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Antiviral candidates for treating hepatitis E virus infection

Running: title: Hepatitis E virus antivirals

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Abstract

Globally, hepatitis E virus (HEV) causes significant morbidity and mortality each year. Despite this burden, there are no specific antivirals available to treat HEV patients, and the only licensed vaccine is not available outside of China. Ribavirin and interferon-α are used to treat chronic HEV infections, however severe side effects and treatment failure are commonly reported. Therefore, this study aimed to identify potential antivirals for further development to combat HEV infection. We selected 16 compounds from the nucleoside and non-nucleoside antiviral classes that range in developmental status from late preclinical to FDA-approved, and evaluated them as potential antivirals for HEV infection, using genotype 1 replicon luminescence studies and replicon RNA quantification. Two potent inhibitors of HEV replication included NITD008 (EC_{50} 0.03 µM, CC_{50} >100 µM) and GPC-N114 (EC_{50} 1.07 µM, CC_{50} >100 µM), and both drugs reduced replicon RNA levels in cell culture (>50% reduction with either 10 µM GPC-N114 or 2.50 µM NITD008). Furthermore, GPC-N114 and NITD008 were synergistic in combinational treatment (combination index 0.4) against HEV replication, allowing for dose reduction indices of 20.42 and 8.82 at 50% inhibition, respectively. Sofosbuvir has previously exhibited mixed results against HEV as an antiviral, both *in vitro* and in a handful of clinical applications, however in this study it was effective against the HEV genotype 1 replicon (EC_{50} 1.97 µM, CC_{50} >100 µM) and reduced replicon RNA levels (47.2% reduction at 10 µM). Together these studies indicate drug repurposing may be a promising pathway for development of antivirals against HEV infection.

Keywords

Hepatitis E virus, direct-acting antivirals, hepatitis therapy development, broad-spectrum antivirals

Introduction

Hepatitis E virus (HEV) is a clinically important emerging virus, causing significant global morbidity and approximately 20 million infections, 44,000 deaths and 3,000 stillbirths each year (1, 2). Despite this substantial disease burden, currently no specific antivirals exist for treating HEV infections, and the sole licensed vaccine (Hecolin®) is only available in China (3). Ribavirin and pegylated interferon-α (PEG-IFN-α) are often used as therapies for chronic HEV infections (4-6), however, treatment failure is common (7-9) and accompanied by severe side effects (6, 10). Therefore, further research is needed to identify safe and effective antivirals to treat HEV patients.

Recovery from acute HEV infection is often protracted, with typical hepatitis symptoms persisting for approximately 4-6 weeks (11). Fulminant hepatitis from HEV infection can prove fatal, with mortality rates reported between 0.5-4%, depending on the geographical region (12-14). Higher mortality rates of up to 27% have been reported in pregnant women (14, 15), with HEV infection particularly severe in the third trimester, and fulminant hepatitis often resulting in stillbirth and maternal death (16, 17). Although HEV infection is usually self-limiting, persistent infections have been frequently reported in immunosuppressed and immunocompromised patients (18-22).

HEV is classified within the *Hepeviridae* family, which is thought to have arisen from an ancient recombination event between viruses from different positive sense RNA superfamilies (23). HEV comprises a single-stranded RNA genome, encapsidated within a 27-34 nm icosahedral, non-enveloped virion (24). The HEV genome is around 7.2 kb, with a 5' cap and a 3' polyadenylated tail (25, 26). The genome consists of three open reading frames (ORFs), with ORF1 encoding the non-structural proteins, ORF2 the capsid protein, while ORF3 is a phosphoprotein thought to act as a viroporin to facilitate viral release from the host cell (27, 28).

Every stage of the HEV replication cycle can be exploited for antiviral design, as many of the non-structural proteins are essential for viral replication, including the RNA-dependent RNA polymerase (RdRp), which makes them ideal antiviral targets. Therapies targeting the RdRp have proven highly successful, with several hepatitis C virus (HCV) antivirals FDA approved for treatment [reviewed in (29)].

The RdRp is highly conserved across all RNA viral families, forming the canonical protein structure resembling a closed right hand, with fingers, palm and thumb domains (30, 31). Two classes of antivirals are employed to target the RdRp; nucleoside analogs (NAs) and non-nucleoside inhibitors (NNIs).

HEV is a largely understudied virus and antiviral development has been hampered by poor viral replication levels in cell culture systems (32), and difficulties purifying the viral polymerase in its active form (25, 33). As such, several HEV replicons have been constructed from various infectious clones (34-38), which have allowed for effective preclinical screening of antiviral candidates (39-41).

As many antivirals have been successfully developed against other viruses such as HCV, repurposing these compounds as potential therapies against emerging infections such as HEV should be considered. This study aimed to identify broad-spectrum antiviral candidates to combat HEV infection. All compounds examined in this study were previously developed against other viruses (Table 1), but have not been reported against HEV before, with the exception of Sofosbuvir. We employed a subgenomic replicon approach to screen 16 compounds belonging to the NA or NNI inhibitor classes of antivirals, and identified three potent compounds that were profiled for dose-responsiveness and cytotoxicity, two of which were further examined for combinational synergism.

84 The potent compounds identified in this work provide a promising platform for the development of
85 antivirals to treat HEV infections.

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Antimicrobial Agents and Chemotherapy

Results

Polymerase-targeted antivirals are examined for anti-HEV activity

Sixteen compounds (Table 1) were selected for examination of HEV replication inhibition and screened at a fixed concentration of 10 µM against the HEV G1 replicon pSK-HEV-2-Luc (Figure 1A). The NA 2CMC was used as a positive control to demonstrate inhibition of HEV replication (Figure 1B). Antiviral efficacy was quantified by the relative luminescence of treated cells (test compounds at 10 µM) compared to mock-treated cells (vehicle control; 0.5% DMSO vol/vol). Four antivirals including NITD008, GPC-N114, Sofosbuvir and Dasabuvir, demonstrated more than 50% inhibition at 10 µM (Figure 1B). These four antivirals were selected for further examination to ascertain effective dose and cytotoxicity profiles, while the other 12 compounds did not reach the threshold of >50% inhibition at 10 µM and were dropped from further investigation in this study.

Three antivirals exhibit dose-dependent inhibition of HEV replication without cytotoxic effects

As NITD008, GPC-N114, Sofosbuvir and Dasabuvir were identified as potential HEV antivirals in the initial screen (Figure 1B), these compounds were further examined for their effects on cell viability and dose-dependent inhibition of HEV replicon replication.

