Class A capsid assembly modulator RG7907 clears HBV-infected hepatocytes through core-dependent hepatocyte death and proliferation

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Contents

Supplementary Materials & methods

Compounds

All compounds used in this study were synthesized by WuXi AppTec (Shanghai, China). Compounds used in animal studies were of pharmacological grade and purity.

Cells and media

HepG2.117 cells were a generous gift from Prof. Michael Nassal (University of Freiburg, Germany) and HepAD38 cells we purchased from Fox Chase Cancer Center (Philadelphia, PA). HepG2.117 and HepAD38 cells were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM, 4.5 g/L D-Glucose, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM stable L-glutamine, 250 µg/mL G418, and 1 µg/mL doxycycline. For HepG2.117 cells, 80 µg/mL hygromycin was added. Cells were incubated at 37°C and 5% CO_2.

HepG2-NTCP cells overexpressing N-terminal HA-tagged HBV antigens were generated as follows. Lentiviral particles were generated by co-transfecting plasmids encoding the N-terminally HA-tagged HBV antigens (HBc or L-HBsAg, from HBV genotype D subtype ayw) with plasmids encoding HIV gag-pol and vesicular stomatitis virus glycoprotein (ratio of 10:10:3) in HEK 293T cells. HepG2-NTCP cells were then plated and transduced with lentivirus. After 3 days, transduced cells were selected with 6 µg/mL of blasticidin. After selection, cells were maintained in high glucose DMEM with 10% FBS, 2 mM stable L-glutamine, non-essential amino acids, gentamicin, 250 µg/mL G418, and 4 µg/mL blasticidin.

Antiviral assay with HBV DNA quantification

HepG2.117 cells were washed with Dulbecco's phosphate-buffered saline (D-PBS), trypsinized and passed through a cell strainer (VWR 21008-952). Cells were then centrifuged, the supernatant was discarded, and the cell pellet was resuspended in high glucose DMEM with 2% Tet-free FBS (Biowest S181T) and 2 mM L-glutamine. Next, 100 µL of cell suspension was seeded in 96-well plates corresponding to 20,000 cells per well. For cell viability assays, white transparent bottom 96-well plates (Corning 3903) were used while transparent 96-well Falcon plates (353072) were used for HBV DNA assays. Cells were incubated overnight. Thereafter,

medium was refreshed, and a serial dilution of compound was added using TECAN D300e digital dispenser later normalized to 2% DMSO. Plates were incubated at 37^{o}C and 5% CO_2 for 3 days.

Next, the medium was removed and cells were washed once with D-PBS and lysed by adding 100 µL of 0.33% 4-nonylphenyl-polyethylene glycol (NP40 substitute, Sigma Aldrich 74385). Plates were incubated at 4^{o}C for 5 min and vortexed vigorously. For DNA extraction, 25 µL of the cell extract was mixed with 46 µL of DNA Quick Extract solution (Lucigen Epicenter QE09050) in a 96-well PCR plate and incubated at 65°C for 6 min followed by 2 min at 98^{o}C. Then, 4 µL of extract was mixed with 16 µL of SsoAdvanced Universal Probes qPCR mix (BioRad) containing 0.4 µM of HBV-Fwd and HBV-Rev primers (5'-GTGTCTGCGGCGTTTTATCA-3' and 5'-GACAAACGGGCAACATACCTT-3', respectively) and 0.2 µM of probe (5'-/56-FAM/-CCTCTKCAT-/ZEN/-CCTGCTGCTATGCCTCATC-/3IABkFQ/3') (IDT, Leuven, Belgium).

qPCR was performed using Bio-Rad CFX96 using the following time-temperature schedule: initiation at 95^{o}C for 3 min followed by 40 cycles of 15" at 95^{o}C and 1 min at 60^{o}C. Data were analyzed using Dotmatics software to determine EC_{50}/EC_{90} values.

Antiviral log drop assay

This assay is a modification of the antiviral assay which includes the addition of a Turbo DNAse step to degrade non-encapsidated HBV DNA. Here, 90 µL of cell extract was mixed with 10 µL of Turbo DNAse buffer and 1 unit of Turbo DNAse (ThermoFisher) and incubated at 37^{o}C for 30 min. Afterwards, another unit of the Turbo DNAse was added to ensure maximum degradation of non-encapsidated DNA for 30 min prior to heat inactivation at 95^{o}C for 5 min. DNA extraction and qPCR were performed as described above.

Cell viability assay

After treatment of cells as described in the antiviral assay section above, cell viability was assessed using CellTiter-Glo 2.0 assay (Promega) 3 days after compound addition. Plates were first placed at room temperature for 30 min. Then, 100 µL of CellTiter-Glo 2.0 reagent was added to each well of a 96-well plate containing 100 µL of culture medium. Plates were

incubated at room temperature with gentle agitation for 10 min before luminescence readout using the Varioskan Lux plate reader (ThermoFisher).

Electron microscopy

The recombinant HBc assembly domain used in this study has an additional C-terminal cysteine and has all its native cysteines replaced by alanine (Cp150).[1(p201)] Cp150 was expressed in Escherichia coli BL21-DE3 strain and purified as previously described.[2] Negative stain transmission EM experiments were performed at Novalix (Strasbourg, France). For each assay condition, 47 µL of Cp150 protein (purified capsid protein) at 43 µM was diluted with 198 µL protein storage buffer and 250 µL NaCl a 150 mM, prior to addition of 5 µL of compound at 2.5 mM (100×) in 100% DMSO. This results in a final volume of 500 µL with Cp150 at 4 µM and 1% DMSO. Negative staining electron microscopy was performed as described elsewhere.[3]

