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Screening of novel drugs for inhibiting hepatitis E virus replication

Takashi Nishiyama[a], Tominari Kobayashi[a], Suljid Jirintai[a,b], Isao Kii[c], Shigeo Nagashima[a], Putu Prathiwi Primadharsini[a], Tsutomu Nishizawa[a], Hiroaki Okamoto[a,*]

[a] Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine, 3311-1 Yakushiji, Shimotsuke-Shi, Tochigi 329-0498, Japan
[b] Division of Pathology, Department of Basic Veterinary Medicine, Inner Mongolia Agricultural University College of Medicine, Hohhot, Inner Mongolia, China
[c] Common Facilities Unit, Integrated Research Group, Compass to Healthy Life Research Complex Program, RIKEN Cluster for Science, Technology and Innovation Hub, Kobe, Japan

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ABSTRACT

Hepatitis E, which is caused by hepatitis E virus (HEV), is generally a self-limiting, acute, and rarely fatal disease. It is sometimes fulminant and lethal, especially during pregnancy. Indeed, it occasionally takes a chronic course in immunocompromised individuals. To cure hepatitis E patients, the broad-spectrum antivirals (ribavirin and pegylated interferon α) are used. However, this treatment is insufficient and unsafe in some patients due to embryoteratogenic effects, leukopenia, and thrombocytopenia. In this study, we constructed an HEV replication reporter system with *Gaussia* luciferase for comprehensively screening anti-HEV drug candidates, and developed a cell-culture system using cells robustly producing HEV to validate the efficacy of anti-HEV drug candidates. We screened anti-HEV drug candidates from United States Food and Drug Administration-approved drugs using the established HEV replication reporter system, and investigated the selected candidates and type III interferons (interferon λ1-3) using the cell-culture system. In conclusion, we constructed an HEV replicon system for anti-HEV drug screening and a novel cell-culture system to strictly evaluate the replication-inhibitory activities of the obtained anti-HEV candidates. Our findings suggested that interferon λ1-3 might be effective for treating hepatitis E.

1. Introduction

Hepatitis E, caused by hepatitis E virus (HEV), is generally self-limiting and rarely fatal, with a lethality of 0.5%–3% in young adults; however, this rate reaches 30% in pregnant women (Hoofnagle et al., 2012; Nimgaonkar et al., 2018; Wang et al., 2016a, 2016b). HEV belongs to the *Hepeviridae* family, which can be divided into two genera: *Orthohepevirus* and *Piscihepevirus* (Purdy et al., 2017). The genus *Orthohepevirus* includes four species (A–D). *Orthohepevirus A* species includes HEV strains of genotypes 1–8; only genotypes 1–4 and 7 HEVs have far been recognized to infect humans (Purdy et al., 2017). Hepatitis E was believed to be a developing country-specific disease caused by the infection of HEV through the fecal-oral route or from polluted water. Recently, however, sporadic cases of hepatitis E have also been observed in industrialized countries as zoonotic food-borne, transfusion-associated, or organ transplantation disease (Emerson and Purcell, 2013; Meng, 2013)

HEV has an approximately 7.2-kilobase (kb) single-stranded, positive-sense RNA genome. This viral genome contains three open reading frames (ORFs). Each of them encodes a nonstructural polyprotein, ORF1; a capsid protein, ORF2; and a multifunctional protein, ORF3 (Holla et al., 2013; Tam et al., 1991). Among them, the ORF2 and ORF3 proteins are both translated from a bicistronic approximately 2.2-kb subgenomic RNA (Graff et al., 2006). HEV is secreted into the culture media and circulating blood as enveloped viruses with the host-derived lipid coat but is shed into feces in a non-enveloped form (Okamoto, 2013). Hence, it was declared to be a quasi-enveloped virus (Yin et al., 2016).

At present, ribavirin (RBV) and pegylated interferon α (PEG-IFN) are administrated to treat hepatitis E (Kamar et al., 2014; Nimgaonkar et al., 2018; Wang et al., 2016a, 2016b). RBV, a purine nucleoside analogue, displays broad-spectrum antiviral activity against several DNA and RNA viruses (Crotty et al., 2000). Interferons (IFNs) are cytokines released by host cells in response to pathogens and are classified

Abbreviations: HEV, hepatitis E virus; RBV, ribavirin; IFN, interferon; PEG-IFN, pegylated interferon α; SOF, sofosbuvir; HCV, Hepatitis C virus; GLuc, *Gaussia* luciferase; HTS, high throughput screening; HBV, Hepatitis B virus; FDA, United States Food and Drug Administration; Mab, monoclonal antibody; MPA, mycophenolic acid; RLU, relative luminescence unit; LMV, lomibuvir; SVR, sustained virological response; VGCV, valganciclovir; GCV, ganciclovir

* Corresponding author.
E-mail address: hokamoto@jichi.ac.jp (H. Okamoto).

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into three types (Type I-III) (de Weerd and Nguyen, 2012). In hepatitis E patients, monotherapy with RBV or PEG-IFN achieves a sustained virological response (SVR) in many cases (Kamar et al., 2014; Nimgaonkar et al., 2018; Todt et al., 2016b). However, this therapy is associated with major side effects, such as leukopenia, thrombocytopenia, risk of organ rejection, and the occasional emergence of RBV-resistant HEV species (Nimgaonkar et al., 2018; Okanoue et al., 1996; Todt et al., 2016b). RBV and PEG-IFN belong to United States Food and Drug Administration (US FDA) categories X and C, respectively. Although HEV infection leads to more fulminant hepatitis in pregnant women, these drugs are not recommended to be given to pregnant women unless substantial benefit can be assured, given the above-mentioned side effects (Spera et al., 2016).

Recently, van der Valk et al. (2017) reported that sofosbuvir (SOF), an oral uridine nucleotide analogue for the treatment of hepatitis C, showed antiviral activity for chronic hepatitis E in combination with RBV. SOF exerts no embryotoxicity in animal studies (*nota bene*: no controlled data on its effects during human pregnancy are available) and belongs to US FDA category B (Cada et al., 2014; Spera et al., 2016). However, another report indicated that SOF without RBV was not effective for treating chronic hepatitis E (Donnelly et al., 2017). This finding reflects the low anti-HEV activity of SOF (IC_{50} = 1.2 μM) compared with its activity against hepatitis C virus (HCV; about 10-fold lower than that of anti-HEV) (Dao Thi et al., 2016; Sofia et al., 2010). These studies suggest that the dose of SOF (400 mg/day) is not sufficient to clear HEV. Thus, more effective and safer anti-HEV drugs as alternatives to RBV, PEG-IFN, and SOF are required.

The HEV genome itself has been shown to stimulate the response of type III IFN (especially, IFN-λ1-3) both in *in vitro* and *in vivo* (Wang et al., 2017b; Wu et al., 2018; Yin et al., 2017). It has recently been suggested that IFN-λ1 and IFN-λ3 inhibit the replication of HEV (Shukla et al., 2012; Todt et al., 2016a; Yin et al., 2017). IFN-λ1-3 are also known as interleukin (IL)-29, IL-28A, IL-28B, respectively (Hemann et al., 2017; Zanoni et al., 2017).

Gaussia luciferase (GLuc) is a 19.9-kDa secretary luciferase that generates a high signal intensity compared with firefly and *Renilla* luciferase (over 2000-fold) and secreted alkaline phosphatase (over 20,000-fold) (Badr et al., 2007; Tannous et al., 2005). GLuc is widely used in high-throughput screening (HTS) assays, including viral replication monitoring systems, such as HBV and HCV (Harada et al., 2017; Hu et al., 2014; Nishitsuji et al., 2015). In addition, GLuc was reported as an effective reporter protein for monitoring the replications of several HEV strains (Debing et al., 2014, 2016; Qu et al., 2017; Wang et al., 2017a). HEV replication reporter systems using GLuc are powerful tools for identifying anti-HEV replication candidates. However, because replication is only an event that occurs during the virus life-cycle, we need to confirm the effectiveness of the obtained candidates in a time-course analysis of HEV growth in living cells.

In this study, we constructed an HEV replication reporter construct using GLuc based on the genotype 3 HEV (JE03-1760F) (Tanaka et al., 2007) and PLC/PRF/5 cells, which supported more efficient propagation of HEV than other hepatoma cells (Tanaka et al., 2007). In addition, we developed a cell-culture system using cells robustly producing HEV for validating the replication-inhibitory activity of selected anti-HEV drugs. We screened the 767 FDA-approved compounds and IFN-λ1-3 with the GLuc reporter construct. We also confirmed the effects of the candidates in the newly developed validating system.

2. Materials and methods

2.1. Compounds

The Screen-Well® FDA approved drug library V2 (BML-2843J-0100, Japanese version) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). RBV (188-02333; Wako Pure Chemical Industries, Ltd., Osaka, Japan), mycophenolic acid (MPA, M2216; Tokyo Chemical Industry Co. Ltd., Tokyo, Japan), SOF (HY-15005; MedChemexpress Co., Ltd., Monmouth Junction, NJ, USA), INF-α2b (HZ-1072; HumanZyme, Inc., Chicago, IL, USA), lomibuvir (LMV, HY-75800; MedChemexpress), ciprofloxacin hydrochloride monohydrate (CPFX, C2227; Tokyo Chemical Industry), IFN-λ1 (IL-29, HZ-1156; HumanZyme), IFN-λ2 (IL-28A, HZ-1235; HumanZyme), and IFN-λ3 (IL-28B, HZ-1245; HumanZyme) were purchased from the sources indicated.

2.2. pJE03-1760F/P10-GLuc and pJE03-1760F/P10/GAA-GLuc plasmid construction

Previously, we reported the generation of the cloned genotype 3 HEV strain (pJE03-1760F/P10) after 10 passages of the wild-type strain pJE03-1760F/wt (Nagashima et al., 2016). pJE03-1760F/P10 (DDBJ/EMBL/GenBank accession no. LC126332) exhibits enhanced HEV production compared with pJE03-1760F/wt. This cell culture-adapted strain was deemed suitable for a reporter assay to screen anti-HEV replication drugs because of its robust production of HEV.