NITD008 was examined over a concentration range of 0.02 – 2.50 µM, while GPC-N114, Sofosbuvir and Dasabuvir were all examined at 0.16 – 25.00 µM (Figure 2). HEV replication was assessed using relative luminescence production compared to mock-treated cells (0.5% DMSO, vol/vol), while cytotoxicity was quantified using a metabolic activity conversion assay.

NITD008 was a potent inhibitor of HEV replication, with a half maximal effective concentration (EC_{50}) value of 0.03 µM (Figure 2A, Table 2). The half maximal cytotoxic concentration (CC_{50}) was not reached when examined up to 100 µM, indicating a therapeutic index for NITD008 of >3,333 (Table 2).

GPC-N114 was also an effective inhibitor of HEV *in vitro*, giving an EC_{50} of 1.07 µM (Figure 2B, Table 2). Huh7 cell viability started to drop at the higher compound concentrations (77.2% of mock-treated cells at 25 µM), however, the CC_{50} was not reached with concentrations up to 100 µM, giving a therapeutic index of >93.

Dasabuvir demonstrated dose-responsive inhibition of HEV replication with an EC_{50} of 1.79 µM (Figure 2C, Table 2), however, evaluation of cell viability indicated that the compound was toxic to Huh7 cells over 2.5 µM, with a CC_{50} of 12.28 µM. This resulted in a poor therapeutic index of 6.86, therefore Dasabuvir was dropped from further investigation in this study.

Sofosbuvir efficacy against HEV G1 and G3 replicons *in vitro* has already been published previously, with an EC_{50} 1.20 µM against G3 and >10 µM against G1 (39, 42). These previously reported results for G1 HEV are in contrast to the findings in this study, as Sofosbuvir inhibited the HEV G1 replicon in a dose-dependent fashion with an EC_{50} of 1.97 µM (Figure 2D, Table 2), therefore demonstrating similar results to the G3 HEV replicon published previously (39). No cytotoxic effects on Huh7 cells were observed up to 100 µM, indicating a therapeutic index of >51 (Table 2).

The positive control 2CMC gave an EC_{50} value of 3.04 µM (Table 2) against the HEV G1 replicon, compared to an EC_{50} of 1.60 µM previously reported for the G3 replicon (43), with no effects on cell viability up to 100 µM (therapeutic index >33, Table 2).

HEV RNA levels are reduced by NITD008, GPC-N114 and Sofosbuvir

NITD008, GPC-N114 and Sofosbuvir all exhibited dose-dependent inhibition of HEV replicon-derived luminescence production, without cytotoxic effects (Figure 2, panels A, B and D). As such, these three antivirals were examined further for their abilities to reduce HEV replicon RNA levels *in vitro*. The positive control 2CMC was assessed at 10 µM and compared to mock-treated RNA levels (0.5% DMSO) to demonstrate a reduction in HEV replicon RNA (Figure 2, panels E and F). 2CMC reduced

RNA levels to 43.6% of the mock-treated samples, from 7.7 x 10^5 replicon copies per well to 3.4 x 10^5 copies per well (Figure 2E and 2F). Sofosbuvir (10 µM) reduced HEV RNA levels to 38.5% of the mock-treated wells, from 7.7 x 10^5 replicon copies per well to 3.0 x 10^5 copies per well, while the NNI GPC-N114 (10 µM) also reduced HEV RNA levels to 42.9% of the mock-treated wells, down to 3.3 x 10^5 copies per well (Figure 2E).

NITD008 was the most potent inhibitor of HEV replication identified in Figures 1 and 2, and was therefore investigated at a lower concentration range, from 0.04 – 2.50 µM. NITD008 reduced HEV replicon RNA levels in a dose-dependent fashion from 7.7 x 10^5 replicon copies per well in mock treated samples to 5.3 x 10^5 per well (69.5% of mock) at 0.04 µM, 4.3 x 10^5 replicon copies per well (56.1% of mock) at 0.16 µM and to 2.8 x 10^5 copies per well (36.7% of mock) at 2.50 µM (Figure 2F).

NITD008 and GPC-N114 exhibited synergism in combination against HEV replication

As GPC-N114 and NITD008 were the most potent HEV replication inhibitors identified in the replicon luciferase assays (Figures 2A, 2B), and also effectively reduced HEV replicon RNA levels (Figures 2E, 2F), these two antivirals were selected for combinational studies against HEV replication.

NITD008 is a broad-spectrum chain-terminating adenosine NA, initially developed as an antiviral against dengue virus (DENV), for which the reported EC_{50} is 0.64 – 1.60 µM (44, 45). In contrast, GPC-N114 is a picornavirus NNI, with an EC_{50} range of 0.13 – 5.44 µM, demonstrating broad-spectrum antiviral activity against multiple genera from within the *Picornaviridae* (46). GPC-N114 binds in the RNA channel of picornavirus polymerases (46), while NAs such as NITD008 bind in the polymerase active site for viruses such as DENV (45), therefore they occupy different binding sites in their original target viruses. For these reasons we assessed for combinational synergy against HEV replicon replication levels.

159 The effects of GPC-N114 (0.16-5.00 µM) and NITD008 (0.005-0.16 µM) were examined in a
160 32:1 ratio, respectively, using the G1 HEV replicon. The Chou-Talalay method (47) was used to
161 analyze the inhibition data and Compusyn software was employed to generate the combinational
162 isobologram (Figure 3A). The mean of the combination indices (CIs) at 50%, 75% and 90% inhibition
163 of HEV replicon replication was 0.4, indicating synergism of NITD008 and GPC-N114 in combination
164 (Figure 3A). This allowed for dose reduction indices at 50% inhibition of 20.42 for GPC-N114 and 8.82
165 for NITD008. The cytotoxic effects of the two drugs were also examined in combination, with no
166 effect on Huh7 cell viability up to 100 µM (Figure 3B).

167

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Discussion

HEV is a major cause of liver disease across the globe, resulting in significant morbidity and mortality (1, 2, 22). Despite this burden, HEV is a largely understudied virus and as such, we currently lack specific therapies to combat HEV infection.

