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# Synergistic antiviral activity of gemcitabine and ribavirin against enteroviruses

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ABSTRACT

Enteroviruses are major causative agents of various human diseases, and some of them are currently considered to be an enormous threat to public health. However, no effective therapy is currently available for the treatment of these infections. We identified gemcitabine, a nucleoside-analog drug used for cancer treatment, from a screen of bioactive chemicals as a novel inhibitor of coxsackievirus B3 (CVB3) and enterovirus 71 (EV71). Gemcitabine potently inhibited the proliferation of CVB3 and EV71, as well as the replication of CVB3 and EV71 replicons, in cells with a low micromolar $IC_{50}$ (1–5 μM). Its strong inhibitory effect was also observed in cells infected with human rhinoviruses, demonstrating broad-spectrum antiviral effects on enteroviruses. Mechanistically, an extensive analysis excluded the involvement of 2C, 3A, IRES-dependent translation, and also that of polyprotein processing in the antiviral effects of gemcitabine. Importantly, gemcitabine in combination with ribavirin, an antiviral drug currently being used against a few RNA viruses, exhibited a synergistic antiviral effect on the replication of CVB3 and EV71 replicons. Consequently, our results clearly demonstrate a new indication for gemcitabine as an effective broad-spectrum inhibitor of enteroviruses and strongly suggest a new therapeutic strategy using gemcitabine alone or in combination with ribavirin for the treatment of various diseases associated with enterovirus infection.



## 1. Introduction

Enteroviruses are members of the picornaviridae family, a large and diverse group of small RNA viruses characterized by a single positive-strand genomic RNA, and are associated with many human and mammalian diseases (van der Schaar et al., 2013). There are four human enterovirus (HEV) species (HEV-A, -B, -C, and -D), comprising more than 100 virus serotypes (Palacios and Oberste, 2005). Coxsackievirus group B type 3 (CVB3), one of most well-studied enteroviruses among those and a member of HEV-B, is one of the main causes of viral meningitis, myocarditis, and pancreatitis (Sawyer, 2002; Whitton et al., 2005). In addition, enterovirus 71 (EV71) is a causative agent of hand-food-mouth disease and also of severe neurological symptoms, such as brainstem encephalitis and poliomyelitis-like paralysis, which can lead to cardiopulmonary failure and even death (Chumakov et al., 1979; McMinn, 2002; Song et al., 2014a; Wang et al., 2003).

Enteroviruses are non-enveloped viruses possessing a single-stranded, positive-sense RNA genome of 7,500–8,000 nucleotides that consists of an open reading frame, flanked by 5′ and 3′

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untranslated regions (UTRs) (McMinn, 2002; Wu et al., 2010b). Upon virus attachment and entry into a host cell, an uncapping event occurs to release the RNA genome into the cytoplasm. The viral RNA genome itself serves as mRNA for the initiation of translation at the internal ribosomal entry site (IRES) in the 5′ UTR, resulting in the production of a large polyprotein. The viral polyprotein is further processed into individual functional proteins (VP4, VP2, VP3, VP1, $2A^{pro}$, 2B, 2C, 3A, 3B, $3C^{pro}$, and $3D^{pol}$) by the action of viral proteases $2A^{pro}$ and $3C^{pro}$. Negative-sense RNA genomes are also generated to serve as templates for the replication of positive-sense RNA viral genomes. Amplified positive-sense RNA genomes and structural proteins (VP1, VP2, VP3, and VP4) are then assembled into virus particles and released from the host cell.

There is an increasing need to develop effective antiviral drugs for the treatment of the various diseases associated with enterovirus infection. To date, many small molecules have been reported to have inhibitory effects against enteroviruses, particularly CVB3 and EV71. Pleconaril (Shang et al., 2013; Zhang et al., 2012), CsA (Qing et al., 2014), BPROZ (Shih et al., 2004), and GPP3-1 (De Colibus et al., 2014) target virus entry. Enviroxime (Heinz and Vance, 1995) and rupintrivir (AG7088) (Dragovich et al., 1999) are potent inhibitors of 3A and $3C^{pro}$, respectively. The peptide LVLQTM inhibits $2A^{pro}$ (Falah et al., 2012), and DTrip-22 and aurintricarboxylic acid target the 3D polymerase (Chen et al., 2009; Urbinati et al., 2008). Although the precise mode of action is not certain, ribavirin, an antiviral drug used against a few RNA viruses, is also reported to have an inhibitory effect on enteroviruses (Song et al., 2014b; Urbinati et al., 2008; Zhang et al., 2012). In addition to synthetic compounds, there are several natural products such as lycorine, raoulic acid (Choi et al., 2009; Liu et al., 2011), and ginsenosides (Song et al., 2014b; Fuzzati, 2004) that possess anti-enteroviral activity. Despite the therapeutic potential of these compounds, their effectiveness needs to be further evaluated *in vivo*. In particular, extensive evaluations of rapidly moving candidate compounds such as pleconaril, enviroxime, and rupintrivir suggest that undesirable side effects *in vivo* are the limiting factors for the therapeutic application of those drugs (Shang et al., 2013; Zhang et al., 2012). Therefore, new therapeutic candidates need to be quickly identified and evaluated. In that regard, the identification of therapeutic candidates among bioactive chemicals is favorable, because a considerable number of chemicals have been proven safe in clinical settings, facilitating their therapeutic application to diseases associated with enterovirus infections in the next stage of development.

In this study, we identified gemcitabine as an effective inhibitor of CVB3 and EV71 from a screen of 1,280 bioactive chemicals (the LOPAC library). Gemcitabine strongly inhibited the proliferation of CVB3 in HeLa cells and moderately inhibited that of EV71 in LLC-MK2 Derivative cells. Its strong inhibitory effect was also shown in three different strains of human rhinovirus (HRV)-infected cells. In addition, gemcitabine showed a strong inhibitory effect on the replication of CVB3 and EV71 replicons in Vero cells, indicating its effect on virion-independent intracellular processes. Further analysis excluded the involvement of polyprotein processing by $3C^{pro}$ and $2A^{pro}$, IRES-dependent translation, and 2C and 3A in the antiviral action of gemcitabine. Instead, gemcitabine, as a nucleoside analog and a potent ribonucleotide reductase inhibitor, is likely to work by interfering with viral RNA polymerization and/or by generating a high number of viral mutations. Importantly, synergistic antiviral activities against CVB3 and EV71 were induced by co-treatment with gemcitabine and ribavirin, a drug frequently used against a few RNA viruses, suggesting a new therapeutic option against a broad spectrum of enteroviruses.

