Sofosbuvir Inhibits Hepatitis E Virus Replication *in vitro* and Results in an Additive Effect when Combined with Ribavirin

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Running title: SOF inhibits HEV replication

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Author contributions: VLDT, YD, XW, CMR, JN, DM and JG designed experiments and conceived the study; VLDT, YD, XW and JG collected and analyzed data; VLDT, YD, XW, CMR, JN, DM and JG interpreted the data and wrote the manuscript.

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Abstract

Infection with hepatitis E virus (HEV) of genotype 3 may result in chronic hepatitis in immunocompromised patients. Reduction of immunosuppression or treatment with ribavirin or pegylated interferon-α can result in viral clearance. However, safer and more effective treatment options are needed. Here, we demonstrate that sofosbuvir inhibits the replication of HEV genotype 3 both in subgenomic replicon systems as well as a full-length infectious clone. Moreover, the combination of sofosbuvir and ribavirin results in an additive antiviral effect. Sofosbuvir may be considered as an add-on therapy to ribavirin for the treatment of chronic hepatitis E in immunocompromised patients.

Keywords: antiviral | chronic hepatitis | immunosuppression | polymerase inhibitor

Abbreviations: gt, genotype; HEV, hepatitis E virus; IC_{50}, 50% inhibitory concentration; iHep, induced pluripotent stem cell-derived hepatocyte-like cell; ORF, open reading frame; RBV, ribavirin; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SOF, sofosbuvir.

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Hepatitis E virus (HEV) is believed to be the most common cause of acute hepatitis and jaundice in the world [1-3]. While HEV genotypes (gt) 1 and 2 infect only humans, gt 3 and 4 are zoonotic viruses that also infect swine and other animal species. Acute hepatitis E is usually self-limited. However, HEV of gt 3 can persist in immunocompromised patients, especially organ transplant recipients, causing chronic hepatitis, which may progress to cirrhosis and liver graft failure [1-4]. Reduction of immunosuppressive therapy, ribavirin (RBV) or pegylated interferon-α have been used with varying success, allowing for viral clearance in up to 78% of patients [1-6]. However, failure of RBV has been described [7]. Hence, safer and more effective treatment options are needed.

A luciferase encoding subgenomic replicon construct derived from the HEV gt 3 Kernow-C1 p6 strain (both kindly provided by Suzanne U. Emerson, NIH, Bethesda, MD) was used to develop a selectable subgenomic replicon in which part of open reading frame 2 (ORF2) and ORF3 was replaced by a neomycin phosphotransferase gene (Fig. 1A) (Suppl. Material and Methods). Transfection of *in vitro*-transcribed replicon RNA into the Huh-7 human hepatocellular carcinoma-derived cell line S10-3 (kindly provided by Suzanne U. Emerson) allowed the establishment of a cell line harboring autonomously replicating HEV RNA, designated as HEV3 Rep/Neo. The full-length and subgenomic HEV RNA species were detectable in this cell line by Northern blot (Fig. 1B). Steady-state HEV RNA replication was quantified by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) (Suppl. Material and Methods).

In line with data previously obtained in a transient HEV Kernow-C1 replicon assay [8], treatment of HEV3 Rep/Neo cells with interferon-α (IFN-α) or RBV for 48 h led to a dose-dependent decrease of HEV RNA levels relative to untreated cells, with 50% inhibitory concentrations (IC_{50}) of 8.5 IU/ml and 8 µM, respectively, without affecting cell viability (Fig. 1C; Suppl. Material and Methods).

We observed that sofosbuvir (SOF), the oral prodrug of a nucleotide hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor [9], efficiently inhibited HEV RNA replication in HEV3 Rep/Neo cells (IC_{50} 1.2 µM; Fig. 1C). This antiviral activity was confirmed in a transient gt 3 reporter replicon system while a gt 1 replicon proved to be less sensitive to inhibition by SOF in this assay (Suppl. Fig. 1A). In addition, two closely related nucleoside analogs with known inhibitory activity on HCV replication, i.e. 2'-*C*-methyladenosine and 2'-*C*-methylcytidine [10], inhibited HEV RNA replication (IC_{50} 3.5 µM and 22 µM, respectively; data not shown). By contrast, the non-nucleoside HCV polymerase inhibitor nesbuvir [11] as well as the HIV/hepatitis B virus reverse transcriptase/DNA polymerase inhibitors lamivudine and tenofovir did, as expected, not show any effect on HEV replication (Fig. 1C and Suppl. Fig. 2).