Immunofluorescent HBc spotting assay

HepG2.117 cells were trypsinized and resuspended in DMEM high glucose with 2% Tet-free FBS and 2 mM L-glutamine. Ten thousand (10,000) cells per well were seeded in 96-well black poly-D-lysine coated plates with clear bottom (Perkin Elmer 6055500) in the absence of doxycycline. After 2 days, the medium was refreshed and compounds were added using the Tecan D300e digital dispenser and normalized to 2% DMSO. Plates were incubated at 37°C and 5% CO_2 for another 5 days. Thereafter, cells were fixed with 5% methanol-free formaldehyde diluted in D-PBS for 15 min and washed three times with D-PBS. Later, cells were permeabilized for 30 min using Triton X-100 (0.5% in D-PBS) and washed again 3 times with D-PBS. Next, cells were covered with blocking buffer containing 5% goat serum (Biowest S2000) in D-PBS for 1h at room temperature. Blocking buffer was removed and cells were incubated with mouse monoclonal anti-HBc antibody (Abcam ab8637) diluted 1:1500 in blocking buffer and incubated overnight at 4°C. Thereafter, cells were washed 3 times with D-PBS and incubated in the dark for 1h with (i) goat anti-mouse AlexaFluor 488 secondary antibody diluted 1:1500 in blocking buffer (ii) Hoechst 33258 (Biotium 40044) diluted 1:5000 and (iii) HCS CellMask Deep Red (ThermoFisher H32721) diluted 1:5000. Cells were washed 3 times with D-PBS and imaged using the Operetta CLS High Content Analysis System (Perkin Elmer, 40x water, room temperature). Subsequent

image analysis was performed with the integrated Operetta Harmony software by determining total HBc spot area per field and normalizing this for the total number of nuclei in that same field, including nuclei with no obvious spotting. The percentage spotting was then calculated relative to the normalized HBc spot area obtained for RG7907 at 10 µM, and absolute EC_{50} values were calculated. For co-staining of nucleoli, primary rabbit monoclonal anti-nucleolin antibody EPR7952 (Abcam ab129200) was used at a 1:100 dilution and goat anti-rabbit Alexa 546 secondary antibody was used at a 1:5000 dilution.

Western blot

HepG2.117 cells were seeded in a 6-well plate at $1x10^6$ cells per well in high glucose DMEM with 2% Tet-free FBS and 2 mM L-glutamine. After 24h, the medium was refreshed and compound was added at a fixed concentration normalized to 2% DMSO. After 3 days, medium and compound were refreshed. Another 3 days later, supernatant was removed and cells were washed once with ice-cold D-PBS and lysed with RIPA extraction buffer (ThermoFisher 89900) supplemented with 1x Halt protease inhibitor cocktail (ThermoFisher 87785). Cell lysates were vortexed for 30" and afterwards centrifuged at 12,000 rpm for 15 min at 4°C. Protein concentration of the extracts was determined using the Pierce BCA protein assay kit (ThermoFisher 23227). Next, 10-20 µL of protein corresponding to 20 µg was mixed with Bolt LDS Sample Buffer (Invitrogen B0007) and Bolt Sample Reducing Agent (Invitrogen B0004); heat inactivated at 70°C for 10 min and loaded onto a 4-12% Bolt Bis-Tris gel (Novex Life Technologies NW04120BOX) and run at 220 volt for 22 min prior to transfer onto nitrocellulose membrane (Invitrogen IB23001) at constant voltage of 20 - 25V for 7 min with the iBlot 2 Dry Blotting System device. After transfer, the nitrocellulose membrane was washed once with Tris-Buffered Saline (TBS) for 5 min at room temperature and blocked with 5% milk in TBS with 0.1% Tween-20 (TBST) blocking buffer at room temperature for 1h. The membrane was washed three times for 5 min each with TBST and incubated with polyclonal anti-HBc primary antibody (Abcam AB115992) diluted 1:500 in blocking buffer with gentle agitation overnight at 4°C. Then the membrane was washed three times for 5 min each with TBST and incubated with goat anti-rabbit IgG HRP-conjugated (G21234) diluted 1:10,000 in blocking buffer with gentle agitation at room temperature for 1h. Next, the membrane was washed 3 times for 5 min each with TBST

and incubated with ECL Western Blotting Detection Reagents (Pierce 32106) with gentle agitation at room temperature for 1 min. Image acquisition was performed on the VWR Imager Chemi Premium. Thereafter, membrane was washed once with TBST and incubated with anti-beta Actin antibody diluted 1:1,000 in blocking buffer prior to incubation with goat anti-rabbit IgG HRP-conjugated diluted 1:10,000 in blocking buffer with gentle agitation at room temperature for 1h. Finally, the membrane was washed three times for 5 min each with TBST and incubated with ECL Western Blotting Detection Reagents (1:1 mixture) with gentle agitation at room temperature for 1 min prior to image acquisition.

HBV infection experiments in primary human hepatocytes

Virus stock used in primary human hepatocytes (PHH) experiments was produced in-house in HepAD38 cells as previously described.[4]

PHH were seeded in collagen-coated black clear-bottom 96-well plates at 80,000 cells per well in 100 µl CP medium with Torpedo antibiotics (BioIVT). Cells were left to attach overnight at 37°C with 5% CO_2. After overnight incubation, the cells were HBV-infected at a multiplicity of infection of 100 in medium containing 4% PEG, 2% DMSO and 10% FBS. After 24 hours of infection, the inoculum was removed and cell layers were washed three times with DMEM (without supplements). Fresh PHH medium (DMEM with 10% FBS, supplemented with HEPES, L-proline, insulin, epidermal growth factor, dexamethasone, and ascorbic acid-2-phosphate)[5] containing 2% DMSO was added to all plates. Plates were incubated at 37°C with 5% CO_2 for 4 days. On day 5 after infection, medium was removed and fresh medium with compound was added. Plates were incubated for another 3 days. On day 8 after infection, medium and compound was refreshed again and cells were incubated for another 4 days. On day 12 post infection, cells were washed three times with D-PBS, fixed, permeabilized, stained, and imaged as described in the *Immunofluorescent HBc spotting assay* section above. An additional masking step to reduce hepatocyte autofluorescence was introduced after permeabilization by incubating cells for 5' with a 1.65% Eriochrome Black T solution in H_2O, followed by 3 washes with D-PBS.

