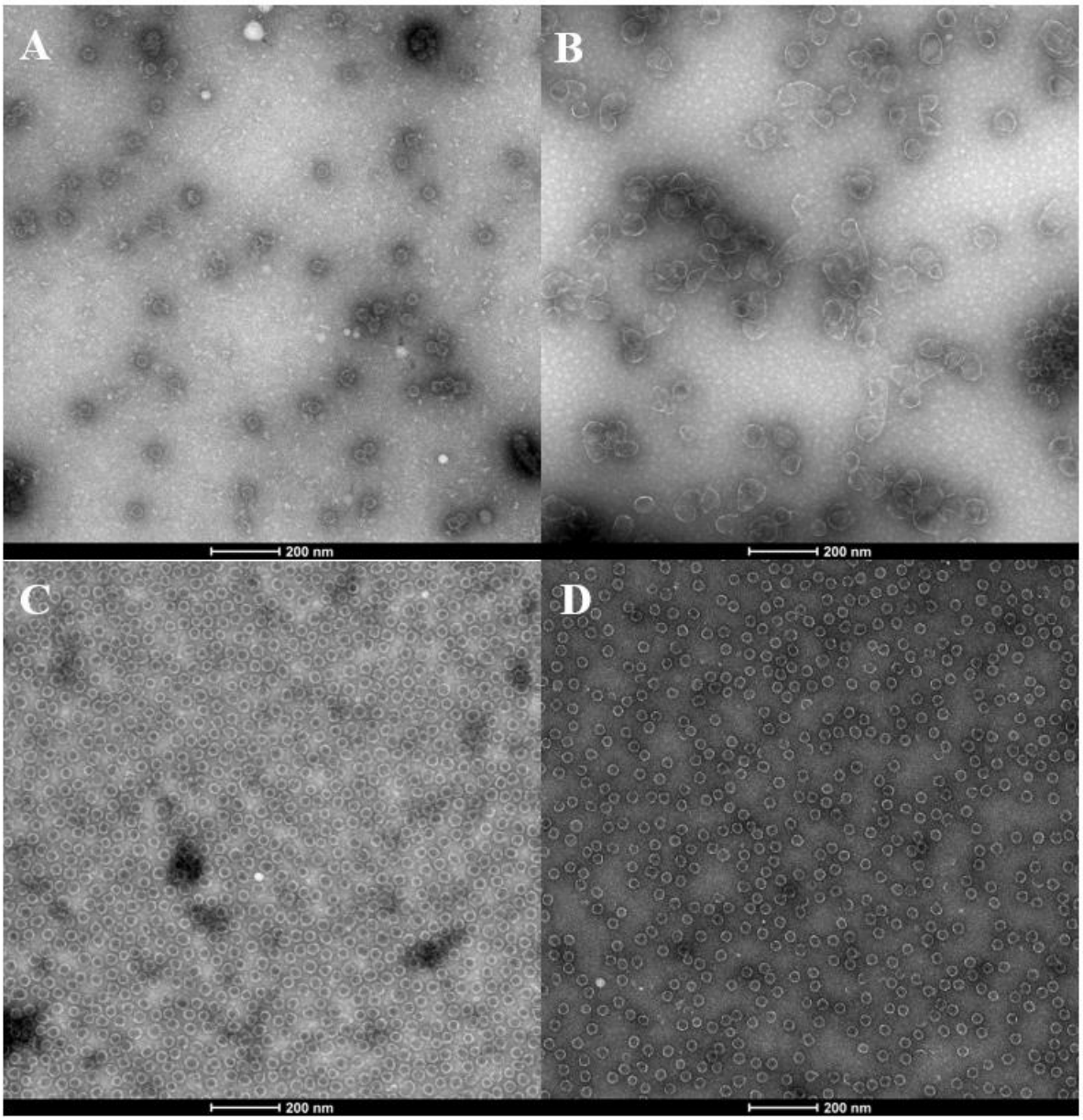
Supplementary Fig. S1 Structure of JNJ-6379.



