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# Antiviral candidates against the hepatitis E virus (HEV) and their combinations inhibit HEV growth in *in vitro*

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ABSTRACT

Hepatitis E is a global public health problem. Ribavirin (RBV) and pegylated interferon alpha are currently administered to cure hepatitis E. Recently, in combination with RBV, sofosbuvir (SOF), an anti-hepatitis C virus nucleotide analog, is also given to patients with chronic hepatitis E. However, this combinatorial therapy sometimes fails to achieve a sustained virological response. In this study, we used 27 antiviral compounds, including 15 nucleos(t)ide analogs, for *in vitro* screening against a genotype 3 HEV strain containing a *Gaussia* luciferase reporter. RBV, SOF, 2′-*C*-methyladenosine, 2′-*C*-methylcytidine (2CMC), 2′-*C*-methylguanosine (2CMG), and two 4′-azido nucleoside analogs (R-1479 and RO-9187) suppressed replication of the reporter genome, while only RBV, SOF, 2CMC and 2CMG inhibited the growth of genotype 3 HEV in cultured cells. Although 2CMG and RBV (2CMG/RBV) exhibited a synergistic effect while SOF/RBV and 2CMC/RBV showed antagonistic effects on the reporter assay, these three nucleos(t)ide analogs acted additively with RBV in inhibiting HEV growth in cultured cells. Furthermore, SOF and 2CMG, with four interferons (IFN-α2b, IFN-λ1, IFN-λ2 and IFN-λ3), inhibited HEV growth efficiently and cleared HEV in cultured cells. These results suggest that, in combination with RBV or interferons, SOF and 2CMG would be promising bases for developing anti-HEV nucleos(t)ide analogs.

## 1. Introduction

Hepatitis E is generally an acute and self-limiting hepatitis. This hepatitis is caused by hepatitis E virus (HEV) from polluted water and via the fecal-oral route in developing countries. Over the last decade, hepatitis E has been increasingly reported—in both developing and developed countries—as a zoonotic food-borne, transfusion-associated, or organ transplantation disease. Hepatitis E is sometimes fulminant and fatal, and is associated with a mortality rate of 0.5–3% in young adults; however, in pregnant women, this rate reaches 30% (Hoofnagle et al., 2012; Nimgaonkar et al., 2018; Wang et al., 2016b).

HEV is classified into *Orthohepevirus* and *Piscihepevirus* genera within the *Hepeviridae* family (Purdy et al., 2017). HEV has an approximately 7.2-kilobase (kb) single-stranded, positive-sense RNA genome (Tam et al., 1991). The HEV genome contains three open reading frames (ORFs), which encode a nonstructural polyprotein involved in viral replication, ORF1; the 660-amino acid virus capsid, ORF2; and a 13-kDa phosphoprotein of 113 or 114 amino acids, ORF3 (Holla et al., 2013; Tam et al., 1991). ORF2 and ORF3 are translated from an approximately 2.2-kb bicistronic subgenomic RNA (Graff et al., 2006; Ichiyama et al., 2009).

At present ribavirin (RBV) and pegylated interferon α (PEG-IFN) are administered to treat hepatitis E (Kamar et al., 2014; Nimgaonkar et al., 2018; Wang et al., 2016b). However, these drugs sometimes fail to achieve a sustained virologic response (SVR) and are associated with major side effects, such as leukopenia, thrombocytopenia, increased 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). As drug repurposing, sofosbuvir (SOF), which is administered to patients with chronic hepatitis C, is also suggested to be effective in

*Abbreviations:* 2CMA, 2′-*C*-methyladenosine; 2CMC, 2′-*C*-methylcytidine; 2CMG, 2′-*C*-methylguanosine; 2CMU, 2′-*C*-methyluridine; GLuc, *Gaussia* luciferase; HCV, hepatitis C virus; HEV, hepatitis E virus; IFN-α2b, interferon α2b; IFN-λ1, interferon-λ1; IFN-λ2, interferon-λ2; IFN-λ3, interferon-λ3; ORF, open reading frame; PEG-IFN, pegylated interferon alpha; RBV, ribavirin; SOF, sofosbuvir; SVR, sustained virological response

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inhibiting HEV replication (Dao Thi et al., 2016). In an *in vitro* assay using a luciferase reporter, Wang et al. (2016a) reported that SOF is not effective for either Sar55 (genotype 1) or KernowC1/p6 (genotype 3), while Dao Thi et al. (2016) reported that SOF is effective for KernowC1/p6 but not Sar55. In our previous study, a genotype 3 HEV (JE03-1760F)-based replicon was sensitive to SOF (Nishiyama et al., 2019). In patients with chronic hepatitis E, SOF failed to achieve an SVR in 4 cases (3 cases with SOF plus RBV) (Donnelly et al., 2017; Todesco et al., 2018, 2017; van der Valk et al., 2017). In contrast, HEV RNA was eradicated with SOF plus RBV in an immunosuppressed kidney transplant recipient with refractory hepatitis E (Drinane et al., 2019), and a patient with acute-on-chronic liver failure due to HEV was successfully treated with SOF plus RBV (Biliotti et al., 2018). SOF is an oral uridine nucleotide analog and is a prodrug of 2′-deoxy-2′-fluoro-2′-*C*-methyluridine monophosphate. Recent reports demonstrated that 2′-*C*-methylcytidine (2CMC) inhibits HEV growth (Qu et al., 2017; van der Valk et al., 2017), suggesting that other 2′-*C*-methyl ribonucleoside class compounds may have an inhibitory effect on HEV growth.

We previously performed anti-HEV drug screening with a *Gaussia* luciferase (GLuc) reporter construct, in which the *orf2* gene of JE03-1760F HEV strain (genotype 3) is replaced with GLuc, and tested candidate anti-HEV drugs/compounds for the ability to suppress HEV growth using a cell culture system with HEV-producing PLC/PRF/5 cells (Nishiyama et al., 2019). In the study, we found that IFN-λ1, IFN-λ2 and IFN-λ3 (collectively, IFN-λ1-3) efficiently inhibit HEV growth in cultured cells over a long time course (Nishiyama et al., 2019), corroborating previous studies in which IFN-λ1 and IFN-λ3 were shown to inhibit HEV replication (Shukla et al., 2012; Todt et al., 2016a; Yin et al., 2017).

Therefore, in the present study, using our previously established anti-HEV screening systems, we tested the HEV growth inhibitory effects of 31 anti-viral drugs/compounds, including RBV, as a control, and interferons (IFN-α2b and IFN-λ1-3), as well as those belonging to the 2′-methyl riboside and the 4′-azido riboside classes (Table 1), and evaluated the combination effects of two compounds/drugs in an attempt to identify anti-HEV candidates that act synergistically and more efficiently in comparison to mono-drugs.