## 2. Materials and methods

### 2.1. Cells, viruses, and reagents

HeLa, 293T, and Vero cells were cultured in DMEM supplemented with 5–10% fetal bovine serum (GE Healthcare Life Sciences or Hyclone) and 1% penicillin-streptomycin. LLC-MK2 Derivative cells (ATCC CCL7.1) were purchased from ATCC and cultured in MEM supplemented with 10% fetal bovine serum. H1HeLa cells (ATCC CRL-1958) were obtained from ATCC and cultured in MEM supplemented with 10% fetal bovine serum. CVB3 (Nancy; ATCC VR-30) was obtained from ATCC and expanded in HeLa cells. EV71 (BrCr; ATCC VR-1775) was purchased from ATCC and expanded in LLC-MK2 Derivative cells. HRV types 14 (ATCC VR-284), 21 (ATCC VR-496), and 71 (ATCC VR-1181) were purchased from ATCC and expanded in H1HeLa cells. The LOPAC library was purchased from Sigma–Aldrich for the screening of antiviral compounds. Gemcitabine (Sigma–Aldrich), tracazolate (Tocris Bioscience), brefeldin A (Sigma–Aldrich), GW5074 (Sigma–Aldrich), fluoxetine (Sigma–Aldrich), BNTX (Sigma–Aldrich), rupintrivir (Santa Cruz), and ribavirin (Sigma–Aldrich) were purchased for further analysis.

### 2.2. Antibodies

Anti-flag and anti-β-actin mouse monoclonal antibodies were purchased from Sigma–Aldrich. Anti-VP1 mouse monoclonal antibody was purchased from Leica (NCL-ENTERO). Anti-3C rabbit polyclonal antibody was generated in house by immunization with recombinant 3C protein. Secondary antibodies conjugated to horseradish peroxidase for Western blotting were purchased from Thermo Fisher Scientific. Secondary antibody conjugated to Alexa Fluor 488 was obtained from Life Technologies.

### 2.3. Replicon assay

Plasmids p53CB3-LUC, pRib-LUC-CB3/T7-wt (van Kuppeveld et al., 1995; van Ooij et al., 2006; Wessels et al., 2006), and pRib-LUC-CB3/T7-(2C-A224V, I227V, 2C-A229V or 3A-H57Y) mutant clones (Ulferts et al., 2013; Wessels et al., 2006), which contain the firefly luciferase gene in place of the P1 capsid-coding region of the CVB3 viral genome, were kindly provided by Frank J. M. van Kuppeveld (Utrecht University, The Netherlands). Plasmid pRibFluc-EV71 wt, which contains the firefly luciferase gene in place of the P1 capsid-coding region of the EV71 viral genome, was also used. CVB3 and EV71 replicon plasmids were used for *in vitro* RNA transcription with the Ribomax large-scale RNA production system (Promega). To perform the screen of 1,280 bioactive chemicals (LOPAC, Sigma–Aldrich), Vero cells ($3 \times 10^5$ cells/well) in a 6-well plate were transfected with 0.4 μg CVB3 or EV71 replicon RNAs using Lipofectamine 2000 (Promega), split into 96-well plates ($2 \times 10^4$ cells/well), and simultaneously treated with 10 μM chemicals. Eight hours after treatment, the cells were assayed for firefly luciferase activity using the One-Glo Luciferase Assay System (Promega). Cell viability was also measured using CellTiter-Glo Luminescent Cell Viability assays (Promega).

### 2.4. Antiviral activity assay

To test the antiviral activity of the compounds, a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich)-based cytopathic effect (CPE) reduction assay was performed as previously described (Kim et al., 2002). Briefly, cells were seeded in 96-well plates at $2 \times 10^4$ cells per well 2 days prior to the assay. Equal volumes of virus [100 cell

culture infected dose 50% ($CCID_{50}$) per well] and compound (double concentrated) were added and incubated at 37 °C (for CVB3 and EV71) or 33 °C (for HRVs) in 5% $CO_2$ to produce an appropriate CPE. For the MTT assay, culture supernatants were removed, and 50 μl MTT solution (2.5 mg/ml in PBS) was added and incubated in a $CO_2$ incubator at 37 °C for 1 h. Then, 100 μl acidified isopropanol/10% Triton X-100 solution was added to dissolve the formazan products. Using a microplate reader (Synergy H1, BioTek), the absorbance at 540 nm (main) and at 690 nm (reference) was measured. Mock-infected, DMSO-treated cells were considered to show 100% survival, and virus-infected, DMSO-treated cells were considered to show 0% survival. The antiviral activity of the compounds was calculated as a percentage of that of the control.

### 2.5. Cell toxicity assay

Cell toxicity was measured using an MTT assay or the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega). All procedures were the same as those used in the antiviral activity assay, except that medium was added instead of virus. For the CellTiter-Glo luminescent assay kit, the assay was performed according to the manufacturer's protocol. Luciferase activity was measured using a luminometer (LB960 centro XS3, Berthold Technologies). Cell toxicity was calculated as a percentage of that of the control.

### 2.6. Immunofluorescence microscopy

Cells infected with CVB3 (5 MOI) and treated with gemcitabine were fixed and permeabilized with a 3:1 mixture of ice-cold methanol-acetone 8 h after infection. Infected cells were stained with anti-3C antibody and anti-rabbit secondary antibody conjugated with Alexa Fluor 488 followed by counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Product # 62248, Thermo Scientific). Images were captured using the Operetta system (Perkin Elmer). Viral infection was quantified by dividing the number of infected cells by the total number of nuclei using the harmony software in the Operetta system. Infection was calculated as a percentage of that of the control.

### 2.7. Western blotting and RT-PCR

Cells were infected and treated with gemcitabine as described in the immunofluorescence microscopy section. Western blotting was performed as previously described (Kim et al., 2014). Eight hours after infection, total cell lysates were harvested and analyzed using anti-3C, anti-VP1, and anti-β-actin antibodies. RT-PCR was performed as previously described (Kang et al., 2014). For RT-PCR of the viral RNA, total cellular RNAs were purified using the QIAGEN RNeasy Mini Kit according to the manufacturer's protocol. Reverse transcription was performed with random hexamers and SuperScript III reverse transcriptase (Invitrogen). The region spanning from 2A to 3C of the CVB3 genome was amplified with primers csp54 (5′-CCGGAATGTACATGTTGGG-3′) and csp57 (5′-GGCTCTGGCTTCACTAAC-3′) and Accupower PCR premix (Bioneer). GAPDH mRNAs were also analyzed as a loading control.

### 2.8. Time-of-addition assay

HeLa cells were infected with CVB3 at 5 MOI and simultaneously treated with 50 μM gemcitabine or 2 μM rupintrivir (0 h). At 1 h intervals, gemcitabine or rupintrivir were added. At 8 h post infection, the cells were fixed and stained with anti-3C antibody as described in the immunofluorescence microscopy section. Viral infection was quantified by dividing the number of infected cells by the total number of nuclei using the harmony software in the Operetta system. Infection was calculated as a percentage of that of the control.

