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Cross Talk between Nucleotide Synthesis Pathways with Cellular Immunity in Constraining Hepatitis E Virus Replication

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Viruses are solely dependent on host cells to propagate; therefore, understanding virus-host interaction is important for antiviral drug development. Since *de novo* nucleotide biosynthesis is essentially required for both host cell metabolism and viral replication, specific catalytic enzymes of these pathways have been explored as potential antiviral targets. In this study, we investigated the role of different enzymatic cascades of nucleotide biosynthesis in hepatitis E virus (HEV) replication. By profiling various pharmacological inhibitors of nucleotide biosynthesis, we found that targeting the early steps of the purine biosynthesis pathway led to the enhancement of HEV replication, whereas targeting the later step resulted in potent antiviral activity via the depletion of purine nucleotide. Furthermore, the inhibition of the pyrimidine pathway resulted in potent anti-HEV activity. Interestingly, all of these inhibitors with anti-HEV activity concurrently triggered the induction of antiviral interferon-stimulated genes (ISGs). Although ISGs are commonly induced by interferons via the JAK-STAT pathway, their induction by nucleotide synthesis inhibitors is completely independent of this classical mechanism. In conclusion, this study revealed an unconventional novel mechanism of cross talk between nucleotide biosynthesis pathways and cellular antiviral immunity in constraining HEV infection. Targeting particular enzymes in nucleotide biosynthesis represents a viable option for antiviral drug development against HEV. HEV is the most common cause of acute viral hepatitis worldwide and is also associated with chronic hepatitis, especially in immunocompromised patients. Although often an acute and self-limiting infection in the general population, HEV can cause severe morbidity and mortality in certain patients, a problem compounded by the lack of FDA-approved anti-HEV medication available. In this study, we have investigated the role of the nucleotide synthesis pathway in HEV infection and its potential for antiviral drug development. We show that targeting the later but not the early steps of the purine synthesis pathway exerts strong anti-HEV activity. In particular, IMP dehydrogenase (IMPDH) is the most important anti-HEV target of this cascade. Importantly, the clinically used IMPDH inhibitors, including mycophenolic acid and ribavirin, have potent anti-HEV activity. Furthermore, targeting the pyrimidine synthesis pathway also exerts potent antiviral activity against HEV. Interestingly, antiviral effects of nucleotide synthesis pathway inhibitors appear to depend on the medication-induced transcription of antiviral interferon-stimulated genes. Thus, this study reveals an unconventional novel mechanism as to how nucleotide synthesis pathway inhibitors can counteract HEV replication.

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus that mainly infects the liver. It is the most common cause of acute viral hepatitis worldwide. In general, HEV infection is a self-limiting disease and is associated with low mortality, but epidemics of hepatitis E occur periodically throughout the developing world, resulting in 70,000 deaths yearly (1). In western countries, HEV primarily affects immunocompromised patients, in particular organ transplant recipients, as well as hematopoietic stem cell transplant recipients (2–5). More than 60% of organ recipients infected with HEV develop chronic hepatitis with rapid progression to cirrhosis (2). Despite being an emerging global health issue, no FDA-approved anti-HEV therapy is currently available. Only alpha interferon (IFN-α), ribavirin, or a combination of these has been used occasionally as an off-label treatment. Thus, further research aimed at understanding its infection biology and developing effective antiviral treatment is urgently required.

Cellular nucleotides, including purines and pyrimidines, are the basic building blocks that form the nucleic acids RNA and DNA. Nucleotides are the fundamental components that are required for cell metabolism, such as genome replication. *In vivo*, nucleotides can be synthesized *de novo* through a series of enzymatic reactions or recycled through salvage pathways. Since viral replication heavily relies on the host cells to supply nucleosides, targeting the nucleotide biosynthesis pathway represents an attractive strategy for antiviral drug development. The nucleotide biosynthesis pathways have been well studied for decades (6–8). Numerous compounds have been developed and well character-

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ized to target particular enzymes of this pathway to inhibit viral infections by depletion or causing an imbalance of nucleotide pools (9–18). Among them, inhibitors of IMP dehydrogenase (IMPDH), a key enzyme of the purine synthesis pathway, have been used successfully in the clinic for decades. These drugs, including ribavirin and mycophenolic acid (MPA), used as antiviral or immunosuppressive medication, respectively, have been demonstrated to have broad antiviral activity against a spectrum of viruses, including dengue virus, yellow fever virus (YFV), and hepatitis B, hepatitis C, and hepatitis E viruses (14, 15, 18–21). Likewise, brequinar (BQ) and leflunomide (LFM), inhibitors of dihydroorotate dehydrogenase (DHODH), an essential enzyme of pyrimidine nucleotide synthesis, have been shown to inhibit human polyomavirus type BK virus, YFV, and dengue virus (12, 22).