AAV-HBV mouse studies

All mouse studies were conducted at Labcorp Drug Development Pharmaceutical Research and Development (Shanghai, China). All animal procedures followed local animal welfare legislation, Labcorp global policies and procedures, ARRIVE guidelines, and the Guide for the Care and Use of Laboratory Animals (permit number SCXK (Hu) 2018-0003, animal health certificate number 20180003000524). Male 5-6-week-old C57BL/6 mice were received from Shanghai Lingchang Bio Tech (Shanghai, China). Mice were kept in polycarbonate cages with corncob bedding under controlled temperature (21 – 25°C), humidity (40 – 70%), and a 12-hour light / 12-hour dark cycle. Food and sterile water were provided *ad libitum*. Mice were injected intravenously with a 10^{11} AAV-HBV viral equivalents (BrainVTA, Wuhan, China). Compound treatment was initiated only after stabilization of viral titers (28 weeks after AAV-HBV injection for the main study). Mice were randomized based on viral titers (HBV DNA, HBsAg, HBeAg) and body weight. Only mice with sufficiently high viral titers were included (based on historical data). Animals were housed in groups of up to 5 per cage and orally administered 20 mg/kg of RG7907 every day for 70 days. RG7907 was formulated in 2% Klucel, 0.1% polysorbate 80 and 0.1% parabens in water. Animals were bled weekly by submandibular vein bleeding and euthanized on days 7, 14, 35, and 70 for serum and organ collection. There were 7 mice in the vehicle group: 3 sacrificed at day 7, the other 4 at day 70. There were 13 mice in the RG7907 group: 3 sacrificed at days 7, 14, 35 each, and 4 sacrificed at day 70. The same procedures were used for untransduced 9-week-old mice treated with vehicle or RG7907, with sacrifice of animals on day 35 (n = 3 mice per study arm).

Blood samples were collected and stored at -60°C to -80°C before ALT assay or viral marker quantification. Serum HBsAg and HBeAg were detected using ARCHITECT i2000 (Abbott Laboratories, Lake Bluff, IL) with supporting kits (HBsAg: 6C36-77; HBeAg: 6C32-77). Serum HBV DNA was analyzed with the QuantStudio 3 System (Applied Biosystems, Foster City, CA) with detection kit (Sansure Biotech Inc., Changsha, Hunan, China). Serum ALT was quantified using the Roche Cobas 6000 c501 Chemistry Analyzer (Roche Diagnostics, Mannheim, Germany) with supporting kit (4467388190).

Liver samples were obtained after perfusion with sterile normal saline and 100 mg was cut from the median into cubes of 1-2 mm and transferred into labelled tubes containing RNAlater solution. Tubes were then placed at 2-8^{o}C overnight and RNAlater was then removed prior to storage -60^{o}C to -80^{o}C before pgRNA analysis. Liver total RNA was extracted using RNA Easy Fast Tissue/Cell Kit (Tiangen Biotech, Beijing, China). Liver HBV pgRNA was measured using QuantStudio 3 with the HBV pgRNA Quantitative Determination Kit (Hotgen Biotech, Beijing, China).

Liver slices of 3-5 mm from the left lateral lobe and the right lateral lobe were collected for each animal and fixed with 10% neutral-buffered formalin. Two slices from different lobes were embedded in paraffin, and cut for HBsAg, HBcAg, and Ki-67 IHC staining, TUNEL staining, and scoring. Liver sections of 3 μm in thickness were cut by using Leica RM2245 (Leica Biosystems, Nussloch, Germany), and used for HBsAg, HBcAg and Ki67 IHC staining. IHC staining was performed by using the Bond RX automatic IHC&ISH system (Leica Microsystems, Nussloch, Germany) with mouse anti-HBcAg antibody (ab8639, Abcam, San Francisco, CA), rabbit anti-HBsAg antibody (20-HR20, Fitzgerald, Concord, MA), or rabbit anti-mouse Ki67 antibody (ab16667, Abcam San, Francisco, CA). BOND Polymer Refine Detection kit (DS9800, Leica Microsystems) was used to detect the antibody staining. ApopTag Fluorescein In Situ Apoptosis Detection Kit (3738620) for TUNEL staining was purchased from Sigma-Aldrich. All the stained slides were scanned using NanoZoomer Digital Slide System NDP2.0-HT from (Hamamatsu Photonics, Hamamatsu, Japan). The images of stained slides were analyzed with HALO platform.

Immunohistochemistry (IHC)

Mounted paraffin-embedded tissue slides were deparaffinized by two washes with graded levels of xylene from 95% to 50% diluted in water, after which the sections were rehydrated by washes in descending (95, 70, 50%) ethanol concentrations ending in water. First, antigen retrieval was performed by boiling the samples in a warm water bath (94^{o}C) using 10 mM sodium citrate buffer (pH6.0) for 10 min. Slides were then washed twice in PBS and once in 1% Tween-20 dissolved in PBS for 15 min. Next, slides were blocked with PBS with 3% bovine serum albumin (BSA) for 1h. Afterwards, the slides were incubated with specific primary antibodies (see antibody overview) at 4°C overnight. Slides were washed three times with PBS-0.1% Triton X-100 and stained with specific secondary antibodies (see antibody overview) for 1h at room temperature. Finally, sections were washed three times with PBS-0.1% Triton X-100 and 2 times with PBS. Sample was covered with mounting medium and coverslip. IHC images were analyzed with the HALO platform and scored based on intensity and percentage of positive cells (H-score). The intensity of positive staining was scored at four levels: 0 (negative), 1+ (weak staining), 2+ (medium staining), 3+ (strong staining). The percentages of cells at different intensity levels were expressed with the H-score = (% at 0)×0 + (% at 1)×1 + (% at 2)×2 + (% at 3)×3. H-scores range from 0 to 300.

Intrahepatic AAV-HBV episome quantification

Total DNA was extracted from liver pieces of compound-treated AAV-HBV mice, collected at different timepoints, with the PureLink kit (Invitrogen) according to the manufacturer's instructions. Total HBV DNA was determined by qPCR as described in the antiviral assay. AAV-HBV episome DNA was quantified by qPCR as well using AAV-HBV Fwd primer (5'-GGGCATGGACATCGACC-3'), probe (5'-/5HEX/-CGAGAGTAA-/ZEN/-CTCCACAGTAGCTCC-/3IABkFQ/-3') and Rev primer (5'-ACTCCATCACTAGGGGTTCC-3'). The Fwd primer and probe target HBV sequences towards the end of the viral genome, whereas the Rev primer is in the AAV inverted terminal repeat (ITR), allowing specific quantification of the AAV-HBV episome.

