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Research paper

Drug screening identified gemcitabine inhibiting hepatitis E virus by inducing interferon-like response via activation of STAT1 phosphorylation

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ABSTRACT

Exposure to hepatitis E virus (HEV) bears a high risk of developing chronic infection in immunocompromised patients, including organ transplant recipients and cancer patients. We aim to identify effective anti-HEV therapies through screening and repurposing safe-in-human broad-spectrum antiviral agents. In this study, a safe-in-human broad-spectrum antiviral drug library comprising of 94 agents was used. Upon screening, we identified gemcitabine, a widely used anti-cancer drug, as a potent inhibitor of HEV replication. The antiviral effect was confirmed in a range of cell culture models with genotype 1 and 3 HEV strains. As a cytidine analog, exogenous supplementation of pyrimidine nucleosides effectively reversed the antiviral activity of gemcitabine, but the level of pyrimidine nucleosides *per se* does not affect HEV replication. Surprisingly, similar to interferon-alpha (IFNα) treatment, gemcitabine activates STAT1 phosphorylation. This subsequently triggers activation of interferon-sensitive response element (ISRE) and transcription of interferon-stimulated genes (ISGs). Cytidine or uridine effectively inhibits gemcitabine-induced activation of ISRE and ISGs. As expected, JAK inhibitor 1 blocked IFNα, but not gemcitabine-induced STAT1 phosphorylation, ISRE/ISG activation, and anti-HEV activity. These effects of gemcitabine were completely lost in STAT1 knockout cells. In summary, gemcitabine potently inhibits HEV replication by triggering interferon-like response through STAT1 phosphorylation but independent of Janus kinases. This represents a non-canonical antiviral mechanism, which utilizes the innate defense machinery that is distinct from the classical interferon response. These results support repurposing gemcitabine for treating hepatitis E, especially for HEV-infected cancer patients, leading to dual anti-cancer and antiviral effects.

1. Introduction

Hepatitis E virus (HEV), a single-stranded positive-sense RNA virus, is the most common cause of acute viral hepatitis worldwide. Globally, it is recently estimated that over 900 million corresponding to 1 in 8 individuals have ever experienced HEV infection. Among those, 15–110 million individuals have recent or ongoing HEV infection (Li et al., 2020). Among the eight classified genotypes, genotype 1 and 2 HEV exclusively infect humans mainly prevalent in developing countries responsible for many water-borne outbreaks. In contrast, genotype 3 and 4 HEV are zoonotic, causing sporadic cases mostly seen in the western world (Zhou et al., 2019).

Although HEV infection is usually acute and self-limiting in healthy individuals, it can cause severe morbidity and even mortality in special populations. Acute infection with genotype 1 HEV in pregnant women imposes a high risk of developing fulminant hepatic failure, leading to a high death rate of up to 30% (Hakim et al., 2017). Infection with genotype 3 and occasionally genotype 4 HEV in immunocompromised patients is prone to develop chronic hepatitis E. This has been well-recognized in organ transplant recipients, as they universally receive immunosuppressive medication (Kamar et al., 2008; Wang et al., 2018; Zhou et al., 2013). Cancer patients, especially those undergoing chemotherapy or radiotherapy, also have a compromised immune system, and have been reported to develop chronic HEV infection (Fuse et al., 2015; Protin et al., 2019; Tavitian et al., 2010; von Felden et al., 2019). Although no FDA-approved medication is available, the general

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antiviral drugs including interferon alpha (IFNα), ribavirin, or their combination have been repurposed as off-label treatment for chronic hepatitis E (Debing et al., 2016; Haagsma et al., 2010; Kamar et al., 2010a, 2010b). Although ribavirin monotherapy is effective in a substantial proportion of treated patients, treatment failure does occur in a subset of patients. Furthermore, many patients are not eligible or do not tolerate IFNα or ribavirin treatment (Debing et al., 2014; Pischke et al., 2013; Rostaing et al., 1995). Thus, there is a clinical need for further developing new antiviral therapies against HEV.

Development of new antiviral drugs usually takes more than ten years requiring enormous investment with a high risk of failure. The fact that only a specific population with HEV infection requires antiviral treatment does not justify the pharmaceutical industry to develop new anti-HEV drugs. Thus, we propose to systematically screen and repurpose the existing drugs that can be readily used in the clinic. In this study, we screened a library of safe-in-human broad-spectrum antiviral agents. These compounds are known to target viruses belonging to two or more viral families, and have been used in the clinic or have passed phase I clinical trials (Andersen et al., 2020; Ianevski et al., 2018). We identified gemcitabine, a widely used anti-cancer drug, potently inhibits HEV infection. Unexpectedly, it functions through the activation of interferon-like response via STAT1 phosphorylation. But the mechanism-of-action is distinct from the classical antiviral interferon response.

2. Materials and methods

2.1. Reagents and antibodies

A library of 94 safe-in-man board-spectrum antiviral agents (https://drugvirus.info) were dissolved in DMSO with a stock concentration of 10 mM. Gemcitabine, ribavirin, human IFNα, mycophenolic acid (MPA), cytidine, uridine, cytidine 5′-triphosphate (CTP) and uridine 5′-triphosphate (UTP) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) and were dissolved in 1 × PBS (Phosphate-Buffered Saline) with the stock concentration of 200 mM. Stocks of JAK inhibitor 1 (SC-204021, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were dissolved in Dimethyl sulfoxide (DMSO, Sigma, Zwijndrecht, The Netherlands) with a final concentration of 5 mg/ml. The HEV ORF2 antibody was purchased from EMD Millipore (MAB8002). Phospho-STAT1 (Tyr701) (58D6, Rabbit mAb, 9167) and STAT1 (Rabbit mAb, 9172) antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). β-actin antibody (mouse monoclonal, sc-47778) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit and anti-mouse IRDye-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NE, USA) were also used.

2.2. Cell culture

Human hepatoma Huh7 and PLC/PRF/5 (PLC) cell lines, human embryonic kidney epithelial cell line (293T), human glioblastoma cell line (U87), and Huh7-STAT1 knockout cells were kindly provided by the Department of Viroscience (Erasmus Medical Center) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 IU/mL streptomycin.

2.3. Viruses and cell culture models

Genotype 3 HEV models are based on a plasmid construct containing the full-length HEV genome (genotype 3 Kernow-C1 p6 clone, GenBank accession number **JQ679013**) or a construct containing subgenomic HEV sequence in which ORF2 was replaced by a Gaussia luciferase reporter gene (p6-Luc). Viral RNA was produced by using the Ambion mMESSAGE nMACHINE *in vitro* RNA transcription kit. Cells were electroporated with p6 full-length HEV RNA or p6-Luc subgenomic RNA to generate infectious or luciferase-based replicon models, respectively. Similarly, the genotype 1 replicon model is based on the Sar 55/S17/luc HEV clone containing a Gaussia luciferase reporter.