Besides their function as building blocks of genetic material, free nucleotides also play important roles in cell signaling. We and others have previously reported the potential interaction of nucleotide deprivation and cellular antiviral immune response, such as provoking the expression of interferon-stimulated genes (ISGs) (19, 23). Given that the liver is a major site for nucleotide synthesis, we comprehensively profiled the role of purine and pyrimidine synthesis pathways in HEV cell culture models aimed at identifying potential antiviral drug targets and understanding the cross talk with cellular antiviral immunity against HEV infection.

MATERIALS AND METHODS

Reagents. Guanosine (CAS 118-00-3), adenosine (CAS 58-61-7), uridine (CAS 58-96-8), 6-thioguanine (6-TG; CAS 154-42-7), lometrexol hydrate (CAS 106400-81-1), methotrexate (MTX) hydrate (CAS 133073-73-1), fludarabine (FA) phosphate (CAS 75607-67-9), brequinar (BQR) sodium salt hydrate (MDL MFCD21363375), leflunomide (LFM) (CAS 75706-12-6), and 6-azauracil (6-AU; CAS 461-89-2) were purchased from Sigma. Twenty-three IMPDH-specific inhibitors were kindly provided by the Center for Drug Design, University of Minnesota. All of the reagents were dissolved in dimethyl sulfoxide (DMSO). The effects of these *de novo* nucleotide biosynthesis inhibitors on host cell viability were determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (see Fig. S7 in the supplemental material). Stocks of JAK inhibitor 1 (CAS 457081-03-7; Santa Cruz Biotech, CA) were dissolved in DMSO at a final concentration of 5 mg/ml. Stocks of CP-690550 (tofacitinib) (Santa Cruz Biotech, CA) were dissolved in DMSO at a final concentration of 10 mg/ml.

Cell culture. Human hepatoma cell line Huh7 and human embryonic kidney epithelial cell line 293T were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin.

Cell culture models. An HEV replication model with subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene and an HEV infection model containing the full-length HEV genome were used in our study. The construction of two models has been described previously (18). Huh7 cells constitutively expressing the firefly luciferase reporter gene driven by the human PGK promoter were used to represent household luciferase activity for normalizing nonspecific effects on luciferase activity (11). Huh7 cells transduced with a lentiviral transcriptional reporter system expressing the firefly luciferase gene under the control of a promoter containing multiple interferon-stimulated response element (ISRE) promoter elements (SBI Systems Biosciences, Mountain View, CA) was established, and luciferase activity represents ISRE promoter activation.

Quantification of HEV replication and infection. The details for examining HEV replication and infection were described before (18). Briefly, for the HEV replication model (p6-Luc), the activity of secreted *Gaussia* luciferase in the cell culture medium was measured using a Bio-Lux *Gaussia* luciferase flex assay kit (New England BioLabs) for the quantification of viral replication, which was normalized by firefly luciferase expression. For the full-length HEV infectious model, SYBR green-based quantitative reverse transcription-PCR (qRT-PCR) was used to quantify the newly formed viral genomic RNA after cell lysis, and the HEV primer sequences are shown in Table S2 in the supplemental material.

Gene knockdown by lentiviral vector-delivered shRNA. Lentiviral vectors targeting PPAT, GART, ATIC, and DHODH were produced in 293T cells as previously described (11). To generate stable gene knockdown cells, Huh7 cells were transduced with lentiviral vectors. Since the vectors also express a puromycin resistance gene, transduced cells subsequently were selected by adding 2.5 μg/ml puromycin (Sigma) to the cell culture medium. After a pilot study, the short hairpin RNA (shRNA) vectors (see Fig. S1 and Table S3 in the supplemental material) exerting optimal gene knockdown were selected by qPCR with the corresponding primers shown in Table S2 in the supplemental material. Meanwhile, shRNA vector expressing green fluorescent protein (shGFP) was used as a control (shCTR). The amount of HEV was assessed after 3 days of HEV infection in medium containing shGFP cells and knockdown cells. For the experiment comparing the activity of compounds between shGFP and knockdown cells, infectious HEV cells were directly transduced with lentiviral shRNA vectors and selected by puromycin.

Statistical analysis. Statistical analysis was performed using the nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism Software). *P* values of less than 0.05 were considered statistically significant.