To construct pJE03-1760F/P10-GLuc, the *orf3* and *orf2* genes of pJE03-1760F/P10 were disrupted and replaced with GLuc. In detail, the first ATG of the *orf3* gene was replaced with GCA, and the 585-base pair (bp) fragment from the first ATG of the *orf2* gene was replaced with the C-terminal FLAG (DYKDDDDK)-tagged GLuc gene with an extra stop codon (585 bp) inserted. The *Psh*AI (R0593; New England Biolabs, Inc., Ipswich, MA, USA)-digested pJE03-1760F/P10 plasmid DNA and the polymerase chain reaction (PCR)-amplified DNA fragments with specific primers (5′-CAACTGGAGAGACCATCGTCCACGGTAAGGTCG-3′ and 5′-TGATCCTGCGGGCGATGCACAAAACATGTTATTC-3′ using the pJE03-1760F/P10 plasmid as a template, 5′-TCGCCCGCAGGATCACC ATGGGAGTCAAAGTTCTG-3′ and 5′-TGTCATCGTCGTCCTTGTAGTCG TCACCACCGGCC-3′ using the pcDNA3-GLuc plasmid (NanoLight Technologies, Prolume, Ltd., Pinetop, AZ, USA) as a template, and 5′-AGGACGACGATGACAAGTAATGACCGCTGGTGCCGAATGC-3′ and 5′-GTAGTGAGGGGACGGCCGTCCAGAGTAACTTTAG-3′ using the pJE03-1760F/P10 plasmid as a template) were fused using an In-Fusion HD cloning kit (Z9633 N; TaKaRa Bio Inc., Shiga, Japan). As a replication-defective (negative) control of JE03-1760F/P10-GLuc, JE03-1760F/P10/GAA-GLuc was constructed. The 4,681GATGAT4,786 of JE03-1760F/P10-GLuc was replaced with GCCGCC (as an amino acid sequence; 1560GDD1562 to GAA). The *Psh*AI-digested pJE03-1760F/P10-GLuc plasmid DNA and the PCR-amplified DNA fragments using specific primers (5′-CAACTGGAGAGACCATCGTCCACGGTAAGGTCG-3′ and 5′-AAGGGTGCCGCCTCGGTGGTCCTCTGTAGTG-3′, and 5′-CGAG GCGGCACCCTTAAAGGCGGCAACCC-3′ and 5′-GTAGTGAGGGGACGG CCGTCCAGAGTAACTTTAG-3′ using the pJE03-1760F/P10-GLuc plasmid as a template) were fused using the In-Fusion HD cloning kit.

2.3. In vitro transcription and capping

pJE03-1760F/P10-GLuc and pJE03-1760F/P10/GAA-GLuc plasmid DNAs were linearized by *Bam*HI-HF (R0136; New England Biolabs) digestion. 1760F/P10-GLuc and 1760F/P10/GAA-GLuc RNAs were transcribed from the linearized pJE03-1760F/P10-GLuc and pJE03-1760F/P10/GAA-GLuc plasmid DNA with an AmpriScribe™ T7-*Flash*™ Transcription Kit (ASF3507; epicentre/Illumina, Inc., San Diego, CA, USA), respectively, according to the manufacturer's protocol. The synthesized RNA was purified by standard phenol-chloroform extraction and ethanol precipitation and then capped with a ScriptCap™ m^7G capping System (C-SCCE0625; CELLSCRIPT, Madison, WI, USA) according to the manufacturer's protocol. The capped RNA was purified by standard phenol-chloroform extraction and ethanol precipitation.

2.4. Cell culture and RNA transfection

PLC/PRF/5 cells (ATCC No. CRL-8024; American Type Culture

Collection, Manassas, VA. USA) were grown in Dulbecco's modified Eagle's medium (DMEM, 12800-058; Gibco/Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, 10270; Gibco/Thermo Fisher Scientific), 100 U/mL of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B (growth medium) at 37 °C in a humid atmosphere saturated with 5% CO_2 (Tanaka et al., 2007). To assess the replication of 1760F/P10-GLuc, 2.0×10^3 cells were seeded onto a 96-well plate (BioLite 96 Well Multidish, 130188; Thermo Fisher Scientific). The next day, 40 ng (for drug screening) and 50 ng (for other assays)/well of capped RNA were transfected with a *Trans*IT-mRNA Transfection Kit (MIR2225; Mirus Bio LLC., Madison, WI, USA) according to the manufacturer's protocol.

2.5. Measurement of Gaussia luciferase activity

Four-microliter culture supernatants were diluted with 36 μL of growth medium in a 96-well microplate (Berthold Technologies, Bad Wildbad, Germany). An equal volume (40 μL) of 2.5 μg/ml coelenterazine (CZ-250; JNC Corporation, Tokyo, Japan) containing reaction buffer (10 mM EDTA, 0.01% Tween20 in PBS) was added to the well, and the luminescence kinetics was measured with a TriStar^2 LB942 multimode plate reader (Berthold Technologies). The measured values for the initial luminescence intensity (I_{max}), represented as relative luminescence units (RLU), were converted to the relative GLuc expression with the standard curve. The obtained values were normalized with that of vehicle control.

2.6. Measurement of cell viability

Cell viabilities were measured using a Cell Counting Kit-8 (WST-8, 341-07761; Dojindo Laboratories, Kumamoto, Japan) with iMark microplate reader (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's protocol. In brief, the cells were pulsed with 10 μL/well of WST-8 solution for 50 min at 37 °C. The absorbance at 450 nm (reference wavelength: at 620 nm) of the reduced WST-8 was measured. The obtained values were normalized with that of vehicle control.

2.7. HEV inoculation and drug assessments

Drug assessments were performed with two different HEV inoculation methods.

In one method, cells producing no virus (naïve; PLC/PRF/5 cells) were seeded at 1.0×10^5 cells/well onto a 24-well plate. The next day, the cells were washed with PBS and inoculated with culture supernatants containing a cell culture-adapted genotype 3 JE03-1760F strain (passage 26; 1.0×10^5 copies/well) (Lorenzo et al., 2008) in 200 μl of plain DMEM. After 4 h, the inoculum was discarded, and the cells were grown in fresh growth medium for 1 day at 37 °C. The cells were rinsed with PBS five times to completely wash out the remaining HEV and then grown in growth medium supplemented with an appropriate concentration of the compounds. Half of the culture medium was exchanged for fresh medium every other day.

In the other method, virus-producing cells (infected-PLC/PRF/5 cells [with a plateau-titer of HEV production] at 1.5×10^3 cells/well) and naïve ones (as in the HEV spreading model; naïve PLC/PRF/5 cells at 3.0×10^5 cells/well) were mixed and seeded onto a 24-well plate (BioLite 24 Well Multidish, 930186, Thermo Fisher Scientific). Two days later, the cells were rinsed with PBS twice, and then the culture medium was exchanged for fresh medium supplemented with appropriated concentrations of the compounds. Half of the culture medium was collected and exchanged for fresh medium every other day. The collected culture medium was subjected to reverse transcription-quantitative PCR (RT-qPCR).

2.8. Quantitation of HEV RNA and housekeeping gene

After treatment of culture supernatant with ribonuclease A (Qiagen) at 37 °C for 40 min, particle-associated viral RNA was purified using TRIzol-LS Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol and quantified by RT-qPCR with a 7900 H T Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). For quantitation of HEV RNA, we used a QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and specific primer and TaqMan probe set targeting the ORF2 and ORF3 overlapping region, as described previously (Takahashi et al., 2008). For quantitation of a housekeeping gene, *TATA-box binding protein* (*TBP*), cellular mRNA was extracted from cell lysates with TRIzol-LS Reagent (Thermo Fisher Scientific) and specific primers, 5′-GCAGCTGCAAAATATTGTATCCAC-3′ and 5′-AACC GCTTGGGATTATATTCGG-3′ and QuantiNova SYBR Green RT-PCR Kit (Qiagen) were used according to the manufacturer's protocol.

2.9. Immunofluorescence

3.0×10^5 cells/well of naïve PLC/PRF/5 cells and 1.5×10^3 cells/well of infected-PLC/PRF/5 cells (with a plateau-titer of HEV production) were seeded on type I-C collagen (631-01011; Nitta Gelatin Inc., Osaka, Japan.) coated coverslip in 24 well plate. 1, 2, 4, 8, and 12 days after, cells were rinsed with PBS and fixed with 4% paraformaldehyde /PBS (paraformaldehyde; TAAB Laboratory Equipment Ltd., Reading, UK). Then cells were permeabilized with 0.2% NP-40/PBS (NP-40, N-6507; SIGMA) and rinsed with PBS. After that, cells were blocked with 2% BSA/PBS (BSA, A4503; SIGMA) following react with 1st antibody, mouse monoclonal antibody (Mab) against HEV ORF2 protein (H6225) (Takahashi et al., 2008), then Alexa 488 labeled 2nd antibody (Life Technologies, Carlsbad, CA) each for 1 h. The stained coverslips were mounted with ProLong Gold mounting medium (Life Technologies).

2.10. Statistics

All values are expressed as mean ± standard deviation (SD). The significance of differences was assessed by one-way analysis of variance (ANOVA), with differences among groups assessed by Tukey-Kramer post-hoc analysis. Probabilities less than 5% ($^*P < 0.05$), 1% ($^{**}P < 0.01$) or 0.1% ($^{***}P < 0.001$) were considered to be statistically significant.