Next, the inhibitory potential of SOF was investigated in the full-length infectious p6 clone [12]. To this end, S10-3 cells were transfected with *in vitro*-transcribed full-length HEV p6 RNA. As the peak of HEV replication is reached 6 d post-transfection [12], inhibitors were added at day 4 post-transfection after which cultures were further incubated for 48 h. Total RNA and protein were analyzed by RT-qPCR and immunoblot, respectively. In agreement with the results obtained in the subgenomic replicon system, IFN-α, RBV and SOF, but not nesbuvir, markedly decreased both HEV RNA levels (Fig. 1D) and the expression of ORF2 protein (Fig. 1E).

Next, we assessed the antiviral effect of combinations of different concentrations of SOF and RBV in HEV3 Rep/Neo cells as well as in human induced pluripotent stem cell-derived hepatocyte-like cells (iHeps) [13, 14] infected with HEV of gt 3 (Suppl. Material and Methods). The addition of RBV allowed for a maximal inhibition of HEV RNA replication at lower concentrations of SOF in both systems (Fig. 2A and C). We demonstrated earlier that the combination of RBV and 2'-*C*-methylcytidine results in an antagonistic effect on HCV replication, which is explained by the fact that RBV results in increased intracellular CTP pools which compete with 2'-*C*-methylcytidine 5'-triphosphate at the polymerase level [15]. However, the combined effect of RBV and SOF on HEV replication was found to be additive in Huh-7-derived replicon cells and in HEV-infected iHeps (Fig. 2B and D). This additive effect was confirmed in the transient replication assay (Suppl. Fig. 3).

In conclusion, using both selectable and transient subgenomic HEV replicons, an infectious cell culture system, and two different cellular systems, we demonstrate that SOF, an approved drug that is successfully used in the treatment of HCV infection, inhibits HEV gt 3 replication *in vitro* and results in an additive effect when combined with RBV. The anti-HEV activity of SOF is markedly less pronounced than the anti-HCV activity of the compound (IC_{50} ranging from 0.014 to 0.11 µM depending on replicon genotype) [16]. However, the concentrations of SOF reached in the liver may be sufficiently high to contribute, together with RBV, to inhibition of HEV replication. Given the additive *in vitro* antiviral effect of both drugs, a combination of the two may result in a faster and more efficient inhibition of HEV replication in immunocompromised patients with chronic hepatitis E as compared to RBV monotherapy.

Acknowledgements

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Legends to Figures

Figure 1. Inhibition of HEV RNA replication in a selectable subgenomic replicon system and an infectious clone. (**A**) Schematic representation of the HEV genome and the selectable subgenomic replicon (Suppl. Materials and Methods). 7mG, 7-methylguanosine. (**B**) Northern blot analysis of HEV3 Rep/Neo cells. Total RNA was extracted from parental S10-3 cells (-) or HEV3 Rep/Neo cells (+) and analyzed by Northern blot using ^{32}P-labelled neomycine phosphotransferase- or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probes (upper and lower panels, respectively). The positions of 28 *S* and 18 *S* ribosomal RNA are indicated. (**C**) Effect of various antiviral agents on HEV RNA replication in HEV3 Rep/Neo cells. HEV RNA (solid circles) and cell viability (triangles) were determined by RT-qPCR and the Cell Proliferation Reagent WST-1 (Roche, Basel, Switzerland), respectively, after 48 h of treatment with increasing concentrations of the indicated antiviral agents. Results are expressed as % relative to the carrier-treated control. Each data point represents the mean ± SEM of at least 3 independent experiments performed in triplicate each. (**D**) HEV RNA in S10-3 cells transfected with the full-length p6 clone. Non-transfected (nt) and cells transfected with full-length p6 (wt) or replication-defective control (GAD) RNA were treated for 48 h with carrier alone (-), 100 IU/ml interferon-α (IFN), 100 µM ribavirin (RBV), 10 µM nesbuvir (Nes) or 10 µM sofosbuvir (SOF). Results are expressed as % relative to the carrier-treated control. Each data point represents the mean ± SEM of at least 2 independent experiments performed in duplicate each. (**E**) ORF2 protein expression. Lysates from cells treated as in (D) were analyzed by immunoblot for the HEV ORF2 (capsid) protein and β-actin. The lower panel represents the mean ± SEM of two independent experiments.