RESULTS

Exogenous guanosine, but not uridine, stimulates HEV replication. Purine and pyrimidine nucleotides are the major cellular energy carriers and constitute the defining subunits of nucleic acids. Two distinct pathways are responsible for the biosynthesis of these two types of nucleotides (Fig. 1A and 2A). Their fundamental role in cellular biochemistry raises the possibility that modifying flux through nucleotide biosynthesis pathways profoundly influences the course of viral infection. Thus, we decided to assess the overall impact of either purine or pyrimidine synthesis on HEV infection. A first indication that such effects exist came from experiments in which we arbitrarily increased the purine and pyrimidine content by supplementation of exogenous guanosine (Fig. 1A) and uridine (Fig. 2A) in human hepatoma cell line (Huh7)-based HEV cell culture models. Guanosine, a purine nucleoside containing guanine attached to a ribose, can be converted to GMP through the purine salvage synthesis pathway and subsequently replenishes the purine nucleotide pool (Fig. 1A). Mechanistically, the cleavage of exogenous guanosine was catalyzed by purine nucleoside phosphorylase (PNP) to form guanine. In the presence of hypoxanthine/guanine phosphoribosyl transferase (HGPRT), guanine was converted to GMP by the addition of ribose 5-phosphate from phosphoribosyl pyrophosphate (PRPP). The supplementation of guanosine dose dependently enhanced HEV replication-related luciferase activity in the subgenomic replicon (p6-Luc) model and increased cellular viral RNA in the full-length (p6) infectious model (Fig. 1B). Likewise, uridine, which is a pyrimidine nucleoside consisting of uracil binding to ribose, commonly presents as UMP to rescue cells from pyrimidine nucleotide depletion (Fig. 2A). In contrast, the supplementation of exogenous uridine had no effect on HEV replication (Fig. 2B). Thus, interaction between at least some of the pathways involved in nucleotide biosynthesis and the HEV infectious process might exist.

Targeting the catalytic steps leading to primary purine nucleotide synthesis (IMP) stimulates HEV replication. Given the

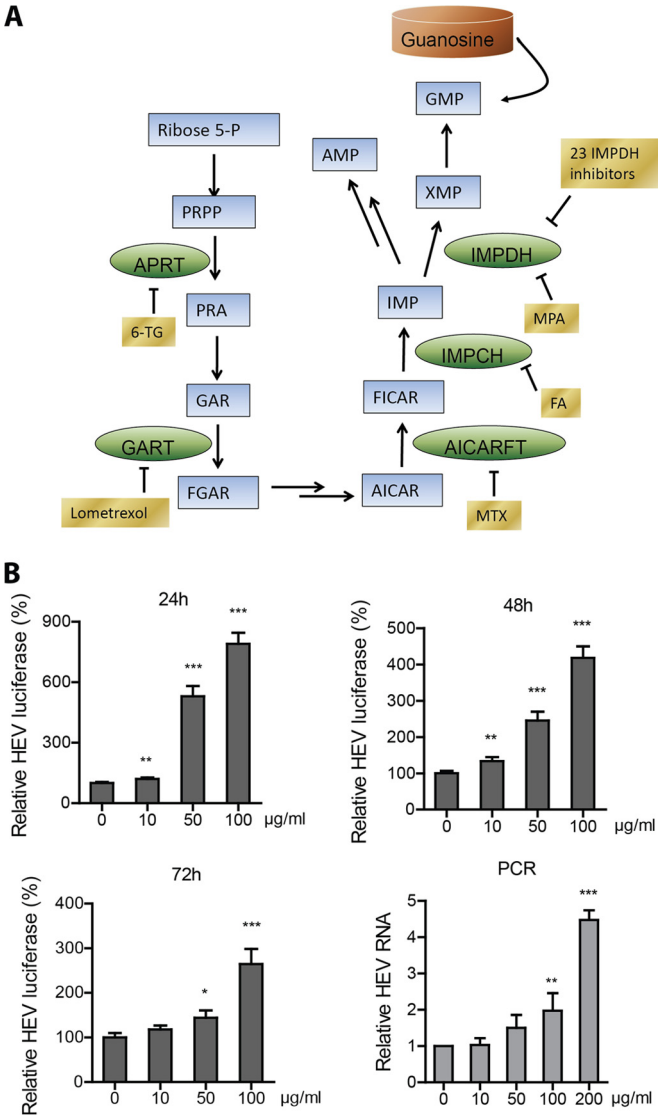

FIG 1 Exogenous guanosine stimulated HEV replication. (A) Schematic overview of *de novo* biosynthesis of purine nucleotide. PRPP, 5-phosphoribosyl-1-pyrophosphate; PRA, 5-phosphoribosylamine; GAR, glycinamide ribonucleotide; FGAR, formyl-GAR; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide. (B) Huh7 cell-based subgenomic HEV replicons containing the luciferase reporter gene were treated for 24 h, 48 h, and 72 h with a dose range of guanosine ($n = 4$). Data are presented as means ± standard errors of the means (SEM). Meanwhile, Huh7 cells with the infectious HEV containing the full-length p6 genome were treated for 48 h with a dose range of guanosine ($n = 5$). Data were normalized to two housekeeping genes (*GAPDH* and *RP2*) and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