### 2.9. IRES assay

For the generation of dual-luciferase reporter plasmids pR/EV71(BrCr)/F-PEST and pR/CVB3/F-PEST, the full-length EV71 and CVB3 5′UTRs were obtained from EV71-infected and CVB3-infected cells using RT-PCR with the appropriate primers [pR/EV71(BrCr)/F-PEST: (forward, 5′- AGCCACCATGGTACCTTAAAACAGCCTGTGGGTTGCACC-3′), (reverse, 5′-GTCCTCCATGGTACCCGCTTCGTGTTCAGCAGTATAATGTAATTG-3′); pR/CVB3/F-PEST: (forward, 5′-AGCCACCATGGTACCTTAAAACAGCCTGTGGGTTGATCCC-3′), (reverse, 5′-GTCCTCCATGGTACCCATTTTGCTGTATTCAACTTAACAATGAAT-3′)] and inserted into the Kpn I restriction site of pR/HCV374/F-PEST (kindly provided by Dr. Jong Heon Kim, National Cancer Center, South Korea). To measure IRES activity in response to gemcitabine, 293T cells ($5 \times 10^5$ cells/well) in 6-well plates were transfected with 2 μg reporter plasmids using X-tremeGENE DNA transfection reagent (Roche). Twenty-four hours post transfection, the transfected cells were split into 96-well plates, incubated with various concentrations of gemcitabine for 24 h, and assayed for firefly and *renilla* luciferase activities.

### 2.10. 3C protease assay

To construct the pcDNA3-flag-3CD plasmid, CVB3 [full-length 3C and the N-terminal part of 3D (amino acids 1–196)] and EV71 (BrCr) [full-length 3C and the N-terminal part of 3D (amino acids 1–218)] were amplified by RT-PCR and inserted into the BamHI restriction site of the pcDNA3-flag vector. The plasmid was transfected into 293T cells using the X-tremeGENE siRNA Transfection Reagent (Roche). The cells were then maintained for 15 h, treated with 10 μM gemcitabine for another 9 h, and then subjected to Western blotting with anti-flag antibody.

## 3. Results

### 3.1. Screening of small-molecule inhibitors against CVB3

In order to identify compounds with antiviral activity against enteroviruses, we screened the LOPAC library, which is a collection of bioactive compounds, using CVB3 subgenomic replicon system. This replicon system contains a firefly luciferase gene in place of the P1 structural genes (VP4–VP1) and allows easy and quantitative measurements of intracellular viral replication (Fig. 1A) (van Kuppeveld et al., 1995; van Ooij et al., 2006). In our preliminary experiments, luciferase activity from Vero cells transfected with *in vitro*-transcribed CVB3-replicon RNAs increased in a time-dependent manner and reached a maximum 10 h after transfection (data not shown). Therefore, we chose to screen the compounds for antiviral activity 8 h after applying them to the cells. Primary screening of 1,280 bioactive compounds (10 μM) in Vero cells transfected with CVB3-replicon RNAs identified 42 compounds that significantly decreased the luciferase activity (more than 80% reduction compared with DMSO-treated control; Fig. 1B). Further analysis found that 16 of the 42 compounds (10 μM) had a considerable cytotoxic effect on Vero cells (reduction of more than 20% compared with DMSO-treated control), which was assessed using CellTiter-Glo reagent. Only 26 compounds exhibited an acceptable level of cell toxicity (reduction of less than 20%

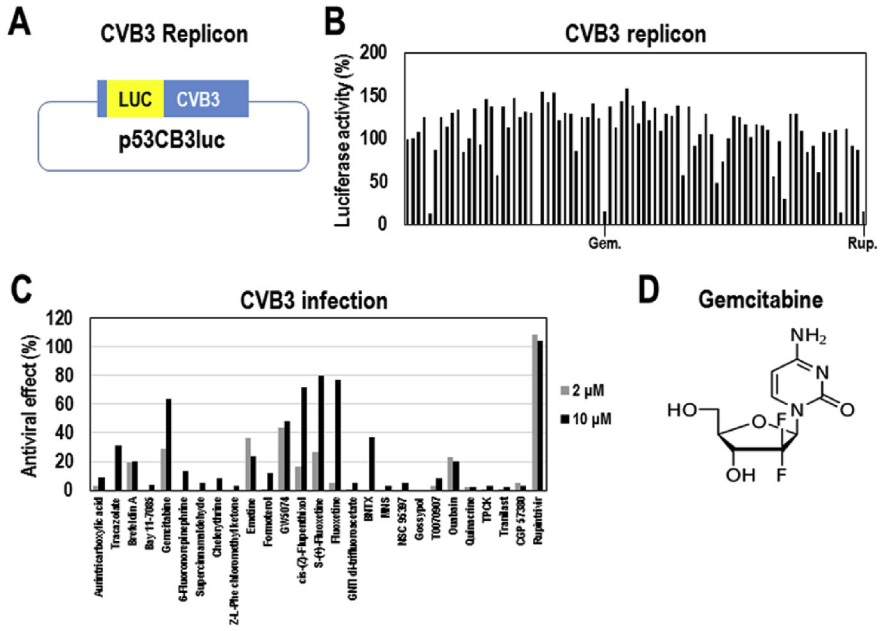


**Fig. 1.** Identification of gemcitabine as an anti-CVB3 inhibitor from a screen of a bioactive chemical library. (A) Schematic diagram of DNA encoding the CVB3 replicon. (B) Vero cells were transfected with *in vitro*-transcribed CVB3-replicon RNAs, immediately treated with 1,280 bioactive chemicals (10 μM) for 8 h, and then assayed for firefly luciferase activity. Rupintrivir (10 μM) was used as a positive control. A representative result from a plate containing gemcitabine is presented. The luciferase activity from DMSO-treated cells was considered to be 100%. (C) The antiviral activities of the primary hits were further evaluated in CVB3-infected HeLa cells. The HeLa cells were infected with CVB3 (100 $CCID_{50}$) and simultaneously treated with the 26 primary hits (2 and 10 μM). Rupintrivir (2 and 10 μM) was used as a positive control. Forty-eight hours after treatment, cell viability was analyzed using an MTT assay. The viability of DMSO-treated cells was considered to be 0%, and that of uninfected cells was considered to be 100%. (D) The chemical structure of gemcitabine.

compared with DMSO-treated control).

