Dual screening uncovers CAD and ESR1 as host factors for HDV infection and antiviral targets

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ONLINE SUPPLEMENTARY MATERIAL

SUPPLEMENTARY FIGURES

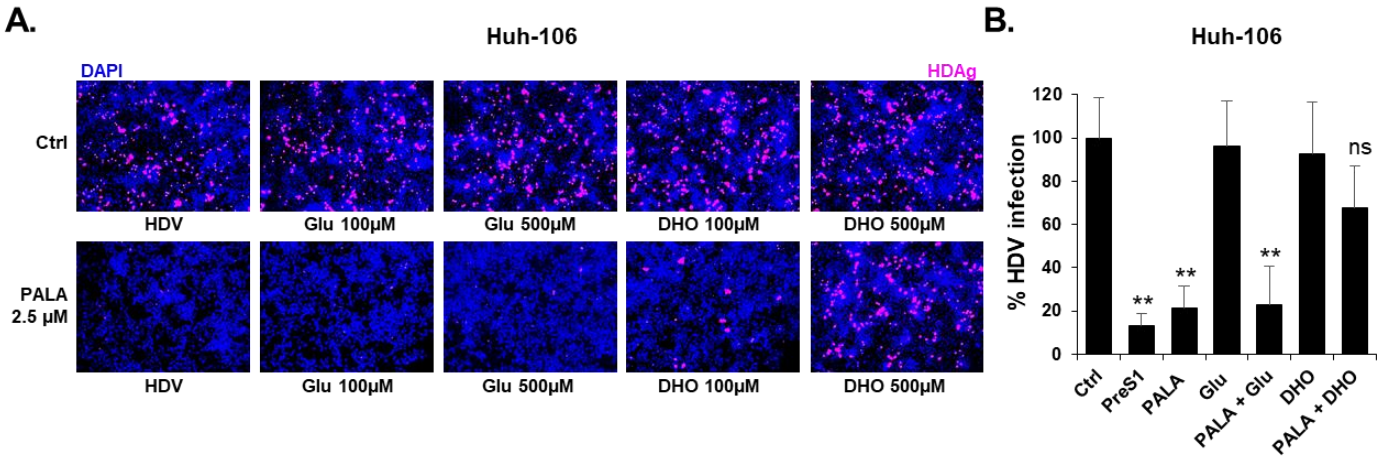

Figure S1. DHO treatment restores HDV infection in PALA-treated cells. Huh-106 cells were treated with PALA 2.5 µM and infected with HDV in presence or absence of L-glutamine (Glu) or dihydroorotate (DHO) at the indicated concentrations. Infection was assessed after 7 days by IF (**A**). One representative experiment is shown. **B**. Summary of three independent experiments using Glu and DHO at 500 µM. Results are expressed as mean ± SEM % HDV infection assessed by qRT-PCR from three experiments (n = 6). ns: non-significant.

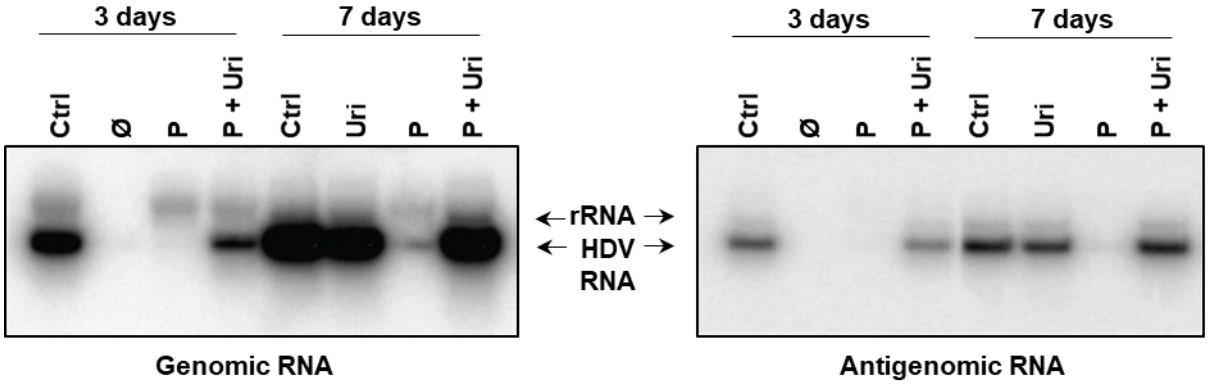

Figure S2. PALA inhibits both genomic and antigenomic HDV RNA synthesis. Huh-106 cells were treated with PALA 2.5 µM (P) in presence or absence of 30 µM uridine (Uri) 24 h prior to infection with HDV. HDV genomic and antigenomic RNAs were detected by Northern blot using specific probes 3 days and 7 days after infection. One experiment is shown. Ctrl: untreated, HDV-infected Huh-106 cells. Ø: empty lane.