RNA extraction and sequencing

RNA extraction

Approximately 30 mg of flash-frozen liver tissue was weighed and homogenized using a Dounce homogenizer. Homogenates were transferred into Eppendorf tubes and centrifuged at 13,000g for 10 min at 4^{o}C. Supernatants were transferred to new Eppendorf tubes on ice and RNA extraction was performed using RNeasy mini kit (QIAGEN) strictly following manufacturer's instructions.

Library preparation

Sequence libraries of mRNA were prepared with the Lexogen QuantSeq 3' mRNA-Seq library prep kit according to the manufacturer's protocol. Samples were indexed to allow for multiplexing. Library quality and size range was assessed using a Bioanalyzer (Agilent Technologies) with the DNA 1000 kit (Agilent Technologies, California, USA). Libraries were diluted to a final concentration of 2 nM and subsequently sequenced on an Illumina HiSeq4000 instrument, producing single end reads of 50 bp length.

Genome alignment

Quality control of raw reads was performed with FastQC v0.11.7, available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Adapters were filtered with ea-utils fastq-mcf v1.05. Splice-aware alignment was performed with HiSAT2 against the mouse reference genome mm10, Ensembl78 version. Reads mapping to multiple loci in the reference genome were discarded and resulting BAM files were handled with Samtools v1.5.[6] Quantification of reads per gene was performed with HT-seq Count v2.7.14. Reported p-values were adjusted for multiple testing with the Benjamini-Hochberg procedure.[7]

RNA-seq gene expression filtering and normalization

Gene read count tables were merged and filtered to retain only genes expressed at 1 cpm or higher in at least three samples to remove low expressed genes. The filtered gene expression data was imported in the R-package edgeR (version 3.36.0)[8] and normalized using the TMM

method (Trimmed Mean of M-values)[9] using the edgeR-function calcNormFactors. logcpm expression values were exported using the edgeR-function cpm.

Multidimensional Scaling

Multidimensional scaling (MDS) was performed using the filtered and normalized logcpm gene expression data from edgeR using the function glimmaMDS from the R-package Glimma (version: 2.4.0)[10].

RNA-seq differential gene expression analysis

The filtered and normalized logcpm expression data from edgeR was imported into the R-package limma/voom (version 3.50.1)[11] using the function voomWithQualityWeights.[12] A linear model was fit with the limma/voom function lmFit with a design matrix containing the grouping for treatment and timepoints. For comparing control timepoints, treatment vs. combined controls (D7 and D70), or treated timepoints, the relevant coefficients were contrasted (limma/voom function makeContrasts) and then applied with empirical Bayes (limma/voom function eBayes). Differential expression analysis results were retrieved unfiltered with limma/voom function topTable. All genes with adjusted p-values below 0.05 after Benjamini-Hochberg correction were considered as differentially expressed.[7] Clustering of gene expression data was performed in R using the hclust function using euclidean distance and ward.D2 clustering method.

Pathway gene set enrichment analysis

The pathway annotations for mouse gene set MH (version: 0.3, mouse-ortholog hallmark gene set from MSigDB)[13] were obtained from gsea-msigdb.org.[14] Pathway gene set enrichment analyses were performed using the R-package fgsea (version 1.20.0)[15] with 10,000 permutations using the gene ranking from differential gene expression of treated samples vs control analyses results. List of pathways that showed significant enrichment for differentially expressed gene ranking in at least one timepoint were combined in a list and filtered used to generate an additional subset listing pathways with significant enrichment for at least two treated timepoints.

Analysis of viral reads

AAV-HBV cloning vector files and mouse genome files were downloaded from GenBank (KX470733.1 and GRCm39, respectively). Unnecessary regions were removed from the cloning vector annotation file. Next, cloning vector sequences were added to the mouse genome fasta and gtf files. Alignment of reads to the custom reference genome was done with STAR (version 2.7.10b). After the alignment, samtools (version 1.6) was used to filter out viral reads and create an indexed bam file (.bam.bai). After the STAR alignment, htseq-count (version 2.0.2) was used to count the number of transcripts mapping to genes with the notable setting to keep all reads, even if they were mapped on multiple genes, due to the overlap between the viral genes. Once the raw count table was generated, normalization by library size was performed to reads per million.

HBc-dependent CAM-A-induced cell death (CCD) assay

In a modified protocol of the antiviral assay, HepG2.117 cells were incubated for prolonged periods with compounds, with weekly medium and compound changes for up to 70 days. Here, compound incubation was done either in the presence or absence or 1 µg/mL doxycycline to induce HBc suppression or expression, respectively. Cell viability was measured at different time points and the percentage of live cells was calculated relative to the untreated DMSO control.

Apoptosis was investigated using the Promega RealTime-Glo Annexin V Apoptosis and Necrosis Assay (JA1012), strictly following the manufacturer's instructions. In brief, 100 µL of diluted HepG2.117 cell suspension corresponding to 10,000 cells/well was seeded in PDL-coated CellCarrier Ultra plates in the presence of 2% Tet-free FBS and 2 mM L-glutamine in DMEM (high glucose). After overnight incubation, cells were treated with a dilution series of compounds in the presence or absence of doxycycline and incubated at 37 °C for 20 days. As control, cells were treated with a dilution series of staurosporine (a known inducer of apoptosis) for 3 days. In addition, intracellular caspase activation was assessed by treating cells with Promega Caspase-Glo 3/7 reagent (G8091) for 1h. Relative caspase activation by

compounds was determined as the percentage change of caspase signal relative to untreated control cells.

CCD assay in HA-HBc-overexpressing HepG2-NTCP cells

HepG2-NTCP cells stably expressing HA-HBc or HA-L-HBsAg were used. The expression of HBV antigens was confirmed by Western blot using a monoclonal peroxidase-conjugated anti-HA antibody (Roche 11867423001) and a monoclonal anti-β-actin antibody (Abcam AB8226). For CCD evaluation, cells were seeded into 96-well plates at a density of 2.5×10^4 per well, in 100 µL of cell culture medium, and incubated for 24h to allow cell adherence. Cells were then incubated in the presence or absence of compound in 2% final DMSO concentration for 7 days. Cellular viability was determined with the resazurin-based PrestoBlue reagent (Invitrogen). Briefly, 10 µL PrestoBlue solution was added into each well; plates were then incubated for 1h prior to absorbance measurement. In addition, lactate dehydrogenase (LDH) released by damaged cells was used to monitor cellular toxicity and was measured with the LDH-Glo Cytotoxicity Assay (Promega J2380) according to the manufacturer's instructions. Cells treated with 0.2% Triton X-100 were used as background control for maximum LDH release. To quantify apoptotic cells, we used the Cell Event Caspase-3/7 Green Detection Reagent (Invitrogen C10723). This reagent relies on a fluorogenic substrate that is edited by activated caspase 3 and 7 inducing a fluorescent signal. After compound treatment, cells were incubated at 37 °C for 30 min in PBS + 2 % FBS + 2 µM of Cell Event Caspase-3/7 Green Detection Reagent and DAPI. Quantification of apoptotic cells was performed using a Celigo Image Cytometer.