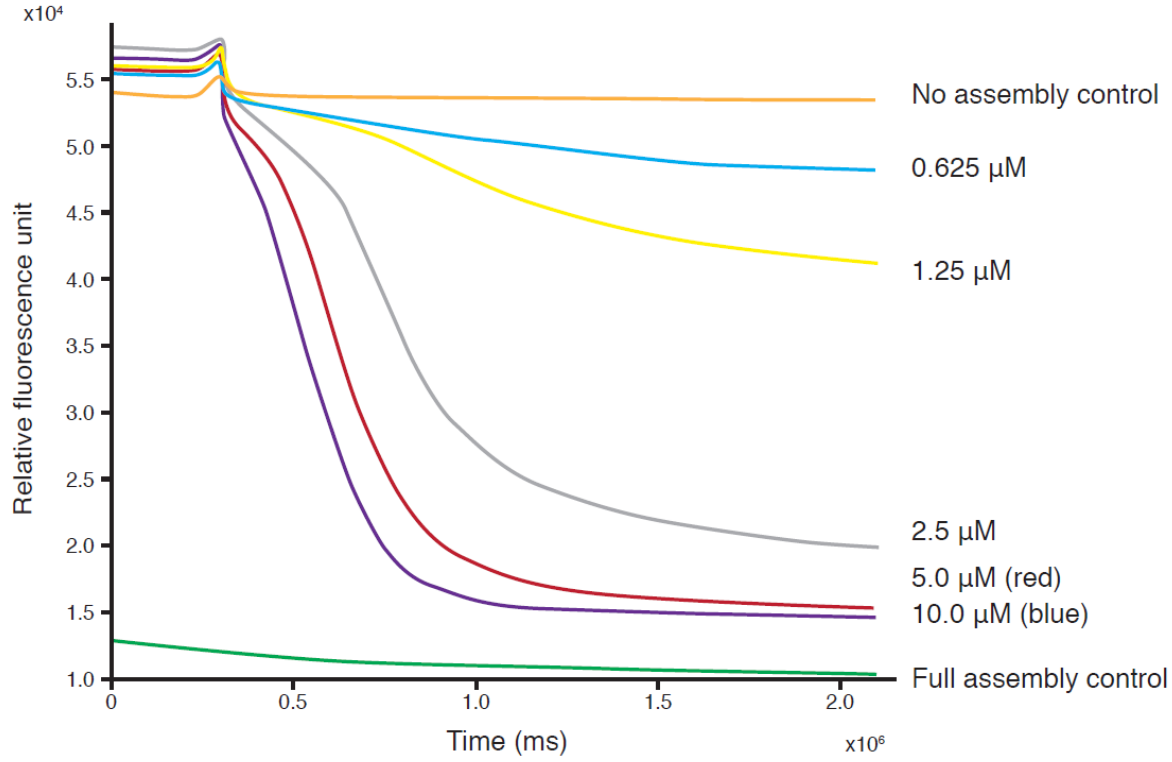
Supplementary Fig. S2 Biochemical mechanism-of-action study using size-exclusion chromatography. Compounds were incubated with the recombinant assembly domain (amino acids 1–149) of HBV core for 24 h in the presence of 150 mM NaCl and separated using size exclusion chromatography. The elution profiles (of capsid and core protein dimer) were quantified based on the absorbance at 280 nM. The red line on the figure is a no assembly control (no compound added).



Supplementary Fig. S3 Biochemical MOA study using electron microscopy. HBV core protein dimers were incubated with 150 mM NaCl A) without compound; B) 20 µM BAY41-4109; C) 20 µM JNJ-6379; D) 30 µM AT-130.

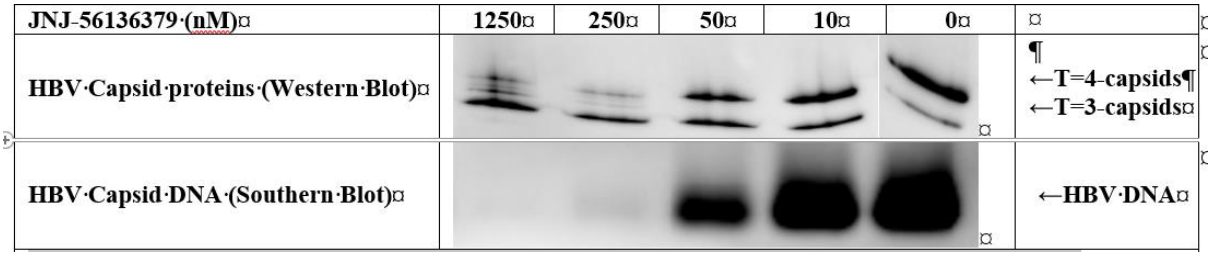


Supplementary Fig. S4 Effect of JNJ-6379 on HBV capsid assembly kinetics *in vitro.*