Next, to assess whether the compounds inhibit the proliferation of intact CVB3 in cells, CVB3-infected HeLa cells were treated with 2 and 10 μM concentrations of the 26 identified compounds for 48 h and then analyzed by MTT assay, which is often employed for the quantitative measurement of cell viability. In this assay, CVB3 infection causes a cytopathic effect (CPE) on the HeLa cells, reducing the cell viability by almost 100%, and any compound with antiviral activity can cause a relative increase in the cell viability. Ten of the 26 compounds significantly increased the cell viability by more than 20% compared with the DMSO-treated control (Fig. 1C). In the same conditions, rupintrivir, a potent 3C inhibitor, showed strong antiviral activity with little cytotoxic effect, as previously reported (Dragovich et al., 1999). Intriguingly, three of the effective compounds were fluoxetine and its analogs, which have already been reported as 2C inhibitors of enteroviruses (Ulferts et al., 2013). GW5074 is a well-known 3A inhibitor of enteroviruses (Arita et al., 2009). Brefeldin A has also been well-characterized for its inhibitory mode of action on enteroviruses through the inhibition of Golgi-specific BFA resistance factor 1 (Claude et al., 1999). Despite noticeable antiviral effects, a few of the compounds showed severe cell toxicity when given alone without CVB3 infection (Supplementary Fig. 2). For that reason, we excluded emetine, a strong inhibitor of protein synthesis, and ouabain, a cardiac glycoside used for the treatment of hypotension and some arrhythmias, from our subsequent analyses. Furthermore, additional analysis with a broad range of concentrations of each compound found that most of the compounds, including the previously reported inhibitors fluoxetine, GW5074, and brefeldin A, had serious cytotoxic effects at higher concentrations (10 or 50 μM; Supplementary Fig. 3). Only gemcitabine had little cytotoxic effect even at the highest concentration of 50 μM, while its antiviral activity was sufficiently strong and dose dependent. Its strong inhibitory effect was also observed in a screen with EV71 replicon-containing cells (Supplementary Fig. 1). Therefore, further studies were conducted only with gemcitabine (Fig. 1D).

### 3.2. Gemcitabine potently inhibits enteroviral proliferation

In order to know how potently gemcitabine inhibits the replication of CVB3 in cells, Vero cells were transfected with *in vitro*-transcribed CVB3-replicon RNAs, simultaneously treated with a broad range of concentrations of gemcitabine for 8 h, and then assayed for luciferase activity. Gemcitabine inhibited the replication of CVB3 replicons in a dose-dependent manner, with an estimated $IC_{50}$ of 0.4 μM (Fig. 2A). As observed in several experiments, it had little effect on the viability of Vero cells, even at the highest concentration, which was assessed using CellTiter-Glo reagent (Fig. 2B). Similar inhibitory effects were also observed in EV71 replicon-containing Vero cells, although the potency was somewhat different from that of the CVB3 replicon (Fig. 2C). Note that the estimated $IC_{50}$ of gemcitabine for the replication of the EV71 replicon was about 1 μM. Consistent with the result for the CVB3 replicon, gemcitabine had little cytotoxic effect on EV71 replicon-containing Vero cells (Fig. 2D). The low cytotoxicity of gemcitabine was further confirmed in an experiment with a longer treatment lasting 24 h (Supplementary Fig. 4).

The antiviral activity of gemcitabine was further analyzed in CVB3-infected HeLa cells. HeLa cells were infected with CVB3, simultaneously treated with a broad range of concentrations of gemcitabine for 48 h, and then analyzed by MTT assay. Gemcitabine showed a clear, dose-dependent antiviral effect, with an estimated $IC_{50}$ of ~5 μM (Fig. 3A), and it had little cytotoxic effect (Fig. 3B). However, it should be noted that gemcitabine exhibited a moderate cytotoxicity on actively growing HeLa cells (Supplementary Fig. 5), as previously reported (Hernandez et al., 2001). Similar antiviral activity was observed in EV71 (BrCr or H)-infected LLC-MK2 Derivative cells and RD cells, with an estimated $IC_{50}$ of ~0.2–1 μM,

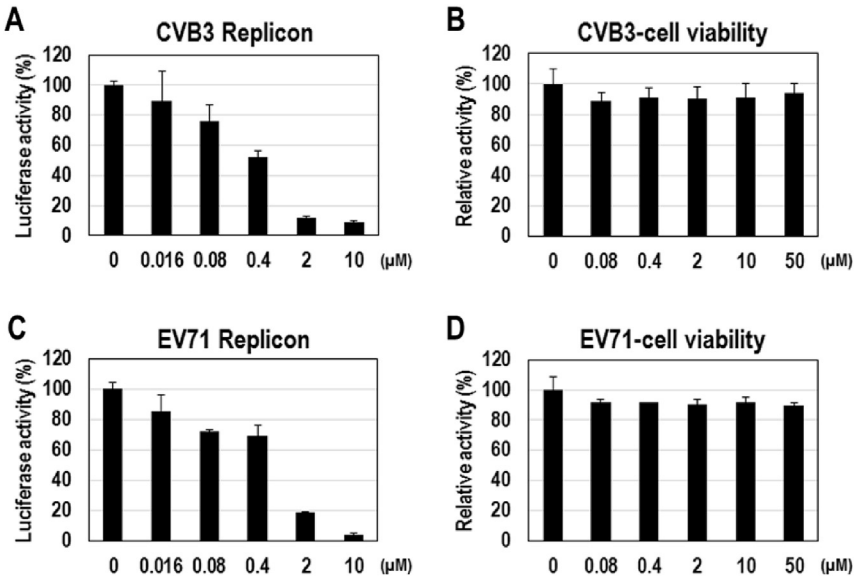


**Fig. 2.** Gemcitabine potently inhibits the replication of the CVB replicon. Vero cells were transfected with *in vitro*-transcribed CVB3-replicon (A) or EV71-replicon (C) RNAs, immediately treated with the indicated concentrations of gemcitabine for 8 h, and then assayed for firefly luciferase activity. The luciferase activity of DMSO-treated cells was considered to be 100%. In the same conditions, another set of CVB3 (C) or EV71 (D) replicon-transfected cells was assayed for cell viability using CellTiter-Glo reagent. The activity of DMSO-treated cells was considered to be 100%.