SUPPLEMENTARY MATERIAL AND METHODS

HDV infection of Huh-106 cells and PHH. Huh-106 cells were plated in 96- or 384-well plates one day prior to infection with HDV, with polyethylene glycol (PEG, Sigma-Aldrich) at 4%. 16 h post-inoculation, cells were washed with PBS and cultured in primary hepatocyte maintenance medium (PMM [1]) containing 2% DMSO to slow cell growth. HDV infection was assessed 7 days post-infection (dpi) by immunofluorescence (IF) using an antibody targeting the hepatitis delta antigen (HDAg) purified from a serum of a HBV/HDV co-infected patient [2] and Alexa Fluor 647-labelled secondary antibody targeting human IgGs (Jackson Research) as described [3]. Cell nuclei were stained with DAPI. Fluorescent imaging was performed using an Axio Observer Z1 microscope (Carl Zeiss, Germany). Alternatively, cells were lysed and total RNA was extracted using the ReliaPrep Kit (Promega). HDV RNA was then detected by Northern blot. The purified RNA (from 1 to 4.5 µg) was subjected to electrophoresis through a 2.2 M formaldehyde, 1.2% agarose gel and transferred to a nylon membrane. The membrane-bound RNA was hybridized to a ^{32}P-labeled RNA probes specific for detection of either genomic HDV RNA or anti-genomic HDV RNA. rRNA specific probes were used as loading control. As a positive control, 5.10^7 HDV RNA genome equivalents extracted from HDV particles produced in Huh7 cells were used (HDV+). Alternatively, HDV infection was assessed 7 dpi by qRT-PCR quantification of HDV RNA using the following primers and probes Forward primer HDV-835-851: 5'-TGGACGTGCGTCCTCCT-3'; Reverse primer HDV-905-889: 5'-TCTTCGGGTCGGCATGG-3'. TaqMan® probe HDV-Pr-856-870: 5'-[FAM]-ATGCCCAGGTCGGAC-[BHQ1]-3'. All values were normalized to *GAPDH* (Applied Biosystems) expression. PHH were plated in 48- or 24-well plates one day prior to incubation with a HBV preS1 or a scrambled peptide control (Bachem) for one hour at 37°C as described [3]. PHH were then infected with recombinant HDV with 4% PEG. 16 h post-inoculation, PHH were washed with PBS and cultured in PMM containing 0.5% DMSO, and HDV infection was assessed after 7 days as described above.

HDV binding assay. Huh-106 were incubated with HDV infectious particles pre-treated or not with heparin (30 µg/mL) in the presence of 4% PEG for 24h at 16°C. Unbound virions were removed by three washes with PBS, and cells and bound virions were lysed. A 24h incubation period was chosen based on previous observations showing the requirement of a greater-than-16h virus/cell exposure [4, 5]. Total RNA was then extracted from Huh-106 cells and HDV RNA was quantified as described above.

RNAi loss-of-function screen. Screening was performed at the High Throughput Screening platform of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) in Illkirch, France. The Human ON-TARGETplus "Druggable Genome" siRNA Library was used for gene expression silencing. Huh-106 cells were first plated in 384-well plates at 1,000 cells per well. siRNA pools targeting the transcripts of 7567 genes were reverse-transfected into Huh-106 cells with InterFERIN-HTS® (Polyplus-transfection, Illkirch, France) at a final concentration of 20nM as described[6]. In each plate, a siRNA targeting *SLC10A1* (NTCP) expression was used as functional positive control, and a siRNA targeting *PLK1* expression was used as a transfection control, as the loss of PLK1 expression induces apoptosis in human cancer cells [7]. Two days after transfection, cells were infected with recombinant HDV (10^6-10^7 HDV copies/µL) without PEG. Cells were then cultured in 2% DMSO-containing PMM and infection was assessed 7 dpi by IF as described above. Nuclei were stained with DAPI. The total number of cells as well as the percentage of HDV-infected cells was then assessed by Cell Health Profiling bioapplication (HCS Studio, Cellomics Thermofisher Scientific). Each siRNA pool was transduced in three biological replicates. Toxicity after each siRNA treatment was determined using AllStars Hs Cell Death Control siRNA (Qiagen). The toxicity parameters of the Cell Death Control siRNA-transfected cells and the non-targeting siRNA control-transfected cells were set at 1 and 0, respectively. The toxicity parameter of each siRNA (from 0 to 1) was obtained by linear regression using the number of DAPI-stained cells at the end of the experiment.