3. Results

3.1. Construction of HEV replicon expressing GLuc

We constructed an HEV replicon based on pJE03-1760F/P10 to express the replication reporter protein GLuc (named to pJE03-1760F/P10-GLuc). This construct disrupted the expression of the capsid protein ORF2 and the multifunctional ORF2-overlapping protein ORF3 (Fig. 1A). In addition, we also constructed the mutant form of pJE03-1760F/P10-GLuc as a replication-deficient (negative) control (pJE03-1760F/P10/GAA-GLuc). pJE03-1760F/P10/GAA-GLuc expresses the functionally disrupted RNA-dependent-RNA polymerase. To confirm the GLuc expression from these synthesized modified HEV genomic RNAs (1760F/P10-GLuc from pJE03-1760F/P10-GLuc, 1760F/P10/GAA-GLuc from pJE03-1760F/P10/GAA-GLuc), we transfected the synthesized 5′-capped RNA as a replicon to PLC/PRF/5 cells. The GLuc activity in the culture supernatant of the 1760F/P10-GLuc-transfected cells plateaued at six days post-transfection but was not detected in the culture supernatant of the 1760F/P10/GAA-GLuc transfected cells (Fig. 1B). We therefore validated the efficient GLuc expression by the HEV replication reporter construct.

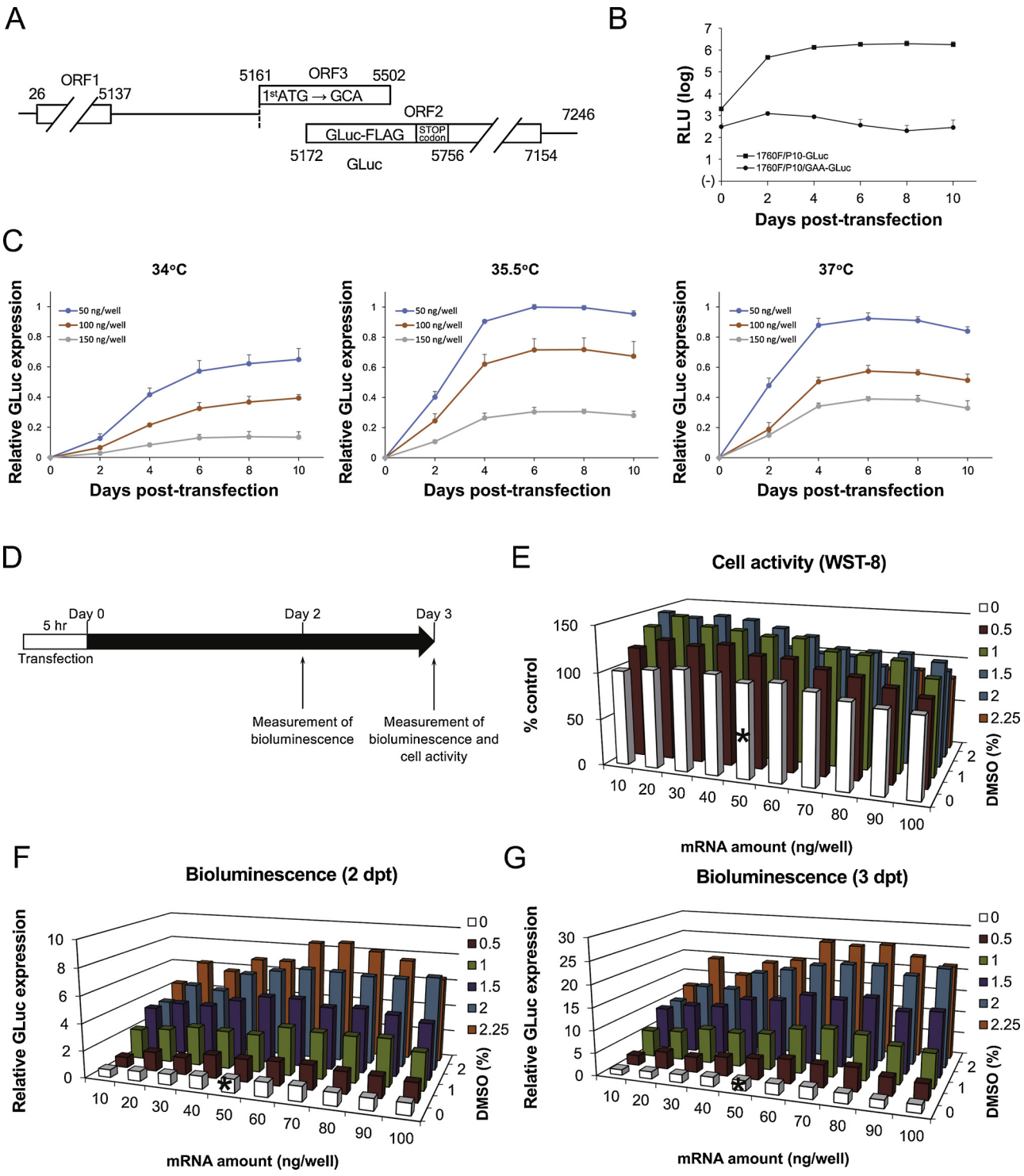

Fig. 1. Construction of the HEV replicon with the specific expression of *Gaussia* luciferase and optimization of the experimental condition to monitor the replication. (A) A schematic illustration of the *Gaussia* luciferase (GLuc)-expressing HEV replicon (1760F/P10-GLuc). The ORF3 protein expression was abolished by mutating its first ATG to GCA. The 5′-terminal 585 bp from the first ATG of the *orf2* gene was replaced with the FLAG-tagged GLuc containing two consecutive STOP codons. (B) The GLuc expression of the construct 1760F/P10-GLuc and its replication defective control 1760F/P10/GAA-GLuc. PLC/PRF/5 cells were transfected with the indicated capped RNAs (100 ng/well, 96-well format) synthesized from the constructs (n = 3). (C) The optimization of the culture temperature (34, 35.5, and 37 °C) and the RNA amount (50, 100, and 150 ng/well) for transfection in PLC/PRF/5 cells (n = 3). The relative GLuc expression was normalized with the mean at 6 days post-transfection at 35.5 °C. Error bars represent the mean ± SD. (D–G) The optimizations of the DMSO concentration and minute amounts of RNA for transfection in PLC/PRF/5 cells. (D) Experimental time-course. (E) The cell activity at three days post-transfection was measured using WST-8 substrate. (F, G) The GLuc activity was measured at 2 (F) and 3 (G) days post-transfection (dpt). The asterisks in (*E*)-(G) indicate the standard (50 ng/well RNA, 0% DMSO) in each of the panels.

3.2. Optimization of the capped 1760F/P10-GLuc RNA replication condition

Screening for anti-HEV replication drugs showing greater efficacy than RBV or PEG-IFN will require the optimization of the monitoring system to exhibit rapid expression of the GLuc from the 1760F/P10-GLuc HEV replicon. To determine the most suitable transfection and culture conditions for the GLuc expression from the replicon, we optimized the amount of capped 1760F/P10-GLuc RNA and the culture temperature. For this replicon, 50 ng/well (96-well format) and 37 °C resulted in the most efficient luciferase activity (Fig. 1C). We further optimized the concentration of dimethylsulfoxide (DMSO), one of the most widely used vehicles of chemical compounds, and the minute amount of capped 1760F/P10-GLuc RNA for 2 and 3 days, as indicated in Fig. 1D. As a result, we found that 1.0% DMSO was suitable for assessing the cell activity (Fig. 1E). Indeed, 40 and 50 ng/well of the capped 1760F/P10-GLuc RNA resulted in the highest levels of GLuc expressions (Fig. 1F and G). We then calculated the Z'-factor, which is widely used to evaluate the quality of HTS, with 40 and 50 ng/well of 1760F/P10-GLuc RNA. The Z'-factor is a statistical parameter and should be between 0.5–1.0 for screening. In our screening systems with 40 and 50 ng/well of the capped 1760F/P10-GLuc RNA, the Z'-factor values were 0.910 and 0.886, respectively. On the other hand, the Relative GLuc expression was higher with 50 ng of RNA (1.11 times higher on day 2) than 40 ng of RNA. We therefore employed 40 ng/well of RNA and 1.0% DMSO at 37 °C for 2 days for the drug screening assay and 50 ng/well of RNA and 1.0% DMSO at 37 °C for the other assays.

3.3. Known anti-HEV drugs suppress the GLuc expression on 1760F/P10-GLuc reporter construct

We next confirmed whether or not the known anti-HEV drugs suppress GLuc expression from 1760F/P10-GLuc RNA transfected cells. We investigated the following drugs: RBV, MPA, SOF, and IFN-α2b. Lomibuvir (LMV, also known as VX-222 and VCH-222), a non-nucleoside HCV NS5B polymerase inhibitor (Yi et al., 2012), was administrated as a negative control. These drugs, except for LMV, sufficiently suppressed the expression of GLuc in a concentration-dependent manner in this reporter system. RBV and MPA at high concentrations were slightly toxic in the GLuc expressing cells (Fig. 2). On SOF, there are conflicting results including ours. Wang et al. (2016a, 2016b) demonstrated that SOF could not suppress HEV replication using Sar-55 based genotype 1 and KernowC1/p6 based genotype 3 replicon assay. However, Dao Thi et al. (2016) also showed poor sensitivity of SOF on Sar-55 based genotype 1 replicon assay but not on KernowC1/p6 based genotype 3 replicon assay. In our study, JE03-1760F (genotype3) based replicon was sensitive for SOF (Fig. 2). These data indicate that our new HEV replication reporter system with GLuc is indeed effective for assessing the anti-HEV drug activity.

3.4. HTS of anti-HEV replication drugs

To identify anti-HEV replication drugs, we screened a library of 767 FDA-approved compounds using this HEV replication reporter system. Abravanel et al. (2015) recently reported that the plasma concentration of RBV was 1.05–6.2 μg/mL (4.28–25 μM) in chronic hepatitis E patients given 200–800 mg of RBV daily, resulting in an SVR. However, in our study, 2 μg/ml (8.2 μM) of RBV exhibited less HEV replication inhibitory activity (Fig. 2). Similarly, although the plasma concentration of RBV is estimated to range from 2.5 to 4.9 μg/mL (10–20 μM) in treated chronic hepatitis C patients (Slavenburg et al., 2011), the anti-HCV effect of RBV was insufficient under 100 μM in an *in vitro* study (Galli et al., 2018).

