A combined small molecule and loss-of-function screen uncovers estrogen receptor alpha and CAD as host factors for HDV infection and antiviral targets

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ABSTRACT

Objective: Hepatitis D virus (HDV) is a circular RNA virus co-infecting hepatocytes with hepatitis B virus. Chronic hepatitis D results in severe liver disease and an increased risk of liver cancer. Efficient therapeutic approaches against HDV are absent.

Design: Here, we combined an RNAi loss-of-function and small molecule screen to uncover host-dependency factors for HDV infection.

Results: Functional screening unraveled the HIF-signaling and insulin resistance pathways, RNA polymerase II, glycosaminoglycan biosynthesis and the pyrimidine metabolism as virus-hepatocyte dependency networks. Validation studies in primary human hepatocytes identified the CAD enzyme and estrogen receptor 1 (ESR1) as key host factors for HDV life cycle. Mechanistic studies revealed that the two host factors are required for viral replication. Inhibition studies using PALA and Fulvestrant, specific CAD and ESR1 inhibitors respectively, uncovered their impact as antiviral targets.

Conclusion: The discovery of HDV host-dependency factors elucidates the pathogenesis of viral disease biology and opens therapeutic strategies for HDV cure.

KEYWORDS: Antiviral therapy; Hepatitis D; Screening; Liver

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Abbreviations: HDV: hepatitis D virus; HBV: hepatitis B virus; HSPG: heparan sulfate proteoglycan; NTCP: sodium taurocholate co-transporting polypeptide; HD-Ag: delta antigen; RNP: ribonucleoprotein; EBV: Epstein-Barr virus; CAD: carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase; ESR1: estrogen receptor 1; PHH: primary human hepatocytes; DHODH: dihydroorotate dehydrogenase; PALA: N-(Phosphonoacetyl)-L-aspartic acid; TFV: tenofovir;

SUMMARY BOX

What is already known about this subject?

- Chronic hepatitis D is the most severe form of viral hepatitis
- Efficient therapeutic strategies are absent
- Hepatitis D virus is a small HBV satellite virus
- Knowledge about HDV-hepatocyte interactions is limited
- HDV host-dependency factors are largely unknown

What are the new findings?

- A RNAi screen identified ESR1 and CAD as novel host factors for HDV infection
- The inhibition of CAD restricts HDV infection through uridine depletion
- ESR1 and CAD are functionally linked by transcriptional activation of gene expression
- ESR1 and CAD inhibitors Fulvestrant and PALA, respectively, specifically inhibit HDV replication in a dose-dependent manner in human hepatocytes
- CAD and ESR1 are previously undiscovered targets for antiviral therapies

Significance of the study

How might it impact on clinical practice in the foreseeable future?

- The discovery of HDV host-dependency factors opens the door for novel therapeutic strategies against chronic hepatitis D – a major unmet medical need
- Fulvestrant and PALA-like molecules are candidate compounds for HDV antivirals to enter preclinical development

INTRODUCTION

Hepatitis delta virus (HDV) infects human hepatocytes and causes acute acerbation of liver disease in patients chronically infected with hepatitis B virus (HBV)[1]. Among HBV infected patients, 5 to 10% are co-infected with HDV[2]. HDV co- or sur-infection in HBV carriers induces more severe disease compared to infection with HBV alone. This includes a greater likelihood of experiencing liver failure and a more rapid progression to liver cirrhosis, with a markedly increased risk of developing hepatocellular carcinoma[3]. Consequently, chronic hepatitis D is considered as one of the most severe forms of viral hepatitis. Treatment with HBV nucleos(t)ide analogues is not effective at reducing HDV replication or disease. Moreover, the virus is only poorly controlled by the current IFN-based therapies[3] and to date, no treatment allows efficient cure of hepatitis D with eradication of the virus and its associated disease[4-6]. Thus, there is a major unmet medical need for curative therapies.

HDV is a small, circular RNA HBV satellite virus related to plant viroids using HBV envelope proteins to assemble its infectious particles[1, 5, 6]. Consequently, it can only be propagated by HBV-infected hepatocytes producing HBV envelope proteins (HBsAg). The HDV genome is a circular 1.7 kb single-stranded negative-sense RNA molecule (for a review on HDV virology and pathogenesis, see [5]). The HDV life cycle starts with virus entry into hepatocyte after attachment to heparan sulfate proteoglycans (HSPG) at the cell surface[5, 7]. As HBV and HDV share the same envelope proteins, they appear to share the same entry pathway and receptor(s), including the sodium taurocholate co-transporting polypeptide (NTCP) and Glypican 5 (reviewed in [5, 6, 8]). The HDV genome is then imported in the nucleus, and three types of RNAs are transcribed by the host RNA polymerase II: replication is initiated by synthesis of an anti-genomic RNA that serves as a template for the production of new HDV genomic RNA, and HDV mRNA from genomic RNA[5]. mRNA transcripts contain one unique open reading frame encoding the delta antigen (HDAg) which exists in two forms (small version S-HDAg and large version L-HDAg) which play differential roles in the HDV life cycle[5, 6]. Indeed, S-HDAg is essential to HDV replication, while prenylation of L-HDAg leads to the inhibition of HDV replication and is crucial for binding to HBV envelope

proteins and virion assembly[6]. In the nucleus, L-HDAgs bind to the *de novo* HDV genomic RNA to form a new ribonucleoprotein (RNPs), which is exported to the cytoplasm and interacts with HBsAgs at the endoplasmic reticulum to form new infectious virions, which are then secreted to propagate further rounds of HDV infection[6]. While the molecular virology of HDV has been elucidated, the role of host-dependency factors for HDV infection remains largely unknown[5].

A unique feature of HDV is the apparent lack of non-structural protein encoded by the viral genome (including viral polymerase), making it highly dependent of the host machinery for its replication. Targeting host factors is an emerging concept in the treatment of infectious diseases including hepatitis viruses[9-12]. Two host compounds targeting HDV factors have been developed up to clinical proof-of-concept: These include Myrcludex B, a small peptide targeting HBV/HDV entry factor NTCP and the prenylation inhibitor Lonafranib[4, 13, 14]. Both compounds demonstrated a significant clinical antiviral effect in chronically infected patients[4, 13, 14], supporting the validity of the concept of host-targeting antivirals for HDV infection. However, data on long-term safety, potential resistance in subsets of patients and sustained virological response remain to be determined. Thus, complementary approaches targeting other host factors and/or steps of the HDV life cycle are needed for curative therapies.

Taking advantage of our recently developed robust HDV infection system for the study of HDV-host factor interactions[7] combined with a dual screening approach, we aimed to uncover HDV host-dependency factors as antiviral targets.

RESULTS

A high-throughput RNAi loss-of-function screen uncovers hepatocyte host-dependency factors required for HDV infection. To identify host factors required for HDV infection, we performed a high-throughput loss-of-function screen using the HDV-susceptible cell line Huh-106[7]. Since one key goal was the discovery of druggable host targets, we used a library comprising siRNAs targeting 7567 genes which have been selected as targets

for therapeutics, including kinases, proteases, phosphatases, G protein–coupled receptor, ion channels, ubiquitin ligases, protein involved in senescence, autophagy, DNA repair, and targetable nuclear receptors. Huh-106 cells were transfected with siRNAs pools 48 hours before inoculation with HDV (Figure 1A) with an optical immunostaining-based viral protein (HDAg) readout 7 days after infection. The silencing of expression of HDV host factor NTCP was used as functional positive control (Figure 1B-C). The results of the primary screen are presented in Table S1.