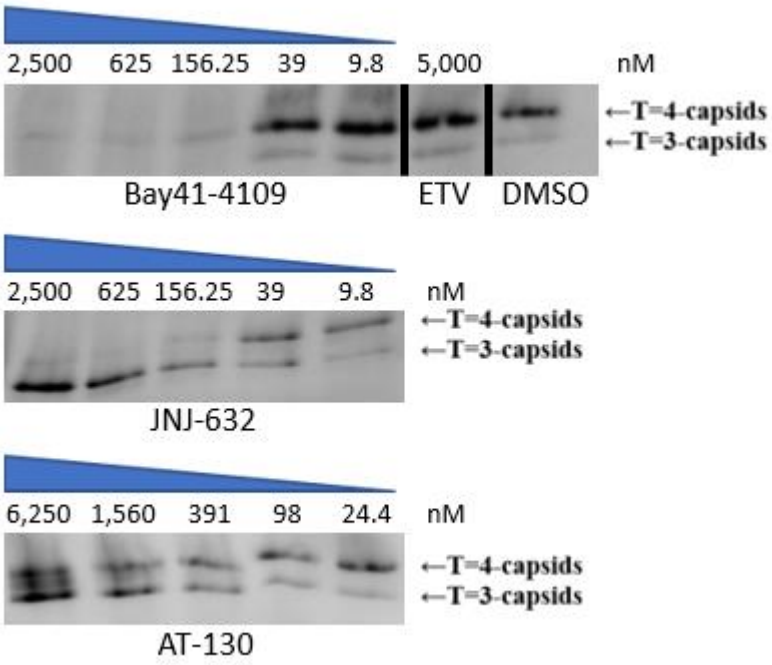
Emission for fluorescence was measured in 10 sec intervals after induction of capsid assembly over a 30 min time period at 540 nm. After a 5-min pre-incubation of labeled HBV core protein dimers in the presence of JNJ-6379, capsid assembly was induced by addition of NaCl to a final concentration of 200 mM. Controls were full assembly control (dimers in presence of 1 M NaCl without JNJ-6379) and no assembly control (dimers without NaCl and without JNJ-6379). The graph shows a representative experiment.

Supplementary Fig. S5 Effect of JNJ-6379 on intracellular HBV capsids and capsid-associated DNA.



JNJ-6379 was added to HBV-replicating HepG2.117 cells at the concentrations shown. After 4 days of incubation, cells were lysed followed by native PAGE and western-blot detection of HBV capsids. HBV capsid-associated DNA was detected by Southern blot.