Algorithm for selection of HDV host-dependency candidates. The impact of gene silencing was defined by an increase or decrease in % HDV positive cell compared to non-targeting siRNA control (siCtrl). The *p-value* and false discovery rate (FDR) for each gene were determined as previously described [8, 9]. For hit selection, a functional threshold (HDV-positive cells < 8.3 %; meaning a 45% inhibition effect compared to siCtrl), a statistical threshold (*p-value* < 0.05; FDR < 0.05), and a toxicity threshold (toxicity < 0.85) were applied. The thresholds were based on the silencing results of positive and negative controls. Finally, the expression of the selected candidates in the liver was assessed using the Illumina Body Map Expression Atlas (https://www.ebi.ac.uk/gxa). Candidates with liver expression > 0.1 Reads per kilobase of exon per million reads mapped (FPKM) were selected for further validations. A total of 191 candidates were selected from the primary screen (Table S2). Functional enrichment pathway analysis of candidates was performed through the ToppGene Suite [10] using Kyoto Encyclopedia of Genes and Genomes (KEGG) database (FDR cutoff: 0.05). Protein-protein interactions were predicted using STRING database [11] and corresponding interaction networks were created using Cytoscape 3.6.0 [12]. Two secondary screens were performed using the 191 candidates and the pools of siRNA. The impact of the silencing of each candidate gene on HDV infection from the primary screen and the two secondary screens was evaluated using an unpaired two-tailed Student's t-test.

Small molecule screen. Screening was performed at the High Throughput Screening platform of the Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) in Illkirch, France. The Chemical Library® containing 1280 FDA-approved molecules was obtained from Prestwick. Huh-106 cells were plated at 750 cells per well one day prior to treatment with the compounds (10 µM). 24 hours post-treatment, cells were infected with HDV (10^6-10^7 HDV copies/µL). Cells were then cultured in PMM in presence of the molecules, and the number of cells as well as the % of HDV-infected cells were assessed 7 dpi by IF. Cyclosporin A (Sandimmun®), a HDV entry inhibitor was used as a functional positive control in each plate (10 µM) and was obtained from Novartis, Switzerland. The *p-value*, false discovery rate (FDR), and viability for each molecule were determined as described above.

Synthesis of sparfosic acid (PALA). Sparfosic acid (L-Aspartic acid, N-(phosphonoacetyl)-, disodium salt (9CI) or PALA, NSC: 224131) was first obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, National Institute of Health, Bethesda, Maryland, USA. For further analyses, PALA was then synthetized at the Institute of Chemistry, University of Strasbourg, using a two-step synthesis involving a peptide coupling between labile esters of L-aspartic acid and diethylphosphonoacetic acid followed by a single mild deprotection [13]. *Step 1:* Triethylamine (0.49 mL, 3.55 mmol, 1 eq) was added to a solution of the commercially available L-aspartic acid di-tert-butyl ester hydrochloride (1 g, 3.55 mmol, 1 eq) in dichloromethane (50 mL). The solution was stirred for 10 min. In another flask, diethylphosphonoacetic acid (0.70 g, 3.55 mmol, 1 eq) was added to 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (1.44 g, 3.55 mmol, 1 eq) and 1-hydroxybenzotriazole (0.48 g, 3.55 mmol, 1 eq) in dichloromethane (8 mL). The latter mixture was then poured into the L-aspartic acid di-tert-butyl ester solution. Then, N,N-diisopropylethylamine (1.24 ml, 7.1 mmol, 2 eq) was added drop-wise at room temperature. After 16 h, the solvent was removed before adding ethyl acetate (90 mL) and successive washing with aqueous HCl (1M; 3 x 30 ml), water (30 ml), aqueous saturated NaHCO3 (3 x 30 ml) and brine (30 ml). The organic phase was then dried over MgSO4 and filtrated before evaporating under reduced pressure, yielding N-(diethoxyphosphinoyl)acetyl-L-aspartic acid di-tert-butyl ester (0.7 g, 85%). *Step 2:* The previously obtained oil (0.52 g, 1.22 mmol) was dissolved in acetonitrile (10 mL) before slowly adding bromotrimethylsilane (1.45 ml, 10.95 mmol). The mixture was then heated at reflux over 40 min under argon atmosphere, cooled down and concentrated under reduced pressure. Water (12 ml) was then added and the resulting solution was vigorously stirred for 10 min at room temperature before evaporating the solvents under reduce pressure, yielding N-(phosphonoacetyl)-L-aspartic acid (0.3 g, 96%).