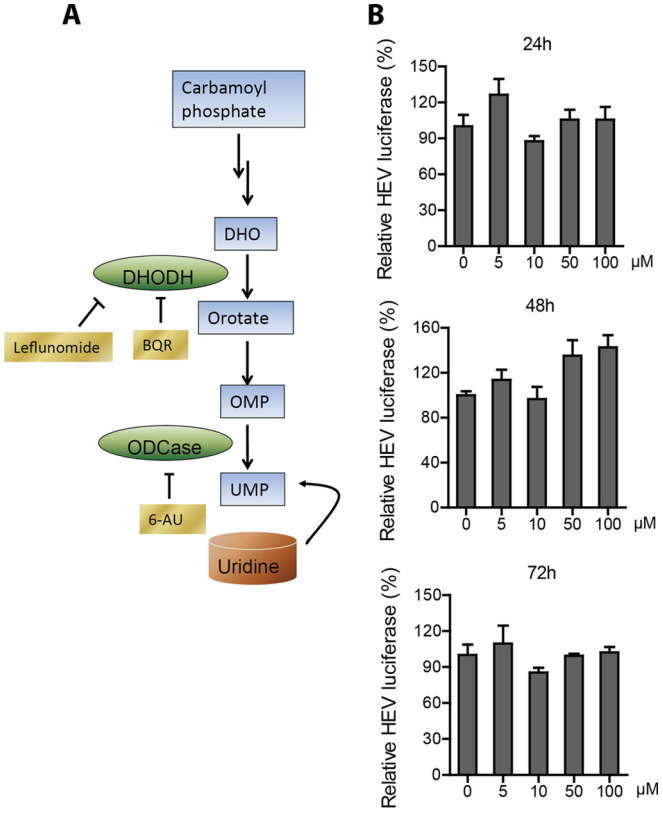

FIG 2 Exogenous uridine does not affect HEV replication. (A) Schematic overview of *de novo* biosynthesis of pyrimidine nucleotide. (B) Huh7 cell-based subgenomic HEV replicon containing the luciferase reporter gene was treated for 24 h, 48 h, and 72 h with a dose range of uridine ($n = 5$). Data are presented as means ± SEM.

clear proviral effect of exogenous guanosine, we explored potential anti-HEV strategies targeting the different enzymes that are involved in purine nucleotide synthesis. *De novo* purine is synthesized mainly in the liver and begins with the starting material PRPP. The first fully formed nucleotide, IMP, is catalyzed through 10 reactions by six enzymes (Fig. 1A). We first selectively targeted three key enzymes of this cascade, including amido phosphoribosyltransferase (APRTase), glycinamide ribonucleotide transformylase (GART), and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (AICARFT), through the use of 6-thioguanine (6-TG), lometrexol, and methotrexate (MTX), respectively. Somewhat counterintuitively, all three compounds increased HEV replication in both cell culture models (Fig. 3). To further clarify the role of their targets, lentivirus-mediated RNA interference (RNAi) was used for the knockdown of the PPAT, GART, and ATIC genes that encode the corresponding enzymes APRTase, GART, and AICARTF, respectively (Fig. 4A). Consistent with the pharmacological results, the downregulation of these enzymes enhanced HEV replication (Fig. 4B). Furthermore, the proviral effects of the pharmacological inhibitors were largely absent in a context in which their targets were silenced, suggesting that pharmacological effects are not due to off-target effects (Fig. 4C).

As a bifunctional enzyme, the N-terminal domain of ATIC has AICARFT activity, and the C-terminal domain has IMP cyclohydrolase (IMPCH) activity. FA, an IMPCH inhibitor, also promoted HEV replication but exerted cytotoxicity concurrently (see Fig. S2 in the supplemental material). Thus, these results highlight the interaction of nucleotide biosynthesis and the HEV infection process but also show that the rational design of therapy aimed at exploiting the nucleotide biosynthesis pathway for treatment of HEV is not straightforward.

IMPDH inhibition counteracts HEV replication by depleting the purine nucleotide pool. As a branching point in purine synthesis, IMP is converted to either AMP or XMP/GMP