We screened these compounds at concentrations of 100, 20, and 10 μM. We administrated these compounds 5 h after transfection of the 1760F/P10-GLuc replicon (Fig. 3A). After two days, we measured GLuc activity and cell viability. First, the compounds were tested at the final concentration of 100 μM (Fig. 3B). Next, the compounds that were highly cytotoxic at 100 μM were retested at concentrations of 20 μM (212 compounds) or 10 μM (237 compounds) (Fig. 3B). A total of 55 compounds containing RBV and MPA, as indicated in the boxed lines in Fig. 3B, inhibited HEV replication. In detail, we chose the compounds; at 100 μM concentration, compound which exhibited less than 10% of GLuc expression and 40–50% of cell activity, and less than 35% of GLuc expression and 50–70% of cell activity, and less than 50% GLuc expression and more than 70% of cell activity to the control. At 20 and 10 μM concentrations, compounds, which exhibited less than 35% GLuc expression and 50–70% of cell activity and less than 50% of GLuc expression and more than 70% cell activity.

3.5. Ciprofloxacin and IFN-λ1-3 inhibit HEV replication

Some of the 55 identified compounds exhibit severe side effects or non-oral and/or -intravenous administration, *e.g.* anti-cancer agent, corticosteroids (airway administration), and so on, and each of them have suitable concentration to be administered to human body. Therefore, we omitted the hit compounds screened at higher concentrations than those suitable for administration to human body and narrowed the compounds down to the 20 with mild side effects (Supplementary Table S1). We confirmed the inhibitory activity against HEV replication of these 20 compounds with the 1760F/P10-GLuc replicon. Our findings showed that a high concentration of ciprofloxacin (CPFX) inhibited HEV replication and displayed the weak toxicity (Fig. 4), while the other compounds did not exhibit sufficient inhibitory activity (data not shown). In addition to the compounds, we also tested whether or not IFN-λ1-3 inhibit HEV replication. All three exerted similar degrees of HEV replication inhibitory activity. Thus, these data suggest that CPFX and all three type III IFNs (IFN-λ1-3) inhibit HEV growth.

3.6. Ciprofloxacin suppresses HEV growth in culture cells

Next, we tested the inhibitory effect of CPFX on the HEV growth in cultured cells. CPFX and RBV (as a positive control) were administrated the day after inoculation of the cell culture-adapted HEV to the PLC/PRF/5 cells. RBV exerted dose-dependent inhibition of HEV growth (Fig. 5A). However, while 40 μg/ml (160 μM) of RBV completely inhibited the HEV growth, a low dose of RBV (< 20 μg/mL) showed insufficient inhibition (Fig. 5A). Indeed, the HEV copy number with 20 μg/ml (80 μM) of RBV plateaued at a lower titer (nearly 5×10^4 copies/ml) than with ≤10 μg/ml (≥1.0×10^8 copies/mL) (Fig. 5A). Similarly, 80 μg/ml (220 μM) of CPFX weakly suppressed the HEV growth in PLC/PRF/5 cells and did not inhibit the viral growth at lower doses (Fig. 5B). HEV expressing cells treated with RBV and those treated with CPFX displayed no significant differences in the *TATA-box binding protein* (*TBP*) mRNA expression, unlike the GLuc replicon transfected cells (Fig. 5A,B). These findings indicate that CPFX can inhibit HEV growth in cell culture, but its effect is insufficient compared to RBV.

3.7. Development of an efficient anti-HEV drug assessment system with culture cells

To further assess the inhibitory activity of anti-HEV drug candidates in cultured cells, we developed a new drug assay model of the *in vitro* spread of HEV. We co-seeded naïve PLC/PRF/5 cells and HEV-infected cells two days before drug administration. As indicated in Fig. 5, HEV growth was suppressed by 40 μg/ml (160 μM) of RBV but not by concentrations of ≤20 μg/ml (80 μM). In Fig. 6, however, the same concentration of RBV failed to suppress HEV production in the cells expressing HEV robustly. In the situation, RBV displayed no significant toxicity in the cell activity test (WST-8 assay) (Supplementary Fig. S1).

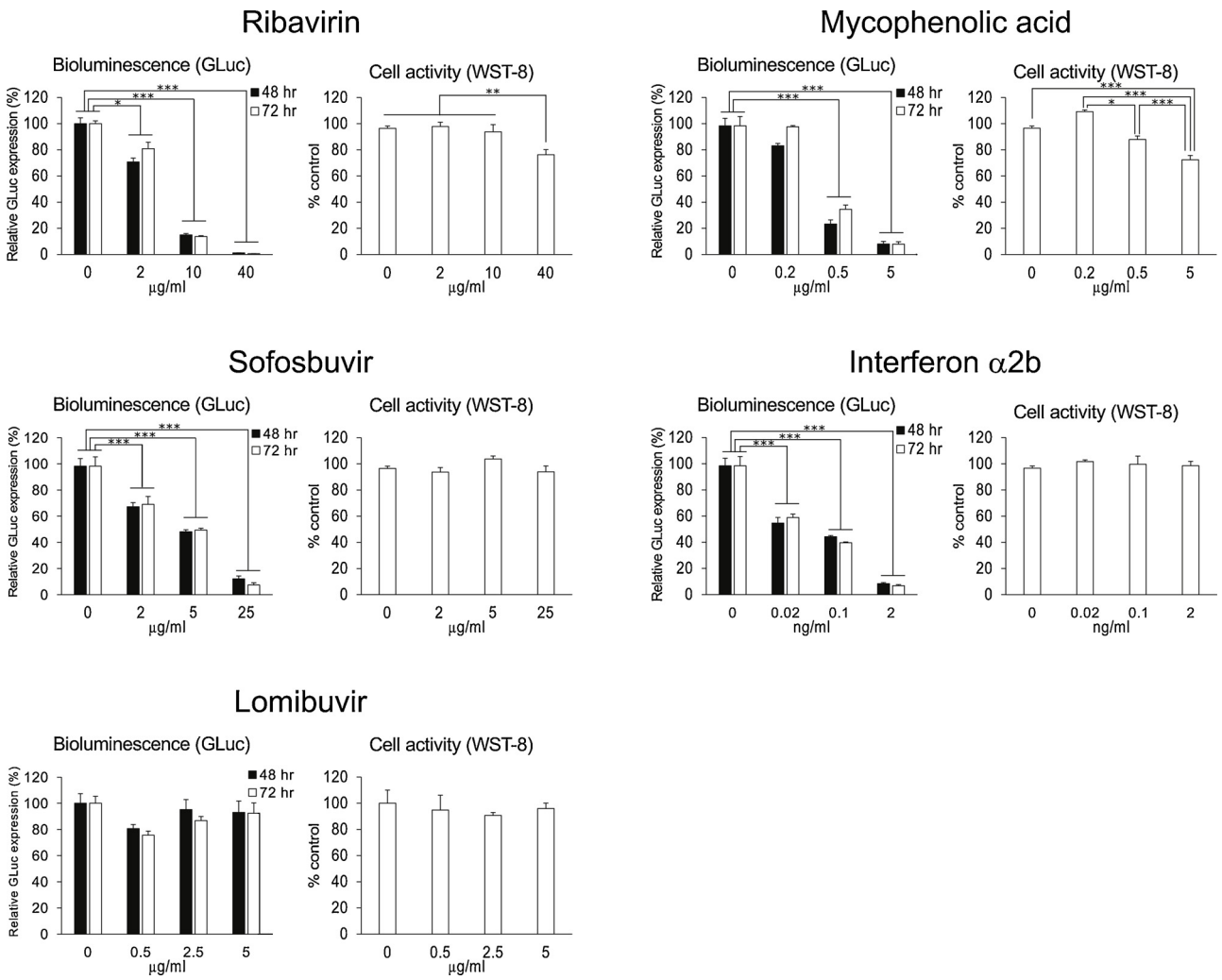

Fig. 2. The effectiveness of the system for monitoring HEV replication. The known compounds and interferon that inhibit HEV replication were tested with this system to evaluate the inhibitory efficiencies of HEV replication. The luciferase activities were measured at 48 and 72 h post-transfection and converted to the relative expression. The cell activities were measured at 72 h post-transfection using WST-8 substrate. Both the relative GLuc expression and the cell activities were normalized with vehicle control. Lomibuvir was used as a negative control (n = 3). Error bars represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA with Tukey–Kramer test.

In addition, immunofluorescence analysis at day 12, detecting HEV ORF2 protein, confirmed the inhibitory effect of RBV (Fig. 6C). These results indicate that this assay system is suitable for strictly confirming the anti-HEV activity of compounds.

3.8. IFN-λ1-3 reduce HEV RNA released from HEV infected PLC/PRF/5 cells

Next, with this new system, we evaluated the anti-HEV ability of CPFX and IFN-λ1-3. CPFX at 80 μg/ml (220 μM) failed to suppress HEV growth, similar to RBV at ≤20 μg/ml (80 μM) (data not shown). All three types of type III IFNs (IFN-λ1-3) suppressed HEV growth with dose dependency (Fig. 6), and the inhibitory effects persisted for at least 28 days. Under the situations, the cell activity tested with WST-8 was upregulated at high concentrations of IFN-λ1-3 (Supplementary Fig. S1). Moreover, the HEV ORF2 protein expressions decreased markedly in IFN-λ1-3 treated cells in the immunofluorescence analysis at day 12 (Fig. 6C). These results indicate that IFN-λ1-3 sufficiently inhibited HEV growth in culture cells, similar to RBV.

4. Discussion

In this study, we developed an HEV replication reporter construct using GLuc with genotype 3 HEV (JE03-1760F strain) and screened 767 compounds to obtain anti-HEV replication drugs using PLC/PRF/5 cells. We obtained 55 drug candidates containing RBV and MPA, which are known to be effective anti-HEV compounds (Nimgaonkar et al., 2018; Wang et al., 2014). We narrowed the 55 candidates down to 20 ones judging from their doses and side effects in clinical use. These 20 candidates were assessed in detail using this reporter construct. Although CPFX exhibited HEV replication inhibitory activity in the reporter system, it did not sufficiently inhibit HEV growth in the *in vitro* culture system using PLC/PRF/5 cells robustly producing HEV. The present study indicated that, although our HEV replication reporter system using replicon with GLuc is suitable for the comprehensive screening of many candidate compounds, our cell culture system using HEV-infected cells robustly producing progeny viruses is more suitable for strictly evaluating the anti-HEV inhibitory activity.