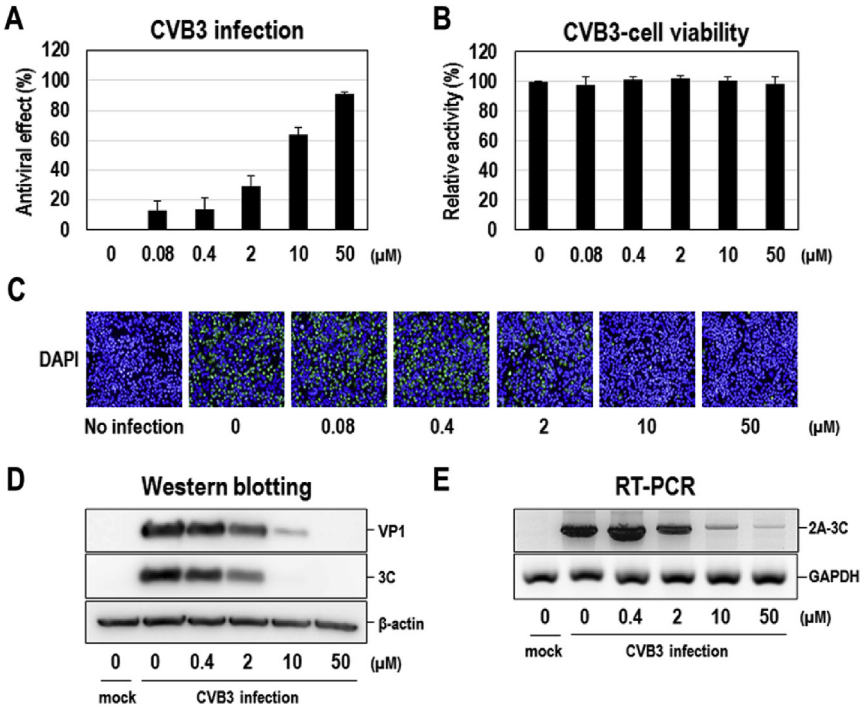


**Fig. 3.** Gemcitabine potently inhibits CVB3 proliferation in HeLa cells. (A) HeLa cells were infected with CVB3 (100 $CCID_{50}$) and simultaneously treated with increasing concentrations of gemcitabine. Forty-eight hours after treatment, antiviral activity was determined by the reduction of the cytopathic effect in an MTT assay. The viability of DMSO-treated cells was considered to be 0%, and that of uninfected cells was considered to be 100%. (B) The same cells treated with the indicated concentrations of gemcitabine without CVB3 infection were also analyzed for cell viability using the CellTiter-Glo assay. (C) HeLa cells were infected with CVB3 (5 MOI) and simultaneously treated with increasing concentrations of gemcitabine. Eight hours after treatment, the viral 3C proteins were stained using a specific antibody and visualized by FITC-conjugated secondary antibody (green). Nuclear DNA was also visualized by DAPI staining (blue). (D) Total cell extracts were prepared from the cells in (C) and subjected to Western blot analysis with anti-VP1 and anti-3C antibodies. β-actin was also analyzed as a loading control. (E) Total RNAs were prepared from the cells in (C) and then subjected to RT-PCR for the 2A-3C region of the CVB3 viral RNA. GAPDH mRNAs were also analyzed as a loading control.

even though the potency was higher and the maximal efficacy was lower than that for CVB3 (Supplementary Fig. 6). In addition, the strong antiviral activity was further confirmed by another approach, in which CVB3-infected HeLa cells were visualized by staining the $3C^{pro}$ protein with an antibody conjugated with a fluorescent dye, and quantified by counting the stained cells. The

fluorescent signal of the $3C^{pro}$ protein was dramatically decreased by the gemcitabine treatment, with an estimated $IC_{50}$ of ~1–2 μM (Fig. 3C and Supplementary Fig. 7). Collectively, these results clearly demonstrated that gemcitabine is a strong inhibitor of enteroviruses.

### 3.3. Inhibition of viral proteins and RNA synthesis in cells by gemcitabine

To further confirm the antiviral activity of gemcitabine in enterovirus-infected cells, viral proteins and RNAs were analyzed. CVB3-infected HeLa cells were treated with increasing concentrations of gemcitabine for 8 h, and then the total cell extracts and RNAs were subjected to Western blot analysis with anti-VP1 and anti-3C antibodies and to RT-PCR with primers corresponding to the 2A–3C regions, respectively. The gemcitabine treatment dramatically reduced the amounts of VP1 and 3C proteins in a dose-dependent manner (Fig. 3D). The effective concentrations for each protein ($EC_{50}$ = ~2 μM) were quite similar to those observed in the cell viability and 3C staining assays ($EC_{50}$ = ~1–5 μM). Similar antiviral effects were also shown in the viral RNA analysis. The amounts of CVB3 RNAs were drastically reduced by the gemcitabine treatment, and the $EC_{50}$ was estimated as 2 μM, which is similar to that observed in the VP1 and 3C protein analysis.

### 3.4. Gemcitabine inhibits a broad spectrum of enteroviruses

In order to know whether gemcitabine inhibits a broad spectrum of enteroviruses, other enteroviruses such as human rhinoviruses (HRV), which are also single-stranded, positive-sense enteroviruses that are pathogenetically significant to human health, were examined. Rhinovirus, the predominant cause of the common cold, is a member of the genus *Enterovirus*, which also includes CVB3 and EV71. H1HeLa cells were infected with three different types of HRV (HRV-14, HRV-21, and HRV-71), treated with increasing concentrations of gemcitabine, and assayed for viability by MTT assay after 72 h. As shown in CVB3-infected or EV71-infected cells, gemcitabine exhibited strong inhibitory activity against all three HRVs, with an estimated $IC_{50}$ of 1–5 μM (Fig. 4). This result indicated that gemcitabine is a broad-spectrum inhibitor of single-stranded, positive-sense enteroviruses.

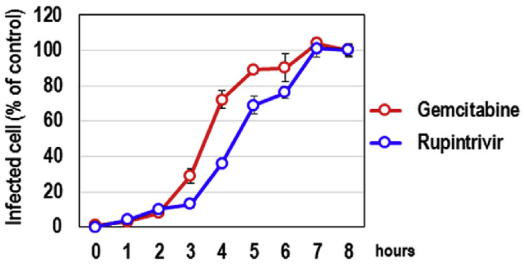


**Fig. 5.** Antiviral activity of gemcitabine depending on the time of addition. HeLa cells were infected with CVB3 at 5 MOI and treated with 50 μM gemcitabine or 2 μM rupintrivir at the indicated times after virus infection. Eight hours post infection, the virus-infected cells were visualized by staining with anti-3C antibody, and the percentage of infected cells among the total cells was calculated. The average and standard deviation were obtained from three independent experiments.

### 3.5. Gemcitabine targets intracellular processes in an early step of CVB3 infection

The inhibitory effect of gemcitabine might affect any step in the infectious cycle, including attachment, entry, uncoating, translation, polyprotein processing, replication, assembly, and release. To know which step is targeted by gemcitabine, gemcitabine was applied to the culture medium at various time points during virus infection (0, 1, 2, 3, 4, 5, 6, 7, and 8 h), and the inhibitory effect of gemcitabine was assessed by quantifying the infected cells showing a fluorescent signal of 3C protein. The strong antiviral effect of gemcitabine was shown even when the drug was added at 1, 2, and 3 h post infection, and those effects were nearly as strong as that obtained when the drug was added at the time of infection (0 h; Fig. 5), indicating that gemcitabine inhibits the proliferation of CVB3 probably by targeting any step(s) after entry.