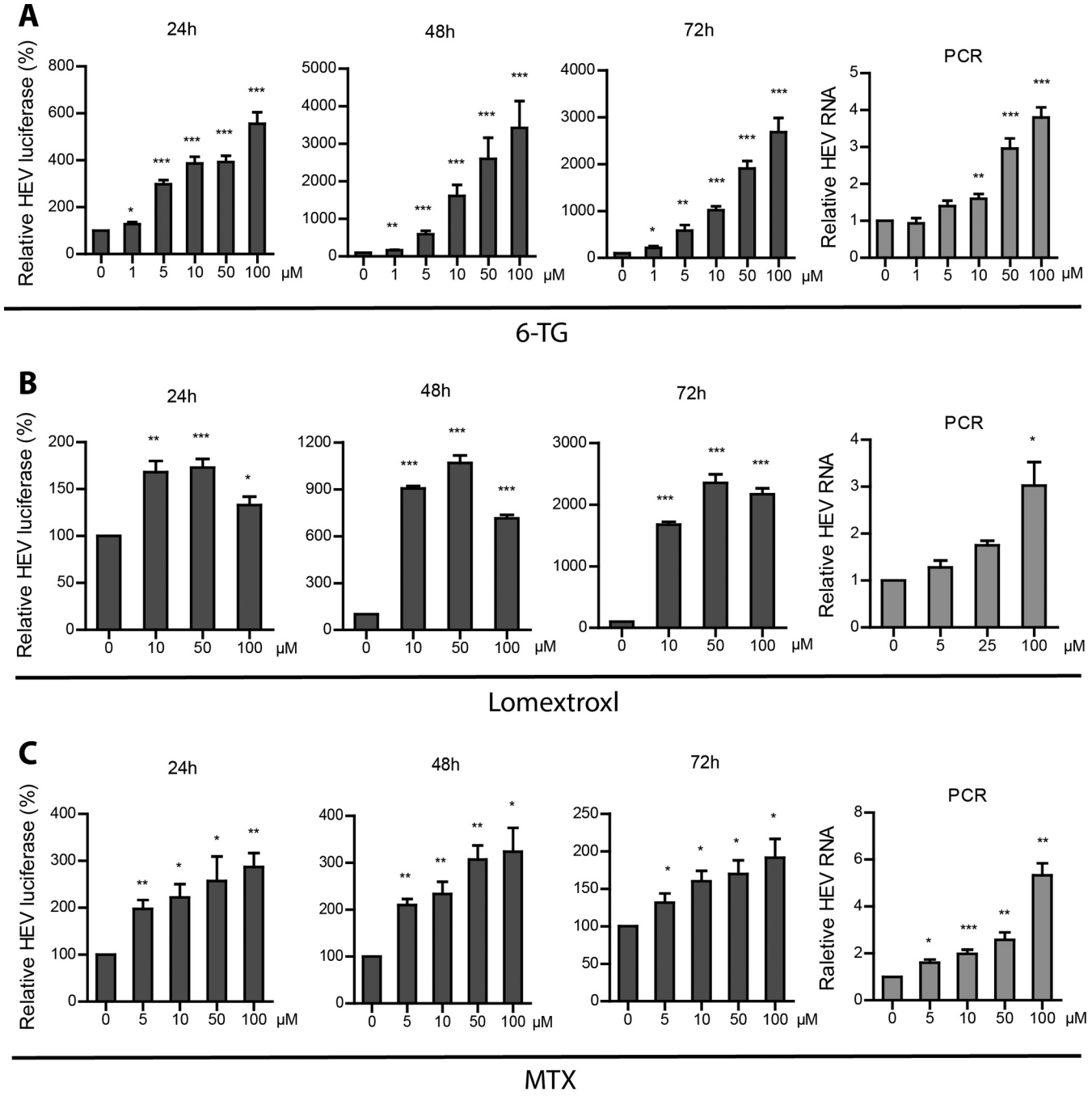

FIG 3 Inhibitors of IMP synthesis cascade stimulate HEV replication. The Huh7 cells containing subgenomic HEV replicons with luciferase reporter genes were incubated with increasing doses of 6-TG (A), lometrexol (B), and MTX (C). The luciferase activity was determined at 24 h, 48 h, and 72 h. Accordingly, Huh7 cells infected with full-length HEV were treated with increasing doses of 6-TG (A), lometrexol (B), and MTX (C). The HEV RNA level was quantified by qRT-PCR after 48 h. Data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM from five to eight experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(Fig. 1A). IMPDH, an enzyme consisting of two isoforms (IMPDH1 and IMPDH2) in humans, catalyzes the reaction of IMP into XMP for further conversion to GMP. We have previously demonstrated that MPA, a clinically used immunosuppressant that preferentially inhibits IMPDH2, has anti-HEV activity (18). To further explore the potential of targeting this enzyme, a panel of 23 inhibitors was custom designed and synthesized with various affinities in inhibiting IMPDH1 or IMPDH2 (see Table S1 in the supplemental material). As shown in Fig. 5A, HEV replication was inhibited by all 23 IMPDH inhibitors at a concentration of 10 μM as measured by luciferase activity. Accordingly, 21 of the 23 inhibitors also suppressed HEV infection as assessed by full-length HEV genome quantification by qRT-PCR (Fig. 5B). Anti-HEV activity also was observed at 2 μM for 20 of the IMPDH