Figure 2. The combination of sofosbuvir and ribavirin results in an additive antiviral effect on HEV RNA replication. (**A, C**) Effect of the combination of various concentrations of sofosbuvir (SOF) and ribavirin (RBV) on HEV RNA replication in (A) HEV3 Rep/Neo cells and (C) HEV gt 3-infected human induced pluripotent stem cell-derived hepatocyte-like cells (iHep). HEV RNA levels were quantified by RT-qPCR after 48 h of treatment with increasing concentrations of SOF (0, 0.1, 1, 10 µM) and RBV (0, 1, 10, 100 µM). Results are expressed as % relative to the carrier-treated control. Each data point represents the mean ± SEM of 2 independent experiments. (**B, D**) Synergy plot representing the % antiviral activity above or below the expected activity for the RBV-SOF combination based on the data depicted in panels A and C, respectively.

References

1. Hoofnagle JH et al. N Engl J Med 2012; 367: 1237-1244.
2. Dalton HR et al. Curr Opin Infect Dis 2013; 26: 471-478.
3. Kamar N et al. Clin Microbiol Rev 2014; 27: 116-138.
4. Behrendt P et al. J Hepatol 2014; 61: 1418-1429.
5. Debing Y et al. Antiviral Res 2014; 102: 106-118.
6. Kamar N et al. N Engl J Med 2014; 370: 1111-1120.
7. **Debing Y**, **Gisa A** et al. Gastroenterology 2014; 147: 1008-1011.
8. Debing Y et al. Antimicrob Agents Chemother 2014; 58: 267-273.
9. Pawlotsky JM. Gastroenterology 2014; 146: 1176-1192.
10. Carroll SS et al. J Biol Chem 2003; 278: 11979-11984.
11. Howe AY et al. Antimicrob Agents Chemother 2008; 52: 3327-3338.
12. **Shukla P**, **Nguyen HT** et al. J Virol 2012; 86: 5697-5707.
13. Wu X et al. PLoS Pathog 2012; 8: e1002617.
14. **Schwartz RE, Trehan K** et al. Proc Natl Acad Sci USA 2012; 109: 2544-2548.
15. Coelmont L et al. Antimicrob Agents Chemother 2006; 50: 3444-3446.
16. Sofia MJ et al. J Med Chem 2010; 53: 7202-7218.

Author names in bold designate shared co-first authors.

Supplementary Material

Material and Methods

Antiviral agents

Interferon-α (IFN-α) was purchased from Roche Pharmaceuticals (Basel, Switzerland), ribavirin and lamivudine from Sigma-Aldrich (St. Louis, MO), sofosbuvir from Alsachim SAS (Illkirch-Graffenstaden, France) and nesbuvir from Selleck Chemicals (Houston, TX). Tenofovir was a kind gift of Gilead Sciences (Foster City ,CA). All compounds except IFN-α were dissolved in DMSO.

Plasmids and cells

Plasmids encoding the HEV genotype 3 Kernow-C1 p6 strain, the genotype 1 Sar55/S17/luc replicon and S10-3 cells were a kind gift from Suzanne U. Emerson, National Institutes of Health, Bethesda, MD [1, 2]. S10-3, Huh-7 and HepG2/C3A cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Life Technologies). HEV3 Rep/Neo cells were cultured in DMEM supplemented with 10% FBS and 500 µg/ml G418 (Life Technologies).

The subgenomic HEV replicon construct harboring a neomycin phosphotransferase selection gene was derived from plasmid p6. Two-step PCR using primers HEVgt3-4487-fd (5'-CCCGTGGTTCCGTGCCATTG-3') and HEV-Neo-rv (5'-AATCCATCTTGTTCAATCATGGTGATCCCATGGGCGATG-3') as well as HEV-Neo-fd (5'-CATCGCCCATGGGATCACCATGATTGAACAAGATGGATT-3') and Neo-rv (5'-CGCAGAATAGCACCACGTGTTAGAAGAACTCGTCAAGAA-3') was performed on plasmids p6 and pcDNA3.1, respectively, followed by overlap extension PCR, digestion of the amplification product with *Afl*II and *Pml*I, and cloning into plasmid p6, yielding plasmid p6/Neo.