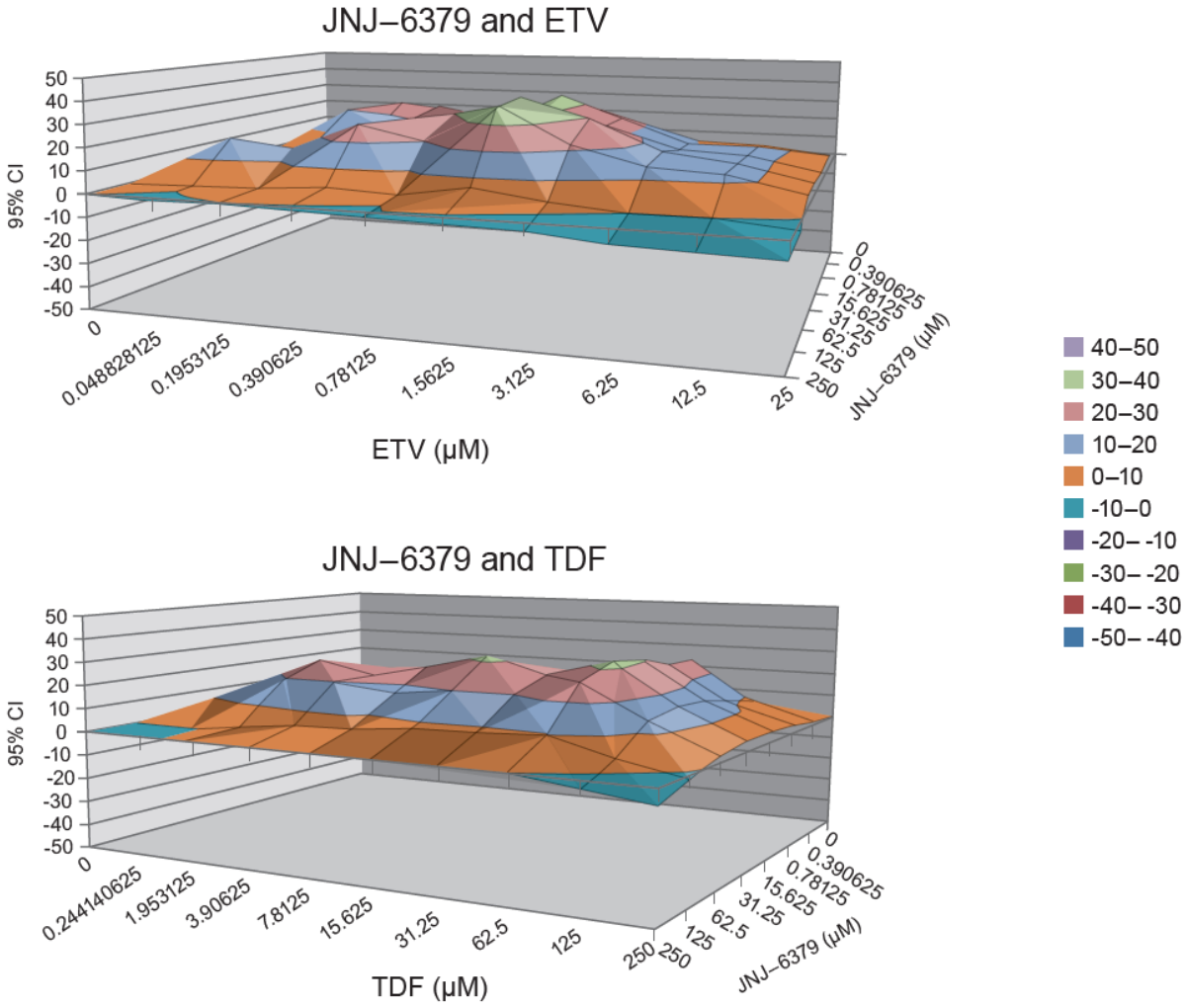
Supplementary Fig. S6 Effect of HBV reference compounds on HBV capsid formation in HepG2.117 cells.



Stable HBV-replicating HepG2.117 cells were incubated in the presence of different concentrations of the CAMs BAY41-4109, JNJ-632 and AT-130 and the NA ETV. After 4 days of incubation, cells were lysed and viral particles were separated on a native PAGE and a Western Blot was performed to detect HBV capsids.