Consistent with that observation, the inhibition of the replication of the CVB3 replicon by gemcitabine, shown in Fig. 2, also

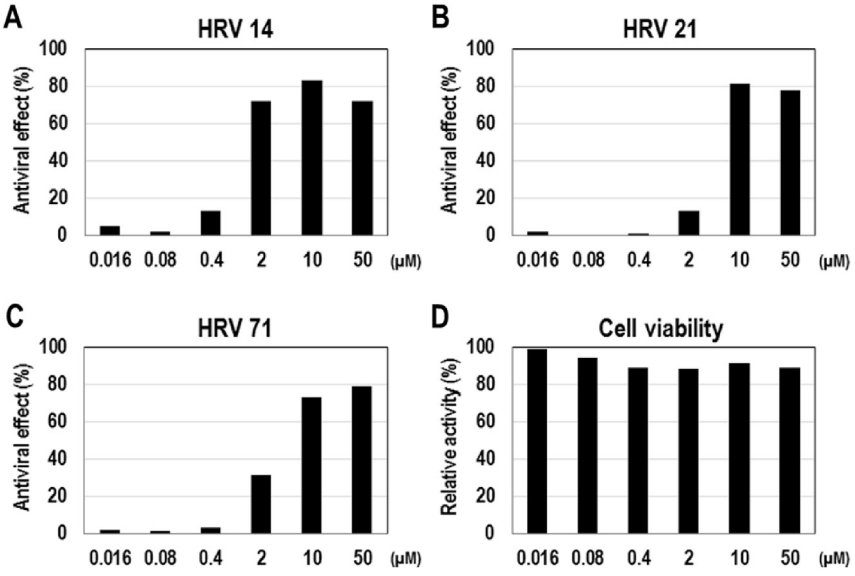


**Fig. 4.** Antiviral effect of gemcitabine on three strains of human rhinovirus. H1HeLa cells were infected with 100 $CCID_{50}$ human rhinovirus type 14 (A), 21 (B), or 71 (C) and simultaneously treated with the indicated concentrations of gemcitabine. Three days after treatment, the antiviral activity was determined by the reduction of the cytopathic effect in an MTT assay. The viability of DMSO-treated cells was considered to be 0%, and that of uninfected cells was considered to be 100%. (D) The same cells treated with the indicated concentrations of gemcitabine without CVB3 infection were also analyzed for cell toxicity using the MTT assay.

provides strong evidence that gemcitabine targets intracellular step(s) that are independent of the virus particle, such as translation, polyprotein processing, replication, or other such processes. To better define which step of CVB3 infection is targeted by gemcitabine, we established various assays and tested the involvement of each process in CVB3 inhibition by gemcitabine. First, we examined the possibility that gemcitabine interferes with translation initiation directed by the IRES in the 5′UTR of the CVB3 RNA. To do that, a dual-luciferase reporter system was generated, in which the expression levels of the firefly and *renilla* luciferases are controlled by CVB3 IRES-dependent and cap-dependent translation, respectively. 293T cells were transfected with dual-luciferase reporter plasmids containing CVB3 IRES (pR/CVB3/F-PEST) and treated with the indicated concentrations of gemcitabine. Twenty-four hours after treatment, the cells were assayed for firefly and *renilla* luciferase activities. Gemcitabine had little effect on firefly luciferase activity, indicating a negligible effect on CVB3 IRES-dependent translation (Fig. 6B). Similarly, EV71 IRES-dependent translation was not affected at all by gemcitabine (Supplementary Fig. 8A). Next, we excluded the possibility that the inhibitory effect of gemcitabine was associated with 2A protease by observing that the cleavage processing of VP1-2A$^{pro}$, supposedly executed by 2A$^{pro}$, was not altered by the gemcitabine treatment in CVB3-infected HeLa cells (data not shown). If gemcitabine worked by targeting 2A protease, we should have seen the accumulation of VP1-2A$^{pro}$ precursor proteins with concomitant decreases of individual VP1 and 2A$^{pro}$ proteins. Similarly, we excluded the association of 3C protease with the inhibitory effect of gemcitabine by showing that the cleavage pattern of 3CD precursor protein exogenously overexpressed in 293T cells was not altered at all by the gemcitabine treatment (Fig. 6C). Similar results were observed with EV71 3CD (Supplementary Fig. 8B). In the same conditions, the level of 3CD precursor protein was dramatically increased with a reciprocal decrease of 3C and 3D levels by treatment with rupintrivir, a well-studied and potent 3C$^{pro}$ inhibitor (Fig. 6C). Moreover, the involvement of 2C and 3A in the inhibitory effect of gemcitabine was also ruled out by the observation that the replication of CVB3 replicons containing well-characterized 2C or 3A mutants was affected by gemcitabine to an extent similar to that, to which wild-type CVB3-replicon replication was affected (Fig. 6A). As exemplified by fluoxetine and GW5074 in our experiment (Supplementary Fig. 9), most inhibitors targeting 2C or 3A have been characterized as less effective on CVB3 replicons containing 2C or 3A mutants than on wild-type CVB3 replicons (Arita et al., 2009; Ulferts et al., 2013). Collectively, our extensive analyses showed that the antiviral effect of gemcitabine is not associated with intracellular processes, such as IRES-dependent translation and polyprotein processing involving 2A$^{pro}$ and 3C$^{pro}$, 2C, and 3A.

### 3.6. The synergistic antiviral effect of gemcitabine and ribavirin

Next, we investigated the inhibitory effect of gemcitabine in combination with ribavirin, a well-known nucleoside-analog antiviral drug currently used for the treatment of several viral infections including HCV and Respiratory syncytial virus (RSV) (Graci and Cameron, 2006; Yin et al., 2009). Ribavirin has also been reported to have an inhibitory effect on enteroviral proliferation in a few cultured cells (Urbinati et al., 2008; Zhang et al., 2012). However, its antiviral activity seems to depend on the cell types and conditions, considering that it has not been observed in other cells (Zhang et al., 2012). Actually, in our cell-based tests, ribavirin consistently had a marginal effect on CVB3 infection, as well as on the CVB3 replicon. Nevertheless, we tested the possibility that gemcitabine, in combination with ribavirin, can induce a synergistic effect. CVB3 replicon-containing Vero cells were treated with increasing concentrations of ribavirin in the absence or presence of 0.4 or 0.08 μM gemcitabine for 8 h and then assayed for luciferase activity. Note that moderate antiviral effects (20–50% reduction of the replication of the CVB3 replicon) were shown with 0.08 and 0.4 μM gemcitabine (Fig. 2A). As expected, ribavirin alone had a marginal antiviral effect (reduction by at most ~10% compared with DMSO-treated control) at only the highest concentration (400 μM), and 0.4 μM gemcitabine alone exhibited a moderate antiviral effect (reduction by ~35%) (Fig. 7A and Supplementary Fig. 10A). Contrary to the small effects of ribavirin or gemcitabine alone, co-treatment with 0.4 μM gemcitabine and increasing concentrations of ribavirin inhibited the replication of the CVB3 replicon by up to 80% (Fig. 7A and Supplementary Fig. 10A), which is much greater than the sum (~45% at the highest concentration of ribavirin) of the antiviral effects of each individual compound. This synergistic effect was