Using the hit selection algorithm described in Methods and Figure 1E, we identified 191 host factor genes showing a robust correlation between gene silencing and level of HDV infection, moderate toxicity of gene-specific siRNA and their expression in the liver (Figure 1D-E and Table S2). The threshold of hit selection (described in Figure 1E) was based on the functional effect of *SLC10A1* silencing (approximately 45% decrease in HDV infection, Table S1). The hit rate of 191 genes corresponds to 2.5% of total genes, which is similar to screens for other virus host factor-dependency screens[15, 16].

Functional pathway analysis using KEGG pathways identified a network of host factors belonging to HSPG biosynthesis, including *EXT1* and *EXT2* (Figure 2A-B). These genes encode two major exostosins, key HSPG-related enzymes exhibiting glycosyltransferase activities and involved in the elongation of HS chains[17]. Moreover, several subunits of the RNA polymerase II were identified in our screen (Figure 2B), including specific subunits such as *POLR2G* or *POLR2I*, encoding Rpb7 and Rpb9, which play key roles in the initiation of transcription and binding to DNA template for an accurate selection of the transcription start sites, respectively[18, 19]. The identification of HSPG enzymes and RNA polymerase II as known HDV-related pathways[5, 7] validates our approach for host factor discovery (Figure 2). Importantly, the screen identified several previously unknown HDV-related networks, such as the hypoxia inducible factor 1 alpha (HIF-1α) signaling pathway, known to enhance the replication of several RNA and DNA viruses such as Epstein-Barr virus (EBV) and HIV[20, 21]. Interestingly, the HIF-1α signaling pathway also plays a key role in hepatocarcinogenesis and liver tumor progression[22, 23]

through its ability to target the expression of oncogenic genes such as the proliferation-specific transcription factor Forkhead box M1[24]. HIF-1α overexpression in HCC has been correlated with worse clinical outcomes and is considered as a poor prognosis factor and molecular target for liver disease therapy[24]. Interestingly, the highly significant scoring of insulin resistance-related pathways highlights the importance of hepatocyte metabolism as host-dependency factors on HDV infection (Figure 2).

Finally, we identified several host factors involved in the biosynthesis of pyrimidine, including *CANT1*, *ENTPD5*, and *CAD.*

The pyrimidine biosynthesis enzyme CAD is a key host factor for HDV infection and antiviral target in human hepatocytes. Given its high impact on HDV infection, functional relevance, liver expression and targetability, we focused on *CAD,* encoding the carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD), an enzyme playing a key role in the pyrimidine biosynthesis (Table S2; Figure 2)[25]. Silencing of *CAD* led to a robust decrease in HDV infection (Figure 3A). CAD is a trifunctional enzyme catalyzing the first steps of pyrimidine biosynthesis[26]. Interestingly, *CAD* expression is known to be regulated by activated estrogen receptor 1 (ESR1) which binds to the transcription factor SP1[27], two other host-dependency factors uncovered by the RNAi screen (Figure 3A). These data suggest a key role of the pyrimidine biosynthesis pathway and its associated nuclear proteins for the HDV life cycle.

We next investigated the functional role of CAD in HDV infection. We first performed additional silencing studies using individual *CAD*-specific siRNA. As shown in Figure 3B-D, a strong reduction of HDV RNA were observed after transfection of Huh-106 cells with the 4 individual siRNAs composing the siRNA pool, validating the key role of CAD expression in HDV infection. Notably, *CAD* expression was not significantly affected by HDV infection (Figure 3E).

Aiming to characterize the potential of CAD as an antiviral target, we synthetized a specific inhibitor of CAD, sparfosic acid or PALA (N-(Phosphonoacetyl)-L-aspartic acid)[26].

PALA is an aspartic acid derivative, which inhibits the aspartate carbamoyltransferase activity of the enzyme. We then investigated the antiviral activity of PALA in HDV-infected Huh-106 cells. As shown in Figure 3F, PALA dose dependently inhibits HDV infection with an IC_{50} = 1.2 µM. Notably, a decrease in cell viability was observed after 7 days of culture.

To validate this result in the most physiological model for HDV infection, we investigated the antiviral activity of PALA using HDV infection of primary human hepatocytes (PHH). Interestingly, while no toxicity was observed in PHH even at high doses (100 µM), the IC_{50} of PALA was slightly higher in PHH (Figure 3G), which may reflect differences in uridine pools and/or CAD expression in rapidly dividing Huh7-based cells versus non-dividing PHH in cell culture[28].

To investigate the mechanism of action of CAD-HDV interaction, we performed a rescue experiment using PALA and uridine-supplemented medium. Northern blot detection of HDV genomic RNA in infected cells revealed that the addition of uridine in PALA-treated cells restored HDV infection, validating the functional relevance of pyrimidine pathway for HDV replication (Figure 4A). Notably, PALA at a concentration of 2.5 µM induced a strong decrease in HDV infection which was efficiently rescued by uridine treatment (Figure 4B-C). This dose was then selected for further functional studies. Importantly, no direct interaction was observed between CAD and the HDV antigens (Figure 4D). As the inhibition of pyrimidine pathway has been shown to stimulate the innate antiviral response with subsequent inhibition of viral propagation [29], we next assessed the expression of *IFNB1* following PALA treatment in Huh-106 cells. As shown in Figure 4E, no *IFNB1* induction was observed after treatment with either PALA or Brequinar, targeting dihydroorotate dehydrogenase (DHODH), suggesting an absence of innate immune stimulation by pyrimidine inhibitors in hepatocyte-derived cells.

To further validate the involvement of the biological function of CAD in HDV infection, cell culture medium was then complemented using either glutamine (Glu, the initial substrate of CAD), or dihydroorotate (DHO, the final product of CAD) (Figure 5A, as described in[25]). As shown in Figure 5B and Figure S1, while glutamine treatment had no effect on

intracellular HDV RNA, DHO treatment in PALA-treated cells restored HDV RNA to non-treated levels, suggesting that PALA antiviral activity is linked to CAD physiological function.

To map the replication steps targeted mediated by CAD and targeted by PALA, we performed Northern blot analyses of HDV RNA allowing to quantify the HDV genome and anti-genome in the presence or absence of PALA. As shown in Figure 5C, PALA treatment effectively decreased the levels of both HDV genomic and antigenomic RNAs. Moreover, this inhibition was already observed during the early stages of viral replication (Figure S2). These data demonstrate that PALA inhibits all the steps of HDV replication including the synthesis of antigenomic RNA (Figure 5C).

Next, we performed kinetic studies adding PALA pre-, peri- and HDV post-infection (Figure 5D). PALA potently inhibited HDV infection given at any time before, during and following initiation of replication. Interestingly, the inhibitory effect was most pronounced when PALA was added prior or during initiation of replication. Finally, to exclude effects on other steps of the early HDV life cycle, we studied the effect of PALA treatment on HDV binding to the Huh-106 cell surface. The absent effect on HDV binding further confirms HDV replication as the life cycle step targeted by PALA (Figure 5E).