## 2. Materials and methods

### 2.1. Compounds

The compounds and interferons used in this study are listed in Table 1. The structures of the nucleos(t)ide analogs tested are shown in Supplementary Fig. S1.

### 2.2. In vitro transcription and capping

pJE03-1760F/P10-GLuc plasmid (Nishiyama et al., 2019) was digested and linearized with BamHI-HF (R0136; New England Biolabs Inc., Ipswich, MA, USA), and subjected to synthesis of 1760F/P10-GLuc RNA with an AmpriScribe™ T7-*Flash*™ Transcription Kit (ASF3507; epicentre/Illumina, Inc., San Diego, CA, USA) and then purified and capped with a ScriptCap™ $m^7G$ capping System (C-SCCE0625; CELLSCRIPT, Madison, WI, USA) according to the manufacturer's protocol.

### 2.3. 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; 10,270; Gibco/Thermo Fisher Scientific), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and

**Table 1**
Compounds and interferons used in this study.

| Compound/interferon | Class of agent | CAS RN | Supplier | Cat. No. |
|---|---|---|---|---|
| Ribavirin (RBV) | nucleoside analog | 36791-04-5 | WAKO (Osaka, Japan) | 182–02331 |
| Sofosbuvir (SOF) | nucleotide analog (2′-methyl class) | 1190307-88-0 | MedChemExpress (Monmouth Junction, NJ, USA) | HY-15005 |
| 2′-C-methyladenosine (2CMA) | nucleoside analog (2′-methyl class) | 15397-12-3 | Carbosynth (Compton, Berkshire, UK) | NM07917 |
| 2′-C-methylguanosine (2CMG) | nucleoside analog (2′-methyl class) | 374750-30-8 | Carbosynth | NM07819 |
| 2′-C-methylcytidine (2CMC) | nucleoside analog (2′-methyl class) | 20724-73-6 | Carbosynth | NM07918 |
| 2′-C-methyluridine (2CMU) | nucleoside analog (2′-methyl class) | 31448-54-1 | Carbosynth | NM07919 |
| R-1656 | nucleoside analog (2′-methyl class) | 817204-33-4 | MedChemExpress | HY-10165 |
| R-7128 (Mericitabine) | nucleoside analog (2′-methyl class) | 940908-79-2 | MedChemExpress | HY-10240 |
| R-1479 (Boceprevir) | nucleoside analog (4′-azido class) | 478182-28-4 | MedChemExpress | HY-10444 |
| RO-9187 | nucleoside analog (4′-azido class) | 876708-03-1 | MedChemExpress | HY-10870 |
| Nucleoside-Analog-1 | nucleoside analog (4′-azido class) | 876707-99-2 | MedChemExpress | HY-77651 |
| Nucleoside-Analog-2 | nucleoside analog (4′-azido class) | 876708-01-9 | MedChemExpress | HY-77652 |
| R-1626 (Balapiravir) | nucleoside analog (4′-azido class) | 690270-29-2 | MedChemExpress | HY-10443 |
| Mizoribine | nucleoside analog (imidazole-type) | 50924-49-7 | WAKO | 138–17,061 |
| T-705 (Favipiravir) | pyrazine analog | 259793-96-9 | Selleck Chemicals (Houston, TX) | S7975 |
| ABT-333 (Dasabuvir)[b] | HCV NS5B polymerase inhibitor | 1132935-63-7 | MedChemExpress | HY-13998 |
| GS-9190 (Tegobuvir)[b] | HCV NS5B polymerase inhibitor | 1000787-75-6 | MedChemExpress | HY-10544 |
| VCH-916[b] | HCV NS5B polymerase inhibitor | 1200133-34-1 | MedChemExpress | HY-13465 |
| HCV-796 (Nesbuvir)[b] | HCV NS5B polymerase inhibitor | 691852-58-1 | MedChemExpress | HY-14775 |
| TMC647055[a, b] | HCV NS5B polymerase inhibitor | 1204416-97-6 | MedChemExpress | HY-15591A |
| VX-222 (Lomibuvir)[b] | HCV NS5B polymerase inhibitor | 1026785-59-0 | MedChemExpress | HY-75800 |
| Amantadine | influenza A virus M2 proton channel inhibitor | 665-66-7 | Sigma-Aldrich (St. Louis, MO) | A1260 |
| Chloroquine | HCV growth inhibitor (antimalarial agent) | 50-63-5 | Sigma-Aldrich | C6628 |
| Fidaxomicin | antibiotics | 873857-62-6 | Selleck Chemicals | S4227 |
| NIK333 (Peretinoin) | HCV RNA replication inhibitor (acyclic retinoid) | 81485-25-8 | MedChemExpress | HY-100008 |
| YK-4-279 | RNA helicase inhibitor | 1037184-44-3 | Selleck Chemicals | S7679 |
| Moroxydine | broad-spectrum antiviral agent | 3160-91-6 | Tokyo Chemical Industry (Tokyo, Japan) | M2443 |
| Interferon α2b (IFN-α2b) | interferon | 98530-12-2 | HumanZyme (Chicago, IL) | HZ-1072 |
| Interferon λ1 (IFN-λ1) | interferon | – | HumanZyme | HZ-1156 |
| Interferon λ2 (IFN-λ2) | interferon | – | HumanZyme | HZ-1235 |
| Interferon λ3 (IFN-λ3) | interferon | – | HumanZyme | HZ-1245 |

[a] Choline salt.
[b] Non-nucleoside inhibitor of HCV polymerase.