Interferon response was monitored by the interferon-stimulated response element (ISRE) reporter (Huh7-ISRE-Luc). Huh7 cells were transduced with a lentiviral transcriptional reporter system expressing the firefly luciferase gene driven by a promoter containing multiple ISRE elements (SBI Systems Biosciences, Mountain View, CA, USA). Luciferase activity indicates ISRE promoter activity.

2.4. Virus production and re-infection assay

Huh7 cells harboring the infectious genotype 3 HEV were seeded into a multi-well plates HEV particles were harvested by repeated freezing and thawing 3 times, and filtered by 0.45 μm filters. Naïve Huh7 cells were seeded into a muti-well plate and culture medium was discarded when cell confluence was approximately 80%, followed by twice 1 × PBS washing. Harvested viruses were added and incubated at 37° C with 5% CO2 for 24 h for re-infection, followed by 3 times washing with 1 × PBS to remove unattached viruses. Then cells were incubated with culture medium for another 48 h. The infectivity of produced HEV particles were analyzed by qRT-PCR, Western blotting, and confocal imaging assays, respectively.

2.5. MTT assay

10 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to the cells seeded in 96-well plate and cells were maintain at 37 °C with 5% CO2 for 3 h. Medium was removed and 100 μL of DMSO was added to each well. The absorbance of each well was read on the microplate absorbance readers (BIO-RAD) at wavelength of 490 nm.

2.6. Quantification of viral replication

Viral replication in HEV replication models was monitored by the activity of secreted Gaussia luciferase measured by BioLux Gaussia Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA, USA). Luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG Lab Tech, Offenburg, Germany). For HEV infectious model, viral RNA was quantified by SYBR-Green-based (Applied Biosystems SYBR Green PCR Master Mix; Thermo Fisher Scientific Life Sciences) real-time PCR (qRT-PCR). GAPDH was used as a housekeeping gene to normalize gene expression using the $2^{-\Delta\Delta Ct}$ method.

2.7. Western blot

Proteins in cell lysates were heated at 95 °C for 5 min, followed by loading onto a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), separated at 90 V for 120 min, and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (pore size: 0.45 mm; Thermo Fisher Scientific Life Sciences) for 120 min with an electric current of 250 mA. Subsequently, the membrane was blocked with blocking buffer (Li-Cor Biosciences). Membrane was followed by incubation with primary antibodies rabbit anti-STAT1 (1:1000), anti-pSTAT1 (1:1000), or mouse anti-HEV ORF2 (1:1000), anti-β-actin (1:1000) overnight at 4 °C. The membrane was washed 3 times followed by incubation for 1 h with anti-rabbit or anti-mouse IRDye-conjugated secondary antibodies (1:5000; Li-Cor Biosciences) at room temperature. After washing 3 times, protein bands were detected with Odyssey 3.0 Infrared Imaging System.

2.8. Statistical analysis

Statistical analysis was performed using the non-paired, non-parametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). All results were presented as mean ±

standard errors of the means (SEM). P values < .05 were considered as statistically significant.

3. Results

3.1. Screening a broad-spectrum antiviral drug library identifies gemcitabine as a potent anti-HEV agent

To identify potential anti-HEV candidates, we screened a library of 94 known safe-in-human broad-spectrum antiviral agents. Huh7 cell-based genotype 3 HEV replicon model (Huh7-p6-Luc) was treated with each compound at a concentration of 10 μM or DMSO vehicle control for 48 h. HEV replication-related luciferase activity and cytotoxicity were determined (Fig. 1A). To minimize off-target effects, 23 candidates with over 50% inhibition on HEV luciferase activity but less than 50% cytotoxicity were selected for subsequent validation (Fig. 1A; Supplementary Table 1). Their antiviral effects were further verified in the full-length HEV infectious model by quantifying viral RNA using qRT-PCR assay (Fig. 1B). In both models, the widely used anti-cancer drug gemcitabine showed potent anti-HEV activity, with inhibition over 70%, and thus was subjected to further detailed study.

3.2. Gemcitabine consistently inhibits HEV in a wide range of cell models

Besides hepatitis, HEV infection associates with a broad range of extrahepatic manifestations. We thus further profiled the antiviral activity of gemcitabine in a variety of cell models, including hepatic and non-hepatic cell lines with genotype 1 or 3 HEV. Gemcitabine treatment inhibited viral replication-related luciferase activity in a dose-dependent manner in the hepatic Huh7 cells harboring genotype 3 HEV replicon (Fig. 2A). The 10% inhibition and cytotoxicity (IC10 and CC10) concentrations of gemcitabine were 0.046 μM and 0.32 mM, 50% inhibition and cytotoxicity (IC50 and CC50) concentrations were 0.42 μM and 2.9 mM, and 90% inhibition and cytotoxicity (IC90 and CC90) concentrations were 3.78 μM and 26.1 mM, respectively (Fig. 2B). Consistently, gemcitabine significantly inhibited HEV at both viral RNA (Fig. 2C) and ORF2 protein (Fig. 2D) levels in Huh7 cells harboring the infectious genotype 3 clone. Furthermore, gemcitabine also does-dependently inhibited genotype 1 HEV replication (Fig. 2E). By harvesting HEV particles from Huh7 cells harboring the infectious genotype 3 clone at 48 h post-treatment, we performed an re-infection assay to determine the relative titers of viruses by re-infecting naïve Huh7 cells. The amount of produced HEV with infectivity was significantly reduced by gemcitabine treatment (Fig. S1A). At the protein level, we found potent inhibition of HEV ORF2 expression determined by Western blotting (Fig. S1B) and confocal imaging (Fig. S1C). These results were further confirmed in hepatic PLC (Fig. S2A), neuronal U87 (Fig. S2B), and kidney 293T (Fig. S2C) cells. Interestingly, HEV replication was more sensitive to gemcitabine with an IC50 of 0.06 μM in 293T cells (Fig. S2D). At the same time, ribavirin, widely demonstrated to inhibit HEV infection, was used as a positive control (Fig. S1; Fig. S3). Furthermore, for the luciferase cell models (GT1 and GT3), the absolute luciferase values were presented in Fig. S4.