Statistical analysis

Data were analyzed using GraphPad Prism v9 software and reported as mean values ± standard error of the mean (SEM). Comparison of groups was performed using one-way ANOVA test, with p-values < 0.05 indicating a statistically significant difference between groups.

CTAT Table

1.1 Antibodies

Name	Citation	Supplier	Cat no.	Clone no.
Anti-HBV core antigen antibody	/	Abcam	ab115992	/
Anti-HBV core antigen antibody	/	Abcam	ab8637	C1
Anti-HBV core antigen antibody	/	Abcam	ab8639	10E11
Anti-HBV surface antigen antibody	/	Fitzgerald	20-HR20	20-HR20
Anti-β-actin antibody	/	Abcam	ab8226	/
Anti-nucleolin antibody	/	Abcam	ab129200	EPR7952
Goat anti-mouse AlexaFluor 488 antibody	/	ThermoFisher	A-11001	/
Goat anti-rabbit Alexa 546 antibody	/	ThermoFisher	A-11035	/
Anti-HA antibody	/	Roche	11867423001	3F10
Goat anti-mouse IgG, HRP	/	ThermoFisher	G-21040	/
Goat anti-rabbit IgG, HRP	/	ThermoFisher	G-21234	/
Recombinant anti-Ki-67 antibody	/	Abcam	ab16667	SP6

1.2 Cell lines

Name	Citation	Supplier	Cat no.	Passage no.	Authentication test method
HepG2.117	[16]	Prof. Michael Nassal, University of Freiburg, Germany	/	/	/
HepAD38	[17]	Fox Chase Cancer Center, Philadelphia, PA	95-13	/	/

1.3 Organisms

Name	Citation	Supplier	Strain	Sex	Age	Overall n number
Mouse	/	Shanghai Lingchang Bio Tech Co. Ltd, Shanghai, China	C57BL/6	Male	5-50 weeks	30

1.4 Sequence based reagents

Name	Sequence	Supplier

HBV Fwd primer	5'-GTGTCTGCGGCGTTTTATCA-3'	IDT, Leuven, Belgium
HBV Rev primer	5'-GACAAACGGGCAACATACCTT-3'	IDT, Leuven, Belgium
HBV probe	5'-/56-FAM/-CCTCTKCAT-/ZEN/-CCTGCTGCTATGCCTCATC-/3IABkFQ/3'	IDT, Leuven, Belgium
AAV-HBV Fwd primer	5'-GGGCATGGACATCGACC-3'	IDT, Leuven, Belgium
AAV-HBV Rev primer	5'-ACTCCATCACTAGGGGTTCC-3'	IDT, Leuven, Belgium
AAV-HBV probe	5'-/5HEX/-CGAGAGTAA-/ZEN/-CTCCACAGTAGCTCC-/3IABkFQ/-3'	IDT, Leuven, Belgium

1.5 Biological samples

Description	Source	Identifier
/	/	/

1.6 Deposited data

Name of repository	Identifier	Link
/	/	/

1.7 Software

Software name	Manufacturer	Version
GraphPad Prism	GraphPad Software	9.0.0
Harmony	Perkin Elmer	4.9
ImageJ	NIH	1.53t

1.8 Other (e.g. drugs, proteins, vectors etc.)

/	/	/

1.9 Please provide the details of the corresponding methods author for the manuscript:

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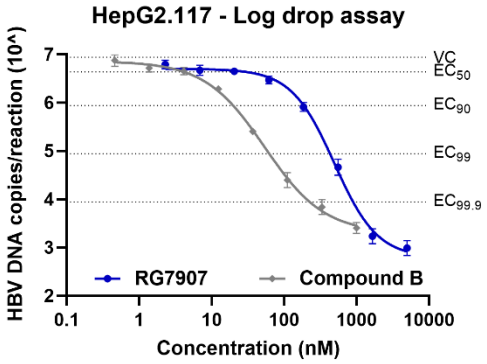
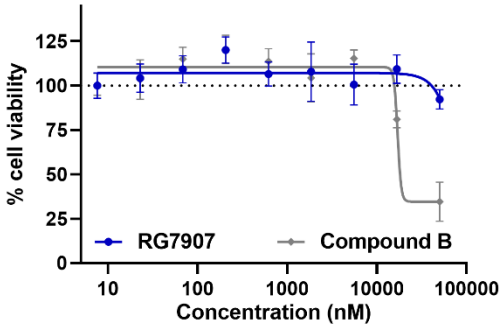
Supplementary Figures

A



	Compound B	RG7907
EC_{50} (nM)	4.12	59.6
EC_{90} (nM)	19.9	204
EC_{99} (nM)	62.6	458
$EC_{99.9}$ (nM)	227	989