Replication reporter systems have some advantages for monitoring the replication of the replicon of the virus in interest: it is a very simple and easy method of obtaining anti-viral drugs. However, this approach is associated with a major point of concern: the obtained drugs may

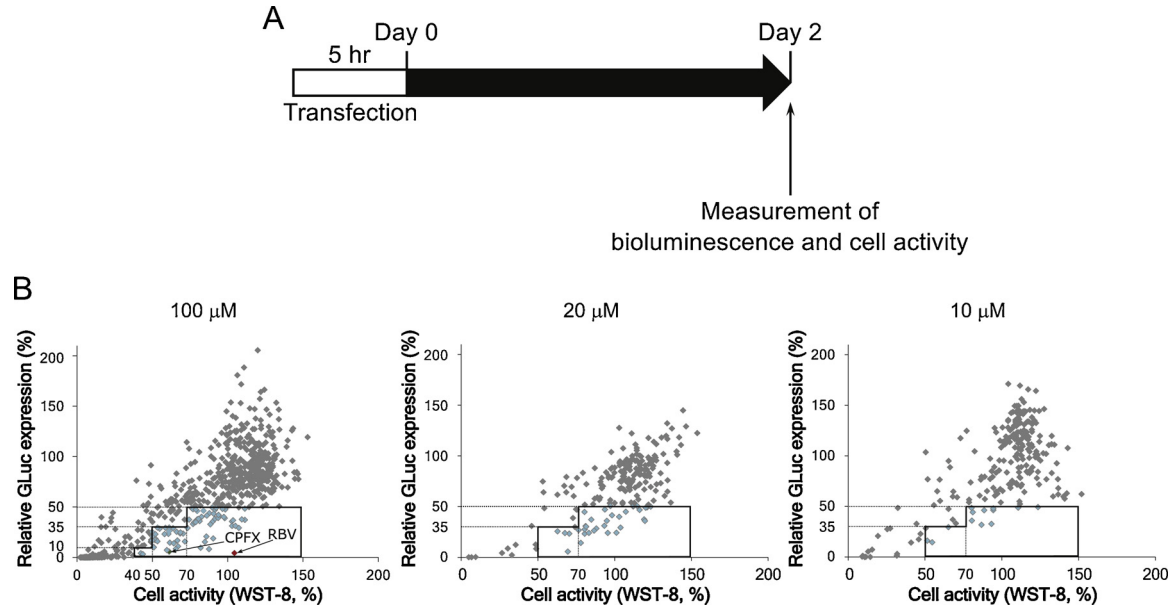

Fig. 3. Anti-HEV replication drug screening. Screening of 767 compounds using the developed replication reporter system. (A) A schematic illustration of the time-course of the assay. The capped 1760F/P10-GLuc RNA was transfected to PLC/PRF/5 cells for 5 h. After two days' exposure of compounds, the luciferase activities in the culture supernatants and the cell activities (WST-8 assay) were measured. (B) The relative GLuc expression (Y-axis) and the cell activity (X-axis), which were normalized to a DMSO (vehicle) control, were plotted (100 μM, 767 compounds; 20 μM, 212 compounds; 10 μM, 237 compounds). The boxed lines indicate the compounds to be assessed in further investigations. The hit compounds were colored in cyan. CPFX and RBV were marked in green and red, respectively.

exhibit inhibitory effects only in this setting, with their effects not being sufficient to inhibit actual viral growth. We therefore need to confirm the obtained compounds using a replication reporter in an *in vitro* viral growth assay. For instance, as described in Fig. 6, 20 ng/ml of IFN-λ1-3 exerted inhibitory effects on HEV growth until 16–20 days post-drug treatment but not thereafter. We therefore must confirm the inhibitory effects of the drugs identified using our *in vitro* virus culture system over a long time-course.

Clinically, although hepatitis E is mostly an acute self-limiting disease, it can progress to fulminant hepatitis, and pregnant women are particularly susceptible (Hoofnagle et al., 2012; Nimgaonkar et al., 2018). Immunocompromised individuals infected with HEV are also at risk of developing chronic hepatitis E (Kamar et al., 2014; Nimgaonkar et al., 2018). In such cases, RBV and PEG-IFN sometimes fail to clear the

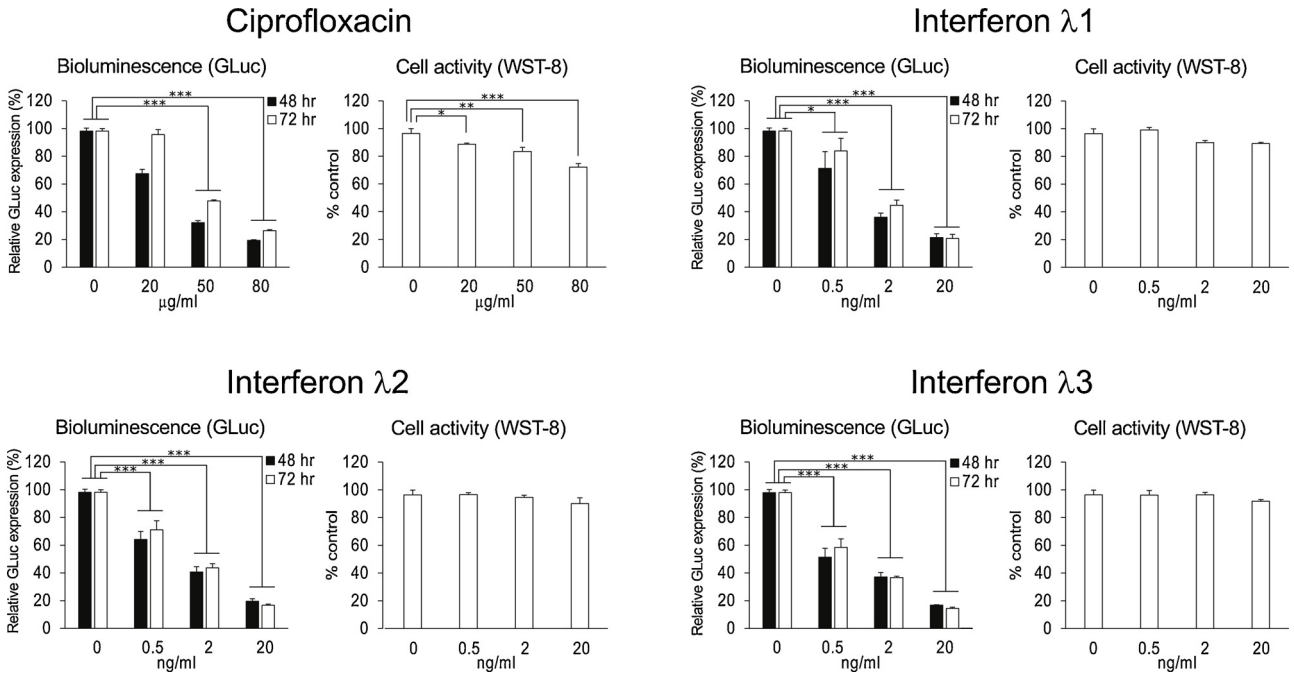

Fig. 4. Ciprofloxacin and interferon λ1-3 inhibit HEV replication. The inhibitory activity of ciprofloxacin (CPFX), a hit compound, and interferon λ1-3 (IFN-λ1-3) on HEV replication. PLC/PRF/5 cells were transfected with 50 ng/well (96-well plate format) of the capped 1760F/P10-GLuc RNA. The cells were administered the indicated amounts of CPFX and IFN-λ1-3. The luciferase activities were measured at 48 and 72 h post-transfection and converted to the relative expression. The cell activities were measured at 72 h post-transfection using WST-8 substrate. Both the relative GLuc expressions and the cell activities were normalized with vehicle control (n = 3). Error bars represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA with Tukey–Kramer test.