3.3. Exogenous supplementation of pyrimidine nucleosides reversed the anti-HEV activity of gemcitabine

As a cytidine analog, gemcitabine depletes the intracellular CTP

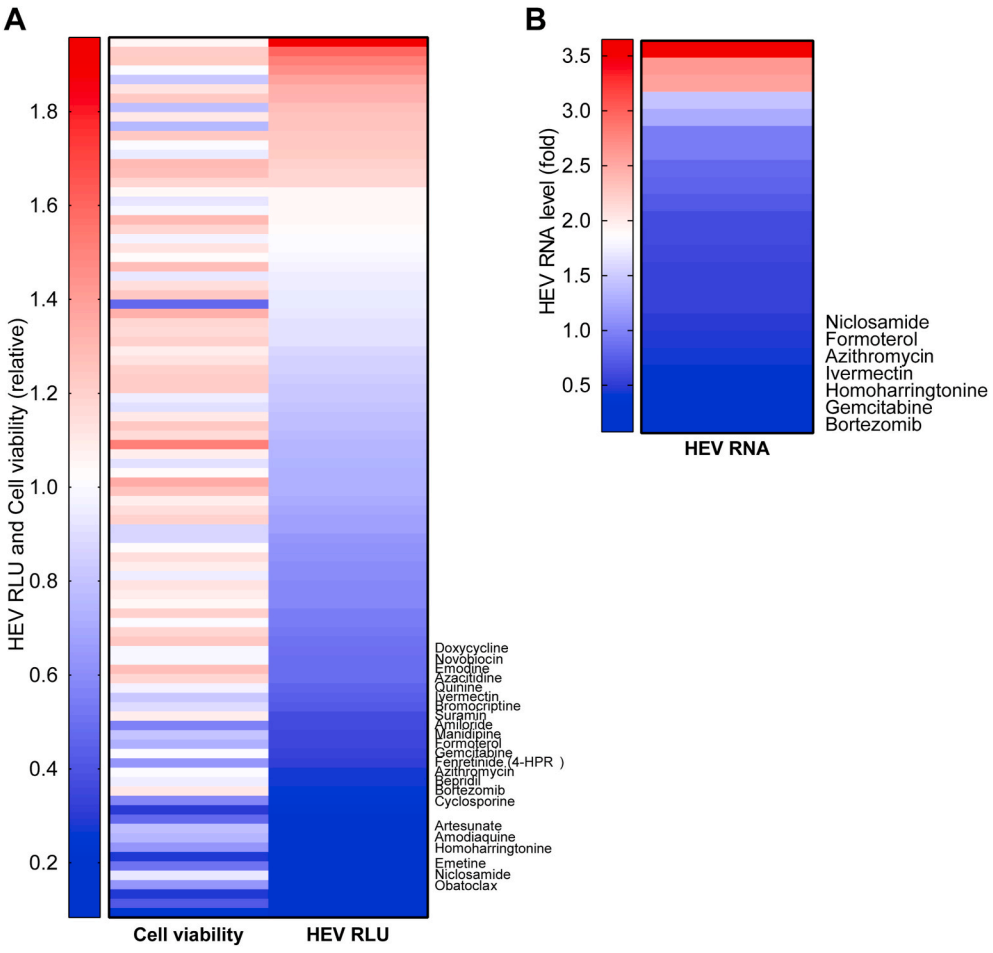

Fig. 1. Drug screening identified gemcitabine as a potent inhibitor of HEV replication. (A) Huh7 cell based HEV replicon model (Huh7-p6-Luc) was treated with 94 broad-spectrum antiviral agents at 10 μM. HEV-related Gaussia luciferase activity was measured 48 h post-treatment. Cell viability was determined by MTT. RLU: relative luciferase unit. (B) HEV infectious model (Huh7-p6) was treated with the 23 drugs selected from the primary screening at 10 μM for 48 h. Viral RNA was quantified by qRT-PCR. Data were normalized to the DMSO vehicle control (set as 1) and presented in heatmap or dot plots.