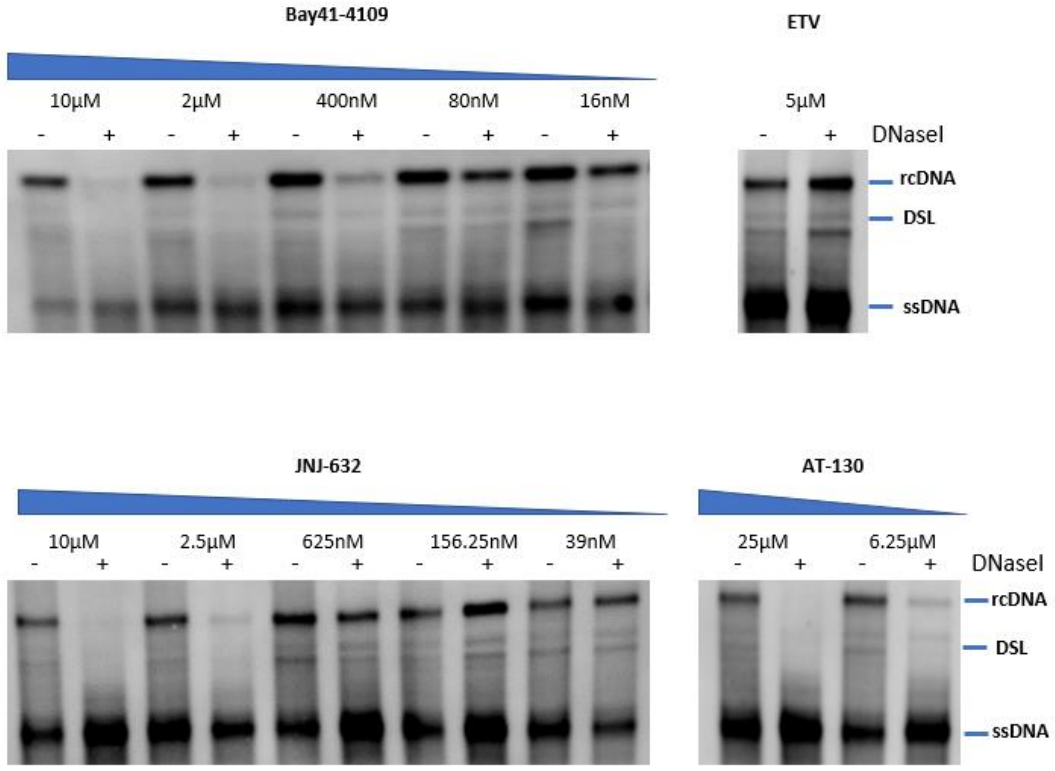
CAM, capsid assembly modulator; DMSO, dimethyl sulfoxide; ETV, entecavir; HBV, hepatitis B virus; NA, nucleos(t)ide analogue; PAGE, polyacrylamide gel electrophoresis.

Supplementary Fig. S7 Representative synergy plots of the combination of JNJ-6379 with ETV and TDF. CI, confidence interval; ETV, entecavir; TDF, tenofovir disoproxil.



One day after seeding HBV-expressing HepG2.2.15 cells, the JNJ-6379 + NA combinations were added at the concentration ranges shown. After three days, the culture medium with test compounds was refreshed and the cells were incubated for another three days. DNA was extracted from 150 µL of supernatant from each well, and detected by quantitative real time polymerase chain reaction. The percentage inhibition for each combination was calculated by averaging values of 3–5 wells/experiment. Synergy plot calculations were based on the lower limit values of the 95% CI.

Supplementary Fig. S8 Effect of BAY41-4109, JNJ-632, and AT-130 on pre-formed mature capsids.



HBV-replicating HepG2.117 cells were cultured for 3 days without doxycycline and on day 4, cells were treated with the appropriate compound at the concentrations shown f for 30 min, in the presence of doxycycline. Cell lysates were incubated with or without DNase I. HBV DNA was assessed by Southern blotting. ETV, entecavir; rcDNA, relaxed circular DNA; ssDNA, single stranded DNA; DSL DNA, double stranded linear DNA.

Supplementary Table S1 Antiviral properties of JNJ-6379 against representative RNA and DNA viruses

Virus class and type	**Family**	**Genome**	**Strain**	**Cell line**	**Median EC_{50} nM**	**Median CC_{50} nM**	**n**
Positive-sense single stranded RNA viruses							
Dengue virus	Flaviviridae	ssRNA(+)	Serotype 2	Vero (ECACC)	21,900	49,000	2
HCV	Flaviviridae	ssRNA(+)	1b	Huh7	71.400	>25,000	2
Human rhinovirus	Picornaviridae	ssRNA(+)	1B, 14, 16	HelaRH	>100,000	77,100	3
Coxsackievirus	Picornaviridae	ssRNA(+)	B4	Vero	>100,000	61,200	1
HIV	Retroviridae	ssRNA(+)	IIIB	MT4-CMV-eGFP	>100,000	47,800	1
Negative-sense single stranded RNA viruses							
Respiratory syncytial virus	Paramyxoviridae	ssRNA(–)	Long strain	Hela	19,100	67,700	1
Parainfluenza virus	Paramyxoviridae	ssRNA(–)	hPIV3	A549	46,900	>100,000	1
Influenza A	Orthomyxoviridae	ssRNA(–)	A/Taiwan/1/86	MDCK	>100,000	>100,000	1
Influenza B	Orthomyxoviridae	ssRNA(–)	B/Singapore/222/79	MDCK	>100,000	>100,000	1
DNA virus							
Cytomegalovirus	Herpesviridae	dsDNA	AD169	Hel299	32,900	>100,000	1