To test the specificity of the pyrimidine pathway for HDV infection, we treated HBV-producing HepAD38 cells with PALA. As shown in Figure 5F, PALA did not modulate HBV antigen production as shown by an absent effect on HBeAg and HBsAg secretion. While tenofovir (TFV) treatment dose-dependently inhibited HBV replication, PALA had no effect on HBV DNA in the supernatant of HepAD38 cells (Figure 5G). Finally, the silencing of *CAD* expression did not affect HBV infection in HepG2-NTCP cells (Figure 5H). These results suggest that the pyrimidine/CAD pathway is relevant for HDV but not HBV replication. Collectively, our results identify CAD as a key HDV host factor involved in HDV replication and target for antiviral therapy.

A small molecule screen uncovers ESR1 inhibitor Fulvestrant as host targeting agent. Next, we performed a small molecule screen using the Prestwick library, containing 1284

FDA-approved drugs (Figure 6A). Among the molecules exhibiting an antiviral activity against HDV we identified Fluvastatin and Cyclosporine A, two well described NTCP inhibitors (Table S3), confirming the validity of the screen. Interestingly, Ribavirin, a broad antiviral nucleoside analog[30] previously used for the treatment of chronic hepatitis C[31] exhibits a marked antiviral activity against HDV (Table S3). In the same vein, Nelfinavir, a protease inhibitor presenting anti-retroviral activity[32] was also identified in the drug screen (Table S3).

Given our discovery of ESR1 as HDV host-dependency factor we next focused on ESR1-targeting agents. The library includes several agonists and antagonists of ESR1, as well as characterized targets for endocrine therapy in breast cancer[33]. Interestingly, the ESR1 antagonist Fulvestrant exhibited a marked, significant and dose-dependent antiviral effect against HDV, whereas Tamoxifen and Toremifen, two ESR1 modulators with estrogenic effects in the liver[34] increased HDV infection, validating the importance of ESR1 in HDV infection (Figure 6B-C). Interestingly, a 48 h-treatment started after virus inoculation induced a marked decrease in HDV-infected cells (Figure 5D), suggesting an effect on HDV replication.

Next, we aimed investigate the mechanism of action of the antiviral activity of Fulvestrant by investigating a functional link of ESR1 and CAD suggested by protein/protein interaction studies[27]. To address this question, CAD expression was analyzed in Huh-106 cells and PHH in the presence or absence of the ESR1-antagonist (Figure 6E-G). As shown in Figure 6G, Fulvestrant treatment resulted in a loss of CAD expression in both Huh-106 cell line and PHH, suggesting that the antiviral activity of ESR1-inhibitor Fulvestrant is mediated by ESR1-mediated down-regulation of *CAD* expression, which in turn is required for HDV replication, confirming the previous observations with PALA.

Taken together, our dual screening approach uncovers the pyrimidine pathway and its associated and regulatory proteins as host factors for HDV infection and targets for antiviral therapy.

DISCUSSION

Chronic hepatitis D is the most severe form of viral hepatitis and at present time no treatment allows robust viral clearance. Using a dual screening approach we identified *ESR1* and *CAD* as host factors and antiviral targets for HDV infection. The functional impact of the pyrimidine biosynthesis for HDV replication is corroborated by (i) a marked decrease in HDV infection after *CAD* and *ESR1* silencing; (ii) a significant antiviral effect of ESR1 and CAD inhibitors PALA and Fulvestrant and (iii) kinetic experiments mapping viral replication as the step of the viral life cycle mediated by these host factors and targeted by the antivirals (vi) validation studies in primary human hepatocytes.

Whereas the molecular virology of HDV is well described, the molecular interactions between the virus and liver host factors are still largely unknown[5]. In mammalian cells, the pyrimidine biosynthesis pathway leads to the production of *de novo* nucleotides and plays a key role in RNA and DNA production, as well as protein glycosylation or cell membrane assembly[25]. Moreover, uridine nucleotides and derivatives regulate key physiological processes, such as lipid metabolism, regulation of normal central nervous system activity or modulation of reproduction[35], making this pathway a major actor of important metabolic processes. As a source of nucleotides for HDV RNA, this pathway is likely to be required for optimal virus replication. Moreover, as a key regulator of viral glycosylation[25], an alteration of this pathway may alter posttranslational modification of host or viral proteins, disrupting viral infection.

CAD is a multifunctional enzyme exhibiting carbamoylphosphate synthetase, aspartate transcarbamoylase, and dihydroorotase activities catalyzing the three first steps of the pyrimidine biosynthesis pathway[36], playing a key role in HDV infection as such. The observed antiviral activity of the CAD-specific inhibitor PALA validates the importance of this factor and pathway in HDV infection. It is of interest to note that another enzyme of the pyrimidine biosynthesis pathway DHODH, which catalyzes the production of orotate from DHO, has been described as an antiviral target by small molecule GSK983[37]. Here, we confirmed that the CAD-mediated DHO and uridine starvation is responsible for the observed

antiviral effect (Figures 4 & 5). Interestingly, HSPG synthesis has been shown to be dependent on the pyrimidine synthesis pathway in a CAD-defective animal model[38]. However, we did not observe any effect of CAD inhibition on either HBV infection or HDV entry (Figure 5 E-H). While recent studies have suggested that the inhibition of pyrimidine biosynthesis can stimulate innate antiviral responses[29], no induction of IFN expression was observed in our model (Figure 4E). Thus, it is likely that the modulation of CAD activity or expression affects HDV infection through its classical enzymatic activity linked to pyrimidine metabolism, mainly affecting viral replication through uridine starvation, affecting both genomic and antigenomic HDV RNAs. In this context, our study suggests that the first steps of replication are particularly susceptible to PALA. These results are consistent with the timing of HDV RNA amplification, much more pronounced during the early phase of replication, before L-HDAg-mediated slow-down of replication[5].

Among the genes regulating the pyrimidine pathway, we identified *ESR1* as a HDV host-dependency factor and antiviral target. ESR1 is a nuclear hormone receptor expressed in the mammary gland and female reproductive track, but also in lung and liver[39]. In our mechanistic analyses, we show that ESR1 inhibition by Fulvestrant led to a decrease in CAD protein expression which most likely explains the antiviral effect of the molecule.

Our screening strategy also identified small molecules exhibiting significant antiviral activity against HDV infection. Among them was found ribavirin, a nucleoside analog previously used for the treatment of chronic HCV infection[40]. Given its concentration in the primary screen (10 µM), HDV inhibition is most likely due to the ability of ribavirin to inhibit *de novo* synthesis of GTP as recently suggested as a possible mechanism for the inhibition of hepatitis E virus (HEV) replication in Huh7 cells (IC_{50} of 18.9 µM for GTP depletion; EC_{50} of 3 µM for HEV replication[41]). However, clinical attempts to treat hepatitis D using ribavirin were largely unsuccessful[4].