B



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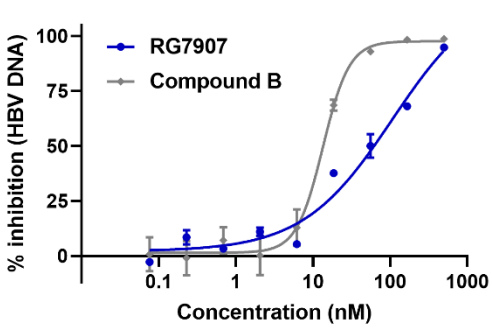
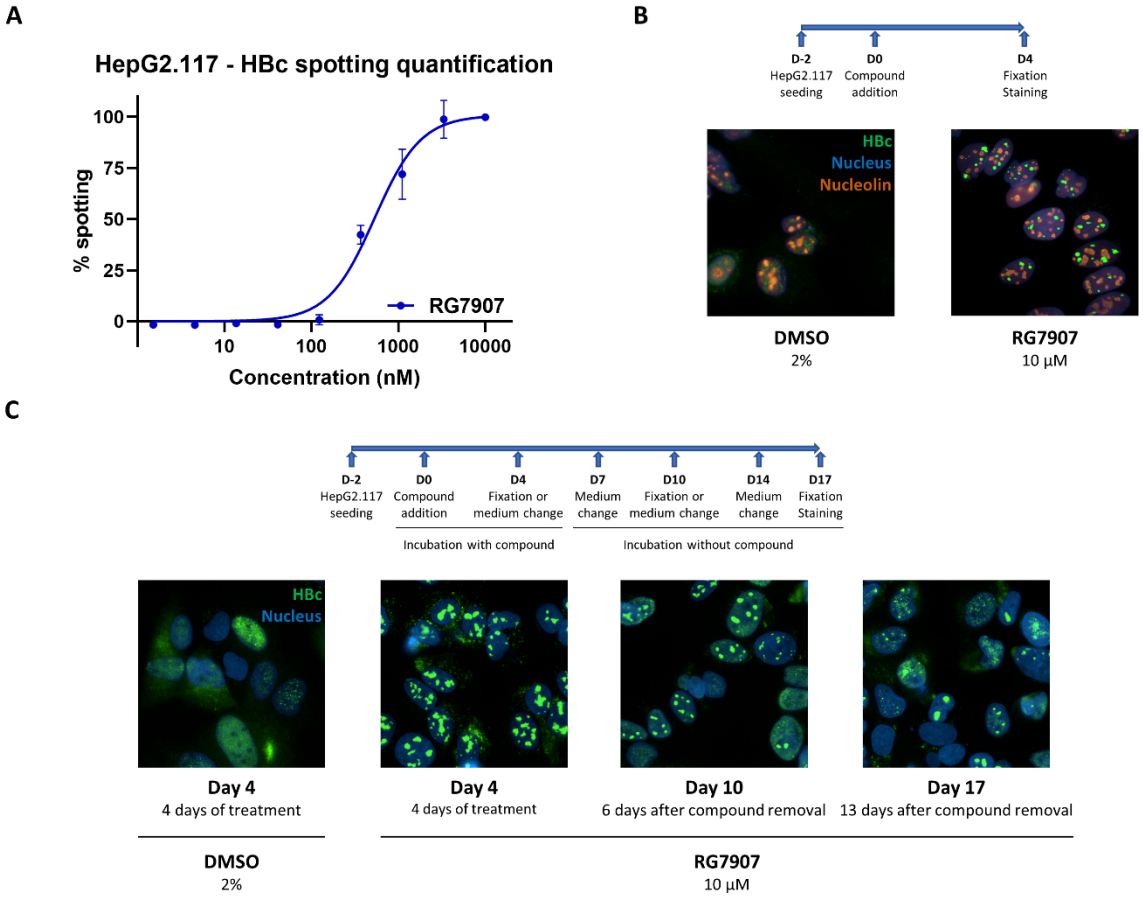
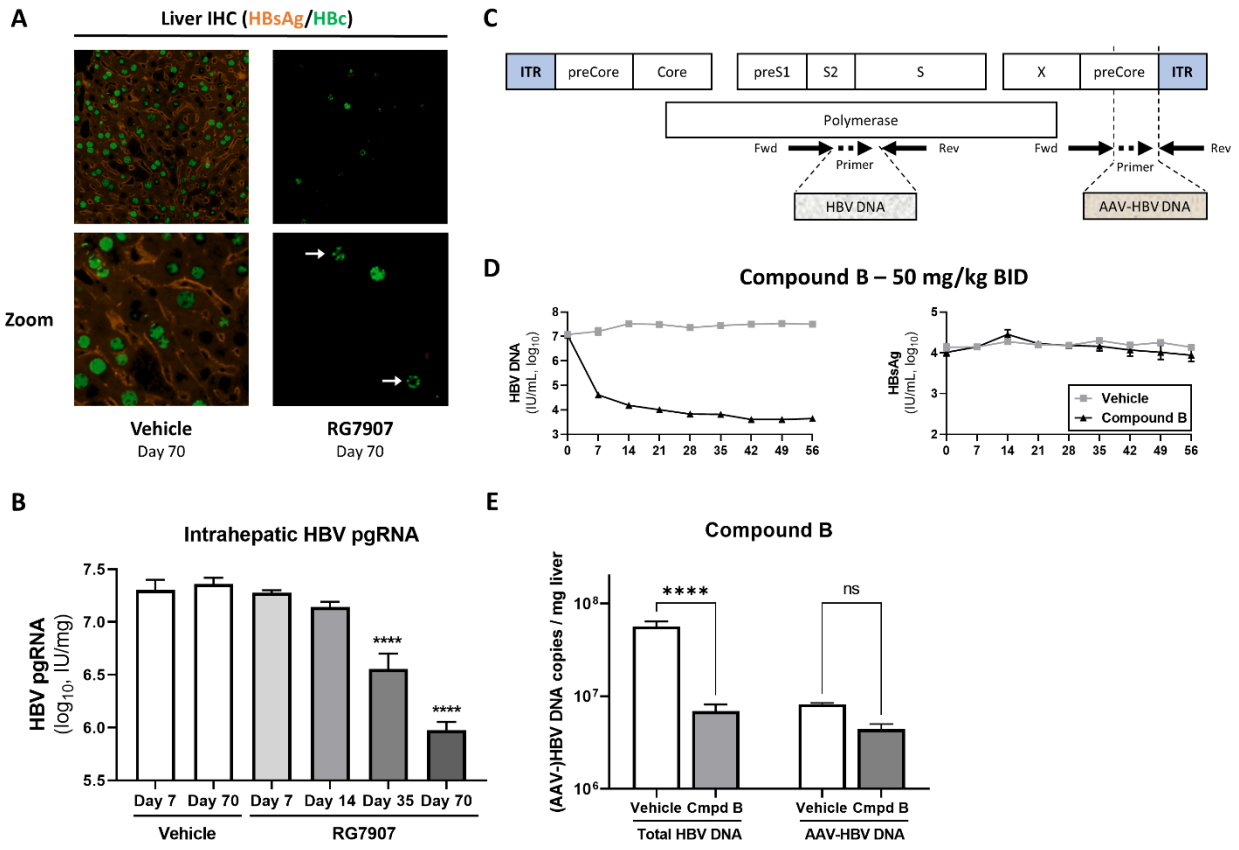