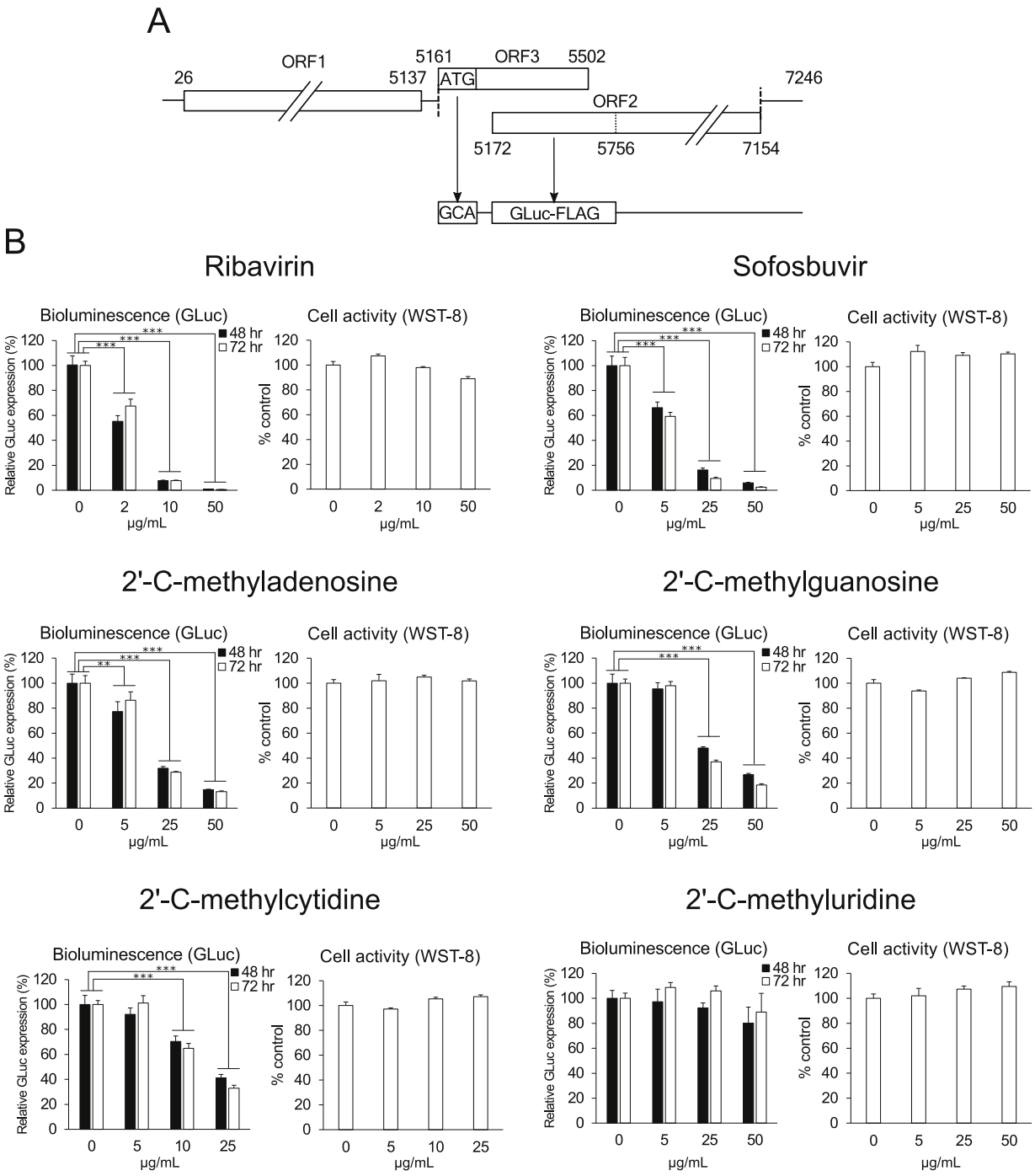



**Fig. 1. Agents of 2′-*C*-methyl ribonucleos(t)ides inhibit HEV replication.** (A) A schematic illustration of the GLuc reporter construct to monitor HEV RNA replication. (B) Inhibitory activities of ribavirin (RBV), sofosbuvir (SOF), 2′-*C*-methyladenosine (2CMA), 2′-*C*-methylguanosine (2CMG), 2′-*C*-methylcytidine (2CMC), and 2′-*C*-methyluridine (2CMU) on HEV replication in PLC/PRF/5 cells were tested with the HEV replication reporter construct. 2CMA, 2CMG, and 2CMC, but not 2CMU, inhibited HEV replication in a dose-dependent manner. RBV is as positive control. Ten μg/mL correspond to 40.1 μM in RBV, 18.9 μM in SOF, 35.6 μM in 2CMA, 33.6 μM in 2CMG, 38.9 μM in 2CMC, and 63.2 μM in 2CMU. Error bars represent the mean ± SD (n = 3).

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 transfect 1760F/P10-GLuc RNA, a *Trans*IT-mRNA Transfection Kit (MIR2225; Mirus Bio LLC., Madison, WI, USA) was used according to the manufacturer's protocol, as described previously (Nishiyama et al., 2019).

### 2.4. Measurement of Gaussia luciferase activity

Four microliters of culture supernatants were diluted with 36 μl of fresh growth medium (Σ = 40 μl) in a 96-well microplate (Berthold Technologies, Bad Wildbad, Germany). An equal volume (40 μl) of reaction buffer (10 mM EDTA, 0.01% Tween20 in PBS, 2.5 μg/mL coelenterazine [CZ-250; JNC Corporation, Tokyo, Japan]) was injected into the well and the luminescence kinetics were measured with a TriStar$^2$ LB942 multimode plate reader (Berthold Technologies). The measured initial luminescence intensity (*I*max) was converted to the relative GLuc expression with the standard curve. The obtained values were normalized to vehicle control. In the drug combination assay, inhibition was determined and modeled with the MacSynergy II software program (Prichard and Shipman, 1990).