Collectively, we identified previously undiscovered pathways and host factors for HDV infection and antiviral targets. Host targeting agents are a promising approach for the development of new antiviral treatments, notably because they limit the emergence of

resistant variants[8-10, 42]. In particular for RNA viruses, which do not integrate into the genome, host targeting agents hold promise for viral cure[43]. Indeed, in chronic HCV infection antibodies targeting a viral host entry factor Claudin-1 cure viral infection in a state-of-the-art mouse model[12], and a miRNA-122 antagonist has been shown to cure chronic HCV infection in monotherapy in a subset of patients[44]. Two host compounds targeting HDV factors have been developed up to clinical proof-of-concept: these include Myrcludex B, a small peptide targeting HBV/HDV entry factor NTCP and the prenylation inhibitor Lonafranib[4, 13, 14]. In randomized clinical trials, both compounds demonstrated a significant and robust antiviral effect in chronically infected patients[4, 13, 14], supporting the validity of the concept of host-targeting antivirals for HDV infection. However, long-term safety, potential resistance in subsets of patients and data on sustained virological response remain to be determined. The discovery of novel host factors described in this study may overcome these limitations by complementary, safer and more efficient approaches. Compounds targeting essential host factors of the viral replication step may be conceptually superior to entry or prenylation inhibitors by targeting the most vulnerable step of the viral life cycle. Indeed, viral replication is the target of many effective clinically licensed antiviral therapies such as nucleoside analogues for HBV infection or polymerase/protease inhibitors for HIV and HCV infection.

A theoretical disadvantage of HTA is their putative adverse effects on physiological processes mediated by the host factors. In this context, given that an HDV treatment will occur in a context of diseased liver, assessment of safety will be a major parameter in development. In this regard, it is of interest to note that pyrimidine synthesis inhibitors, such as Leflunomide or Teriflunomide, are currently used for the treatment of rheumatoid arthritis and multiple sclerosis[45, 46]. The safety profile of these compounds suggests that pyrimidine biosynthesis is a targetable pathway for antiviral strategies. Indeed, the FDA-approved Leflunomide, targeting DHODH, induces uridine starvation at concentrations similar to PALA concentrations we used in this study (0-100 µM)[47]. Although detailed toxicity analyses in animal model and human tissues will be required to assess the

therapeutic window of PALA, the dose-response effects of Leflunomide suggests that PALA doses required to inhibit HDV infection are most likely in a range that will have an acceptable clinical safety profile.

In conclusion, by uncovering key host factors for HDV infection our results significantly improve the understanding of the HDV life cycle and contribute to the development of novel antiviral strategies for HDV cure.

MATERIAL AND METHODS

Human subjects. Human serum from patients with chronic HBV/HDV infection followed at the Strasbourg University Hospitals, Strasbourg, France was obtained with informed consent. PHH were obtained from liver tissue from patients undergoing liver resection for liver metastasis at the Strasbourg University Hospitals with informed consent. Protocols were approved by the local Ethics Committee of the Strasbourg University Hospitals (CPP) and the Ministry of Higher Education and Research of France (DC 2016 2616).

Cell lines and human hepatocytes. Huh-106[7], HepAD38[7] and HepG2-NTCP[7] cells have been described. PHH were isolated and cultured as described[48].

Key Reagents. Fulvestrant (I4409), DL-dihydroorotic acid (DHO, D7003), L-glutamine (59202C), uridine (U3003), and tenofovir (1643601) were purchased from Sigma-Aldrich® (Merck). Sparfocid 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 synthetized at the Institute of Chemistry, University of Strasbourg, as described in Supplementary Material and Methods. PreS1 peptide was synthetized by Bachem[7].

HDV and HBV production and infection. The production of recombinant HDV and HBV infectious virus as well as the protocol for HDV infection of Huh-106 cells and PHHs and for HBV infection of HepG2-NTCP cells have been described[7, 49, 50]. A detailed protocol is presented in Supplementary Material and Methods.

RNAi loss-of-function and small molecule screens. 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. For the small molecule screen, the Chemical Library® containing 1280 FDA-approved molecules was obtained from Prestwick. Detailed information about both screening strategies as well as the algorithm for selection of HDV-dependency candidates are presented in Supplementary Material and Methods.

Validation of CAD as a HDV host factor using individual siRNAs. Huh-106 cells were reverse-transfected with the 4 individual siRNAs from the siCAD pool, a pool of siRNA targeting SLC10A1 (siNTCP), or a non-targeting siRNA control (siCtrl) using Lipofectamin RNAi max as described[7, 50]. Gene expression was assessed, two days after transfection, by Western blot detection of CAD protein using a Rabbit monoclonal anti-CAD antibody (Abcam ab40800) as previously described[7, 50]. β-actin expression was assessed as a loading control using a mouse monoclonal anti-β-actin antibody (Sigma, A5441). Two days after transfection, cells were infected by HDV and infection was assessed after 7 days as described in Supplementary Material and Methods.

Statistical analysis. All experiments were performed at least twice in an independent manner. Statistical analyses were performed using a two-tailed Mann-Whitney U test unless otherwise stated; $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) were considered statistically significant. Significant *p*-values are indicated by asterisks in the individual figures and figure legends. The number of biological replicates is indicated in the figure legends (n).

Additional methodological information are available in Online Supplementary Material.

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AUTHOR CONTRIBUTIONS

TFB initiated the study. TFB and ERV designed and supervised research. ERV, AW, LB and TFB set up, designed and performed the siRNA and small molecule screens. TG and PPa produced the Sparfosic acid. ERV, CB, LH, VTL and EC performed the validation experiments. CSu performed the HDV Northern blots. PPe provided human hepatocytes. ERV, AW, CB, LH, EC, MBZ, CSu, CSc, LB, TFB analyzed the data. ERV, HES and AK performed the bioinformatical analyses of the screens. ERV and TFB wrote the manuscript. All the authors approved the study.

COMPETING INTEREST

The authors have no competing interest to disclose.

DATA AVAILABILITY

The datasets generated in this study, including the results from both RNAi and small molecule primary screens are available within Online Supplementary Material files. The rest of the data is available from the corresponding authors upon reasonable request.