The development of safe and effective antivirals to combat HEV is needed to treat both chronically infected patients and also for acute infections, to reduce the lengthy recovery period, and to prevent fulminant hepatitis. Additionally, antivirals could be useful as a prophylactic measure in the case of outbreaks and epidemics, which are often reported in refugee camps (48, 49) and military troops (50-52). Furthermore, if safety and efficacy could be achieved without teratogenic side effects, then treating HEV-infected pregnant women to prevent the significant mortality rates for both mother and child may also be possible.

Current HEV treatment options include the reduction of immunosuppressants for HEV-infected organ transplant patients as the first choice of action, followed by courses of PEG-IFN-α and/or ribavirin (53). The reduction of immunosuppressants is effective in clearing the virus in around 30% of solid organ transplant cases (22), leaving around 70% of patients requiring further action. PEG-IFN-α cannot be used in most organ donor recipients due to the risk of transplant rejection, therefore ribavirin is prescribed in the majority of cases (9). Ribavirin monotherapy is usually required for at least three months, and results in significant and undesirable side effects including severe anemia (4, 6). Furthermore, ribavirin and PEG-IFN-α are both contraindicated during pregnancy (54).

Clinical resistance to ribavirin resulting in treatment failure has been associated with several HEV G3 polymerase mutations, including Y1320H, K1383N and G1634R, and an insertion into the HEV hypervariable region (8, 9, 55). The effects of these RdRp mutations have been studied *in vitro* using mutant replicons, cell culture of HEV isolates, and deep sequencing, and it was found that HEVs bearing Y1320H and G1634R mutations were still sensitive to ribavirin, but were associated

194 with enhanced replicative capacities, while the effects of K1383N mutations could not be elucidated
195 *in vitro* (9). Further studies using deep sequencing of HEV G3 isolates from chronically infected
196 patients revealed that viral heterogeneity was increased following ribavirin treatment, and that the
197 RdRp C-terminal mutation G1634R was particularly associated with ribavirin treatment failure (56).

198 As the current HEV therapies have several reported issues ranging from drug resistance to
199 severe side effects, further research is warranted to identify safe and efficacious antivirals to treat
200 HEV patients.

201

202 G1 HEV infection results in the highest number of fetal and maternal mortalities, while G3 causes the
203 majority of chronic infections worldwide (22). In addition, G1, G4 and G7 human HEVs have also
204 caused chronic infections in immunocompromised and immunosuppressed patients (18-21), so
205 effective, broad-spectrum therapeutics to combat all human HEV genotypes would be beneficial.

206

207 This study evaluated 16 antivirals (Table 1) previously developed against other viruses, for inhibition
208 of HEV replicon replication *in vitro*. Four of the 16 antivirals examined inhibited the HEV G1 replicon
209 with more than 50% inhibition at 10 µM (Figure 1B). Of these four antivirals, three exhibited potent,
210 dose-dependent and nontoxic antiviral activity against HEV replicon replication in Huh7 cells (Table
211 2), with EC_{50} values of 0.03 µM for NITD008 (Figure 2A), 1.10 µM for GPC-N114 (Figure 2B) and 1.97
212 µM for Sofosbuvir (Figure 2D). Moreover, the same three antivirals effectively reduced HEV replicon
213 RNA levels (Figures 2E-F), albeit not as efficiently as observed in the luciferase assays. However,
214 discrepancies in antiviral efficacy examined by RNA quantification compared to luciferase assays are
215 widely reported (57-61) and the trend of inhibition of HEV replication was the same across the two
216 methodologies.

217

Two HCV NAs, 2CMC and Sofosbuvir, have already been evaluated for antiviral efficacy against HEV (43). The HCV developmental drug 2CMC is a chain terminating NA, and has been shown to inhibit the G3 HEV replicon *in vitro*, with a half maximal effective concentration (EC_{50}) of 1.6 µM (43). However, development of the oral prodrug for 2CMC (Valopicitabine) was halted following reports of undesirable side effects, and further development of this NA as an antiviral is now unlikely.

The HCV NA Sofosbuvir has also been previously evaluated against HEV G1 and G3 replicons *in vitro*, with inconsistent reports of antiviral efficacy (39, 42). One study reported efficacy of Sofosbuvir against the HEV G3 replicon, with an EC_{50} of 1.2 µM, but it was unexpectedly ineffective against the G1 replicon at concentrations up to 10 µM (39). However, another study reported Sofosbuvir to be ineffective against both G1 and G3 replicons *in vitro*, even at concentrations as high as 10 µM (42). In both these studies where Sofosbuvir had no effect on G1 HEV, the Sar55/S17/luc replicon was utilized, which harbors an insertion from the human ribosomal S17 protein within the hypervariable region (37, 62). This S17 insertion dramatically enhances replication levels (37), and as such, likely renders it less susceptible to Sofosbuvir inhibition compared to wildtype viruses and other replicons which lack this insertion, as previously observed with G1634R-associated ribavirin failure (63, 64).

In contrast to previous reports, in the present study using the pSK-HEV2-Luc replicon, we show that Sofosbuvir can inhibit HEV G1 replication *in vitro*, and further investigation as a potential HEV antiviral may be warranted if known mutations associated with enhanced replication are absent. The discrepancies between the observed effects of Sofosbuvir against HEV G1 in this study compared to previous studies may also be attributed with the use of a stable replicon cell line versus the transient replicon. However, previous studies using both stable and transient HEV replicons to assess the antiviral effects of IFNα or ribavirin have revealed very similar results, indicating that these systems are comparable for screening potential antivirals (4, 39). Additionally, Sofosbuvir may demonstrate

variable antiviral efficacy across the different HEV genotypes, and further preclinical work would be required to ascertain its cross-genotypic activities.

Clinically, Sofosbuvir has been used in a handful of individual cases with or without ribavirin to treat HEV G3 infected patients with variable success (63, 65-68), ranging from a reduction of HEV RNA to undetectable levels (63, 66), through to treatment failure or relapse following treatment (65, 67, 68). These mixed reports of efficacy have raised questions around the pursuit of Sofosbuvir as a HEV antiviral (69), indicating that further work is required to ascertain its suitability as an anti-HEV therapeutic. Clinical treatment failure of Sofosbuvir has been attributed to the HEV phylogenetic subtype, patient immune status (63) and presence of HEV RdRp mutations known to confer increased replication levels that cause ribavirin treatment failure, particularly G1634R (63, 64).