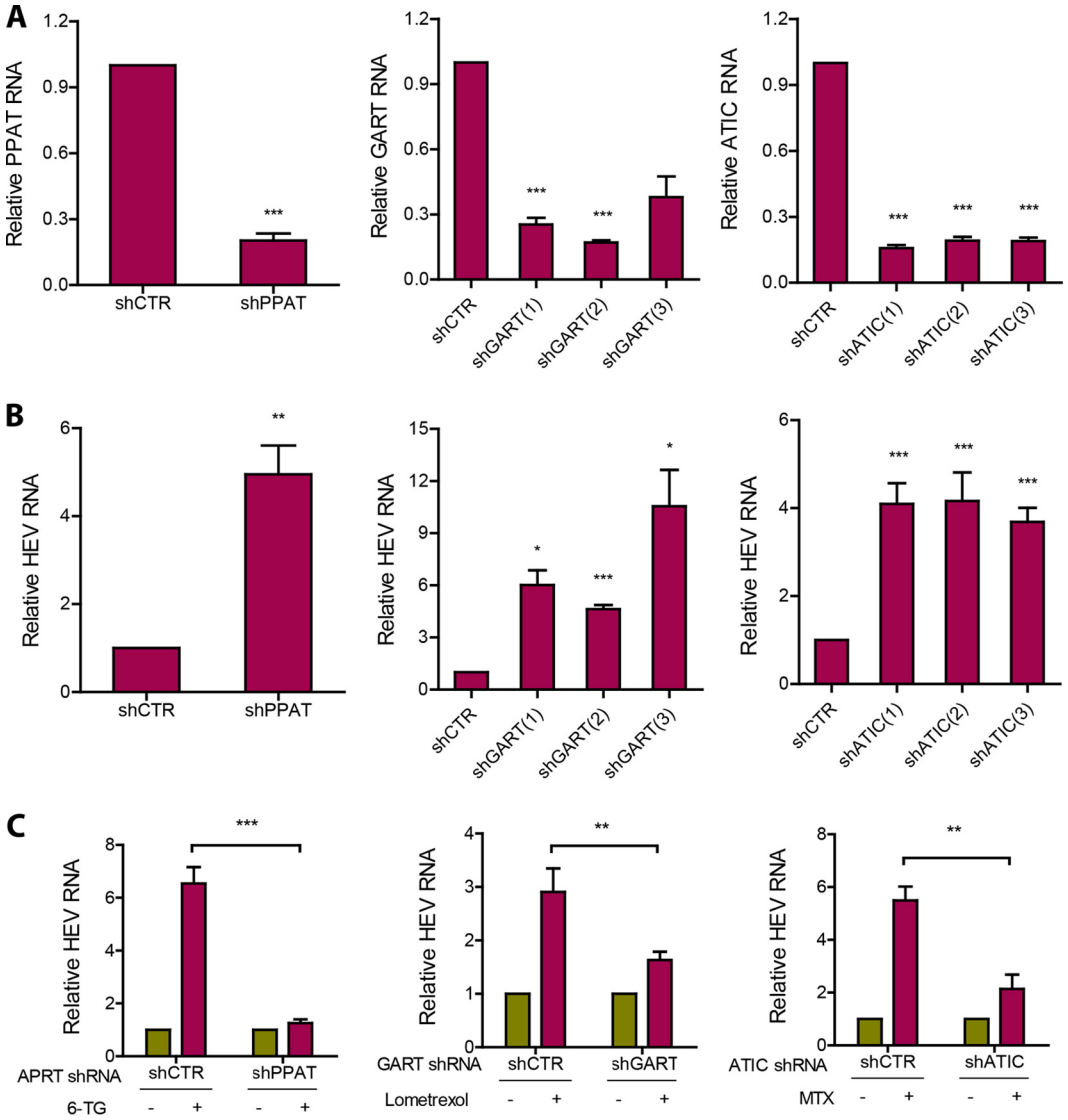

FIG 4 Silencing of enzymes involved in IMP synthesis cascade facilitates HEV replication. (A) Huh7 cells were transduced with lentiviral shRNAs to stably silence the corresponding genes for PPAT, GART, and ATIC (a set of independent shRNA clones targeting each gene was used). Huh7 cells transduced with lentiviral shRNA targeting GFP (shCTR) were used as a control. The efficiency of gene knockdown was analyzed by qRT-PCR. (B) Silencing of PPAT, GART, and ATIC resulted in significant elevation of viral RNA upon inoculation of HEV. HEV RNA levels were determined 72 h after inoculation. (C) Silencing of PPAT, GART, and ATIC abrogated the pro-HEV effects of 6-TG, lometrexol, and MTX. Data were normalized to that for cells without treatment with the three compounds (green bar; set as 1). All data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1) (means ± SEM from four to eight experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

inhibitors (see Fig. S3). To further characterize the inhibition, we selected three representative compounds with anti-HEV activity in both models. Similar to ribavirin and MPA, guanosine supplementation abrogated the anti-HEV activity of these compounds (Fig. 5C), suggesting that the depletion of the purine nucleotide pool is responsible for the antiviral action. Thus, inhibitors with anti-HEV potential exert their action in this respect through targeting nucleotide synthesis.