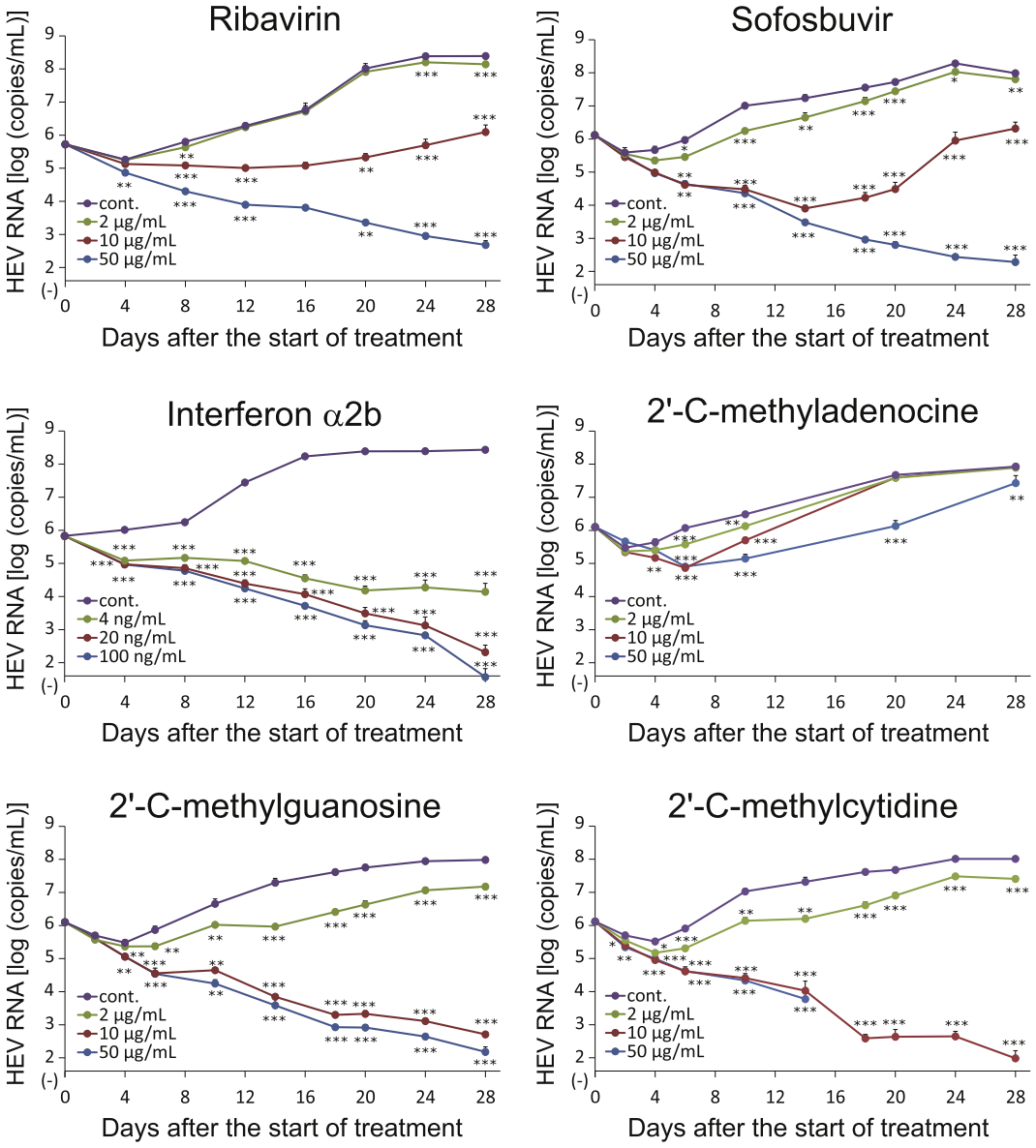



**Fig. 2. 2′-*C*-methyl ribonucleoside and other antiviral agents inhibit HEV growth in cultured cells.** As an *in vitro* HEV spreading model, naïve PLC/PRF/5 cells and HEV-infected cells (with a plateau-titer of HEV production) were co-seeded. The selected antiviral agents with the HEV replication reporter system were applied to the *in vitro* spreading model for 28 days. Ribavirin and interferon α2b were used as a positive control. The panels show the means of three independent experiments. Ten μg/mL correspond to 40.1 μM in RBV, 18.9 μM in SOF, 35.6 μM in 2CMA, 33.6 μM in 2CMG, and 38.9 μM in 2CMC. In the 2CMC panel, the treatment with 50 μg/mL was stopped to due to cell detachment. Error bars represent the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA with Tukey-Kramer test.

### 2.5. 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.

### 2.6. HEV inoculation and drug assessment

To prepare HEV-producing cells, virus-producing cells (HEV-infected PLC/PRF/5 cells [with a plateau-titer of HEV production] at $1.5 \times 10^3$ cells/well) and naïve PLC/PRF/5 cells (at $3.0 \times 10^5$ cells/well) were mixed and seeded onto a 24-well plate (BioLite 24 Well Multidish; 930,186, Thermo Fisher Scientific). Two days later, the cells were rinsed with PBS twice, and then fresh growth medium supplemented with appropriate concentrations of compound(s) was added. Half of the growth medium was collected and supplemented with fresh medium containing one or two kinds of compounds every other day. The collected growth medium was subjected to a reverse transcription-quantitative PCR (RT-qPCR).

### 2.7. Quantitation of HEV RNA

RNA was purified using TRIzol-LS Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Then, HEV RNA was quantitated by an RT-qPCR with a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) and specific primers and

TaqMan probe set targeting the ORF2 and ORF3 overlapping region, as described previously (Takahashi et al., 2008).

### 2.8. Statistical analysis

All values are described as the mean ± standard deviation (SD). The significance of differences was assessed by a one-way analysis of variance (ANOVA), with differences among groups assessed by Tukey-Kramer post-hoc analysis. Probability of < 5% ($*P < 0.05$), 1% ($**P < 0.01$) or 0.1% ($***P < 0.001$) was considered to indicate statistical significance.

## 3. Results

### 3.1. 2′-C-methyl ribonucleoside class suppressed HEV RNA replication in the reporter assay

To screen anti-HEV compounds from 27 candidate antivirals (Table 1; excluding four interferons that exhibited anti-HEV activities in our previous study [Nishiyama et al., 2019]), we employed a system in which HEV RNA replication was monitored using GLuc based on the JE03-1760F (genotype 3) strain (Fig. 1A). As a result, 2′-methyl class of nucleos(t)ide analogs exhibited inhibitory activity on HEV RNA replication (Fig. 1B, Supplementary Fig. S2). For short time-courses (48 h and 72 h), SOF, 2′-*C*-methylcytidine (2CMC), 2′-*C*-methylguanocyne (2CMG), and 2′-*C*-methyladenocine (2CMA) but not 2′-*C*-methyluridine (2CMU) exhibited an inhibitory effect on HEV RNA replication, similar to RBV (a positive control) (Fig. 1B). In addition to 2′-*C*-methyl nucleos(t)ide analogs, 4′-azido nucleoside analogs also exhibited an inhibitory effect on HEV RNA replication (R-1479 and RO-9187 but not nucleoside-analog-1, nucleoside-analog-2, and R-1626) (Supplementary Fig. S2). Unfortunately, the development of 4′-azido nucleoside analogs had been stopped due to toxicity, lack of efficacy, and other reasons (Sofia, 2014). None of the six non-nucleos(t)ide type inhibitors of hepatitis C virus (HCV) NS5B polymerase tested (Table 1) were effective for inhibiting HEV RNA replication (Supplementary Fig. S2).

### 3.2. 2CMC and 2CMG inhibit HEV growth in vitro

Next, we tested whether those four compounds (SOF, 2CMA, 2CMG, and 2CMC), belonging to the 2′-*C*-methyl nucleos(t)ide analog class, inhibit HEV growth in our previously reported *in vitro* HEV spreading model (Nishiyama et al., 2019). In this model, we used HEV-infected PLC/PRF/5 cells propagating HEV at $10^5$-$10^6$ copies/mL in culture medium, half of which was replaced with growth medium supplemented with appropriate compounds/drugs every other day (Nishiyama et al., 2019). SOF, 2CMG, and 2CMC efficiently suppressed HEV growth, similar to RBV and interferon α2b (IFN-α2b), while 2CMA inhibited HEV growth less efficiently (Fig. 2). In addition to 2CMA, neither 4′-azido nucleoside analog (R-1479 and RO-9187) could sufficiently suppress HEV growth (Supplementary Fig. S3). Moreover, none of the six HCV NS5B (non-nucleos(t)ide) polymerase inhibitors listed in Table 1 inhibited HEV growth (data not shown). Thus, we used 2′-*C*-methyl nucleos(t)ide analogs in the subsequent assays.