In contrast to Sofosbuvir, the antiviral efficacy of GPC-N114 has not been evaluated against HEV replication before. Under preclinical development as a picornavirus antiviral, GPC-N114 has broad-spectrum activity against multiple viruses from the *Picornaviridae*, with potency ranging from 0.13 µM against human enterovirus 71 through to 5.44 µM against mengovirus (46). It has been reported to affect the viral RNA-template duplex from binding to the picornavirus RdRp template channel, thus inhibiting replication (46).

Similarly, NITD008 has also not been previously evaluated as a HEV antiviral. A broad-spectrum NA, NITD008 effectively inhibits the replication of a number of viruses, including the Norwalk (human norovirus) replicon (unpublished data), enterovirus 71 (70, 71), and several flaviviruses, such as DENV (44, 45), HCV (72), tick-borne encephalitis (73), Zika virus (57), and Japanese encephalitis virus (74). Reported potency ranges between 8.7 nM against HCV G2a (72), and 3.31 µM in the tick-borne flavivirus Alkhurma hemorrhagic fever virus (73).

265 During development of NITD008 against DENV, toxic side effects were observed after two
266 weeks of *in vivo* treatment in rats and dogs (45). This reported NITD008 toxicity might not be
267 clinically significant for a short-term therapeutic course to treat human HEV infection, however
268 reduced treatment doses may in turn reduce the risk of toxic side effects. As such, NITD008 and
269 GPC-N114 were evaluated together for combinational synergy.

270 NITD008 and GPC-N114 were synergistic in combinational treatment against HEV G1
271 replicon-derived luminescence levels (Figure 3A: mean combination index of 0.4). This suggests that
272 the two compounds occupy different HEV binding sites, as they do in their original target viruses,
273 and allowed for dose reductions to achieve the same antiviral effect *in vitro*. Combinational therapy
274 is of great importance to overcome viral resistance to direct-acting antivirals in addition to allowing
275 dose-reductions of drugs to reduce side effects. Combinations of at least two therapeutics have
276 been used to successfully treat many HCV and HIV patients [reviewed in (75-77)] and should be
277 considered for HEV antivirals to safeguard against viral evolution that can confer drug resistance.

278

279 In this study, we have identified two novel HEV antiviral candidates, NITD008 and GPC-N114, that
280 demonstrated potent antiviral activity and combinational synergy against G1 HEV *in vitro*. These
281 compounds could provide useful scaffolds for further antiviral development against HEV infection.
282 Additionally, we have shown that in the absence of known polymerase mutations that confer
283 increased replication levels, Sofosbuvir demonstrates antiviral efficacy against G1 HEV replicon
284 replication.

285 Further preclinical evaluation is required for these compounds as potential HEV antivirals
286 before clinical assessment is undertaken. Firstly, it would be prudent to test NITD008, GPC-N114 and
287 Sofosbuvir against other human-infecting HEV genotypes (G3, G4, and G7) using replicons and live
288 virus in culture where available, to ascertain the cross-genotypic activities of these compounds.

Secondly, structure-activity relationship studies for these antivirals may reveal structural moieties that confer antiviral activity, allowing for the development of more potent and less toxic derivatives, particularly in the case of NITD008 as a potential HEV therapeutic. Thirdly, longer term treatment could be evaluated in cell culture to ascertain toxicity over time and to reveal if any resistance mutations arise. Finally, further assessment of the potential synergy between NITD008 and GPC-N114 against HEV in humanized mice, or another animal model such as swine, may be able to confirm the potential to reduce the required dose for the same non-toxic antiviral effects as we observed in this study. These compounds represent promising candidates for further HEV antiviral development to combat this pervasive virus.

299 ## Methods and materials

300 **Test compounds**

301 Compounds examined in this study included Dasabuvir, Sofosbuvir and Favipiravir
302 (MedChemExpress New Jersey, NJ, USA), GPC-N114 (kind gift from Gerhard Pürstinger (46), formerly
303 University of Innsbruck, Innsbruck, Austria), JTK-109 (Dalton Pharma Services, Toronto, Canada),
304 Lomibuvir (Selleckchem, Houston, TX), Nesbuvir and Tegobuvir (Haoyuan Chemexpress, Shanghai,
305 China), NITD008 (collaboration with Subhash Vasudevan, Duke-NUS Graduate Medical School,
306 Singapore), Quercetagetin and 7-deaza-2'-C-methyladenosine (Santa Cruz Biotechnology, Dallas, TX),
307 Filibuvir and Setrobuvir (Acme Biosciences, Palo Alto, CA), Beclabuvir and TMC-647055 (Taizhou
308 Crene Biotechnology, Zhejiang, China), Triazavirin (Mcule, Palo Alto, CA) and 2'-C-Methylcytidine
309 (2CMC; Sigma-Aldrich, St. Louis, MO). All compounds were >95% pure and dissolved in 100%
310 dimethyl sulfoxide (DMSO), then freshly diluted on the day of the experiment. Details of the 16
311 compounds examined in this study are shown in Table 1.

312

313 **HEV replicon**

314 The HEV pSK-HEV-2-Luc replicon plasmid was a kind gift from Sue Emerson (National Institute of
315 Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The pSK-HEV-2-Luc
316 replicon is based on a human genotype (G) 1 HEV infectious clone (Sar55, accession number
317 AF444002.1) within the ORF2 capsid gene (nucleotides 5148-5816) disrupted by a luciferase reporter
318 gene (38).

319

320 **Transcription of HEV replicon**

321 The HEV replicon plasmid was linearized with BglII and RNA synthesized using mMessage *in vitro*
322 transcription kits (Ambion, Austin, TX). Unless stated otherwise, 100 µl capping transcription

reactions contained approximately 7.5 µg linearized template, 10 µl of 10x transcription buffer, 50 µl of 2x capping dNTP mix (containing 15 mM of ATP, CTP and UTP, 3 mM of GTP and 12 mM of cap analog), 5 µl of 30 mM GTP, and 10 µl of the T7 RNA polymerase mix, and were incubated for 2 hours at 37°C. Reactions were then DNAse treated for 15 min at 37°C and RNA purified using RNeasy kits (Qiagen, Hilden, Germany). RNA integrity was confirmed using agarose gel electrophoresis and quantified using spectrophotometry before transfection.

Cell culture

The human hepatoma Huh7 cell line was a kind gift from Mark Douglas (Westmead Institute for Medical Research, Sydney, Australia). Cell culture was carried out as previously described (78). Briefly, Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1x Glutamax (Life Technologies), 10mM HEPES buffer (ThermoFisher Scientific, Waltham, MA) and 100 U/ml penicillin-streptomycin (Life Technologies).