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FIGURE LEGENDS

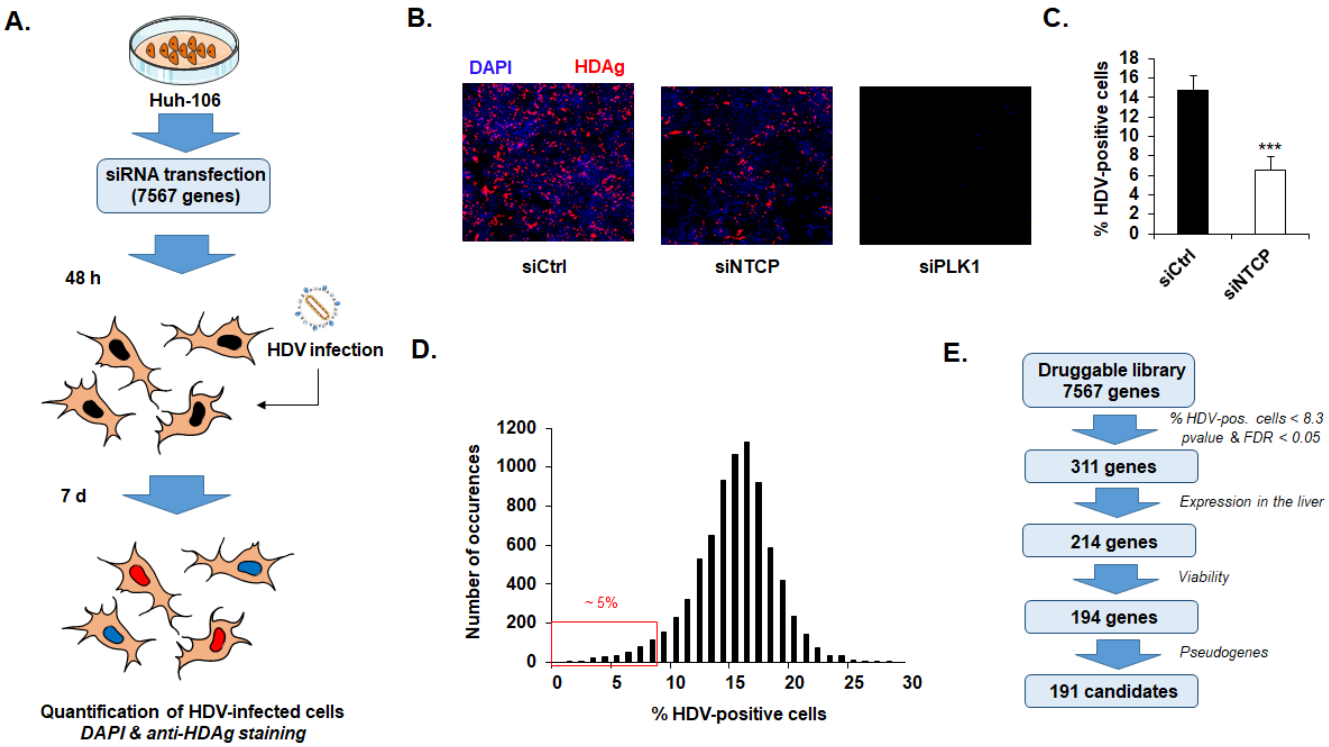
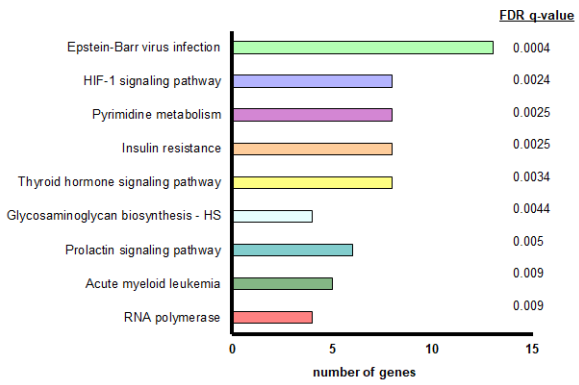


Figure 1. Identification of host-dependency factors for HDV infection using a high-throughput RNAi screen. A. Approach with flow chart of the screen. NTCP-overexpressing Huh7 cells (termed Huh-106) were transfected with pools of 4 siRNAs per target of the Dharmacon "Druggable genome" library 48 h before HDV infection. Infection was assessed after 7 days by immunofluorescence. Each siRNA pool was tested in triplicate. As controls, Huh-106 cells were transfected with a non-targeting siRNA control (siCtrl), siRNA targeting *SLC10A1* (siNTCP) and *PLK1* (siPLK1, lethal for the cells) expression. Representative images of HDV infection in Huh-106 cells from the primary screen are shown in **B**. As readout for infection, cells were stained with an anti-HDAg antibody and cell nuclei were stained with DAPI. **C.** General effect of the siNTCP on HDV infection at the screen level. Results are expressed as means ± SD % HDV positive cells from siCtrl-treated cells (n = 318) and siNTCP-treated cells (n = 265). *** *p*value < 0.001 (unpaired Student's t-test). **D**. Distribution of host-factor candidates according to their inhibitory effect on HDV infection. The top 5% (% HDV positive cells < 8.3) were selected as candidates for further work-up. **E**. Selection of candidate genes from the primary screen. From the 311 genes inducing a > 45% decrease in HDV infection after silencing, candidate genes were selected depending on their robustness (p-value & FDR < 0.05), their expression in the liver (Illumina Body Map tool) and their toxicity. Toxicity was evaluated as the percentage of viability compared to the siCtrl, quantified by counting the DAPI-positive nuclei in the wells at the end of infection. From the 194 selected candidates, *RNF130* was present twice (subset 8 and subset 10) and 2 pseudogenes (tAKR/*AKR1C6P* and *LOC402164*) were removed. 191 candidates (top 2.5%) were further worked-up.

A.



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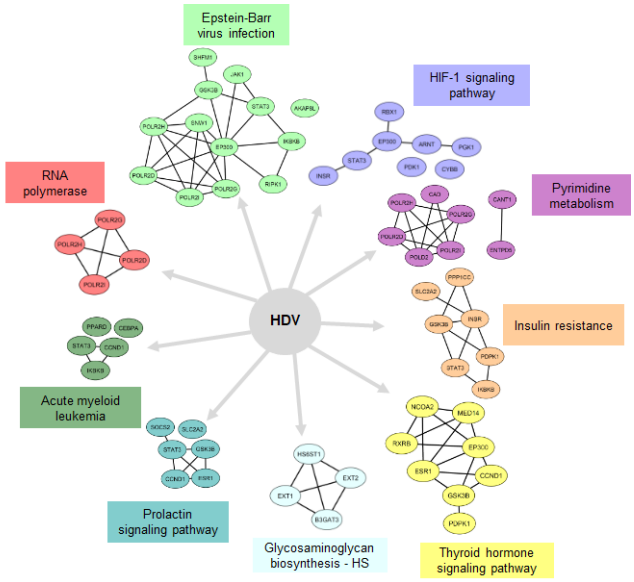


Figure 2. Pathway analysis of HDV host factors identified within the RNAi screen. The identified host genes were subjected to functional enrichment pathway analysis through ToppGene Suite (https://toppgene.cchmc.org) using Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Pathways scoring at a FDR value of < 0.05 were considered significant. **A**. Representation of the number of genes and FDR values of different KEGG pathways significantly enriched within the primary siRNA screen candidates. **B**. Individual genes contributing to the enrichment of the significant KEGG pathways were further analyzed for protein-protein interactions using STRING database. Interaction networks were represented using Cytoscape 3.6.0.