The full-length HEV p6 genome carrying a GAD mutation in the polymerase active site was prepared similarly. Two-step PCR using primers HEVgt3-4487-fd (see above) and HEVp6-GAD-rv (5'-AGGACCACCGAATCAGCACCCTTAAAGGC-3') as well as HEVp6-GAD-fd (5'-GCCTTTAAGGGTGCTGATTCGGTGGTCCT-3') and HEV-5897-rv (5'-GATGCCTCAGTAGCCATGAT-3') was performed on plasmid p6, followed by overlap extension PCR, digestion of the amplification product with *Afl*II and *Pml*I, and cloning into plasmid p6, yielding plasmid p6/GAD.

Northern blot

Total RNA was extracted from HEV3 Rep/Neo and parental S10-3 cells using the ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI). Twenty µg of total cellular RNA each were separated by 1% denaturing agarose gel electrophoresis, followed by transfer onto a nitrocellulose membrane (Life Technologies) and Northern blot using a ^{32}P-labelled neomycine phosphotransferase- or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific probe.

Measurement of antiviral activity in HEV replicon cells

HEV3 Rep/Neo cells were seeded into 24-well plates at a density of 8 x 10^4 cells per well. Antiviral agents were added the next day. Fourty-eight h later, cells were washed with PBS and total RNA was extracted as above, followed by reverse transcription using the PrimeScript RT Reagent Kit (Takara, Shiga, Japan). HEV positive-strand RNA and GAPDH expression were quantified with specific primers (HEV3-4740fd 5'-CAGAGCCGCAATGCGGCAGC-3' and HEV3-4968rv 5'-ATCAACACAGACCTGCGCAAC-3' or GAPDH-fd 5'-GAAGGTGAAGGTCGGAGTC-3' and GAPDH-rv 5'-GAAGATGGTGATGGGATTTC-3', respectively) using the SYBR Select Master Mix (Life Technologies) in a StepOne Real-Time PCR cycler (Life Technologies). GraphPad Prism6 software was used to fit response curves with nonlinear regression analysis and to calculate IC_{50} values.

Transient antiviral assays using the gt 3 p6/luc and gt1 Sar55/S17/luc replicons, combination studies with p6/luc and assessment of cell viability through the MTS/PMS method were performed as described previously [3].

Data from combination studies were analyzed with the MacSynergy II template [4].

Viral RNA preparation and transfection

HEV RNA was *in vitro* transcribed from *Mlu*I-linearized plasmids p6/Neo, p6 or p6/GAD by the use of the mMESSAGE mMACHINE Kit (Life Technologies). Two-hundred ng of *in vitro* transcribed viral RNA was transfected into 10^5 S10-3 cells per well of a 12-well cell culture plate using the TransIT-mRNA transfection kit (Mirus Bio LLC, Madison, WI). Cells transfected with the subgenomic replicon construct were subjected to selection with 500 µg/ml G418 (Life Technologies) 3 d post-transfection. Cells transfected with the full-length HEV RNA were washed 4 d later and antiviral agents added for 48 h prior to cell lysis. RNA and protein levels were analyzed by RT-qPCR and immunoblot, respectively.

Generation of human induced pluripotent stem cell-derived hepatocyte-like cells

Human induced pluripotent stem cells were differentiated into hepatocyte-like cells as described previously [5], with minor modifications. In brief, the base defined medium (DM) consisted of RPMI 1640, 1% B27 serum-free supplement, and 0.5% non-essential amino acids (all from Life Technologies). To induce endoderm differentiation, an iPS.C3A cell subclone [5, 6] was harvested with gentle cell dissociation reagent (Stemcell Technologies, Vancouver, Canada) and plated into Matrigel-coated culture dishes (Corning, New York, NY) in mTeSR1 medium (Stemcell Technologies). The next day, culture medium was changed to medium A (DM/activin-A/basic fibroblast growth factor [bFGF]/Wnt-3A) for 24 h, followed by 72 h of culture in medium B (DM/activin-A/bFGF). To induce hepatic differentiation, definitive endoderm cells were re-seeded and cultured in the presence of medium C (DM/bone morphogenic protein 4 [BMP-4]/bFGF) for five days and then in the presence of medium D (DM/epidermal growth factor [EGF]/hepatocyte growth factor [HGF]) for five more days. Finally, cells were matured in Hepatocyte Culture Medium (Lonza, Basel, Switzerland) supplemented with oncostatin-M for five to seven days before being used for infection.