CC_{50}, 50% cytotoxic concentration; dsDNA: double stranded deoxynucleic acid; EC_{50}, 50% effective concentration; HCV, hepatitis C virus; HIV, human immunodeficiency virus; n, number of experiments; ssRNA, single stranded ribonucleic acid.

Suppliers of the viruses: Dengue virus and HCV: University of Heidelberg, Germany; human rhinovirus: American Type Culture Collection, Manassas, VA, USA; coxsackievirus: LGC Promochem, Teddington, UK; HIV: Institute of Tropical Medicine, Antwerp, Belgium; respiratory syncytial virus: National Institutes of Health, Bethesda, MD, USA; parainfluenza virus: Institute for Antiviral Research Department, Utah State University, UT, USA; influenza A: University of Virginia, School of Medicine, Charlottesville, USA; influenza B: MRC National Institute for Medical Research, London, UK; Cytomegalovirus: Institute of Clinical and Molecular Virology, Erlangen, Germany.

Suppliers of cell lines: Vero (ECACC): European Collection of Cell Cultures; Huh7-Luc: Universitätsklinikum Heidelberg; HeLa RH: in-house; Vero Bayer cells: Institute of Tropical Medicine, Antwerp, Belgium; MT4-CMV-EGFP cells were generated by stably transforming MT4 cells from National Institute of Infectious Diseases, AIDS Research Center, Tokyo, Japan; HeLa: ATCC; A549: Tibotec, Mechelen, Belgium; MDCK: ATCC.

Supplementary Table S2 Antiviral properties (EC_{90}) in HBV-infected PHHs

Day and compound	Median (range) EC_{90} (nM), n			
	Extracellular HBV DNA	**Total intracellular HBV RNA**	**Extracellular HBe/cAg**	**Extracellular HBsAg**
Day 0				
JNJ-6379	378 (304–556) 8	4,019 (1,917–4,867) 6	1,118 (2,576–4,617) 8	5,768 (4,079–7,478) 6
TDF	<8.0 (ND) 1	>5,000 (ND) 1	>5,000 (ND) 1	>5,000 (ND) 1
ETV	0.18 (0.09–0.65) 4	>5,000 (>1.0–>5,000) 17	>5,000 (>1.0–>5000) 19	>5,000 (>1.0–>5,000) 22
Day 4/5				
JNJ-6379	376 (247–422) 8	>25,000 (>5,000–>25,000) 8	>25,000 (>5,000–>25,000) 8	>25,000 (>5,000–>25,000) 8
TDF	<8.0 (ND) 1	>5,000 (ND) 1	>5,000 (ND) 1	>5,000 (ND) 1
ETV	0.19 (0.06–0.80) 6	>5,000 (>1.0–>5,000) 23	>5,000 (>1.0–>5,000) 23	>5,000 (>1.0–>5,000) 23

Stable HBV-replicating HepG2.2.15 cells were the source of HBV for the infection of cryopreserved PHH. The antiviral properties of each compound were tested in a dose-response assay in HBV-infected PHHs. HBV DNA (extracellular) and HBV RNA (intracellular) levels were assessed using qPCR. HBe/cAg and HBsAg in the supernatant were evaluated by AlphaLISA.

HBV, hepatitis B virus; EC_{50}, 50% effective concentration; ETV, entecavir; HBe/cAg, hepatitis B e antigen and hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; n: number of experiments; ND, not determined; qPCR, quantitative polymerase chain reaction; TDF, tenofovir disoproxil.