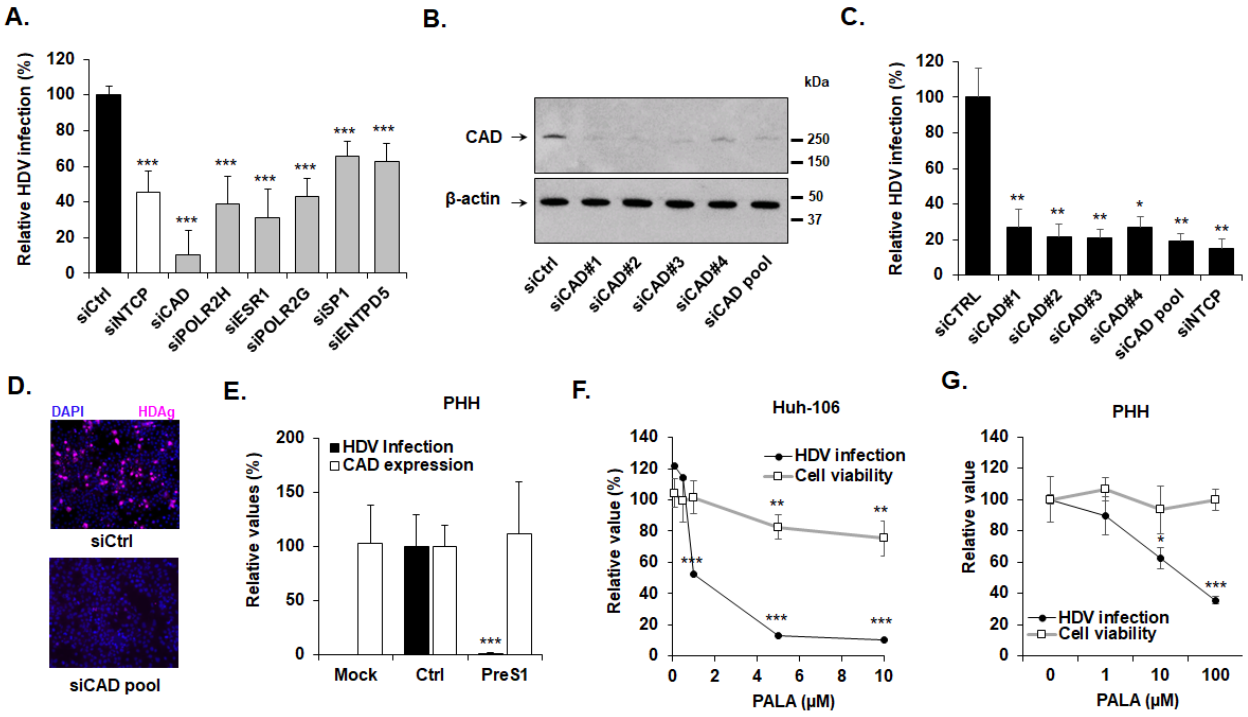
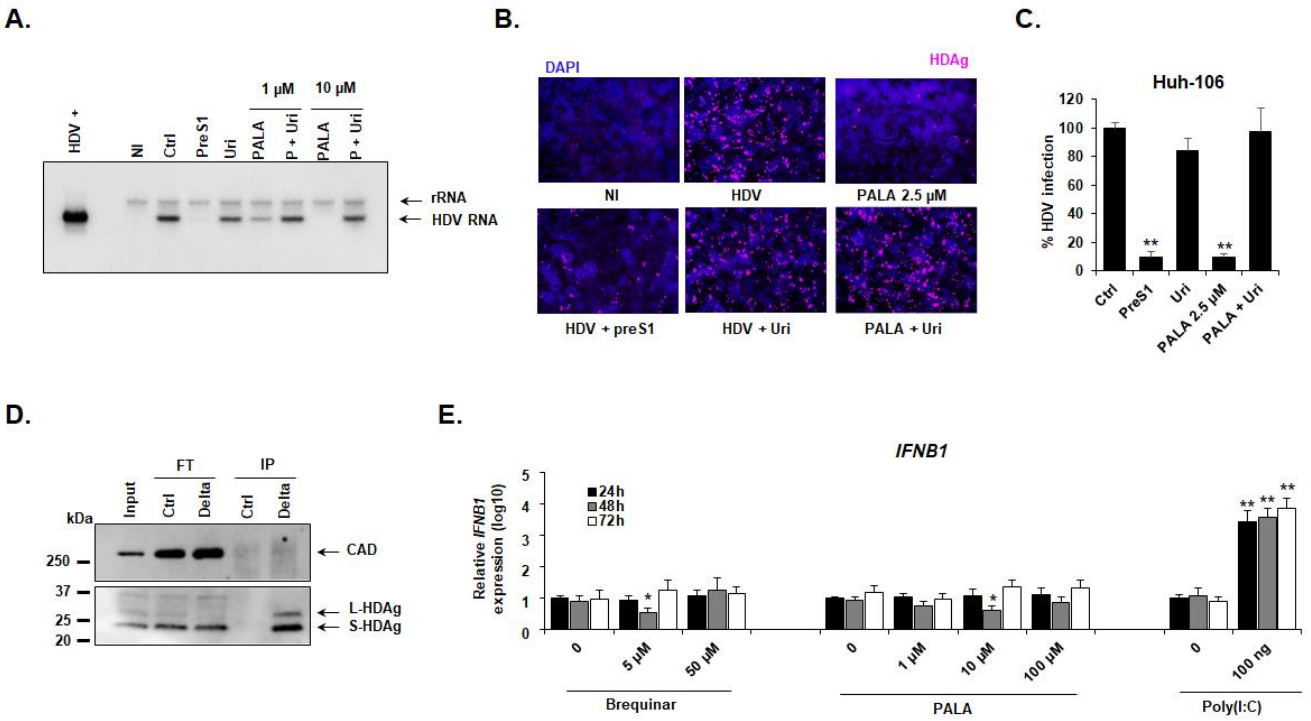


Figure 3. CAD is a key host factor required for HDV infection. A. Functional validation of host factors belonging to the pyrimidine biosynthesis using perturbation studies. Results are presented as means ± SD % HDV infection compared to control siRNA (siCtrl, set at 100%) from three independent screens (n = 9, primary screen and two validation screens performed in triplicate). *** *p*value < 0.001 (unpaired Student's t-test compared to siCtrl samples). **B-D**. CAD is required for HDV infection. Huh-106 cells were reverse-transfected with 4 individual siRNAs targeting *CAD* mRNA or with the pool of 4 siRNAs. Silencing efficacy was assessed by Western blot after two days (**B**). One representative experiment is shown. Cells were then infected with HDV and virus infection was assessed after 7 days by qRT-PCR. Results are expressed as means ± SEM % HDV infection compared to siCtrl (set at 100%) from three independent experiments (n = 8) (**C**). Alternatively, HDV infection was assessed by immunofluorescence using a patient-derived anti-HDAg antibody (**D**). One representative experiment using the pool of siCAD is shown. **E**. HDV infection has no effect on CAD expression in PHH. PHH were treated with preS1 peptide (preS1) or a peptide control (Ctrl) for one hour prior to infection with HDV for seven days. Results are expressed as means ± SD % HDV infection (assessed by HDV RNA levels) or *CAD* expression compared to control peptide-treated cells (Ctrl, set at 100%) from four independent experiments (n = 8). **F-G**. The CAD inhibitor PALA dose-dependently inhibits HDV infection in Huh-106 cells (**F**) and PHH (**G**). Cells were treated with PALA at the indicated concentrations 24 h before infection with HDV. Cells were then cultured for 7 days in presence of PALA. HDV infection was assessed by qRT-PCR. Cell viability was assessed by Presto Blue. Results are expressed as means ± SD % HDV infection or cell viability compared to untreated cells (0, set at 100%) from three independent experiments (Huh-106, **F**, n = 9) or as means ± SEM % HDV infection or cell viability from three independent experiments (PHH, **G**, n = 8).