Targeting pyrimidine biosynthesis inhibits HEV replication. Even though the supplementation of exogenous uridine has no effect on HEV, inhibitors of pyrimidine synthesis have been widely reported to inhibit the infection of a broad spectrum of other viruses, prompting further exploration of the role of pyrimidine biosynthesis in HEV replication. Thus, we selected two catalytic enzymes involved in *de novo* pyrimidine synthesis for further study. DHODH, which localizes to the mitochondria, is a critical enzyme that converts dihydroorotate to orotate. Brequinar (BQR) and leflunomide (LFM) are well-known clinically tested DHODH inhibitors. Treatment with BQR (10 to 500 nM) results in a significant reduction of HEV replication-related luciferase activity in the subgenomic replicon assay system (Fig. 6A). Concordantly, BQR also dose dependently inhibits cellular viral RNA in our infectious HEV model. Treatment with 500 nM BQR for 48 h resulted in 78% ± 17% (means ± standard deviations [SD]; $n = 7$; $P < 0.001$) inhibition of HEV genomic RNA level (determined by qRT-PCR) compared with that of the control (Fig. 6A). Similar

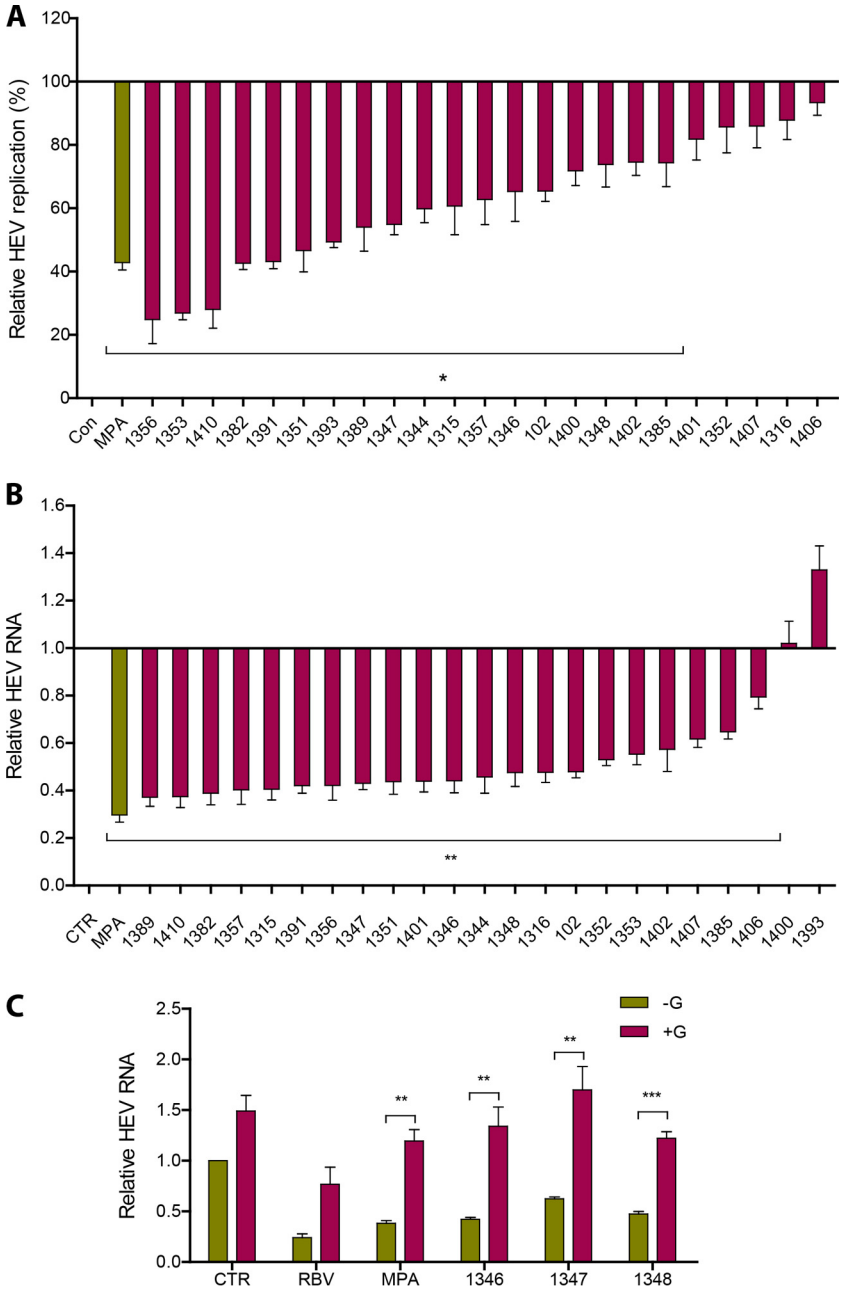

FIG 5 IMPDH inhibitors potently inhibit HEV replication by depletion of the purine nucleotide pool. (A) Huh7 HEV replicon luciferase cells were treated with 23 specific IMPDH inhibitors (10 μM) with MPA as a positive control. Luciferase activity was quantified at 24 h after treatment ($n = 3$). (B) Huh7 cells harboring full-length HEV were treated with 23 specific IMPDH inhibitors with MPA as a positive control. HEV RNA levels were measured by qRT-PCR at 48 h after treatment ($n = 5$). (C) Supplementation of guanosine abrogated the anti-HEV effects of 3 representative IMPDH inhibitors (1346, 1347, and 1348) ($n = 5$). Ribavirin (RBV) and MPA served as positive controls. Data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1) (means ± SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

results were observed with treatment of LMF (Fig. 6B). The specificity of these effects was confirmed in experiments in which we examined the cognate target of these inhibitors, DHODH, by lentiviral RNAi-mediated silencing. Consistent with previous results, the knockdown of DHODH inhibited HEV replication and abrogated the anti-HEV effect of BQR (Fig. 7), and this enzyme emerged as a relevant target in anti-HEV therapy.