Activin-A, Wnt-3A and oncostatin-M were purchased from R&D Systems (Minneapolis, MN), bFGF was from Life Technologies, and BMP-4, EGF and HGF were purchased from Peprotech (Rocky Hill, NJ).

HEV infection of iHeps

Full-length HEV genotype 3 Kernow-C1 p6 strain RNA was prepared and transfected into S10-3 cells as described above. Six days post-transfection S10-3 cells were frozen and thawed three times to release infectious HEV, followed by centrifugation for 2 min at 10,000 x *g*. to remove cell debris. iHeps were infected using the HEV-containing recovered S10-3 cell lysate for 12-16 h, followed by removal of the inoculum by thorough but gentle washes with culture medium (Wu X, Dao Thi VL *et al*., manuscript in preparation). Antiviral agents were added to the culture medium 3 d post-infection. Cells were harvested for RT-qPCR analysis at 3 d post-treatment with antiviral agents.

Immunoblot

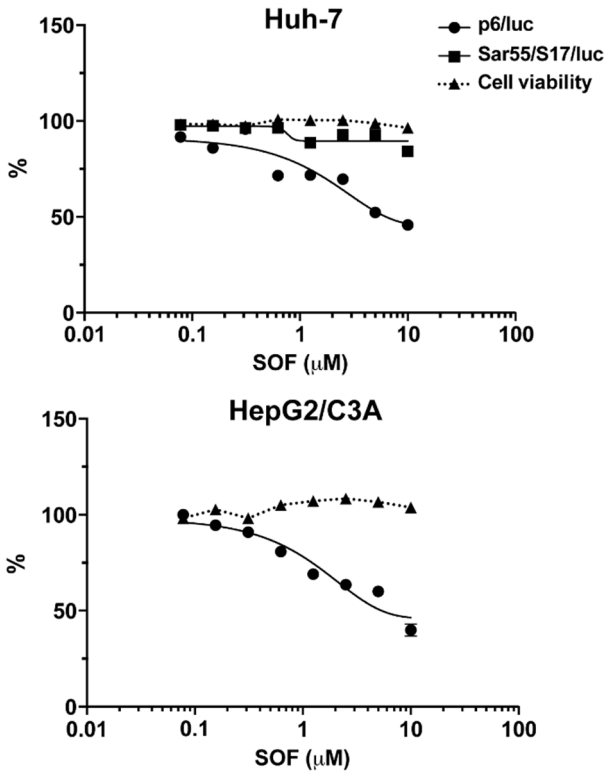
Cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by transfer onto polyvinylidene fluoride membrane (Merck-Millipore), as described previously [7]. HEV ORF2 protein was detected using monoclonal antibody 1E6

(Merck-Millipore) and β-actin using monoclonal antibody AC-74 (Sigma-Aldrich). ORF2 band intensities were quantified using ImageJ software.