Transfection

Huh7 cells were grown to 80% confluency in 96 well plates and media was changed 1 h prior to transfection. HEV RNA transcripts (75 ng/well) were chemically transfected into Huh7 cells using the TransIT-mRNA transfection kit (Mirus Bio LLC, Madison, WI), as per manufacturer's instructions.

Cytotoxicity assays

Compound cytotoxicity was assessed using monolayers of Huh7 cells, seeded into 96-well plates at a density of 5,000 cells/well. The next day, fresh media (without antibiotics) was added 1 h prior to

adding compounds. All compounds were freshly diluted in complete DMEM, added to cells and then incubated for a total of 72 h. Cytotoxicity was quantified using a metabolic conversion assay (CellTiter-Blue; Promega, Madison, WI), as per the manufacturer's instructions, and fluorescence was measured on a Fluostar Optima microplate reader (BMG Labtech, Ortenberg, Germany). Half-maximal cytotoxic concentration (CC_{50}) values were determined by nonlinear regression in GraphPad Prism, version 7.02. Mean values were calculated from quadruplicate datasets from at least two independent experiments.

Luciferase assays

Antiviral activities of test compounds were examined by adding a fixed concentration (10 µM) or increasing concentrations of each drug; 0.02-2.50 µM for NITD008, and 0.16-25.00 µM for GPC-N114, Dasabuvir and Sofosbuvir, to replicon-transfected cells. The NA 2'-C-methylcytidine (2CMC) was used as a positive control at 10 µM to demonstrate inhibition of replicon replication (43). Huh7 cells were seeded into 96-well plates at a density of 5,000 cells/well. The next day, fresh antibiotic-free DMEM was added to the cells 1 h prior to transfection. Compounds were freshly diluted in complete DMEM, added to the cells 4 h post transfection and incubated for 72 h. Mock treated cells were incubated with 0.5% (vol/vol) DMSO, the compound vehicle. Replication of the pSK-HEV-2-Luc replicon was determined by luciferase-derived luminesce, using Luciferase Assay System kits (Promega), as per manufacturer's instructions. Luminescence was measured on a Fluostar Optima microplate reader (BMG Labtech). HEV replicon luminesce levels in treated cells were compared to those from mock-treated cells (0.5% vol/vol DMSO) to calculate the percentage of HEV replication. All compounds were also tested in the absence of the HEV replicon to ensure that they did not interfere with the luciferase signal. Half-maximal effective concentration (EC_{50}) values were determined by nonlinear regression in GraphPad Prism, version 7.02. Mean values were calculated from quadruplicate datasets from at least two independent experiments.

Combinational compound treatment

Synergism was calculated using Compsyn software V1.0 which employs the Chou-Talalay method (47). GPC-N114 (concentration range 0.16-5.00 µM) and NITD008 (concentration range 0.005-0.16 µM) were examined alone and together in a 32:1 ratio, respectively, against the HEV replicon, with luminescence quantified as described above. Data was generated from quadruplicate datasets.

RNA extraction

Viral and cellular RNA was extracted from transfected Huh7 cell monolayers 72 h post transfection with TRIzol LS (Invitrogen, Carlsbad, CA, USA) using phase separation, as per the manufacturer's instructions. RNA was further purified using the RNeasy Mini Kit (Qiagen), which included DNA removal using RNase-free DNase (Qiagen). RNA was quantified using spectrophotometry, and RNA integrity was assessed by agarose gel electrophoresis.

HEV RNA level quantitation

HEV replicon RNA levels were measured from transfected Huh7 cells by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Briefly, cDNA was synthesized using a SuperScript VILO cDNA Synthesis Kit (Life Technologies). Replicon RNA was measured using an iTaq Universal SYBR green Supermix (Bio-Rad, Hercules, CA), as described previously (79). Samples were normalized to the house-keeping gene β-actin and the fold change was analysed using the ΔΔCT method, as described in (80). HEV-specific primers were used for quantitation targeting the RdRp encoding region and included the forward primer (HEV replicon Fwd 5'-TGTCCTGATTGCTGGCTGTG-3') and the reverse primer (HEV replicon Rev 5'-GAGAAGAATTGGGGCCCTGG-3'). Mean values were

394 calculated from triplicate datasets and two independent experiments were performed. All statistical
395 calculations were performed using Graphpad Prism software (v7.02). Data were analysed using an
396 unpaired t-test: $p>0.05$, * = $p\leq0.05$, ** = $p\leq0.01$ and *** = $p\leq0.001$.

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Abbreviations: HCV: hepatitis C virus; HEV: hepatitis E virus; G: genotype; NA: nucleoside analog; NNI: non-nucleoside inhibitor; ORF: open reading frame, PEG-IFNα: pegylated-interferon alpha, RdRp: RNA-dependent RNA polymerase

Author contributions: NEN and PAW conceived the study, DET assisted NEN with experimental design, NEN designed and performed the experiments under the supervision of PAW. SGV and JMM provided expertise and materials. NEN and PAW analysed and interpreted the data. NEN wrote the manuscript with the help of PAW, which was edited by DET, SGV and JMM.

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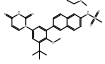
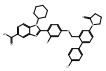
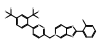
421 ## Figures and Tables