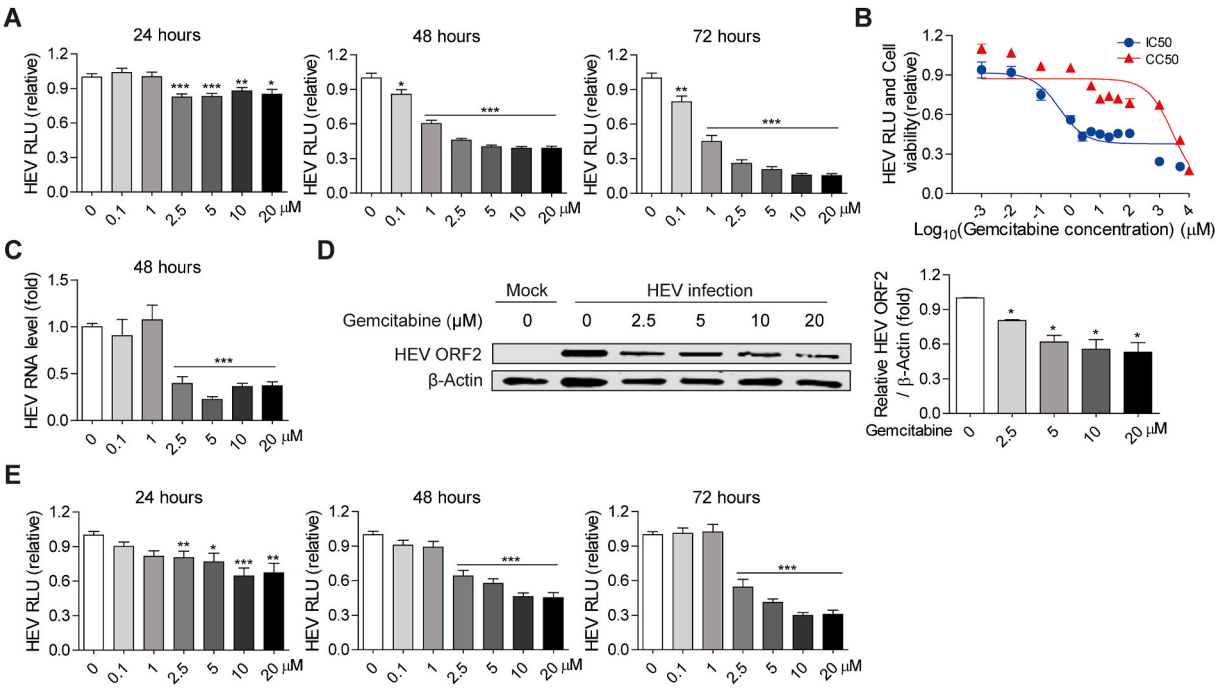

Fig. 2. Anti-HEV activity of gemcitabine in different cell models. (A) The effects of gemcitabine treatment on viral replication related luciferase activity in Huh7-p6-Luc cell model. The untreated group serves as control (set as 1) (n = 18). RLU: relative luciferase unit. (B) The 50% inhibitory concentration (IC50) and 50% cytotoxic concentration (CC50) of gemcitabine in Huh7-p6-Luc cell model and Huh7 cell line was calculated using GraphPad Prism 5 software (n = 6–12). (C) Huh7-p6 cell model was treated with concentrations of gemcitabine for 48 h. The effects on viral RNA was quantified by qRT-PCR (n = 10). (D) Western blot analysis of HEV capsid ORF2 protein level in Huh7-p6 cells treated with gemcitabine for 48 h. The uninfected group (mock) serves as a negative control, and the infected but untreated group serves as a positive control (set as 1) (n = 4). (E) Huh7 cell based genotype 1 HEV replicon was treated with gemcitabine for 24, 48 or 72 h and viral replication related luciferase activity was measured (n = 10). Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

(Heinemann et al., 1995). We investigated whether exogenous supplementation of pyrimidine nucleosides could affect the anti-HEV activity of gemcitabine. Addition of cytidine, uridine, CTP, or UTP dose-dependently reversed the antiviral activity of gemcitabine in HEV replicon, and 200 μM of these nucleosides completely blocked the antiviral activity of gemcitabine (Fig. 3A). This effect was confirmed in the HEV infectious model and significantly reversed the antiviral activity of gemcitabine albeit to a less extent (Fig. 3B). In contrast, pyrimidine nucleosides had no effect on the anti-HEV activity of IFNα (Fig. S5). Intriguingly, adding these nucleosides alone hardly affects HEV replication (Fig. 3), suggesting that depletion of the pyrimidine nucleosides *per se* does not explain the anti-HEV activity of gemcitabine.

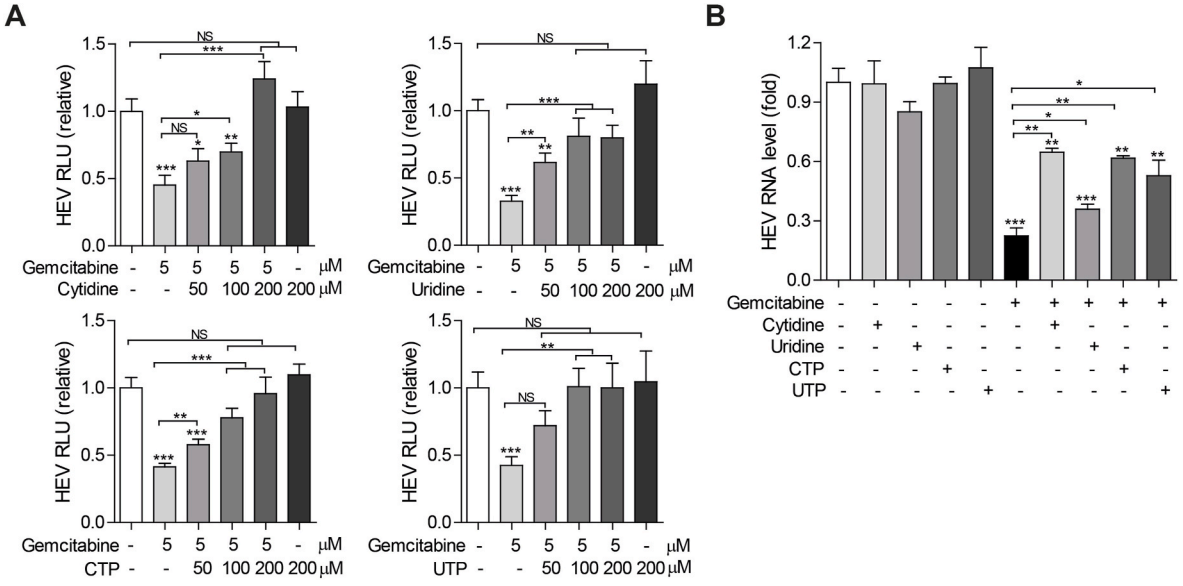

Fig. 3. Supplement of exogenous pyrimidine nucleosides reversed the anti-HEV effect of gemcitabine. (A) HEV replication related Gaussia luciferase activity in Huh7-p6-Luc cells was measured after 72 h of treatment with 5 μM gemcitabine by adding exogenous cytidine, uridine, CTP, or UTP (n = 9–12). (B) HEV RNA in Huh7-p6 cells was quantified by qRT-PCR after 48 h of treatment with 5 μM gemcitabine by adding 200 μM exogenous cytidine, uridine, CTP, or UTP (n = 4–10). Data were normalized to the untreated control (set as 1). RLU: relative luciferase unit. Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

3.4. Gemcitabine activates interferon-like response

We and others have previously found that nucleoside synthesis pathways crosstalk with cellular innate immunity. Several nucleosides analogs are capable to activate the transcription of antiviral interferon-stimulated genes (ISGs), although the underlying mechanisms remain unknown (Lucas-Hourani et al., 2013; Pan et al., 2012; Shin et al., 2018; Wang et al., 2016c). ISGs as the ultimate antiviral effectors are usually activated by interferons. Upon binding to receptors, interferons initiate the Janus kinase signal transducer and activator of transcription (JAK-STAT) cascade to recruit the ISGF3 complex, which binds to ISRE motifs in the nucleus to induce ISG transcription (Xu et al., 2017). Similar to IFNα treatment, we found that treatment with different concentrations of gemcitabine dose-dependently activated the transcription of a panel of ISGs. Particularly, 20 μM of gemcitabine resulted in over a hundredfold increase of ISG15 and more than tenfold increase of MX1, IFIT1, or DDX58 gene expression (Fig. 4A, Fig. S6). In an ISRE reporter mimicking interferon response, gemcitabine robustly triggered the transcriptional activity indicated by increased luciferase activity (Fig. 4B). For instance, treatment with 20 μM of gemcitabine for 72 h resulted in a 3.9 ± 0.2 (mean ± SEM, $n = 15$, $p < 0.0001$) -fold increase of ISRE related luciferase activity. Thus, similar to interferons, gemcitabine is capable to trigger the antiviral innate immune response.