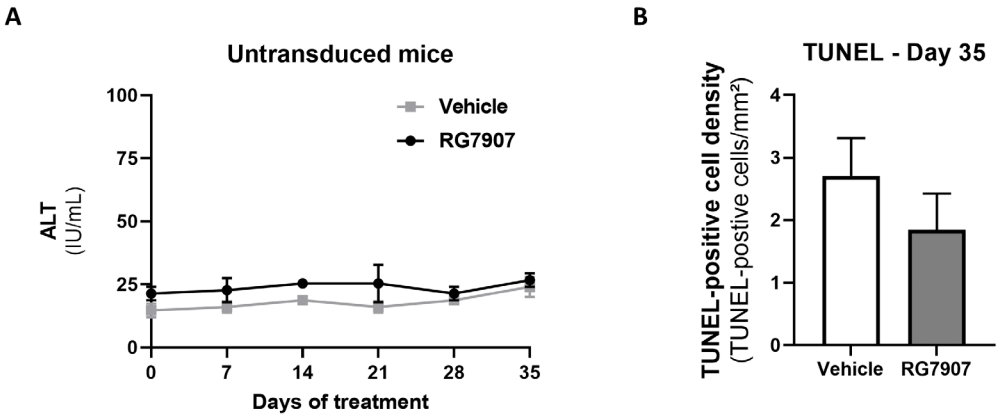
Supplementary Fig. 1. Antiviral and cell viability data of RG7907 and compound B in HepG2.117 and HepAD38 cells. (A) Both RG7907 and compound B decreased HBV DNA titers by several log_{10}s, as assessed in the log drop assay (HBV DNA antiviral assay in HepG2.117 cells with the addition of DNase digestion steps to remove background non-encapsidated HBV DNA, n = 2). (B) Cell viability for RG7907- or compound B-treated HepG2.117 cells (after 14 days) as assessed by intracellular ATP quantification (n = 3). (C) Antiviral activity of RG7907 (n = 1) and compound B (n = 4) in HepAD38 cells, as assessed by intracellular HBV DNA quantification by qPCR.



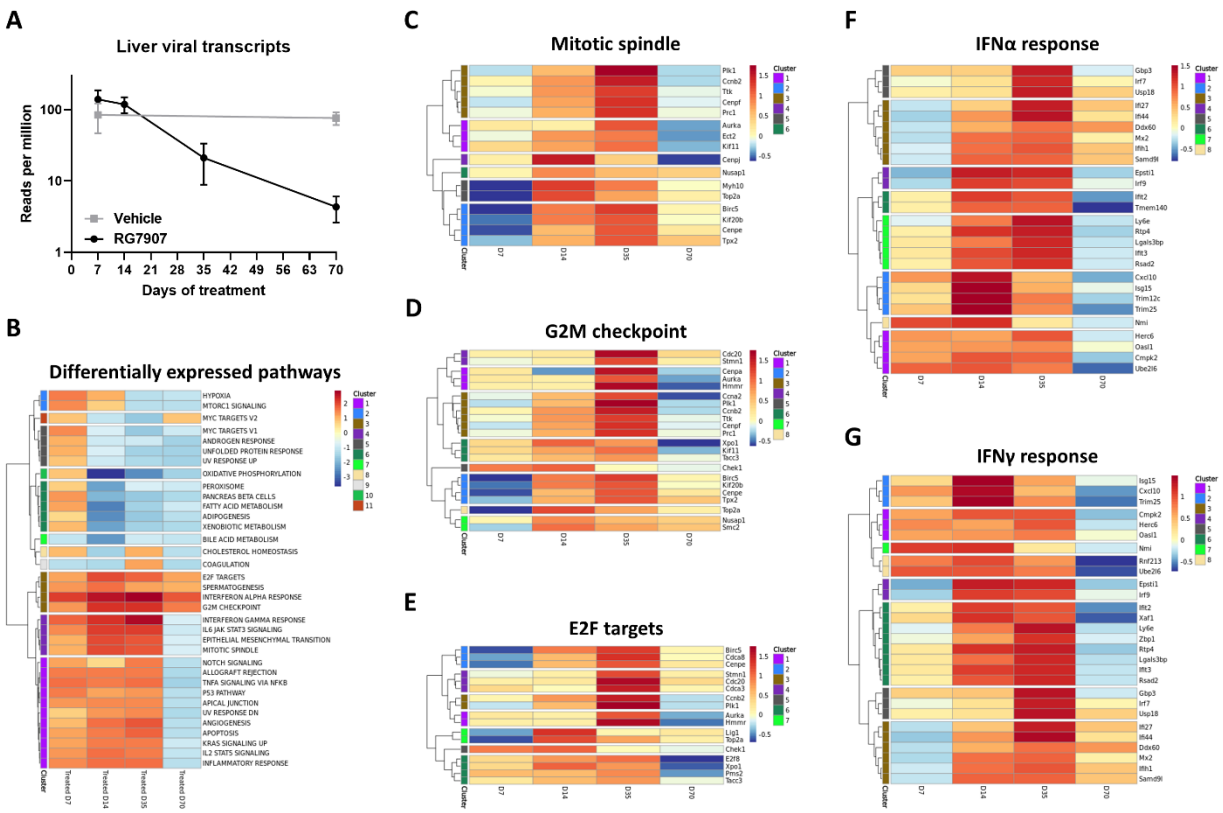
Supplementary Fig. 2. RG7907 induced nuclear HBc aggregates that did not colocalize with nucleoli and remained present for several weeks after compound removal. (A) Quantification of HBc aggregation after IF staining by automated image analysis. Total nuclear spot area was determined, normalized for cell count, and expressed relatively to the signal at 10 µM RG7907 (n = 4). (B) IF staining for HBc (green), Hoechst-stained nuclei (blue), and nucleolin (orange) in HepG2.117 cells either mock-treated with DMSO or treated with RG7907 for 4 days, showing a lack of colocalized HBc and nucleoli. (C) IF staining of RG7907-treated HepG2.117 cells (HBc in green, nuclei in blue) with compound removal and subsequent incubation after an initial 4 days of treatment. Nuclear aggregates could be observed up to 13 days after compound removal, although they decreased in number. The re-occurrence of free (unaggregated) nuclear HBc could be observed from 6 days after compound removal on.