422 **Table 1. Antiviral compounds examined in this study**

Compound class / name	Chemical Structure	Molecular mass (g/mol)	Original target virus	RdRp binding site	Developmental stage	Reference
Non-nucleoside inhibitors						
Beclabuvir (BMS-791325)		659.8	Hepatitis C virus	Thumb I	Phase II clinical trials	(81)
Dasabuvir (ABT-333)		493.6	Hepatitis C virus	Palm I	FDA approved	(82)
Filibuvir (PF868554)		503.6	Hepatitis C virus	Thumb II	Halted after Phase II trials	(83)
GPC-N114		436.8	Picornaviruses	RNA channel	Preclinical	(46)
JTK-109		638.1	Hepatitis C virus	Thumb I	Halted after Phase II trials	(84)
Lomibuvir (VX-222)		445.6	Hepatitis C virus	Thumb II	Halted after Phase II trials	(85)
Nesbuvir (HCV-796)		446.5	Hepatitis C virus	Palm II	Halted after Phase II trials	(86)
Setrobuvir (ANA-598)		560.6	Hepatitis C virus	Palm I	Halted after Phase II trials	(87)
Tegobuvir (GS-9190)		517.4	Hepatitis C virus	Palm β	Halted after Phase II trials	(88)
TMC-647055		606.7	Hepatitis C virus	Thumb I	Halted after Phase II trials	(59)
Triazavirin		228.2	Influenza	ND[B]	Preclinical	(89)
Nucleoside analogs						
2′-C-Methylcytidine[A] (2CMC)		257.2	Hepatitis C virus	Active site	Halted after Phase II clinical trials	(90)
7-deaza-2′-C-methyladenosine (7DMA)		280.3	Hepatitis C virus	Active site	Preclinical	(91)
Favipiravir (T705)		157.1	Influenza	Active site	FDA approved	(92)
NITD008		290.28	Dengue virus	Active site	Preclinical	(45)
Sofosbuvir (PSI-7977)		529.5	Hepatitis C virus	Active site	FDA approved	(93)
Other						
Quercetagetin		318.23	Herpesviruses	ND[B]	Preclinical	(94)

423 [A] Positive control compound
424 [B] ND: Not determined

Table 2. Potency and cytotoxicity of antiviral compounds with efficacy against the HEV G1 replicon

Test Compound	Half maximal effective concentration (EC^{50}) [µM] *(95% CIs[B])*	Half maximal cytotoxic concentration (CC^{50}) [µM] *(95% CIs[B])*	Therapeutic Index (TI) [CC^{50}/EC^{50}]
Dasabuvir	1.79 *(1.38-2.32)*	12.28 *(11.13-13.59)*	6.86
GPC-N114	1.07 *(0.13-1.35)*	>100	>93
NITD008	0.03 *(0.02-0.04)*	>100	>3,333
Sofosbuvir	1.97 *(1.58-2.45)*	>100	>51
2CMC[A]	3.04 *(1.52-4.51)*	>100	>33

[A]Positive control compound

[B]Confidence Intervals

Figure legends:

Figure 1. Screening of broad-spectrum antivirals against the HEV G1 replication. A) A schematic representation of the HEV genome (top) is shown for comparison with the subgenomic replicon (bottom), with non-structural proteins encoded within ORF1 and the structural and phospho-proteins encoded within ORF2 and ORF3 respectively. Nucleotide positions and 5' untranslated regions (UTR) and 3' polyadenylated tails (polyA) are indicated. The HEV G1 subgenomic replicon pSK-HEV-2-Luc (bottom) (38) shows the disruption of the ORF2 capsid gene (nucleotides 5148-5816), with the firefly luciferase gene as described in (38). B) An initial screen of 16 broad-spectrum antivirals (Table 1) for inhibitory activities against the human HEV G1 subgenomic replicon pSK-HEV-2-Luc through quantitation of luminescence is shown. All compounds were examined at a fixed concentration of 10 µM, and the percentage of mock treated HEV replication (compound vehicle only, 0.5% vol/vol DMSO) for each compound are plotted. The black horizontal dotted line represents 100% HEV replication (0% inhibition), while the red dotted line represents 50% HEV

replication (50% inhibition). The NA positive control, 2CMC is used to demonstrate effective inhibition of HEV replication. Mean values are shown ± SEM.

Figure 2. Dose-response curves and cytotoxicity profiles of lead inhibitory compounds against HEV replication. The HEV inhibitory and cytotoxicity effects of four compounds identified in Figure 1B are shown. Dose-response graphs were generated by quantification of luminescence (red bars, left Y axis) and effects on cell viability were examined using a fluorescent resazurin to resorufin assay (blue lines, right Y axis). The black dotted horizontal lines represent 50% inhibition. The EC_{50} and CC_{50} values are shown on the graphs and in Table 2 for compounds A) NITD008 (0.019 µM – 2.5 µM), B) GPC-N114 (0.16 µM – 25.0 µM), C) Dasabuvir (0.16 µM – 25.0 µM), and D) Sofosbuvir (0.16 µM – 25.0 µM). The synthesis of HEV replicon RNA was reduced by three broad-spectrum antivirals compared to the mock-treated control (0.5% DMSO vol/vol), as quantified by qRT-PCR, using primers to detect the HEV RdRp. E) Sofosbuvir and GPC-N114 were examined 10 µM, and F) NITD008 was examined at concentrations 0.04 – 2.50 µM. The NA 2CMC (10 µM) was used as a positive control, and RNA levels were normalized to the housekeeping gene β-actin, while the relative fold of expression was calculated using the ΔΔCt method, *** indicates a *P* value of <0.001, ** <0.01, and * <0.05. Mean values are shown ± SEM.

Figure 3. GPC-N114 and NITD008 exhibited synergistic inhibition of HEV replication. GPC-N114 and NITD008 were examined in combination against HEV replication *in vitro*, quantified by relative luminescence. GPC-N114 (0.16 – 5.00 µM) and NITD008 (0.005 – 0.16 µM) were evaluated in a 32:1 concentration ratio respectively. A) Isobologram of GPC-N114 and NITD008 in combination. Data was analysed using the Chou-Talalay method (Compusyn software) with an average combination index of 0.4 over 50% (blue dots), 75% (red squares) and 90% inhibition (green triangles), indicating

467 synergism. B) Cytotoxicity effects of the two drugs in combination over the same concentration
468 range as A) were assessed using a fluorescent resazurin to resorufin assay. Mean values are shown ±
469 SEM.