3.5. Supplementation of pyrimidine nucleosides abrogates gemcitabine-induced innate immune response

As addition of pyrimidine nucleosides reversed the anti-HEV effects of gemcitabine (Fig. 3), we further tested the effect on the innate immune response. Supplementation of cytidine or uridine (200 μM) significantly abrogated gemcitabine-triggered induction of ISRE activity and ISG transcription (Fig. 5A). Among the tested ISGs, except for ISG15, the induction of others including MX1, IFIT1, DDX58, CXCL10, and STAT1 was significantly attenuated (Fig. 5B). In contrast, cytidine and uridine had no effect on IFNα induced ISRE activation and ISG transcription (Fig. S7). These results suggest that gemcitabine triggers interferon-like antiviral response, but through distinct mechanisms as compared to IFNα.

3.6. Gemcitabine activates Janus kinase-independent STAT1 phosphorylation

In the classical interferon pathway, Janus kinases phosphorylate STATs to initiate the response. Interestingly, gemcitabine dramatically enhanced the protein expression of STAT1, in particular at the phosphorylation level, which is a hallmark of interferon response. Treatment with 10 μM gemcitabine increased STAT1 and pSTAT1 protein levels up to 3.4 ± 0.9 (mean ± SEM, $n = 5$, $p < 0.01$) and 2.2 ± 0.2 (mean ± SEM, $n = 5$, $p < 0.01$) -fold, respectively (Fig. 6A). As expected, blocking the function of Janus kinases by JAK inhibitor 1 almost completely reversed IFNα induced STAT1 phosphorylation, ISRE activation, ISG transcription, and anti-HEV activity. In contrast, JAK inhibitor 1 hardly affected the functions of gemcitabine in this respect (Fig. 6B–E). These results further confirm a non-canonical mechanism-of-action of gemcitabine in triggering interferon-like antiviral response.

3.7. STAT1 is essentially required for the action of gemcitabine

Although Janus kinases are not required, the activation of STAT1 phosphorylation by gemcitabine is intriguingly prominent (Fig. 6). To dissect the functional implication, we used STAT1 knockout Huh7 cells (Fig. 7A). In STAT1$^{-/-}$ compared to wild type Huh7 cells, both IFNα and gemcitabine failed to trigger ISG transcription (Fig. 7B) and lost the anti-HEV activity (Fig. 7C). Therefore, STAT1 phosphorylation is functionally required for the anti-HEV action of gemcitabine.

3.8. Gemcitabine antagonizes ribavirin and MPA, but partially synergizes IFNα

The nucleoside analogs, ribavirin and MPA targeting purine nucleoside synthesis, have been widely demonstrated to inhibit HEV infection (Wang et al., 2014). Surprisingly, combination with gemcitabine antagonizes the anti-HEV effects of ribavirin (Fig. S8A) and MPA (Fig. S8B). In contrast, although not with other concentrations, combining 10 μM gemcitabine with 10 IU IFNα resulted in a mild synergistic effect (Fig. 8A). This was mechanistically supported by the enhanced transcription of ISGs (Fig. 8B).

4. Discussion

In approximately 80% of patients treated, ribavirin is effective for treating chronic hepatitis E. However, treatment failure has been frequently reported, probably attributed to resistance development or poor tolerance (Debing et al., 2016). The substantial side effects limit ribavirin applications in pregnant women, young children, and elderly patients. Thus, great efforts have been dedicated to looking for new anti-HEV drugs. For example, sofosbuvir as a direct-acting antiviral against hepatitis C virus (HCV) has been widely tested for treating chronic hepatitis E. Unfortunately, both experimental and clinical results are contradictive and inconclusive (Dao Thi et al., 2016; Donnelly

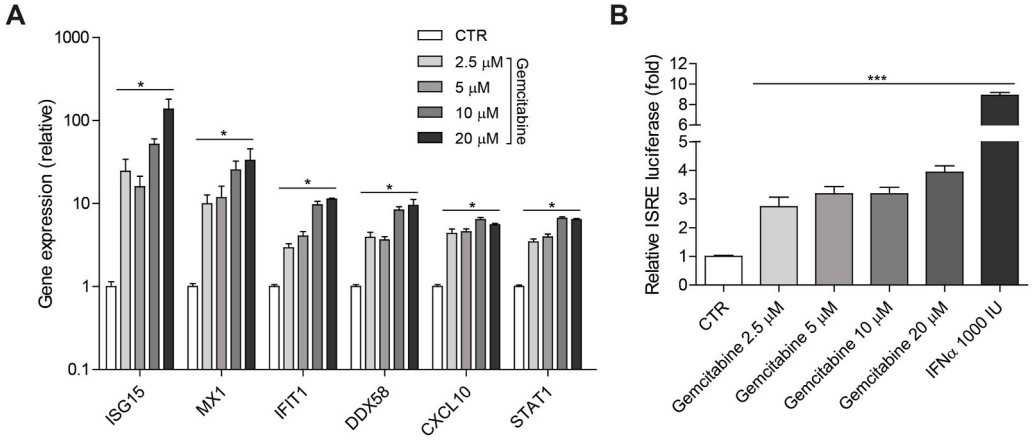

Fig. 4. Gemcitabine activates an interferon-like response. (A) Gene expression of ISGs in Huh7-p6 cells was quantified by qRT-PCR after treatment with gemcitabine for 48 h (n = 4). (B) Analysis of ISRE related firefly luciferase activity in Huh7-ISRE-Luc cells treated with gemcitabine or IFNα for 72 h (n = 15–20). Data were normalized to the untreated control (set as 1). Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

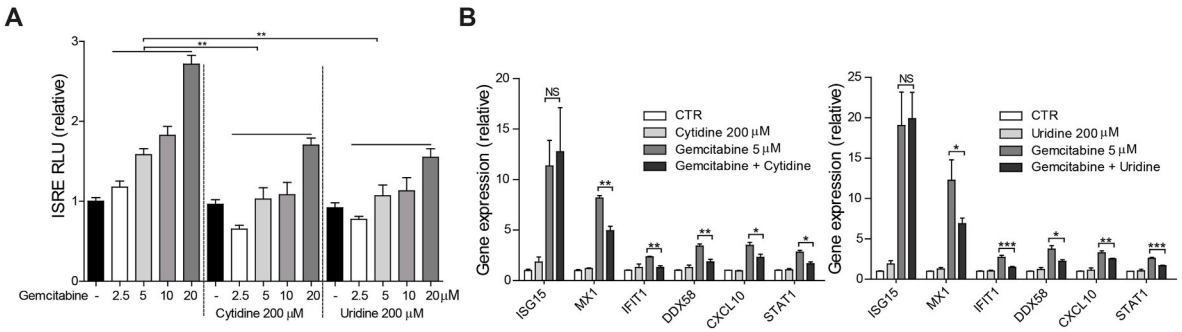

Fig. 5. Supplement of exogenous cytidine or uridine abrogated gemcitabine-induced interferon-like response. (A) ISRE related firefly luciferase activity in Huh7-ISRE-Luc cells was measured after 72 h of treatment with gemcitabine by adding 200 μM exogenous cytidine or uridine (n = 5–10). (B) Gene expression of ISGs in Huh7 cells was quantified by qRT-PCR after 24 h treatment with 5 μM gemcitabine by adding 200 μM exogenous cytidine or uridine (n = 6). RLU: relative luciferase unit. Data were normalized to the untreated control (set as 1). Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