### 3.3. 2CMG and RBV exhibited a synergistic inhibitory effect on HEV RNA replication

In the GLuc reporter HEV replication assay, the combined administration of 2CMC and RBV (2CMC/RBV) and that of SOF/RBV exhibited a weak antagonistic effect on HEV RNA replication (Supplementary Figs. S4 and S5, respectively). Of interest, the combination of 0–50 μg/mL of 2CMG and 0–10 μg/mL of RBV dose-dependently inhibited HEV RNA replication, as shown in Fig. 3A. The synergistic effect of 2CMG/RBV on the inhibition of HEV RNA replication was observed with MacSynergy II (Prichard and Shipman, 1990) (Fig. 3B). These data suggest that RBV acts synergistically with 2CMG, but antagonistically with pyrimidine nucleos(t)ides such as 2CMC and SOF, on HEV RNA replication.

### 3.4. In addition to 2CMG, SOF and 2CMC act additively with RBV in inhibiting HEV growth in HEV-infected cultured cells

Although SOF and 2CMC acted antagonistically with RBV in the GLuc reporter assay, the mono-administration of SOF, 2CMG, and 2CMC effectively and dose-dependently suppressed *in vitro* HEV growth in cultured cells (Fig. 2). Then, we tested the inhibitory effect of these three drugs in combination with RBV on *in vitro* HEV growth. At a dose of 25 μg/mL, the mono-drugs of 2CMG and 2CMC could inhibit HEV growth but not SOF (Fig. 4, left column). In combination with RBV, all three compounds more effectively inhibited HEV growth (Fig. 4, right column). These data support the synergistic effect of 2CMG/RBV on HEV RNA replication as indicated in Fig. 3. Contrary to the antagonistic effects by 2CMC/RBV and SOF/RBV found in the HEV replication reporter assay, these combinations exhibited an additive effect on HEV growth in cultured cells (Fig. 4). No cytotoxicity was observed in the tested combination treatments (Supplementary S6A). Of note, the ORF2 proteins were not detected by Western blotting in culture supernatants (day 60 in Fig. 4) of mono (2CMG or 2CMC)- or combinatorially (RBV/SOF, RBV/2CMG or RBV/2CMC)-administered cells with drugs at high concentration (25 μg/mL) (Supplementary Fig. S7). In addition, the HEV genomes were not detected in the lysates of the cells recovered on day 60 (data not shown). As a result, HEV RNA continued to be undetectable, even in plain media (Supplementary Fig. S8).

### 3.5. SOF and 2CMG efficiently inhibit in vitro HEV growth in combination with interferons

We previously reported that IFN-λ1-3 inhibited HEV growth in PLC/PRF/5 cells, similar to IFN-α2b (Nishiyama et al., 2019). In addition, it has been reported that in the treatment of genotype 1 HCV infected patients, combination therapy with PEG-IFN and RBV plus SOF resulted in a higher SVR rate in comparison to conventional PEG-IFN and RBV combination therapy (Dolatimehr et al., 2017). We therefore tested whether SOF and 2CMG inhibit *in vitro* HEV growth more efficiently in combination with interferons (IFN-α2b, IFN-λ1-3). SOF (10 μg/mL) or 2CMG (10 μg/mL) was tested in combination with interferons at concentrations of 4, 20, and 100 ng/mL. As shown in Fig. 5, SOF and 2CMG exhibited highly inhibitory and additive effects on HEV growth in cultured cells in combination with either of the four interferons. No cytotoxicity was observed in the tested combination treatments (Supplementary S6B,C). The ORF2 proteins in culture supernatants were not detected in mono- (IFNα2b, IFN-λ1, IFN-λ2 or IFN-λ3)-, and combinatorially (SOF/IFNα2b, 2CMG/IFNα2b, SOF/IFN-λ1, 2CMG/IFN-λ1, SOF/IFN-λ2, 2CMG/IFN-λ2, SOF/IFN-λ3 or 2CMG/IFN-λ3)-administered cells with drugs at high concentration (100 ng/mL) (Supplementary Fig. S9). In addition, the HEV genomes were not detected in the lysates of any combinatorially-treated cells (data not shown). To test whether HEV RNA became completely undetectable in these combinatorially-treated cells, media containing the compounds were changed to plain media and cultured for an additional 32 days. As a result, HEV RNA continued to be undetectable, even in plain media (Supplementary Fig. S8), although newly inoculated HEV could grow efficiently in cells that had been cultivated for 60 days in the presence of the compounds and eradicated HEV, reaching the viral loads of $10^8$ copies/well during 30 days of cultivation (data not shown), indicating that the cells were still viable and susceptible to HEV growth on day 60.

## 4. Discussion

In this study, we tested the inhibitory activity of 27 antiviral compounds on HEV RNA replication using the GLuc reporter HEV replicon