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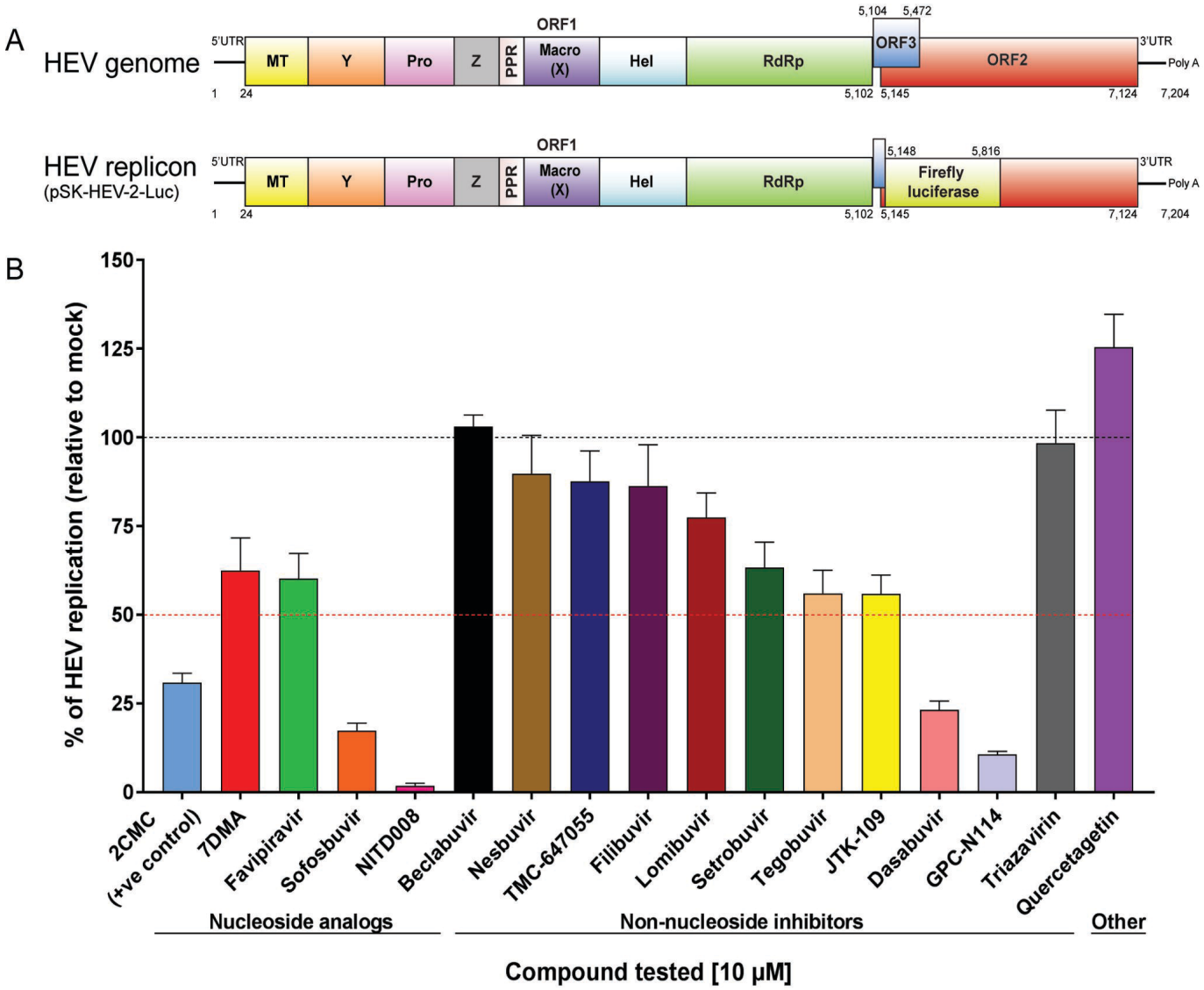

Figure 1. Screening of broad-spectrum antivirals against the HEV G1 replication. A) A schematic representation of the HEV genome (top) is shown for comparison with the subgenomic replicon (bottom), with non-structural proteins encoded within ORF1 and the structural and phospho-proteins encoded within ORF2 and ORF3 respectively. Nucleotide positions and 5' untranslated regions (UTR) and 3' polyadenylated tails (polyA) are indicated. The HEV G1 subgenomic replicon pSK-HEV-2-Luc (bottom) (38) shows the disruption of the ORF2 capsid gene (nucleotides 5148-5816), with the firefly luciferase gene as described in (38). B) An initial screen of 16 broad-spectrum antivirals (Table 1) for inhibitory activities against the human HEV G1 subgenomic replicon pSK-HEV-2-Luc through quantitation of luminescence is shown. All compounds were examined at a fixed concentration of 10 μM, and the percentage of mock treated HEV replication (compound vehicle only, 0.5% vol/vol DMSO) for each compound are plotted. The black horizontal dotted line represents 100% HEV replication (0% inhibition), while the red dotted line represents 50% HEV replication (50% inhibition). The NA positive control, 2CMC is used to demonstrate effective inhibition of HEV replication. Mean values are shown ± SEM