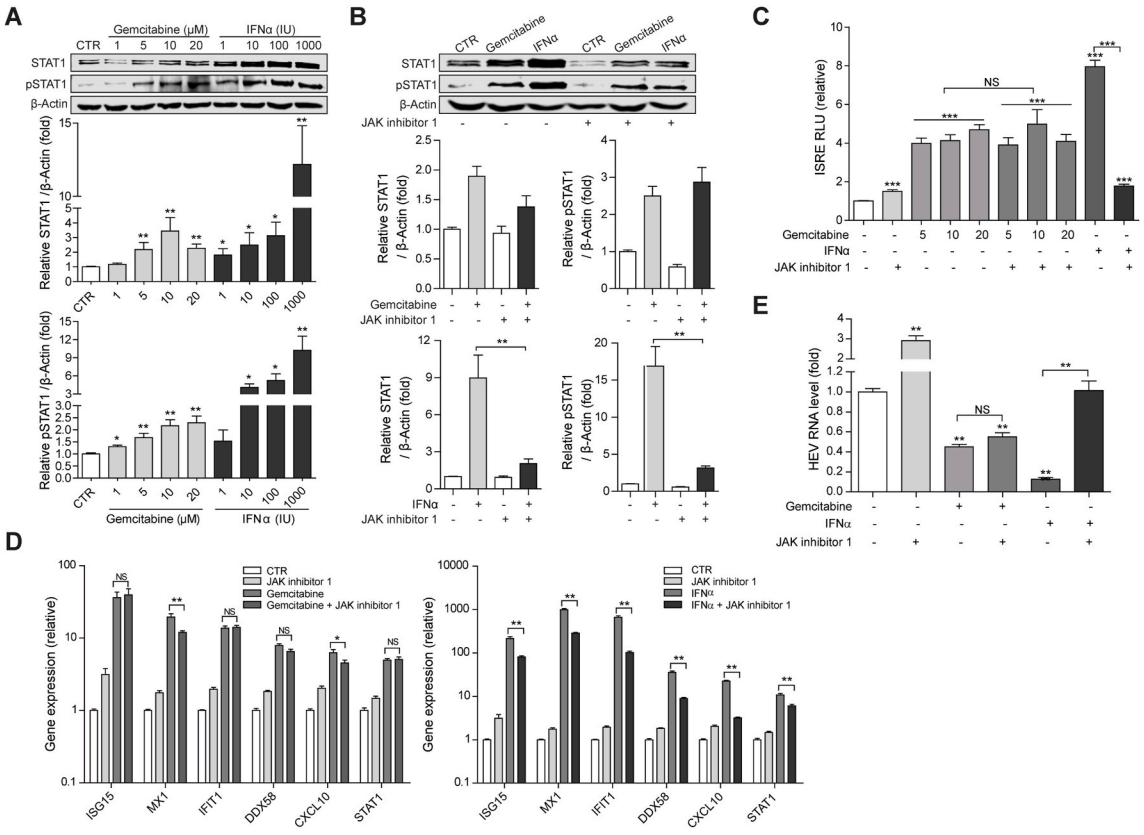

Fig. 6. JAK inhibitor 1 does not affect gemcitabine-induced interferon-like response and anti-HEV activity. (A) Western blot analysis of total STAT1 or phosphorylated STAT1 (pSTAT1) expression in Huh7 cells treated with indicated concentrations of gemcitabine or IFNα for 48 h. β-actin served as an internal reference (n = 3–5). (B) Western blot analysis of STAT1 and phosphorylated STAT1 (pSTAT1) expression in Huh7 cells treated with gemcitabine (10 μM) or IFNα (1000 IU/mL) and/or JAK inhibitor 1 (10 μM) for 48 h (n = 6). (C) Analysis of ISRE related firefly luciferase activity in Huh7-ISRE-Luc cells treated with gemcitabine or IFNα (1000 IU/mL) and/or JAK inhibitor 1 (10 μM) for 72 h (n = 14–16). (D) qRT-PCR analysis of ISGs in Huh7 cells treated with gemcitabine (10 μM) or IFNα (1000 IU/mL) and/or JAK inhibitor 1 (10 μM) for 48 h (n = 6). (E) qRT-PCR analysis of HEV RNA in Huh7-p6 cells treated with gemcitabine (10 μM) or IFNα (1000 IU/mL) and/or JAK inhibitor 1 (10 μM) for 48 h (n = 6). RLU: relative luciferase unit. Data were normalized to the untreated control (set as 1). Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

et al., 2017; Wang et al., 2016a). As it is highly optimized to target HCV polymerase, sofosbuvir is likely not potent against HEV (Kamar et al., 2017; Wang et al., 2016b). In contrast to empirically testing individual candidates, we hypothesize that high-throughput drug screening shall enable systematic and unbiased identification of potential anti-HEV drugs. We have recently screened a library comprising of over 1000 FDA-approved drugs, and identified the anti-histamine drug deptropine inhibiting HEV in cell culture models (Qu et al., 2019). Although deptropine has been widely prescribed for treating asthmatic symptoms in the past, it is currently rarely used in patients because of severe side effects (Vaessen and Koopmans, 1992).

To increase the probability of identifying new anti-HEV agent that can immediately treat patients, this study screened a library of known safe-in-human broad-spectrum antiviral agents. These compounds have been proven to inhibit multiple viruses, and have been used in the clinic or passed phase I trials (Andersen et al., 2020; Ianevski et al., 2018). In this study, we identified several candidates with novel activities agents HEV, but we focused on gemcitabine. As a chemotherapeutic agent with