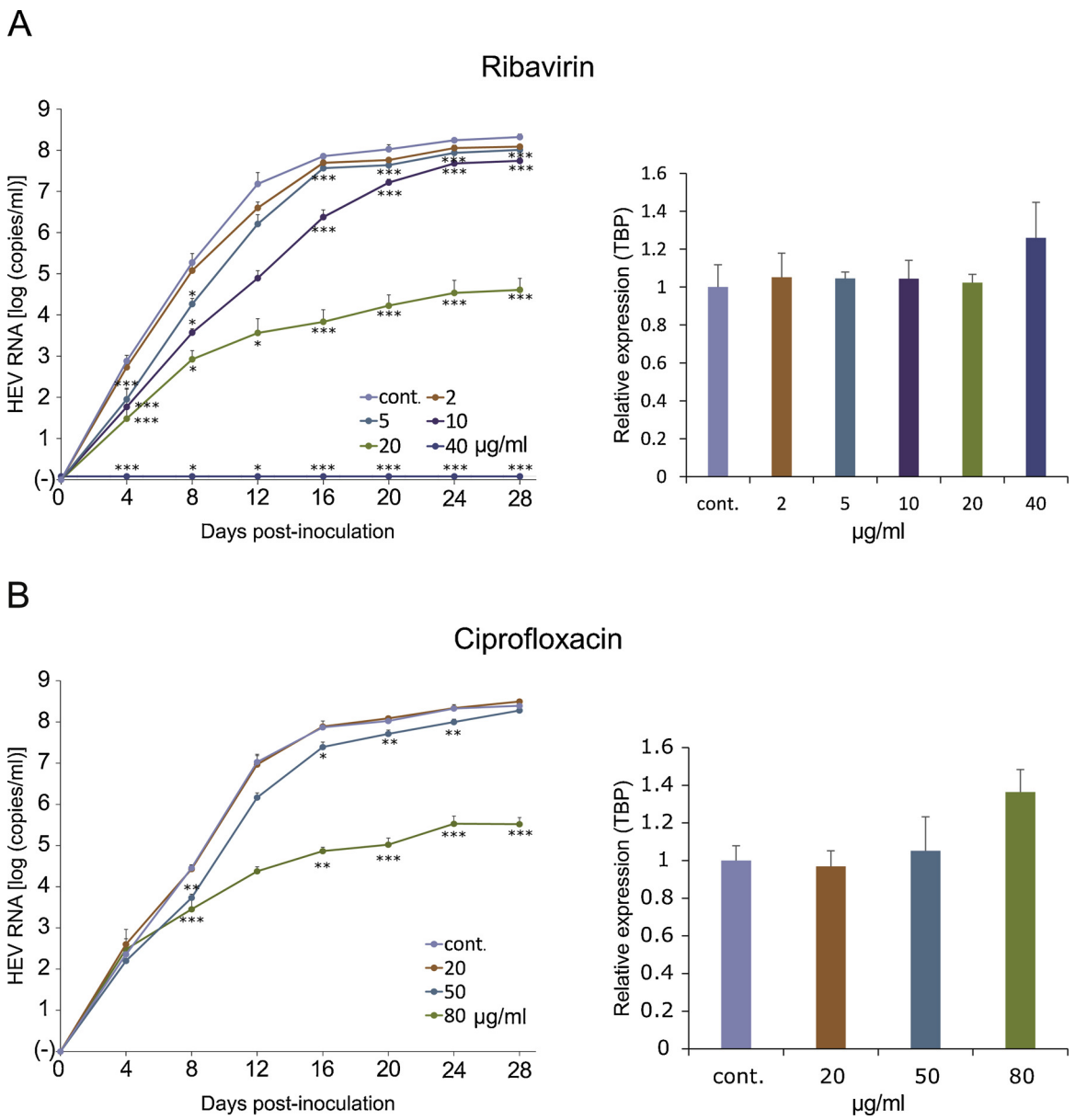

Fig. 5. Ciprofloxacin suppresses HEV growth. The inhibitory effect of ciprofloxacin (CPFX) on HEV growth was tested with the HEV culture system using PLC/PRF/5 cells. PLC/PRF/5 cells were inoculated with cell-culture derived HEV and then treated with the indicated compounds. (A) Ribavirin (RBV) as a positive control inhibited HEV growth in a dose-dependent manner. The mean values were plotted (n = 3). (B) CPFX suppressed HEV growth in a dose-dependent manner. As internal control, mRNA expression of *TATA-box binding protein*; *TBP* were displayed. No significant differences were observed. Shown in both (A) and (B) are the means of three independent experiments. Error bars represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA with Tukey–Kramer test.

HEV from the patient's serum due to ineffectiveness and/or the emergence of drug-resistant HEV (Nimgaonkar et al., 2018). To achieve an SVR in hepatitis E patients with a high virus titer, more effective alternatives to RBV and PEG-IFN are needed. For this reason, it is unsuitable to evaluate anti-HEV drug candidates using immediately inoculated cells, which start with a very low (nearly 0) titer of HEV RNA. We therefore searched for candidates using a new *in vitro* system to evaluate the anti-HEV activity using cells robustly producing HEV. In the previous system (drug treatment immediate after inoculation), RBV exhibited anti-HEV growth activity at doses exceeding 20 μg/ml (80 μM); however, this result was not achieved with the new assay system (Fig. 6). Our new assay system therefore seems able to evaluate the anti-HEV activity of candidate drugs more strictly than the old system.

CPFX is a member of the fluoroquinolone class and one of the most widely used antibiotics (Naeem et al., 2016). This antibiotic is classified into pregnancy category C by the FDA because of insufficient studies on its effects in pregnant women. As such, its administration during pregnancy should be avoided unless ample benefit can be demonstrated, such as in the treatment of anthrax (Meyerhoff et al., 2004). CPFX acts on extracellular pathogens and slightly penetrates tissues, include the liver and lung (Wong et al., 2003). For this reason, several reports have described liposome encapsulation or other methods of delivering CPFX inside cells to combat intracellular infections (Cipolla et al., 2016; Drulis-Kawa and Dorotkiewicz-Jach, 2010; Wong et al., 2003). The inhibitory effect of high-dose CPFX on HEV replication observed in this study might therefore be ascribable to its indirect effects, which is in contrast to the direct activities of nucleoside analogues such as RBV and SOF on HEV genome replication.

IFN-λ1-3 are type III IFNs. Yin et al. (2017) and Wu et al. (2018) found that HEV infection of culture cells induce a type III IFN response. In addition, Wang et al. (2017b) indicated that the HEV genome also triggers a type I IFN response. Indeed, Yin et al. (2017) and Todt et al.

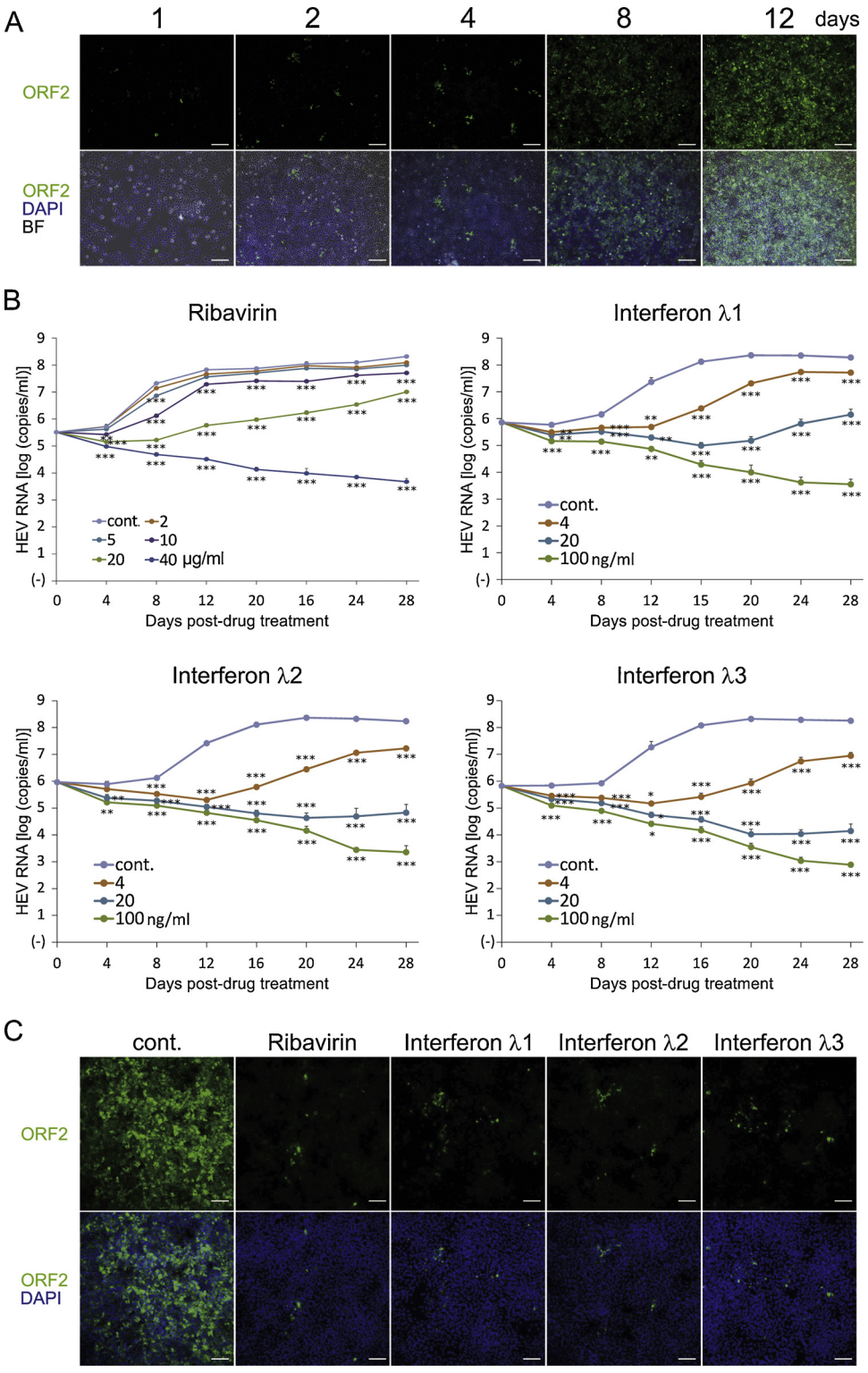

Fig. 6. Ribavirin and interferon λ1-3 decrease the HEV propagation in cultured cells. (A) Time-course infection and spreading of HEV. Immunofluorescence analysis was performed with anti-HEV ORF2 Mab. The green, blue, and gray signals indicated HEV ORF2, DAPI, and bright field (BF). Bars = 200 μM. (B) The anti-HEV inhibitory efficiencies of RBV and IFN-λ1-3 were tested in HEV-producing PLC/PRF/5 cells. HEV-producing PLC/PRF/5 cells in the plateau phase and naïve ones were co-cultured and treated with the indicated drugs. RBV 40 μg/ml and IFN-λ1-3 100 ng/mL inhibited HEV production. Shown in the panels are the means of three independent experiments. (C) Immunofluorescence analysis at day 12, detecting HEV ORF2 protein, in HEV-infected PLC/PRF/5 cells treated with RBV (40 μg/mL) or IFN-λ1-3 (100 ng/mL). Error bars represent the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA with Tukey–Kramer test.

(2016a) indicated that IFN-λ1 and IFN-λ3 inhibit HEV replication using Kernow-C1/p6 genotype 3 HEV replicon. Todt et al. (2016a) showed that IFN-λ3 moderately inhibits HEV growth in cultured cells (HepG2 cells) for a short period (48 h). Their data suggest that IFN-λ3 inhibits HEV growth *in vitro*. We similarly observed that IFN-λ3 suppressed HEV growth in a dose-dependent manner in PLC/PRF/5 cells. Furthermore, in our study, the inhibitory effect persisted for a long period of time (at least 28 days) (Fig. 5). Moreover, IFN-λ1 and IFN-λ2 also reduced the amount of HEV RNA, indicating that IFN-λ1 and IFN-λ2 sufficiently inhibited HEV growth in culture cells, similar to RBV and IFN-λ3.