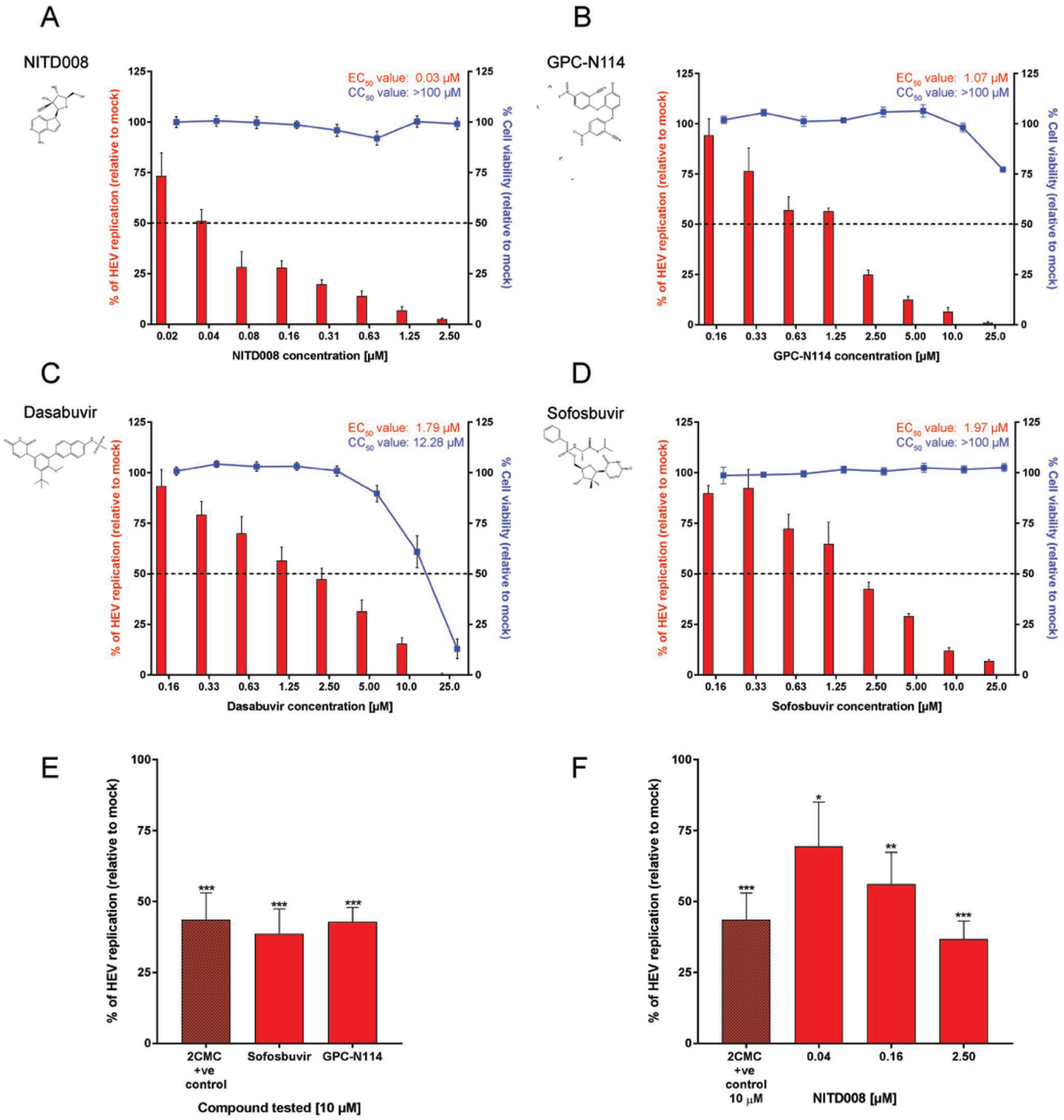

Figure 2. Dose-response curves and cytotoxicity profiles of lead inhibitory compounds against HEV replication. The HEV inhibitory and cytotoxicity effects of four compounds identified in Figure 1B are shown. Dose-response graphs were generated by quantification of luminescence (red bars, left Y axis) and effects on cell viability were examined using a fluorescent resazurin to resorufin assay (blue lines, right Y axis). The black dotted horizontal lines represent 50% inhibition. The EC50 and CC50 values are shown on the graphs and in Table 2 for compounds A) NITD008 (0.019 µM – 2.5 µM), B) GPC-N114 (0.16 µM – 25.0 µM), C) Dasabuvir (0.16 µM – 25.0 µM), and D) Sofosbuvir (0.16 µM – 25.0 µM). The synthesis of HEV replicon RNA was reduced by three broad-spectrum antivirals compared to the mock-treated control (0.5% DMSO vol/vol), as quantified by qRT-PCR, using primers to detect the HEV RdRp. E) Sofosbuvir and GPC-N114 were examined 10 µM, and F) NITD008 was examined at concentrations 0.04 – 2.50 µM. The NA 2CMC (10 µM) was used as a positive control, and RNA levels were normalized to the housekeeping gene β-actin, while the relative fold of expression was calculated using the ΔΔCt method, *** indicates a P value of <0.001, ** <0.01, and * <0.05. Mean values are shown ± SEM.

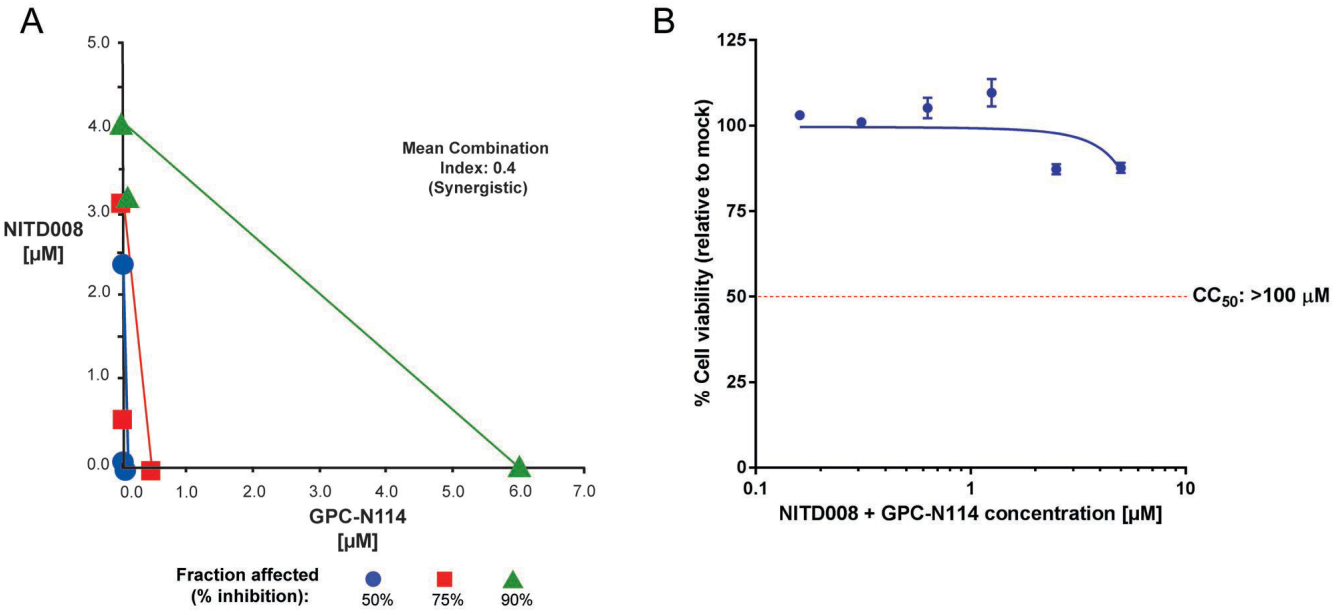

Figure 3. GPC-N114 and NITD008 exhibited synergistic inhibition of HEV replication. GPC-N114 and NITD008 were examined in combination against HEV replication in vitro, quantified by relative luminescence. GPC-N114 (0.16 – 5.00 μM) and NITD008 (0.005 – 0.16 μM) were evaluated in a 32:1 concentration ratio respectively. A) Isobologram of GPC-N114 and NITD008 in combination. Data was analysed using the Chou-Talalay method (Compusyn software) with an average combination index of 0.4 over 50% (blue dots), 75% (red squares) and 90% inhibition (green triangles), indicating synergism. B) Cytotoxicity effects of the two drugs in combination over the same concentration range as A) were assessed using a fluorescent resazurin to resorufin assay. Mean values are shown ± SEM.