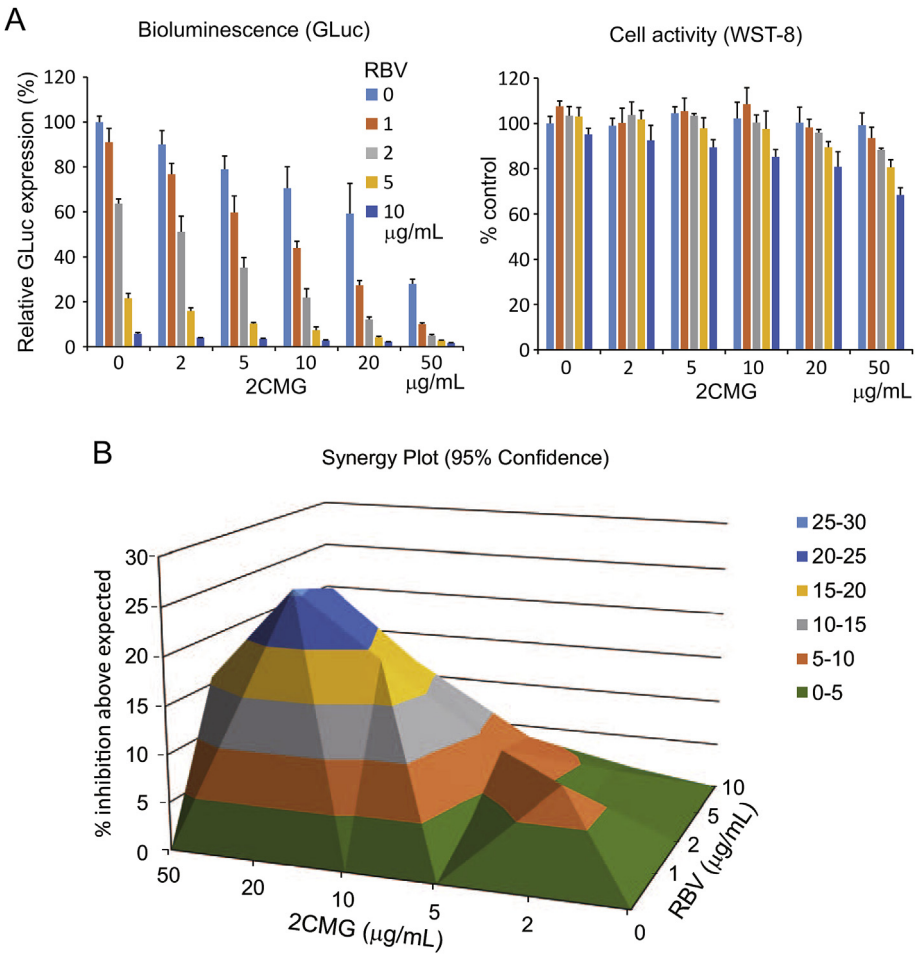



**Fig. 3. The combined administration of 2CMG and RBV exhibited a synergistic effect on HEV RNA replication.** (A) 2CMG (0–50 μg/mL) and RBV (0–10 μg/mL) were administered in combination for 3 days with the HEV RNA replication reporter system. (B) The synergistic effect was calculated with the MacSynergy II software program. Ten μg/mL correspond to 40.1 μM in RBV and 33.6 μM in 2CMG. See Supplementary Table S2 for statistical analysis.

assay. In addition to RBV, four 2′-methyl (SOF, 2CMA, 2CMG and 2CMC) and two 4′-azido (R-1479 and RO-9187) nucleos(t)ide analogs suppressed HEV RNA replication. Among the four 2′-methyl nucleos(t)ide analogs, SOF, 2CMG, and 2CMC inhibited HEV growth in PLC/PRF/5 cells, and exhibited additive inhibitory activity on *in vitro* HEV growth in combination with RBV, although SOF/RBV and 2CMC/RBV acted antagonistically to each other in the GLuc reporter assay. Moreover, SOF and 2CMG exhibited higher inhibitory activity on *in vitro* HEV growth in combination with four interferons (IFN-α2b and IFN-λ-3). In contrast, two 4′-azido nucleoside analogs, R-1479 and RO-9187, exhibited only weak inhibitory activity on HEV growth in cultured cells, although showed strong inhibitory effects on HEV RNA replication in the GLuc reporter assay (Supplementary Figs. S2 and S3). Our results suggest that SOF and 2CMG would be promising bases for candidates to cure hepatitis E, and that the combinations of 2′-*C*-methyl nucleos(t)ide analogs and RBV or interferons are likely to be more effective for inhibiting HEV growth than the mono-drugs.

Among the four 2′-methyl-class nucleoside analogs tested in the present study (2CMA, 2CMG, 2CMC and 2CMU), 2CMU did not exhibit an inhibitory effect on HEV RNA replication in the GLuc reporter assay and 2CMA did not inhibit HEV growth in cultured cells, despite the strong inhibitory activity found in the GLuc reporter assay. 2CMA is reportedly susceptible to enzymatic conversion by adenosine deaminase and purine nucleoside phosphorylase (Eldrup et al., 2004a, 2004b). Thus, the intracellular concentration of 2CMA (-triphosphate; -TP) may not have been sufficient to suppress HEV RNA replication in comparison to 2CMC (-TP) and 2CMG (-TP). Moreover, 2CMA could not suppress the GLuc expression over a long time-course (for 9 days) in the HEV replication reporter assay using GLuc (data not shown), as ineffective HEV growth inhibition was observed in cultured cells (Fig. 2).

Qu et al. (2017) reported that 2CMC inhibits genotype 3 HEV (KernowC1/p6) replication but antagonizes RBV. Indeed, Coelmont et al. (2006) showed an antagonistic effect of 2CMC/RBV on HCV replication inhibition. Corroborating this report, we observed that 2CMC inhibited the replication of another genotype 3 HEV strain of JE03-1760F/p10 and acted antagonistically with RBV in our GLuc reporter assay system (Fig. 1 and Supplementary Fig. S4), while 2CMC acted additively with RBV on HEV growth in cell culture. The same phenomenon was also observed in the combination of SOF/RBV. Notably, 2CMG displayed the synergistic inhibitory effect with RBV on genotype 3 HEV (JE03-1760F/p10) replication in both the GLuc reporter assay (Fig. 3) and HEV-producing cells (Fig. 4). RBV has been reported to have some direct/indirect inhibitory effects on virus growth; RNA-dependent RNA polymerase inhibition, translation inhibition, upregulation of interferon signaling, and reducing viral fitness (Graci and Cameron, 2006; Paeshuyse et al., 2011). The observed antagonistic effect of 2CMC/RBV and SOF/RBV in GLuc reporter assay seemingly led to reduced viral fitness (Manrubia et al., 2010).

Unfortunately, the development programs for all 4 anti-HCV 2′-methyl and 2′-deoxy-2′-fluoro-2′-methyl guanosine nucleotide prodrugs were discontinued due to toxicity, as shown in Supplementary Table S1 (Gentile et al., 2015; Sofia, 2014). These toxicities resulted from the impairment of the mitochondrial activity by these compounds (Dousson, 2018). In addition, Jin et al. (2017) displayed that the