Recently, Te et al. (2013) reported an interesting case in which valganciclovir (VGCC) cleared HEV in a chronic hepatitis E patient who received an organ graft and was infected with genotype 3 HEV. VGCC, a prodrug of ganciclovir (GCV), which is a synthetic analogue of 2′-deoxy-guanosine, is incorporated into viral DNA and inhibits viral DNA polymerases (Ambrose et al., 2016). In our HEV replication reporter system, VGCC did not inhibit GLuc expression (data not shown). A recent study suggested that GCV also functions *via* other mechanisms to

reduce the titer of the RNA virus HCV, just as with HEV, such as by improving the body's antiviral immunity (Li et al., 2016; Miszewska-Szyszkowska et al., 2015). These previous findings suggest that these compounds might be suitable for treating chronic hepatitis E *in vivo*. A chronically HEV-infected animal model using immuno-deficient rats may help clarify the effects of these compounds against HEV (Debing et al., 2016).

In the present study, we constructed an HEV replication reporter system using GLuc with the genotype 3 JE03-1760F strain and screened FDA-approved drugs. The candidate compound CPFX suppressed the expression of reporter protein in the HEV replication reporter system. However, CPFX only moderately inhibited HEV growth in our cell-culture system. These results suggest that we need to perform assessments not only in an HEV replicon reporter model but also in a strict HEV growth model using cells robustly producing HEV over a long time period in order to obtain more plausible data on candidates for treating HEV infection. Using this assay model, we found that the Type III IFNs (IFN-λ1-3) sufficiently inhibit HEV growth. These cytokines may be useful for clearing HEV *in vivo*.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.017.

References

Abravanel, F., Lhomme, S., Rostaing, L., Kamar, N., Izopet, J., 2015. Protracted fecal shedding of HEV during ribavirin therapy predicts treatment relapse. Clin. Infect. Dis. 60, 96–99. https://doi.org/10.1093/cid/ciu742.

Ambrose, T., Sharkey, L.M., Louis-Auguste, J., Rutter, C.S., Duncan, S., English, S., Gkrania-Klotsas, E., Carmichael, A., Woodward, J.M., Russell, N., Massey, D., Butler, A., Middleton, S., 2016. Cytomegalovirus infection and rates of antiviral resistance following intestinal and multivisceral transplantation. Transplant. Proc. 48, 492–496. https://doi.org/10.1016/j.transproceed.2015.09.070.

Badr, C.E., Hewett, J.W., Breakefield, X.O., Tannous, B.A., 2007. A highly sensitive assay for monitoring the secretory pathway and ER stress. PLoS One 2, e571. https://doi.org/10.1371/journal.pone.0000571.

Cada, D.J., Cong, J., Baker, D.E., 2014. Sofosbuvir. Hosp. Pharm. 49, 466–478. https://doi.org/10.1310/hpj4905-466.

Cipolla, D., Blanchard, J., Gonda, I., 2016. Development of liposomal ciprofloxacin to treat lung infections. Pharmaceutics 8. https://doi.org/10.3390/pharmaceutics8010006.

Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R., Cameron, C.E., 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat. Med. 6, 1375–1379. https://doi.org/10.1038/82191.

Dao Thi, V.L., Debing, Y., Wu, X., Rice, C.M., Neyts, J., Moradpour, D., Gouttenoire, J., 2016. Sofosbuvir inhibits hepatitis E virus replication *in vitro* and results in an additive effect when combined with ribavirin. Gastroenterology 150, 82–85. https://doi.org/10.1053/j.gastro.2015.09.011. e4.

de Weerd, N.A., Nguyen, T., 2012. The interferons and their receptors-distribution and regulation. Immunol. Cell Biol. 90, 483–491. https://doi.org/10.1038/icb.2012.9.

Debing, Y., Emerson, S.U., Wang, Y., Pan, Q., Balzarini, J., Dallmeier, K., Neyts, J., 2014. Ribavirin inhibits *in vitro* hepatitis E virus replication through depletion of cellular GTP pools and is moderately synergistic with alpha interferon. Antimicrob. Agents Chemother. 58, 267–273. https://doi.org/10.1128/AAC.01795-13.

Debing, Y., Mishra, N., Verbeken, E., Ramaekers, K., Dallmeier, K., Neyts, J., 2016. A rat model for hepatitis E virus. Dis. Model. Mech. 9, 1203–1210. https://doi.org/10.1242/dmm.024406.

Donnelly, M.C., Imlach, S.N., Abravanel, F., Ramalingam, S., Johannessen, I., Petrik, J., Fraser, A.R., Campbell, J.D.M., Bramley, P., Dalton, H.R., Hayes, P.C., Kamar, N., Simpson, K.J., 2017. Sofosbuvir and daclatasvir anti-viral therapy fails to clear HEV viremia and restore reactive T cells in a HEV/HCV co-infected liver transplant recipient. Gastroenterology 152, 300–301. https://doi.org/10.1053/j.gastro.2016.05.060.

Drulis-Kawa, Z., Dorotkiewicz-Jach, A., 2010. Liposomes as delivery systems for antibiotics. Int. J. Pharm. 387, 187–198. https://doi.org/10.1016/j.ijpharm.2009.11.033.

Emerson, S.U., Purcell, R.H., 2013. Hepatitis E virus. Fields Virol., 6th Ed. pp. 2242–2258.

Galli, A., Mens, H., Gottwein, J.M., Gerstoft, J., Bukh, J., 2018. Antiviral effect of ribavirin against HCV associated with increased frequency of G-to-A and C-to-U transitions in infectious cell culture model. Sci. Rep. 8, 4619. https://doi.org/10.1038/s41598-018-22620-2.

Graff, J., Torian, U., Nguyen, H., Emerson, S.U., 2006. A bicistronic subgenomic mRNA encodes both the ORF2 and ORF3 proteins of hepatitis E virus. J. Virol. 80, 5919–5926. https://doi.org/10.1128/JVI.00046-06.

Harada, K., Nishitsuji, H., Ujino, S., Shimotohno, K., 2017. Identification of KX2-391 as an inhibitor of HBV transcription by a recombinant HBV-based screening assay. Antiviral Res. 144, 138–146. https://doi.org/10.1016/j.antiviral.2017.06.005.

Hemann, E.A., Gale, M., Savan, R., 2017. Interferon lambda genetics and biology in regulation of viral control. Front. Immunol. 8, 1707. https://doi.org/10.3389/fimmu.2017.01707.

Holla, R.P., Ahmad, I., Ahmad, Z., Jameel, S., 2013. Molecular virology of hepatitis E virus. Semin. Liver Dis. 33, 3–14. https://doi.org/10.1055/s-0033-1338110.

Hoofnagle, J.H., Nelson, K.E., Purcell, R.H., 2012. Hepatitis E. N. Engl. J. Med. 367, 1237–1244. https://doi.org/10.1056/NEJMra1204512.

Hu, Z., Lan, K.-H., He, S., Swaroop, M., Hu, X., Southall, N., Zheng, W., Liang, T.J., 2014. Novel cell-based hepatitis C virus infection assay for quantitative high-throughput screening of anti-hepatitis C virus compounds. Antimicrob. Agents Chemother. 58, 995–1004. https://doi.org/10.1128/AAC.02094-13.

Kamar, N., Dalton, H.R., Abravanel, F., Izopet, J., 2014. Hepatitis E virus infection. Clin. Microbiol. Rev. 27, 116–138. https://doi.org/10.1128/CMR.00057-13.

Li, J., Pan, C.-W., Zhou, G.-Y., Zhuge, L., Fang, P.-P., Jin, L.-X., Lin, W., Lin, X.-Z., Zheng, Y., 2016. Studies on the interference of ganciclovir to HCV liver fibrosis. Eur. Rev. Med. Pharmacol. Sci. 20, 4343–4347.

Lorenzo, F.R., Tanaka, T., Takahashi, H., Ichiyama, K., Hoshino, Y., Yamada, K., Inoue, J., Takahashi, M., Okamoto, H., 2008. Mutational events during the primary propagation and consecutive passages of hepatitis E virus strain JE03-1760F in cell culture. Virus Res. 137, 86–96. https://doi.org/10.1016/j.virusres.2008.06.005.

Meng, X.-J., 2013. Zoonotic and foodborne transmission of hepatitis E virus. Semin. Liver Dis. 33, 41–49. https://doi.org/10.1055/s-0033-1338113.

Meyerhoff, A., Albrecht, R., Meyer, J.M., Dionne, P., Higgins, K., Murphy, D., 2004. US Food and Drug Administration approval of ciprofloxacin hydrochloride for management of postexposure inhalational anthrax. Clin. Infect. Dis. 39, 303–308. https://doi.org/10.1086/421491.

Miszewska-Szyszkowska, D., Mikołajczyk, N., Komuda-Leszek, E., Wieczorek-Godlewska, R., Świder, R., Dęborska-Materkowska, D., Szmidt, J., Durlik, M., 2015. Severe cytomegalovirus infection in a second kidney transplant recipient treated with ganciclovir, leflunomide, and immunoglobulins, with complications including seizures, acute HCV infection, drug-induced pancytopenia, diabetes, cholangitis, and multi-or. Ann. Transplant. 20, 169–174. https://doi.org/10.12659/AOT.892076.

Naeem, A., Badshah, S.L., Muska, M., Ahmad, N., Khan, K., 2016. The current case of quinolones: synthetic approaches and antibacterial activity. Molecules 21, 268. https://doi.org/10.3390/molecules21040268.