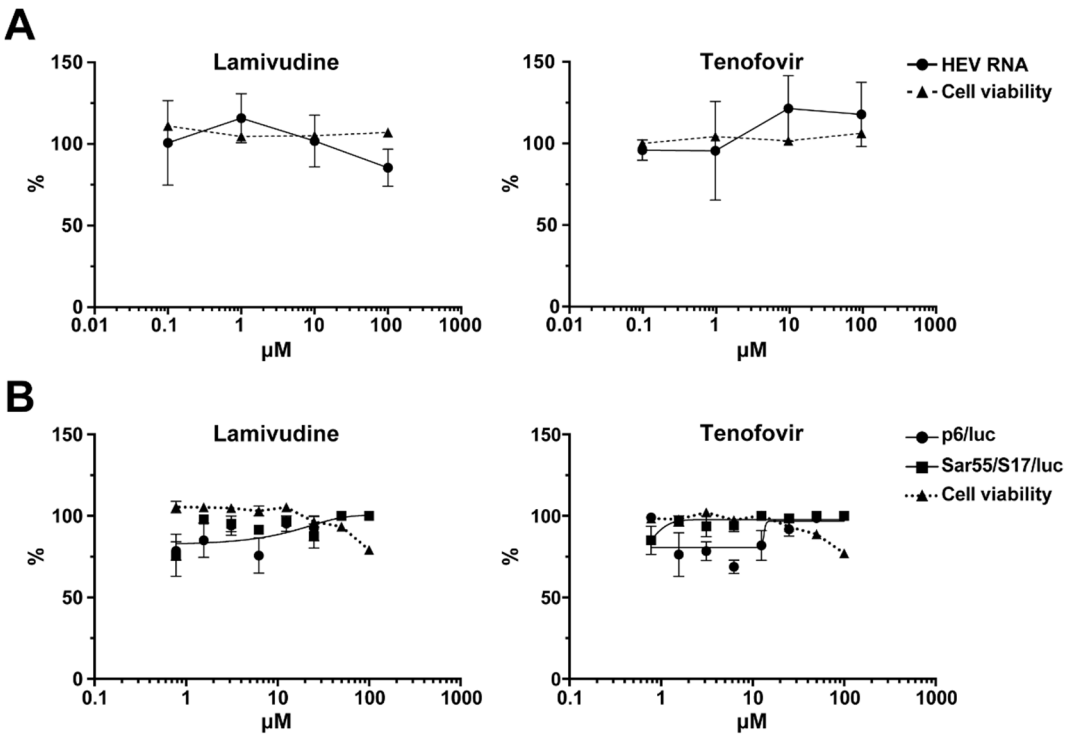
Supplementary references

1. **Shukla P**, **Nguyen HT** et al. J Virol 2012; 86: 5697-5707.
2. Nguyen HT et al. J Virol 2014; 88: 868-877.
3. Debing Y et al. Antimicrob Agents Chemother 2014; 58: 267-273.
4. Prichard MN et al. Antiviral Res 1990; 14: 181-205.
5. Wu X et al. PLoS Pathog 2012; 8: e1002617.
6. **Si-Tayeb K, Noto FK** et al. Hepatology 2010; 51: 297-305.
7. Moradpour D et al. Hepatology 1998; 28: 192-201.

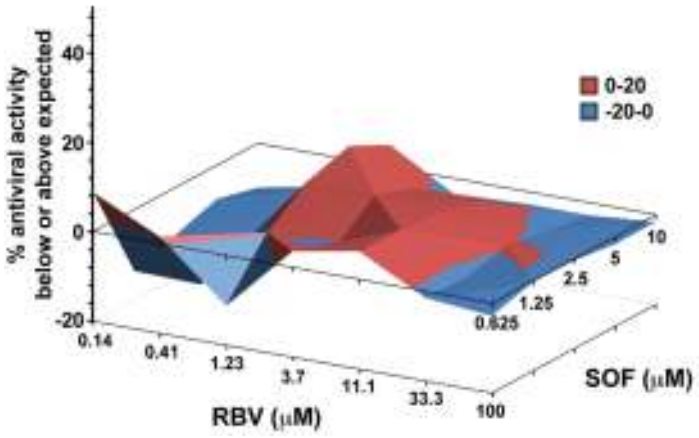
Author names in bold designate shared co-first authorship