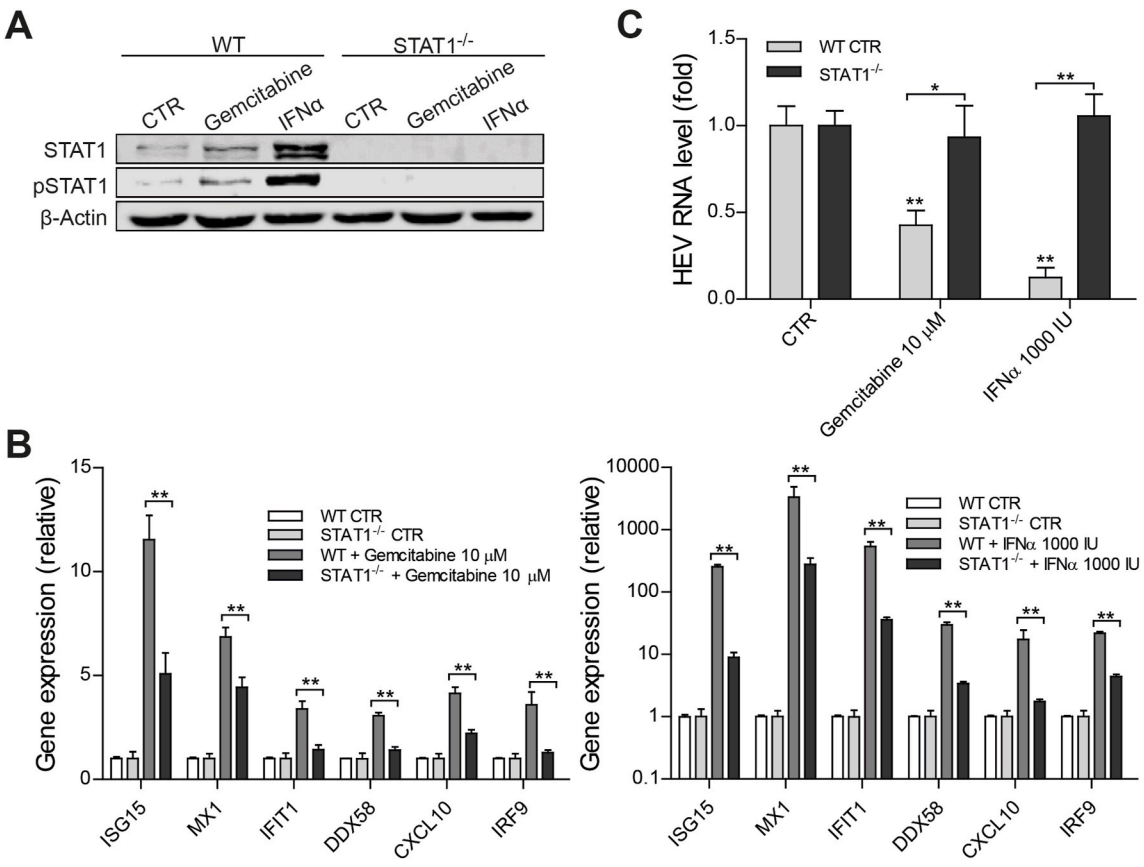

Fig. 7. STAT1 knockout completely abrogated gemcitabine-induced ISG transcription and anti-HEV activity. (A) Western blot analysis of STAT1 and phosphorylated STAT1 (pSTAT1) expression in WT and STAT1$^{-/-}$ Huh7 cells. (B) qRT-PCR analysis of ISGs in WT and STAT1$^{-/-}$ Huh7 cells treated with gemcitabine (10 μM) or IFNα (1000 IU/mL) for 24 h (n = 6). (C) qRT-PCR analysis of HEV RNA in Huh7-p6 and Huh7-STAT1$^{-/-}$-p6 cells treated with gemcitabine (10 μM) or IFNα (1000 IU/mL) for 48 h (n = 6). Data were normalized to the untreated control (set as 1). Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

generic versions widely available, gemcitabine has been extensively used to treat many types of cancer (Cerqueira et al., 2007; Zhang et al., 2019). It is on the list of essential medicines of WHO, among the safest and most effective medicines needed in healthcare. In experimental models, gemcitabine has been reported to inhibit a broad range of RNA viruses including enterovirus with an estimated IC50 of ~5 μM (Lee et al., 2017), human rhinovirus with IC50 from 0.81 to 1.92 μM for different virus strains (Song et al., 2017), HCV (IC50 of 12 nM) (Beran et al., 2012), influenza a (Denisova et al., 2012), HIV (Clouser et al., 2012), and MERS-CoV and SARS-CoV with micromolar IC50s (1.2 μM and 4.9 μM, respectively) (Dyall et al., 2014) and ZIKA virus (Kuivanen et al., 2017). Furthermore, various studies have been reported the broad antiviral activity of gemcitabine in animal models, such as infected with human rhinovirus (Song et al., 2017), leukemia virus (Clouser et al., 2011) or HIV-1 (Clouser et al., 2012).

In this study, we have demonstrated potent anti-HEV activity of gemcitabine in multiple cell models with both genotype 1 and 3 strains that are causing major clinical burden. These results support the potential of clinical application, in particular for treating HEV infected cancer patients. In this study, the IC50 of gemcitabine in inhibiting HEV was 0.42 μM. This concentration exerting anti-HEV activity in our models is easily achievable in cancer patients treated with gemcitabine (Keith et al., 2003). Cancer patients, especially when treated with chemotherapy or radiotherapy, have a weakened immune system. They are prone to infections with worse outcomes (Hotchkiss and Moldawer, 2014). Chronic HEV infection has been frequently reported in cancer patients, but many of them were not treated with antiviral therapy. For ribavirin treated individuals, a subset of patients did not tolerate the medication or failed to clear HEV (Fuse et al., 2015; Protin et al., 2019; Tavitian et al., 2010; von Felden et al., 2019). Our results open a unique opportunity for these patients that gemcitabine may simultaneously combat the cancer and the virus. Of note, a recent study has nicely demonstrated HEV infection in primary human hepatocytes (Todt et al., 2020). Another recent study has shown HEV infection in human stem cell-derived hepatocyte-like cells (Dao Thi et al., 2020). There are also multiple animal models available for HEV (Corneillie et al., 2019). In this study, we did not further validate our findings in these emerging models due to the lacking of essential techniques and expertise, but these models highly valuable for HEV drug development.

The primary anti-cancer mechanism of gemcitabine is depletion of pyrimidine nucleosides to block DNA synthesis, thereby killing proliferating cancer cells (Heinemann et al., 1995). This explains the antiviral activity at least against some viruses such as enterovirus and rhinovirus that supplementation of pyrimidine nucleosides reverses the effect of gemcitabine (Lee et al., 2017; Song et al., 2017). Similarly, we also found that exogenously adding pyrimidine nucleosides largely abrogated the anti-HEV activity of gemcitabine. Surprisingly, pyrimidine nucleosides alone did not affect HEV replication. In contrast, we previously found that adding purine nucleosides enhanced HEV replication (Wang et al., 2016c). Thus, the mechanisms of how nucleotide biosynthesis regulates viral infection are highly context-dependent, namely the type of targeted virus and the type of affected nucleotide. Based on the results from this study, we postulate that the intracellular level of pyrimidine nucleosides *per se* does not affect HEV replication, but indirectly mediates the antiviral action of gemcitabine.