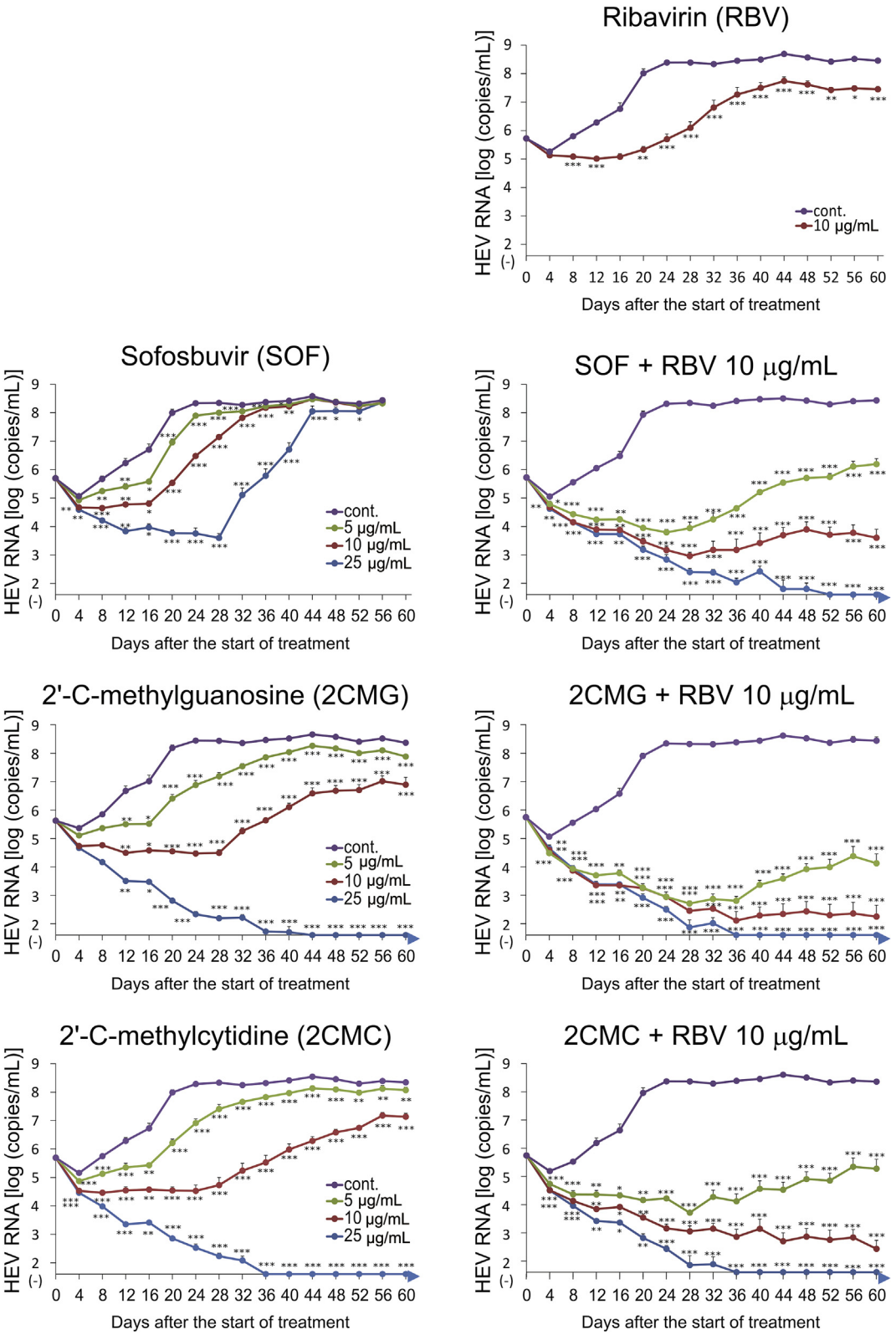
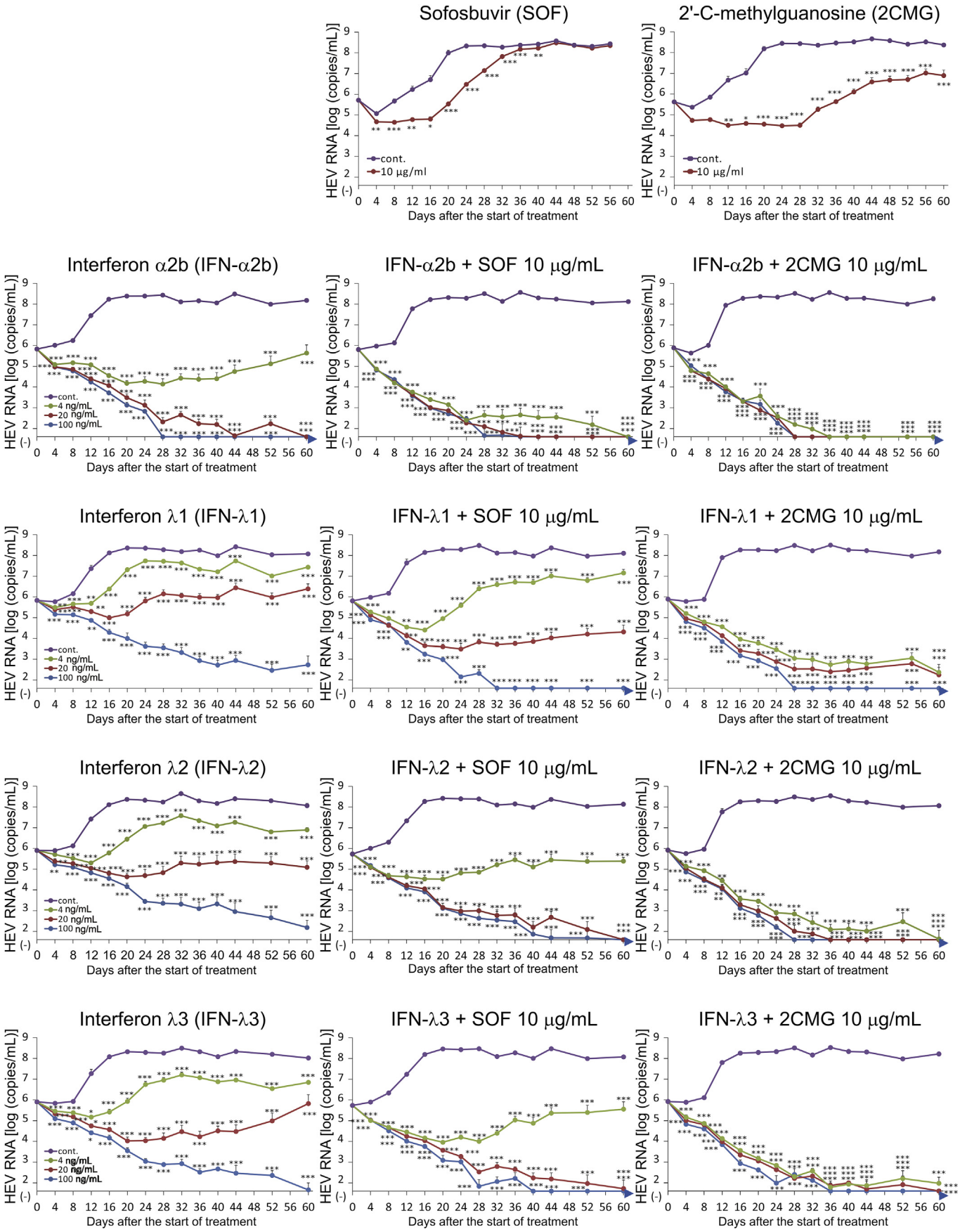



**Fig. 4. The combined administration of ribavirin and other antiviral agents significantly inhibited HEV growth in culture cells.** The combined administration of RBV (10 μg/mL) and SOF, 2CMC, or 2CMG (each 5–25 μg/mL) effectively inhibited HEV growth in comparison to each of the mono-compounds in an *in vitro* HEV spreading model. The cells were cultured and the compound was applied for 60 days. (−) indicates below limit of detection. Ten μg/mL correspond to 40.1 μM in RBV, 18.9 μM in SOF, 33.6 μM in 2CMG, and 38.9 μM in 2CMC. Arrow heads indicate that the HEV growth after the cessation of treatment is shown in Supplementary Fig. S8. Error bars represent the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA with Tukey-Kramer test.