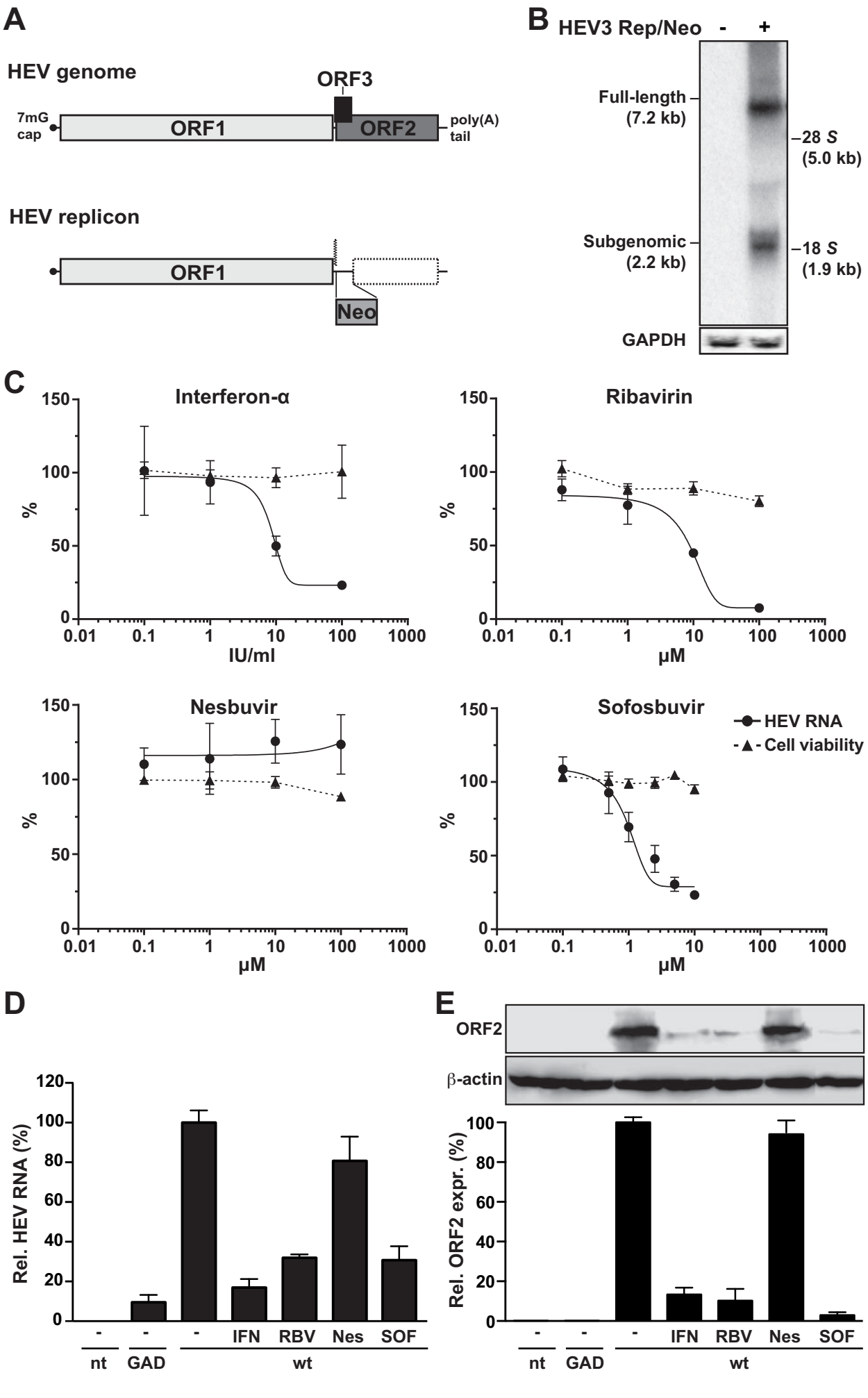
Supplementary Figure 1. Sofosbuvir inhibits replication of a transient HEV gt 3 reporter replicon. Effect of sofosbuvir (SOF) in a transient HEV RNA replication assay. HEV RNA replication in Huh-7 cells transfected with genotype 3 p6/luc and genotype 1 Sar55/S17/luc replicon RNA was determined by *Gaussia* luciferase-derived luminescence and cell viability was assessed using the MTS/PMS method as described previously [3]. Each data point represents the mean ± SEM of 3 independent experiments.



Supplementary Figure 2. Lamivudine and tenofovir do not inhibit HEV replication. (**A**) Effect of lamivudine and tenofovir on HEV RNA replication in HEV3 Rep/Neo cells. HEV RNA (solid circles) and cell viability (triangles) were determined by RT-qPCR and the Cell Proliferation Reagent WST-1 (Roche, Basel, Switzerland), respectively, after 48 h of treatment with increasing concentrations of the indicated antiviral agents. Results are expressed as % relative to the carrier-treated control. Each data point represents the mean ± SEM of 2 independent experiments performed in triplicate each. (**B**) Effect of lamivudine and tenofovir in a transient HEV RNA replication assay. HEV RNA replication in Huh-7 cells transfected with genotype 3 p6/luc and genotype 1 Sar55/S17/luc replicon RNA was determined by *Gaussia* luciferase-derived luminescence and cell viability was assessed using the MTS/PMS method as described previously [3]. Each data point represents the mean ± SEM of 3 independent experiments.