Nagashima, S., Kobayashi, T., Tanaka, T., Tanggis, Jirintai, S., Takahashi, M., Nishizawa, T., Okamoto, H., 2016. Analysis of adaptive mutations selected during the consecutive passages of hepatitis E virus produced from an infectious cDNA clone. Virus Res. 223, 170–180. https://doi.org/10.1016/j.virusres.2016.07.011.

Nimgaonkar, I., Ding, Q., Schwartz, R.E., Ploss, A., 2018. Hepatitis E virus: advances and challenges. Nat. Rev. Gastroenterol. Hepatol. 15, 96–110. https://doi.org/10.1038/nrgastro.2017.150.

Nishitsuji, H., Ujino, S., Shimizu, Y., Harada, K., Zhang, J., Sugiyama, M., Mizokami, M., Shimotohno, K., 2015. Novel reporter system to monitor early stages of the hepatitis B virus life cycle. Cancer Sci. 106, 1616–1624. https://doi.org/10.1111/cas.12799.

Okamoto, H., 2013. Culture systems for hepatitis E virus. J. Gastroenterol. 48, 147–158. https://doi.org/10.1007/s00535-012-0682-0.

Okanoue, T., Sakamoto, S., Itoh, Y., Minami, M., Yasui, K., Sakamoto, M., Nishioji, K., Katagishi, T., Nakagawa, Y., Tada, H., Sawa, Y., Mizuno, M., Kagawa, K., Kashima, K., 1996. Side effects of high-dose interferon therapy for chronic hepatitis C. J. Hepatol. 25, 283–291.

Purdy, M.A., Harrison, T.J., Jameel, S., Meng, X.-J., Okamoto, H., Van der Poel, W.H.M., Smith, D.B., ICTV Report Consortium, 2017. ICTV virus taxonomy profile: hepeviridae. J. Gen. Virol. 98, 2645–2646. https://doi.org/10.1099/jgv.0.000940.

Qu, C., Xu, L., Yin, Y., Peppelenbosch, M.P., Pan, Q., Wang, W., 2017. Nucleoside analogue 2'-C-methylcytidine inhibits hepatitis E virus replication but antagonizes ribavirin. Arch. Virol. https://doi.org/10.1007/s00705-017-3444-8.

Shukla, P., Nguyen, H.T., Faulk, K., Mather, K., Torian, U., Engle, R.E., Emerson, S.U., 2012. Adaptation of a genotype 3 hepatitis E virus to efficient growth in cell culture depends on an inserted human gene segment acquired by recombination. J. Virol. 86, 5697–5707. https://doi.org/10.1128/JVI.00146-12.

Slavenburg, S., Huntjens-Fleuren, H.W.H.A., Dofferhoff, T.S.M., Richter, C., Koopmans, P.P., Verwey-Van Wissen, C.P.W.G.M., Drenth, J.P.H., Burger, D.M., 2011. Ribavirin plasma concentration measurements in patients with hepatitis C: early ribavirin concentrations predict steady-state concentrations. Ther. Drug Monit. 33, 40–44. https://doi.org/10.1097/FTD.0b013e318205f892.

Sofia, M.J., Bao, D., Chang, W., Du, J., Nagarathnam, D., Rachakonda, S., Reddy, P.G., Ross, B.S., Wang, P., Zhang, H.-R., Bansal, S., Espiritu, C., Keilman, M., Lam, A.M., Steuer, H.M.M., Niu, C., Otto, M.J., Furman, P.A., 2010. Discovery of a β-d-2'-deoxy-2'-α-fluoro-2'-β-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus. J. Med. Chem. 53, 7202–7218. https://doi.org/10.1021/jm100863x.

Spera, A.M., Eldin, T.K., Tosone, G., Orlando, R., 2016. Antiviral therapy for hepatitis C:

has anything changed for pregnant/lactating women? World J. Hepatol. 8, 557–565. https://doi.org/10.4254/wjh.v8.i12.557.

Takahashi, M., Hoshino, Y., Tanaka, T., Takahashi, H., Nishizawa, T., Okamoto, H., 2008. Production of monoclonal antibodies against hepatitis E virus capsid protein and evaluation of their neutralizing activity in a cell culture system. Arch. Virol. 153, 657–666. https://doi.org/10.1007/s00705-008-0045-6.

Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.C., Bradley, D.W., Fry, K.E., Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185, 120–131.

Tanaka, T., Takahashi, M., Kusano, E., Okamoto, H., 2007. Development and evaluation of an efficient cell-culture system for Hepatitis E virus. J. Gen. Virol. 88, 903–911. https://doi.org/10.1099/vir.0.82535-0.

Tannous, B.A., Kim, D.-E., Fernandez, J.L., Weissleder, R., Breakefield, X.O., 2005. Codon-optimized *Gaussia* luciferase cDNA for mammalian gene expression in culture and *in vivo*. Mol. Ther. 11, 435–443. https://doi.org/10.1016/j.ymthe.2004.10.016.

Te, H.S., Drobeniuc, J., Kamili, S., Dong, C., Hart, J., Sharapov, U.M., 2013. Hepatitis E virus infection in a liver transplant recipient in the United States: a case report. Transplant. Proc. 45, 810–813. https://doi.org/10.1016/j.transproceed.2012.08.020.

Todt, D., François, C., Anggakusuma, Behrendt P., Engelmann, M., Knegendorf, L., Vieyres, G., Wedemeyer, H., Hartmann, R., Pietschmann, T., Duverlie, G., Steinmann, E., 2016a. Antiviral activities of different interferon types and subtypes against hepatitis E virus replication. Antimicrob. Agents Chemother. 60, 2132–2139. https://doi.org/10.1128/AAC.02427-15.

Todt, D., Gisa, A., Radonic, A., Nitsche, A., Behrendt, P., Suneetha, P.V., Pischke, S., Bremer, B., Brown, R.J.P., Manns, M.P., Cornberg, M., Bock, C.T., Steinmann, E., Wedemeyer, H., 2016b. In vivo evidence for ribavirin-induced mutagenesis of the hepatitis E virus genome. Gut 65, 1733–1743. https://doi.org/10.1136/gutjnl-2015-311000.

van der Valk, M., Zaaijer, H.L.H.L., Kater, A.P.A.P., Schinkel, J., 2017. Sofosbuvir shows antiviral activity in a patient with chronic hepatitis E virus infection. J. Hepatol. 66, 242–243. https://doi.org/10.1016/j.jhep.2016.09.014.

Wang, Y., Zhou, X., Debing, Y., Chen, K., Van Der Laan, L.J.W., Neyts, J., Janssen, H.L.A., Metselaar, H.J., Peppelenbosch, M.P., Pan, Q., 2014. Calcineurin inhibitors stimulate and mycophenolic acid inhibits replication of hepatitis E virus. Gastroenterology 146, 1775–1783. https://doi.org/10.1053/j.gastro.2014.02.036.

Wang, W., Hakim, M.S., Nair, V.P., de Ruiter, P.E., Huang, F., Sprengers, D., Van Der Laan, L.J., Peppelenbosch, M.P., Surjit, M., Pan, Q., 2016a. Distinct antiviral potency of sofosbuvir against hepatitis C and E viruses. Gastroenterology 151, 1251–1253. https://doi.org/10.1053/j.gastro.2016.09.061.

Wang, Y., Zhao, C., Qi, Y., Geng, Y., 2016b. Hepatitis E virus. Adv. Exp. Med. Biol. 948, 1–16. https://doi.org/10.1007/978-94-024-0942-0_1.

Wang, W., Wang, Y., Debing, Y., Zhou, X., Yin, Y., Xu, L., Herrera Carrillo, E., Brandsma, J.H., Poot, R.A., Berkhout, B., Neyts, J., Peppelenbosch, M.P., Pan, Q., 2017a. Biological or pharmacological activation of protein kinase C alpha constrains hepatitis E virus replication. Antiviral Res. 140, 1–12. https://doi.org/10.1016/j.antiviral.2017.01.005.

Wang, W., Wang, Y., Qu, C., Wang, S., Zhou, J., Cao, W., Xu, L., Ma, B., Hakim, M.S., Yin, Y., Li, T., Peppelenbosch, M.P., Zhao, J., Pan, Q., 2017b. The RNA genome of hepatitis E virus robustly triggers antiviral interferon response. Hepatology. https://doi.org/10.1002/hep.29702.

Wong, J.P., Yang, H., Blasetti, K.L., Schnell, G., Conley, J., Schofield, L.N., 2003. Liposome delivery of ciprofloxacin against intracellular Francisella tularensis infection. J. Control. Release 92, 265–273.

Wu, X., Dao Thi, V.L., Liu, P., Takacs, C.N., Xiang, K., Andrus, L., Gouttenoire, J., Moradpour, D., Rice, C.M., 2018. Pan-genotype hepatitis E virus replication in stem cell-derived hepatocellular systems. Gastroenterology 154 (663–674), e7. https://doi.org/10.1053/j.gastro.2017.10.041.

Yi, G., Deval, J., Fan, B., Cai, H., Soulard, C., Ranjith-Kumar, C.T., Smith, D.B., Blatt, L., Beigelman, L., Kao, C.C., 2012. Biochemical study of the comparative inhibition of hepatitis C virus RNA polymerase by VX-222 and filibuvir. Antimicrob. Agents Chemother. 56, 830–837. https://doi.org/10.1128/AAC.05438-11.

Yin, X., Ambardekar, C., Lu, Y., Feng, Z., 2016. Distinct entry mechanisms for nonenveloped and quasi-enveloped hepatitis E viruses. J. Virol. 90, 4232–4242. https://doi.org/10.1128/JVI.02804-15.

Yin, X., Li, X., Ambardekar, C., Hu, Z., Lhomme, S., Feng, Z., 2017. Hepatitis E virus persists in the presence of a type III interferon response. PLoS Pathog. 13, e1006417. https://doi.org/10.1371/journal.ppat.1006417.

Zanoni, I., Granucci, F., Broggi, A., 2017. Interferon (IFN)-λ takes the helm: immunomodulatory roles of type III IFNs. Front. Immunol. 8, 1661. https://doi.org/10.3389/fimmu.2017.01661.