638 **Figure 4. Characterization of CAD-HDV functional interaction**. **A-B-C**. Uridine
639 complementation restores HDV infection in PALA-treated cells. Huh-106 cells were treated
640 with PALA (1 µM or 10 µM) in presence of absence of 30 µM uridine 24 h prior to infection
641 with HDV and compound treatment was maintained for 7 days. HDV infection was assessed
642 by Northern blot (**A**). HDV+ corresponds to approximately 5.10^7 HDV RNA genome
643 equivalents extracted from HDV particles produced in Huh7 cells. rRNA corresponds to
644 ribosomal RNA. One representative experiment is shown. Alternatively, Huh-106 cells were
645 treated with PALA 2.5 µM in presence of absence of 30 µM uridine. HDV infection was
646 assessed after 7 days by IF (**B**) or qRT-PCR (**C**) Results are expressed as means ± SEM %
647 HDV infection compared to HDV-infected untreated cells (Ctrl, set at 100%) from three
648 independent experiments (n = 6). **D**. Absent direct interaction between HDV antigens and
649 CAD. Huh-106 were transfected with pSVL(D3) plasmid encoding the HDV genome. Three
650 days after transfection, cells were lysed and HDAg-specific co-IP was performed using an
651 anti-HDAg antibody (Delta) or with a control antibody (Ctrl). HDV antigens and CAD
652 expression in the original cell lysate (Input), in Flow-through control samples (FT) and in IP
653 eluates (IP) were assessed by Western blot. One experiment is shown. **E.** No IFN induction
654 after inhibition of pyrimidine biosynthesis in Huh-106 cells. Huh-106 cells were treated with
655 Brequinar or PALA at the indicated concentrations. Alternatively, Huh-106 cells were
656 reverse-transfected with Poly(I:C) (100 ng). Cells were then lysed every day for three days,
657 and *IFNB1* expression was assessed by qRT-PCR. Results are expressed as means ± SD
658 relative *IFNB1* expression (log10) compared to untreated or non-transfected cells (0, all set
659 at 1) from three independent experiments (n = 6).
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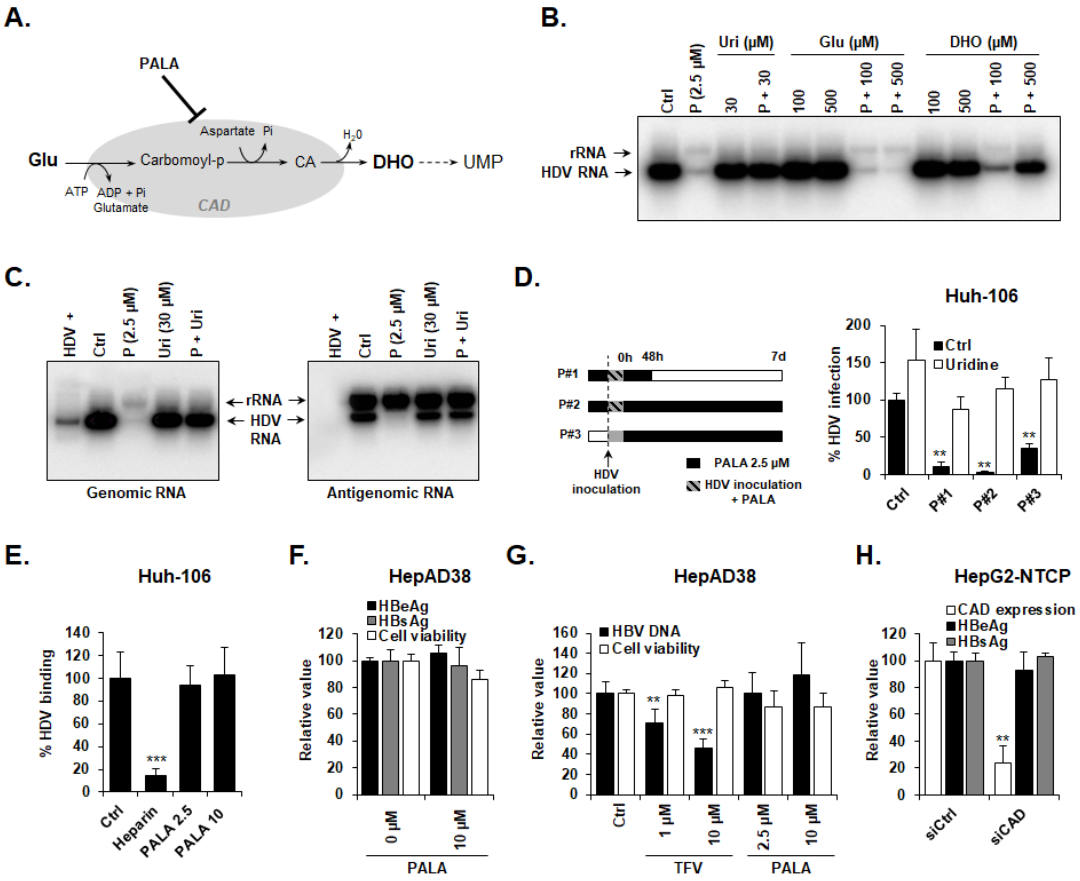

Figure 5. PALA specifically inhibits HDV replication by targeting CAD biological function. A. Schematic representation of reactions catalyzed by CAD enzyme. Glu: glutamine; Carbomoyl-p: carbomoyl phosphate; Pi: inorganic phosphate; CA: Carbamoyl aspartic acid; DHO: dihydroorotate; UMP: uridine mono-phosphate. **B.** DHO treatment restores HDV infection in PALA-treated cells. Huh-106 cells with treated with PALA 2.5 µM and infected with HDV in presence or absence of L-glutamine (Glu) or DHO at the indicated concentrations. Infection was assessed after 7 days by Northern blot detection of HDV genomic RNA. rRNA corresponds to ribosomal RNA. One experiment is shown. **C.** PALA inhibits both HDV genomic and antigenomic production. Huh-106 cells were treated with PALA 2.5 µM 24 h prior to infection with HDV. HDV genomic and antigenomic RNAs were detected by Northern blot using specific probes 7 days after infection. HDV+ corresponds to approximately 5.10^7 HDV RNA genome equivalents extracted from HDV particles produced in Huh7 cells. One experiment is shown. **D.** Kinetics of HDV infection by PALA. Huh-106 cells were pretreated with PALA at 2.5 µM and infected with HDV (conditions P#1 and P#2). Cells were then cultured in presence of PALA for 48 h (P#1) or for 7 days (P#2) in presence or absence of uridine (30 µM). Alternatively, Huh-106 cells were infected with HDV with no PALA pre-treatment (P#3). 16 h after viral inoculation, cells were cultured in presence of PALA 2.5 µM with or without uridine (30 µM). HDV infection was assessed after 7 days by qRT-PCR. Results are expressed as means ± SEM % HDV infection compared to HDV-infected untreated cells (Ctrl, set at 100%) from three independent experiments (n = 6). **E.** PALA does not affect HDV binding. Huh-106 cells were culture for 24 h à 16°C in presence of HDV particles which were pre-treated or not with heparin (30 µg/mL). HDV binding was measured by qRT-PCR quantification of total HDV RNA bound to cells after 24h. Results are expressed as means ± SD % HDV binding relative to control untreated cells (Ctrl, set at 100%) from three independent experiments (n=9). **F-H.** CAD inhibition or silencing does not affect HBV infection and replication. **F.** HBV-producing HepAD38 cells were treated with PALA (10 µM) for three days. HBeAg and HBsAg secretion in culture supernatant was then quantified by chemiluminescent immunoassay (CLIA). Cell viability was assessed by Presto