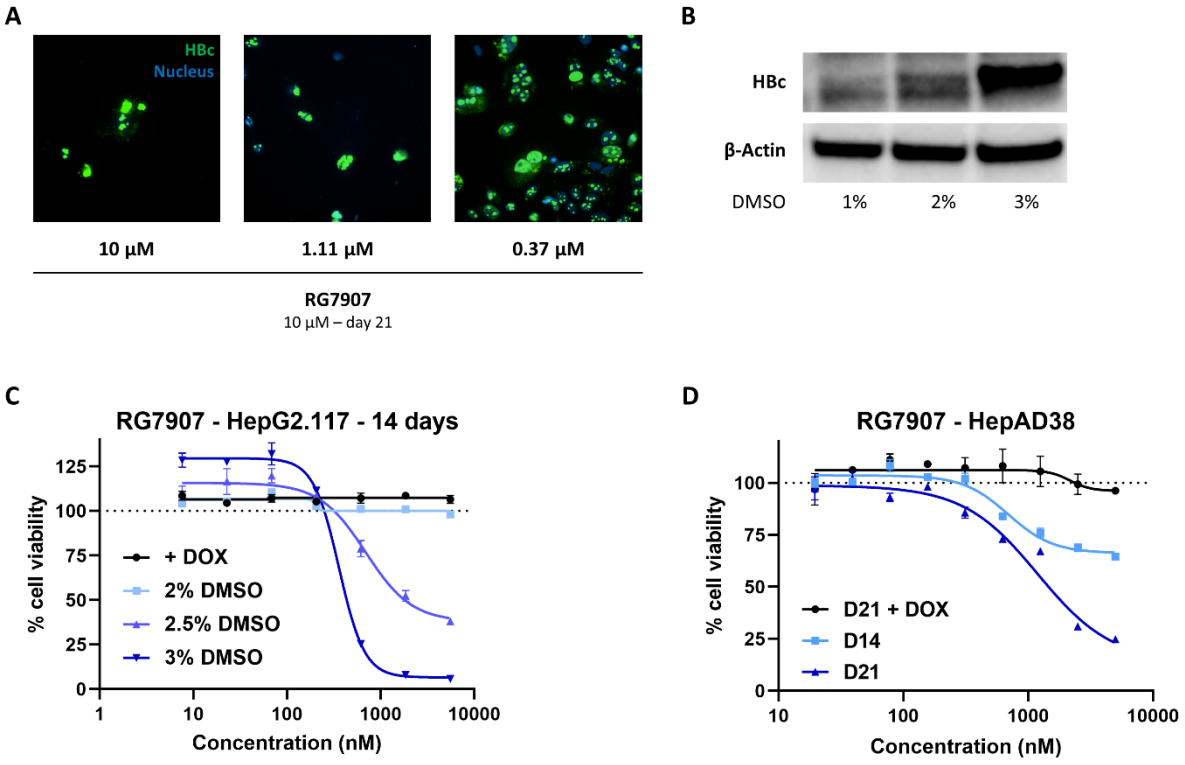
Supplementary Fig. 3. Effects of RG7907 and compound B in the AAV-HBV mouse model. (A) Extended treatment of AAV-HBV mice with RG7907 lead to a strong reduction in hepatic HBsAg and HBc, as demonstrated by fluorescent IHC, but also lead to the formation of nuclear spots (HBc aggregates, white arrows) in the few cells that remained HBc-positive. (B) RG7907 induced a gradual reduction in intrahepatic pgRNA, in line with reductions in its transcription template, the AAV-HBV episome. (C) Schematic representation of the AAV-HBV DNA-specific qPCR setup. (D) Evolution of serum HBV DNA and HBsAg levels in compound B-treated AAV-HBV mice (50 mg/kg, BID, *per os*), showing a pronounced reduction in HBV DNA, but no relevant effect on HBsAg levels. (E) Quantification of intrahepatic total HBV DNA and AAV-HBV DNA showed a pronounced reduction in total HBV DNA, but no statistically significant effect on AAV-HBV DNA, in line with a mechanism of suppression of replication, as opposed to the clearance observed for RG7907. Data are represented as mean ± SEM ($n \geq 3$). Statistical analysis was performed by ordinary one-way t-test or ANOVA test followed by Dunnett's multiple comparisons test: **** $p < 0.0001$, ns = not significant. For panel B, vehicle day 7 and day 70 data were pooled as a control group to perform multiple comparisons.



Supplementary Fig. 4. Effects of RG7907 in untransduced mice. (A) Untransduced mice treated with vehicle or RG7907 (20 mg/kg QD per os) for 35 days showed no ALT elevation over the course of the study. (B) Normalized quantification of TUNEL liver staining at the end of treatment.



Supplementary Fig. 5. RNAseq of RG7907-treated AAV-HBV mouse. (A) Evolution of viral transcript reads (aligning to the HBsAg gene) in the liver over time. (B) Heatmap of pathways differentially expressed at minimally 1 timepoint during RG7907 treatment, as compared to vehicle controls. (C-G) Heatmaps of individual genes involved in the mitotic spindle (C), G2M checkpoint (D), E2F targets (E), interferon-α response (F), and interferon-γ response (G).



Supplementary Fig. 6. Characterization of the CCD effect in different cellular settings. (A) IF staining for HBc (green) and nuclei (blue) in HepG2.117 cells treated for 21 days with RG7907 (10 µM), showing dose-dependent toxicity and extensive nuclear HBc aggregation and accumulation. (B) Western blot of HepG2.117 cells treated with different DMSO concentrations (1-3%), showing remarkably higher HBc levels at 2% and especially 3% DMSO. (C) Cell viability assessments for HepG2.117 treated with RG7907 for 14 days in the presence of different DMSO concentrations. While no CCD was observed at 2% DMSO at this early timepoint, CCD clearly manifested already at 2.5% and especially 3% DMSO. (D) CCD assessment in HepAD38 cells, displaying slightly faster kinetics than those observed in HepG2.117 cells.

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