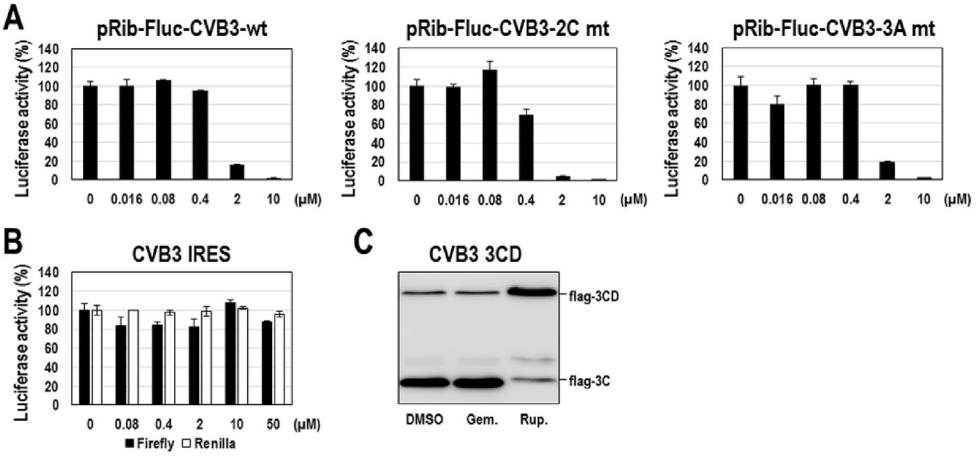


**Fig. 6.** The anti-CVB3 effect of gemcitabine is not related to 2C, 3A, IRES-dependent translation or polyprotein processing. (A) Vero cells were transfected with *in vitro*-transcribed CVB3-wt, CVB3–2C mt, or CVB3-3A mt replicon RNAs and simultaneously treated with the indicated concentrations of gemcitabine. Eight hours after treatment, the cells were assayed for luciferase activity. For each replicon, the luciferase activity from DMSO-treated cells was considered to be 100%. (B) 293T cells were transfected with the dual-luciferase reporter DNA measuring CVB3 IRES-dependent translation and then treated with the indicated concentrations of gemcitabine. Twenty-four hours after treatment, the cells were assayed for firefly and *renilla* luciferases. The luciferase activity from DMSO-treated cells was considered to be 100%. (C) 293T cells were transfected with a plasmid expressing flag-3CD and then treated with 10 μM gemcitabine. Nine hours after treatment, total cell extracts were prepared and subjected to Western blot analysis with anti-flag antibody. Rupintrivir (10 μM) was included as a positive control.

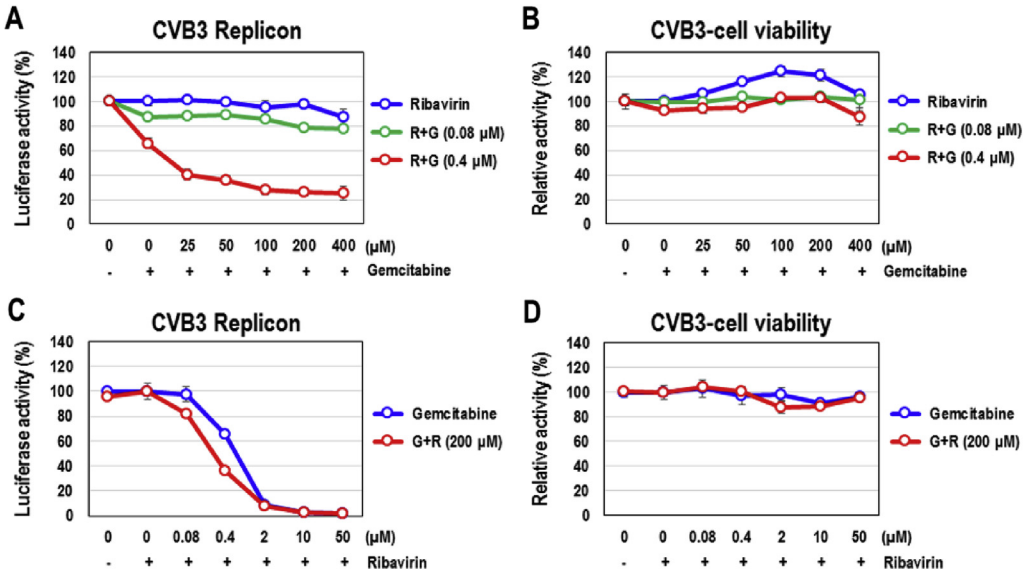


**Fig. 7.** Synergistic anti-CVB3 effect of gemcitabine and ribavirin. (A) Vero cells were transfected with *in vitro*-transcribed CVB3-replicon RNA and simultaneously treated with the indicated concentrations of ribavirin with or without 0.08 or 0.4 μM gemcitabine. Eight hours after treatment, the cells were analyzed for luciferase activity. The luciferase activity from DMSO-treated cells was considered to be 100%. The average and standard deviation were obtained from three independent experiments. (B) The same cells used in (A) were assayed for cell viability using the CellTiter-Glo assay. (C) Vero cells were transfected with *in vitro*-transcribed CVB3-replicon RNA and simultaneously treated with the indicated concentrations of gemcitabine with or without 200 μM ribavirin. Eight hours after treatment, the cells were analyzed for luciferase activity. (D) The same cells used in (C) were assayed for viability using the CellTiter-Glo assay.

quantitatively confirmed by analyzing the antiviral effects by using CompuSyn software. The combination index (CI) values for the combined effect of 0.4 μM gemcitabine and increasing concentrations of ribavirin were much smaller than 1 (CIs < 0.28), indicating an obvious synergism (Supplementary Fig. 10B) (Chou, 2006). Note that the CI values quantitatively define synergism (CI < 1) and additive effect (CI = 1). It is noteworthy that a very low concentration of ribavirin (less than 25 μM) was sufficient to induce half the efficacy of the antiviral effect when combined with gemcitabine. The synergistic effect of gemcitabine and ribavirin was also observed when increasing concentrations of gemcitabine were co-treated with a fixed concentration of ribavirin (200 μM; Fig. 7C). The estimated $IC_{50}$ of gemcitabine in combination with ribavirin was ~0.2 μM, representing 5 times greater efficacy compared with that of gemcitabine alone (estimated $IC_{50}$ of ~1 μM). Importantly, despite the significant antiviral effects, the combined treatment of gemcitabine and ribavirin had little cytotoxic effect (Fig. 7B and D). Moderate synergistic effects appeared on the replication of the EV71 replicon (Supplementary Fig. 11), which was quantitatively confirmed by small CI values (Supplementary Fig. 12). Collectively, these results indicate that gemcitabine in combination with ribavirin is more effective against enteroviruses than gemcitabine or ribavirin alone.