690 Blue. Results are expressed as means ± SEM % HBeAg production, HBsAg production or
691 cell viability compared to untreated cells (0 µM, all set at 100%) from three independent
692 experiments (n = 6). **G.** HepAD38 cells were treated with either tenofovir (TFV) or PALA at
693 the indicated concentrations for three days. HBV replication was assessed by quantification
694 of HBV DNA copies in the supernatant of treated cells by qPCR. Results are expressed as
695 means ± SD % HBV DNA in the supernatant or cell viability compared to untreated cells (Ctrl,
696 all set at 100%) from three independent experiments (n = 9) **H**. HepG2-NTCP cells were
697 reverse-transfected with siCAD or siCtrl for two days prior to infection with HBV. HBV
698 infection was assessed after 10 days by quantification of HBeAg and HBsAg production
699 (CLIA). Results are expressed as means ± SD % CAD expression, HBeAg production or
700 HBsAg production compared to siCtrl-transfected cells (siCtrl, all set at 100%) from three
701 independent experiments (n = 6).
702

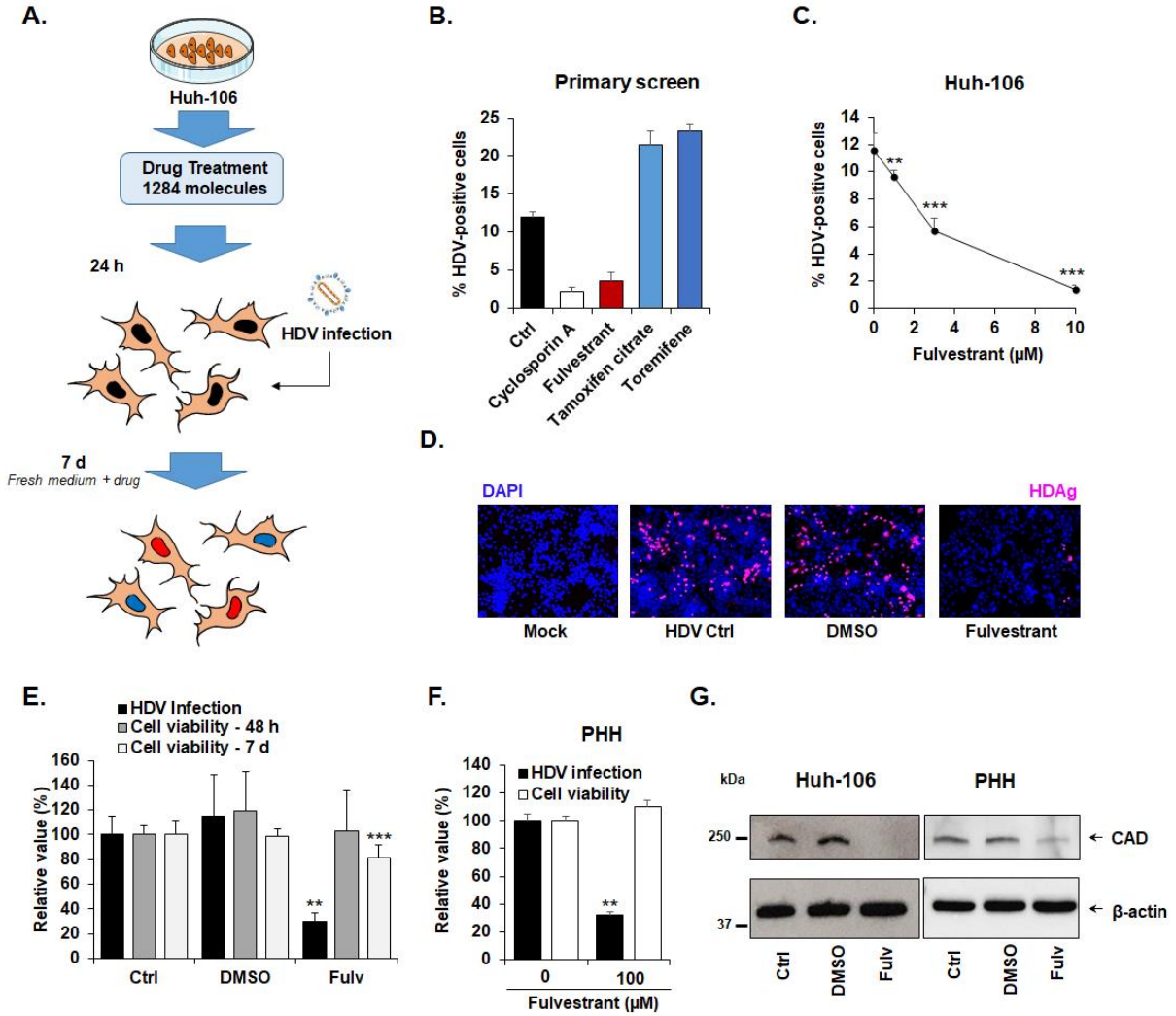

Figure 6. ESR1 inhibitor Fulvestrant inhibits HDV replication by suppression of CAD expression. **A**. Small molecule screen flow chart. Huh-106 cells were treated with individual compounds belonging to the Prestwick Chemical library (10 µM) one day prior to infection with HDV. Infection was assessed after 7 days by immunofluorescence. Every compound was tested in triplicate. **B**. ESR1 modulators affect HDV infection. From the primary screen, Fulvestrant (an ESR1 inhibitor) from the one hand, Tamoxifen and Toremifen from the other hand (two agonists/modulators of ESR1) exhibited antiviral or proviral activity against HDV, respectively. Results are presented as means ± SD % HDV infected cells (n = 3). **C**. Fulvestrant dose-dependently inhibits HDV infection. Huh-106 cells were treated with Fulvestrant at the indicated concentrations and then infected with HDV for seven days. Results are expressed as means ± SD % HDV infected cells from three independent experiments (n = 9). **D.** Short Fulvestrant treatment for 48 h following virus inoculation inhibits HDV infection. Huh-106 cells were infected with HDV and then treated with Fulvestrant (10 µM) or DMSO for 48 h. Cells were then culture in absence of drug and infection was assessed after 7 days by IF. One representative experiment is shown. **E**. Fulvestrant antiviral activity is associated with slight cytotoxicity. Cell viability was assessed after 48 h and after 7 days by Presto blue. HDV infection was assessed after 7 days by qRT-PCR. Results are expressed as means ± SD % HDV infection (HDV RNA) or cell viability compared to untreated HDV-infected cells (Ctrl, set at 100%) from three independent experiments (n = 9). **F**. Fulvestrant antiviral activity in PHH. PHH were infected with HDV and then treated with Fulvestrant for 48 h at the indicated concentrations. Results are expressed as means ± SD % HDV infection (HDV RNA) or cell viability compared to untreated HDV-infected PHH (Ctrl, set at 100%) from two independent experiments (n = 6). **G**. Fulvestrant inhibits CAD expression. Huh-106 cells and PHH were treated with Fulvestrant (Fulv, 10 µM) or DMSO for 72h. CAD expression was assessed by Western blot.