*(caption on next page)*

**Fig. 5. The combined administration of SOF or 2CMG and interferons significantly inhibited HEV growth in culture cells.** The combined administration of SOF or 2CMG (each 10 μg/mL, corresponding to 18.9 μM or 33.6 μM, respectively) and IFN-a2b, -λ1, -λ2, or -λ3 (each 4–100 ng/mL) effectively inhibited HEV growth in comparison to each of the mono-compounds in an *in vitro* HEV spreading model. The cells were cultured and the compound was applied for 60 days. (−) indicates below limit of detection. Data in the SOF and 2CMG panels at the top were retrieved from Fig. 4. Arrow heads indicate that the HEV growth after the cessation of treatment is shown in Supplementary Fig. S8. Error bars represent the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA with Tukey-Kramer test.

prodrug moiety itself also results in cellular toxicity by changing the prodrug moiety of SOF (Supplementary Fig. S10) and INX-189 (Supplementary Table S1). At present, although there are no promising 2′-methyl guanosine nucleos(t)ide prodrugs, their derivatives that may be made available in the future drug developments would be effective anti-HEV drugs in combination with/without RBV and/or interferons.

SOF is an oral uridine nucleotide analog for the treatment of hepatitis C. Although HCV and HEV are distinct viruses belonging to different families, they both have a single-stranded, positive-sense RNA as their viral genome and cause hepatitis that can be resolved by RBV with PEG-IFN. The anti-HEV potential of one of the HCV polymerase inhibitors, SOF, has been evaluated in *in vitro* assays and clinical cases. In an *in vitro* assay, Dao Thi et al. (2016) reported that SOF could inhibit the replication of the KernowC1/p6 (genotype 3)-based replicon but not that of the Sar55 (genotype 1)-based replicon, similar to our result with JE03-1760F/p10-based replicon (Fig. 1). In contrast, Wang et al. (2016a) reported that SOF slightly inhibited the growth of KernowC1/p6 HEV but could not inhibit the replication of either Sar55-or KernowC1/p6-based replicons, suggesting that the effectiveness of SOF on HEV, evaluated by replicon, depends on the genotype and genomes of HEV. As described above, 2CMC sufficiently inhibited genotype 3 HEV in both viral RNA replications in the GLuc reporter assay and viral growth in cultured cells (Figs. 1 and 2) but not 2′-deoxy-2′-fluoro-modified 2CMC, R-1656 (Supplementary Figs. S2 and S10) (data not shown). The 2′-deoxy-2′-fluoro-modification is known to stabilize the glycosidic linkage (Stuyver et al., 2006; Watanabe et al., 1983, 1979) and improve the specificity of HCV RNA-dependent RNA polymerase (NS5B protein). Paradoxically, this modification spoils the broad spectrum antiviral activity of 2CMC on various viruses, including bovine viral diarrhea virus, West Nile virus, yellow fever virus, dengue virus, and human immunodeficiency virus (Clark et al., 2005; Dapp et al., 2014; Dousson, 2018; Gong et al., 2008; Julander et al., 2010; Lee et al., 2015; Stuyver et al., 2006). This reflects the lower inhibitory activity of SOF (prodrug of 2′-deoxy-2′-fluoro-modified 2′-*C*-methyluridine monophosphate) on HEV in comparison to HCV ($IC_{50}$ = 1.2 μM on HEV, $IC_{50}$ = 0.014–0.11 μM on HCV; reported by Dao Thi et al., 2016). The clinical use of SOF against hepatitis E gave contradicting results. Biliotti et al. (2018) observed that SOF/RBV combination therapy cleared HEV in patients with acute hepatitis E. Drinane et al. (2019) also reported that the combined administration of SOF/RBV eradicated refractory HEV, which is not fully sensitive to RBV alone, in an immunosuppressed individual. However, other groups reported that the combined administration of RBV/SOF could not clear HEV in chronic hepatitis E patients with organ transplantation or human immunodeficiency virus infection (Donnelly et al., 2017; Todesco et al., 2017; van der Valk et al., 2017). A phase 2 multicenter clinical trial evaluating the treatment of hepatitis E with SOF is currently ongoing and will clarify whether SOF is effective against HEV in patients (Kinast et al., 2019).

Some anti-HCV compounds are under development: for example, uprifosbuvir and ACH-3422 (Dousson, 2018). Uprifosbuvir is a phosphoramidate prodrug of 2′-deoxy-2′-chloro modified 2′-*C*-methyluridine (Supplementary Fig. S10) (Alexandre et al., 2017), while ACH-3422 is a deuterium incorporated phosphoramidate prodrug of 2CMU monophosphate (Supplementary Fig. S10). Since 2′-hydroxy group remains in this compound, it would have inhibitory activities on many viruses including HEV.

A variety of HEV genome modifications, including new single nucleotide variations in the RNA-dependent RNA polymerase region or insertions in the hypervariable region of ORF1, that cause RBV treatment failure have been identified in solid-organ transplant patients (Todt et al., 2018). It would be interesting to detect escape mutants in the long-term cell culture under treatment with antiviral compounds by sequencing viral RNAs in the supernatants.

Although the present study was conducted using a genotype 3 HEV strain of JE03-1760F, mono-drugs of SOF, 2CMC and 2CMG and combinations with RBV inhibited growth of rat HEV (Jirintai et al., 2014) in cultured cells (Supplementary Fig. S11). These results suggest that the compounds identified in this study inhibit other genotypes of HEV.

In conclusion, we found that 2CMG (but not 2CMC or SOF) has a synergistic effect with RBV in inhibiting HEV replication with an HEV replication reporter assay using GLuc. In cultured cells, 2CMG, 2CMC, and SOF in combination with RBV showed additive effects in inhibiting HEV growth and eradicated HEV in cultured cells. Moreover, 2CMG and SOF with four interferons acted additively in inhibiting HEV growth and cleared HEV genomes in cultured cells. Our results suggest that the phosphoramidate prodrug of both 2CMU and 2CMG monophosphates, which have the 2′-hydroxy group, would be promising anti-HEV drugs with/without RBV and/or interferons.

## Conflicts of interest

The authors declare no conflicts of interest in association with the present study.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104570.

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