## 4. Discussion

There is increasing demand for antiviral drugs to treat the various diseases caused by enteroviruses. A great deal of effort has been put towards the discovery and development of antiviral drugs since the advent of enteroviruses. Two major strategies to achieve that goal have been: 1) the utilization of pre-existing antiviral drugs or drug candidates and 2) the search for new compounds. Unfortunately, none of the pre-existing antiviral drugs, which have been effective against HIV, HCV, influenza, herpesvirus, and other viruses, have shown any antiviral activity against enteroviruses (Clouser et al., 2010; Rawson et al., 2013). Pleconaril, a promising drug candidate that inhibits virus attachment by binding to the viral coat protein, has been dropped from further clinical development because of concerns about viral resistance and side effects in patients (Pevear et al., 1989; Zeichhardt et al., 1987). Rupintrivir, another promising drug candidate that potently inhibits 3C protease, was halted from further clinical development because of low effectiveness in naturally infected patients. Moreover, ribavirin, which is currently used against a few RNA viruses, has shown weak and inconsistent antiviral activity against enterovirus infections in cultured cells, and hardly in animal models (Graci and Cameron, 2006). Hence, considerable efforts were made to develop new antiviral drugs based on the screening of synthetic and natural chemical libraries. From the various approaches, a great variety of compounds have been shown to have some antiviral activity against broad or narrow ranges of enteroviruses (Wu et al., 2010a). However, none of them has demonstrated effectiveness at the clinical level. Hence, the development of new antiviral drugs against broad ranges of enteroviruses is urgently required before the enteroviruses cause more severe health problems in human beings. To more efficiently achieve that goal, we sought to identify new compounds among those in the LOPAC bioactive chemical library, which consists partly of FDA-approved drugs, with which further possible clinical applications for enterovirus-associated diseases would be likely and feasible. In the present study, we identified gemcitabine, a drug currently in use for cancer therapy, as an effective anti-enteroviral agent. Extensive analysis in various assay systems firmly demonstrated that gemcitabine is a potent inhibitor of CVB3 and EV71. Moreover, we demonstrated its antiviral effects against three different strains of HRV, which are members of genus *Enterovirus* and closely related to CVB3 and EV71, suggesting that gemcitabine could be a potential therapeutic application for a broad spectrum of viruses in the genus *Enterovirus*.

Gemcitabine is an FDA-approved anti-cancer drug used for chemotherapy against various cancers: non-small cell lung cancer, pancreatic cancer, bladder cancer, and breast cancer (Clouser et al., 2010). In the present study, we found a new indication for gemcitabine as an agent controlling enterovirus infections. Previously, antiviral activities of gemcitabine were reported in a few virus

infections (Denisova et al., 2012). Most of those reports are related to RNA viruses such as HIV (Clouser et al., 2010) and influenza (Denisova et al., 2012). To the best of our knowledge, ours is the first work to report the antiviral effects of gemcitabine on single-stranded, positive-sense, non-enveloped RNA viruses, although nucleoside analogs similar to gemcitabine have been reported to have antiviral activity against poliovirus and foot-and-mouth disease virus, which are quite similar to CVB3 and EV71 (Pariente et al., 2003, 2001; Ruiz-Jarabo et al., 2003).

Gemcitabine is a nucleoside analog and a potent ribonucleotide reductase inhibitor that can interfere with normal DNA/RNA-associated processes (Graci and Cameron, 2006; Yin et al., 2009). That is why gemcitabine is classified in the family of drugs called antimetabolites and explains how gemcitabine exerts its antiviral effects. There might be two possible mechanisms to explain the antiviral effects of gemcitabine on enteroviruses. First, as a nucleoside analog, gemcitabine can be directly incorporated into the newly synthesized enteroviral RNAs during the polymerization process. Because viral RNA replication is generally more active than cellular DNA/RNA synthesis, gemcitabine would be preferentially incorporated into viral RNA, which is similar to what happens to gemcitabine in cancerous cells. Alternatively, gemcitabine can simply bind to some place in the nucleotide-binding region of the 3D polymerase and thus block the entry of incoming nucleotides. Whichever scenario is correct, gemcitabine blocks further elongation and eventually suppresses viral proliferation. Second, the inhibition of ribonucleotide reductase by gemcitabine could be involved in the antiviral effect. Impaired ribonucleotide reductase activity causes a decrease in the ribonucleoside triphosphate (NTP) pool, leading to an increase in the mutation rate during viral polymerization (Baba et al., 1987; Balzarini et al., 1987; Gao et al., 1999; Meyerhans et al., 1994). The increased mutation rate in the viral genome can cause the viruses to lose the tolerances that enable them to retain their proliferative capability. Indeed, an inverse correlation between the mutation frequency and infectivity has been clearly shown for a number of viruses, including HIV (Clouser et al., 2010). Further investigation in a future study will clarify the precise mode of action for the anti-enteroviral effects of gemcitabine.

As aforementioned, no therapeutic drug is available for the treatment of the diseases associated with enterovirus infection. Among the many antiviral drugs currently being used for other viral diseases, ribavirin is the only drug that exhibits a noticeable antiviral effect in cultured, enterovirus-infected cells, although the effect has not been demonstrated in an animal model (Graci and Cameron, 2006). Ribavirin, an antiviral drug used against RNA viruses such as HCV and RSV, is a nucleoside analog and is regarded as a nucleoside antimetabolite drug similar to gemcitabine (Urbinati et al., 2008; Zhang et al., 2012). Intriguingly, many previous studies have shown that combinations of nucleoside analogs, particularly those acting as viral mutagens and ribonucleotide reductase inhibitors, can have synergistic antiviral effects on viral infection (Clouser et al., 2012, 2010). Therefore, although the antiviral effect of ribavirin was not significant in our enterovirus systems, we tested whether gemcitabine and ribavirin could show any synergy. Surprisingly, when a low dose of gemcitabine (0.4 μM), which caused a moderate antiviral effect when administered alone, was combined with various concentrations of ribavirin, we observed a considerable increase in antiviral activity (Fig. 7A). In terms of therapeutic applications, it is noteworthy that a very low concentration of ribavirin (less than 25 μM) was sufficient to induce half the efficacy of inhibition when combined with gemcitabine. A similarly enhanced effect was seen when increasing concentrations of gemcitabine were co-administered with a single dose of ribavirin (Fig. 7C). Importantly, both combinations had few cytotoxic effects (Fig. 7B and D). Moreover, similar phenomena were observed in EV71 systems (Supplementary Fig. 11). Those observations indicated that gemcitabine and ribavirin work, at least partly, through different mechanisms for anti-enteroviral effects, although both are nucleoside antimetabolite drugs. More importantly, our results suggest new therapeutic strategies for the control of enterovirus infections using gemcitabine alone or combined with a very low dose of ribavirin. Considering that many clinical studies currently underway, especially those for HCV therapy, involve using ribavirin in combination with other drug candidates to achieve better efficacy, this new potential therapeutic option appears to be very promising.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2015.10.011.

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