Inhibition of HDV infection using small molecules. Huh-106 cells were treated with Fulvestrant and PALA at the indicated concentrations in presence or absence of uridine, glutamine, or DHO one day prior to infection with HDV in presence of the molecules unless otherwise stated. Cells were then cultured as described above for 7 days in presence of the molecules (with medium change and freshly added compound every 2/3 days). For validation experiments with Fulvestrant in PHH, PHH were infected with HDV 24 h before treatment with Fulvestrant for 48 h at the indicated concentrations. For validation experiments with PALA in PHH, PHH were treated with PALA at the indicated concentration 24 h prior to infection with HDV. Cells were then cultured as described above for 7 d in presence of the molecule (with medium change and freshly added compound every 2/3 days). For testing the effect of Fulvestrant on CAD expression, Huh-106 cells and PHH were treated with Fulvestrant for three days. CAD expression was assessed by Western blot as described above.

HDAg immunoprecipitation. Huh-106 cells seeded in 6 well plates and then transfected with pSVL(D3) (1 µg). Three days after transfection, HDV delta antigens (HDAg) immunoprecipitation was performed using Pierce™ Crosslink Magnetic IP/Co-IP Kit. Briefly, confluent cells were washed with cold PBS and lysed in ice-cold IP Lysis/Wash Buffer on ice. Lysate was cleared by centrifugation at 13,000 x g for 10 minutes. Cleared cell lysate was incubated with 5 µg of patient-derived anti-HDV antibody covalently bound to Pierce Protein A/G Magnetic Beads, or 5 µg of human control IgG (PALI IgG4, described in [14]) used as negative control. The beads were washed extensively to remove non-bound material and bound proteins were eluted in a low-pH elution buffer according to manufacturer's instructions. Eluted samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed by immunoblot using anti-CAD or anti-HDV antibodies.

Quantification of *IFNB1* expression in Huh-106 cells. Brequinar and Poly(I:C) were purchased from Sigma-Aldrich. Huh-106 cells were treated with Brequinar (5 µM; 50 µM), PALA (1 µM; 10 µM; 100 µM) or transfected with Poly(I:C) (100 ng/well of 96-well plate). Cells were then lysed every day for three days and RNA was extracted as described above and *IFNB1* expression was assessed by qRT-PCR as described [15]. Gene expression was normalized to GADPH expression. Primers and TaqMan® probe for *GAPDH* and *IFNB1* mRNA detection were obtained from ThermoFisher (TaqMan® Gene Expression Assays).

HBV infection of HepG2-NTCP cells. HBV production from HepAD38 cells have been described [3]. HepG2-NTCP cells were reverse-transfected with a pool of siRNA targeting *CAD* expression (siCAD) or a non-targeting siCtrl two days prior to infection with HBV in presence of 4% PEG. Cells were then cultured in 3.5% DMSO-containing PMM and HBV infection was assessed after 10 days by quantification of secreted HBsAg and HBeAg in the supernatant of infected cells by chemiluminescent immunoassay (CLIA, Autobio CL0310-2 and CL0312-2, respectively) following manufacturer's instructions.

Quantification of HBV replication in HepAD38 cells. HBV-producing HepAD38 cells have been described [16]. HepAD38 cells were treated with PALA (10 µM) for three days. HBeAg and HBsAg secretion in culture supernatant were then quantified by CLIA. Alternatively, HepAD38 cells were treated with either tenofovir (TFV) or PALA at the indicated concentration. HBV DNA in the supernatant was quantified by qRT-PCR as described [3, 15].

SUPPLEMENTARY TABLES

Table S1: HDV siRNA screen. The % of HDV positive cells as well as the level of HDV infection compared to control siRNA from three replicates per gene are shown. *p*-value, FDR, and the toxicity parameter are presented. This table refers to Figure 1 of the main manuscript.

Table S2: HDV candidate host factors. 191 candidate genes were selected from the primary screen according to the level of HDV inhibition after silencing, the gene expression in the liver and the measured toxicity after silencing. The expression level of each candidate according to Illimuna Body Map is presented. FPKM: Fragments Per Kilobase per Million mapped reads. This table refers to Figure 1 of the main manuscript.

Table S3: HDV small molecule screen. The % of HDV positive cells as well as the level of HDV infection compared to control cells from three replicates per molecule are shown. *p*-value, FDR, and the toxicity parameter are presented. This table refers to Figure 5 of the main manuscript.

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