Supplementary Figure 3. The combination of sofosbuvir and ribavirin results in an additive antiviral effect on HEV RNA replication in a transient replication assay. Synergy plot representing the % antiviral activity above or below the expected activity for the combination of sofosbuvir (SOF) and ribavirin (RBV) based on the data depicted in Supplementary Figure 1.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HEV genome
ORF3
7mG cap
ORF1
ORF2
poly(A) tail
HEV replicon
ORF1
Neo
B
HEV3 Rep/Neo
-
+
Full-length (7.2 kb)
28 S (5.0 kb)
Subgenomic (2.2 kb)
18 S (1.9 kb)
GAPDH
C
Interferon-α
Ribavirin
Nesbuvir
Sofosbuvir
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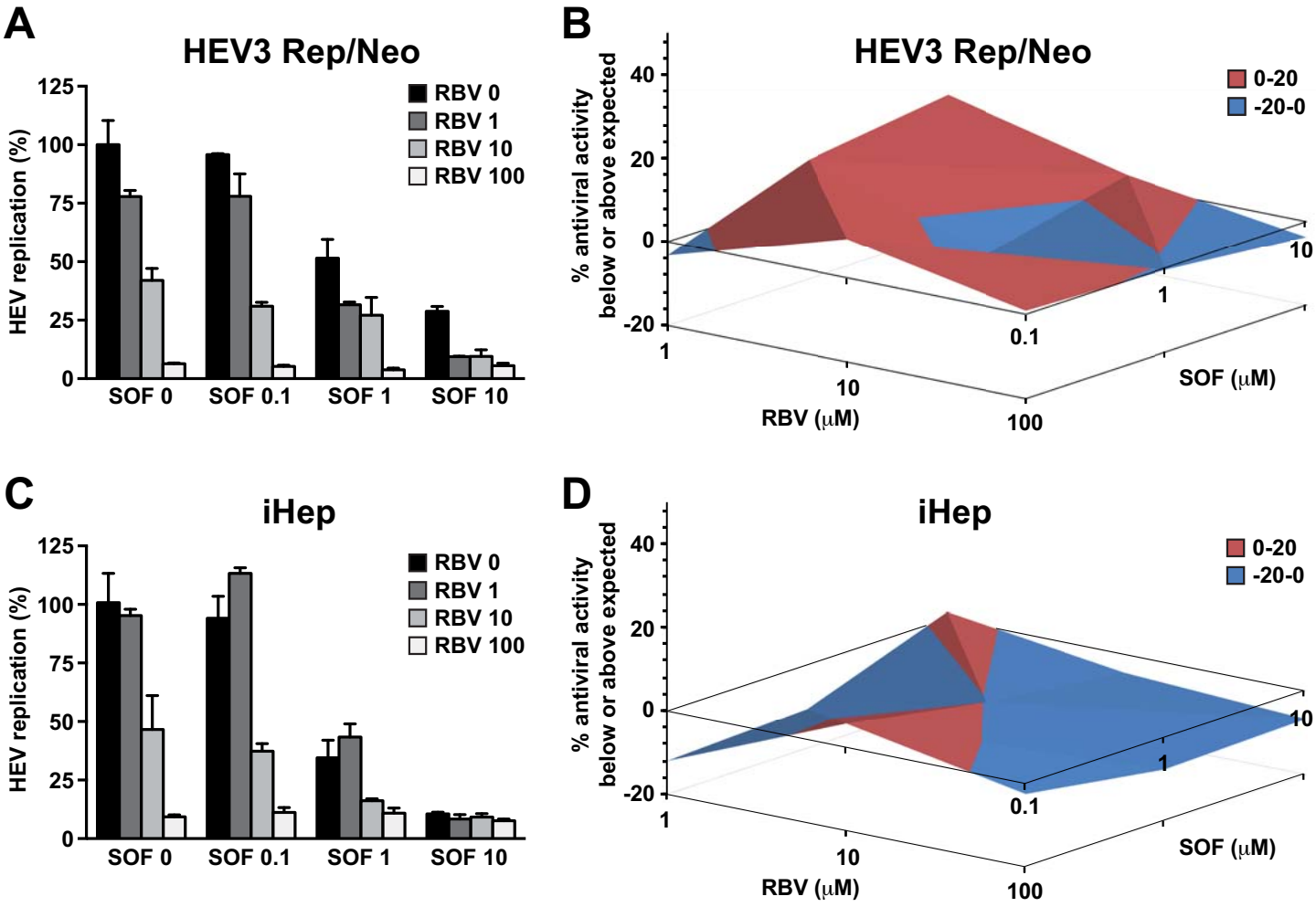
A
HEV3 Rep/Neo
HEV replication (%)
125
100
75
50
25
0
RBV 0
RBV 1
RBV 10
RBV 100
SOF 0
SOF 0.1
SOF 1
SOF 10
B
HEV3 Rep/Neo
% antiviral activity below or above expected
40
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-20
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100
RBV (μM)
0.1
1
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SOF (μM)
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HEV replication (%)
125
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