We and other groups have extensively demonstrated that some nucleoside analogs are capable of activating cellular innate immunity, in particular ISG induction (Chung et al., 2016; Lucas-Hourani et al., 2013;

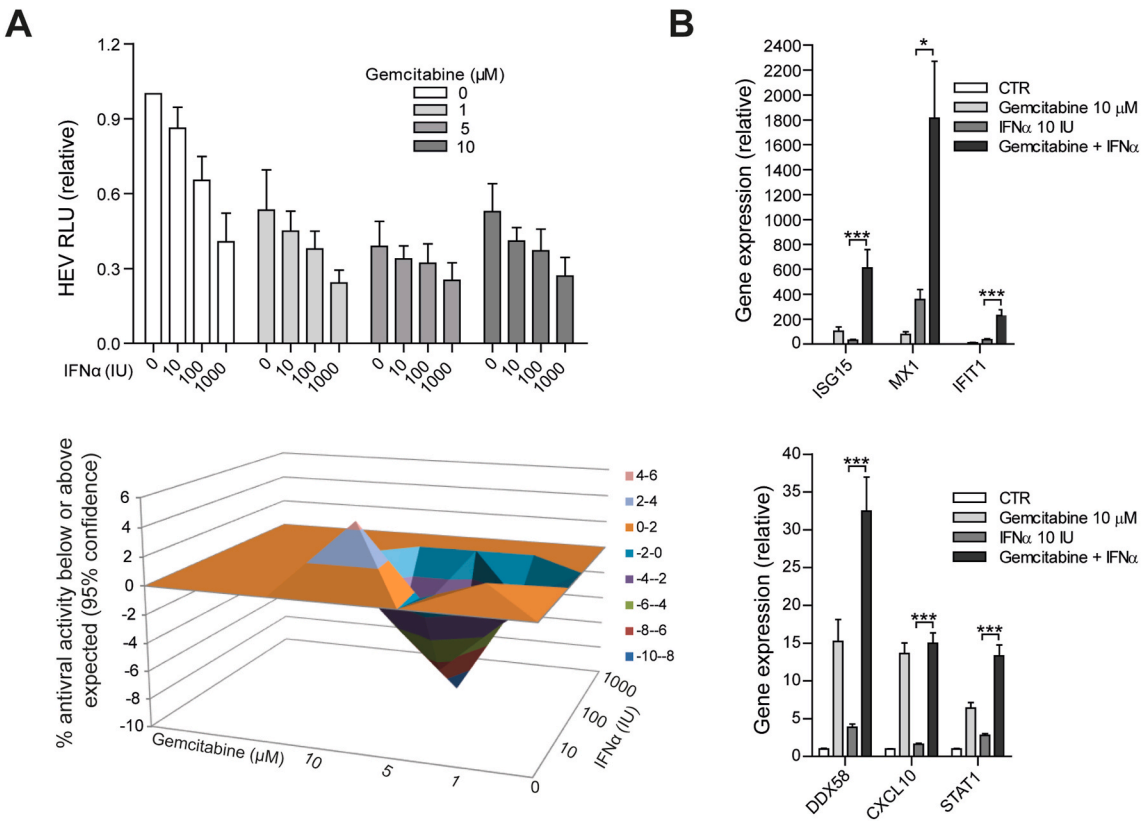

Fig. 8. The combinatory effects of gemcitabine with IFNα on anti-HEV activity and ISG transcription. (A) The antiviral effects of gemcitabine in combination with IFNα were analyzed by MacSynergyII model. The three-dimensional surface plot represents the differences (within 95% confidence interval) between actual experimental effects and theoretical additive effects of the combination at various concentrations (n = 4). (B) qRT-PCR analysis of ISGs in Huh7 cells treated with gemcitabine (10 μM) and/or IFNα (10 IU/mL) (n = 8). RLU: relative luciferase unit. Data were normalized to the untreated control (set as 1). Data are presented as means ± SEM. (*P < 0.05; **P < 0.01; ***P < 0.001).

Pan et al., 2012; Wang et al., 2016c; Yeo et al., 2015). Similar to IFNα treatment, we found that gemcitabine effectively activated ISRE transcriptional activity and ISG expression. Consistently, supplementation of pyrimidine nucleosides blocked this interferon-like response. To our knowledge, there is no evidence that nucleosides can directly regulate the production of interferon cytokines. A plethora of recent studies have reported a variety of non-canonical pathways activating ISG transcription, but their mechanisms remain largely elusive (Wang et al., 2017). Although intuitive, we speculate that lowering intracellular level of pyrimidine nucleosides may act as a sensor of pathogen invasion, and thereby signal host cells to initiate defense machinery.

Classically, interferon activates Janus kinases to phosphorylate STATs, which subsequently recruits the ISGF3 complex to drive ISG transcription (Xu et al., 2017). Similar to IFNα treatment, we observed robust activation of STAT1 phosphorylation by gemcitabine, which is a hallmark of the antiviral interferon response. As expected, blocking Janus kinases by pharmacological inhibitor completely abrogated the antiviral activity of IFNα. In contrast, Janus kinase inhibitor hardly affected gemcitabine triggered STAT1 phosphorylation, ISRE transcription, ISG expression, and anti-HEV activity. These results concretely confirm a non-canonical action of gemcitabine in triggering interferon-like response independent of Janus kinases. Using loss-of-function assay, we demonstrated that STAT1 is essentially required for the antiviral function of gemcitabine. Nevertheless, we still do not have a mechanistic clue how gemcitabine can activate STAT1 phosphorylation dispensable of Janus kinases, which deserves future investigation.

In summary, by screening a library of known safe-in-human broad-spectrum antiviral agents, we identified gemcitabine as a potent inhibitor against HEV infection. It functions by triggering interferon-like response through STAT1 phosphorylation but independent of Janus kinases. This represents a non-canonical antiviral mechanism utilizing the innate defense machinery overlapping with the interferon pathway, but is distinct from the classical interferon response. As a widely used anti-cancer drug, gemcitabine is extremely appealing for treating HEV infected cancer patients that will likely lead to simultaneous anti-cancer and antiviral effects. Whether gemcitabine is also applicable for treating hepatitis E in non-cancer patients remains to be carefully assessed, particularly considering the potential side effects as a chemotherapeutic agent.

Declaration of competing interest

The authors declare that they have no competing interests.

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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.2020.104967.

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