To further identify potential anti-HEV targets, we also examined orotidine-5′-monophosphate decarboxylase (ODCase), the downstream enzyme of DHODH that catalyzes the decarboxylation of OMP to UMP. To this end, we employed 6-azauracil (6-AU), a potent inhibitor of ODCase. As shown in Fig. 6C, HEV replication was dose dependently inhibited by 6-AU. Conversely, supplementation with uridine fully restored the HEV infectious potential despite the presence of BQR, LMF, or 6-AU (Fig. 8). In

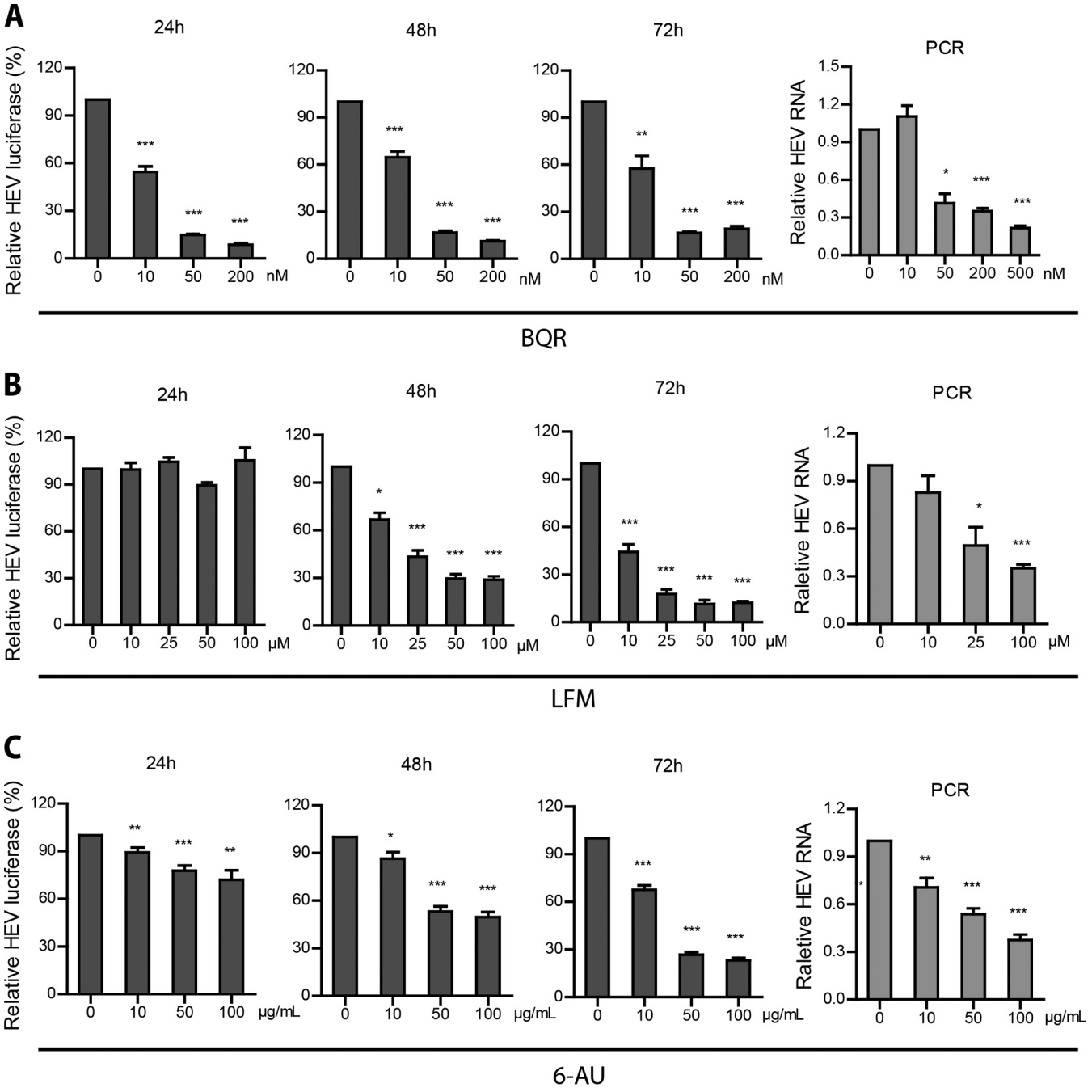

FIG 6 Inhibition of pyrimidine nucleotide synthesis suppresses HEV replication. Huh7 cells containing subgenomic HEV replicons with luciferase report genes were treated with increasing doses of BQR (A), LFM (B), and 6-AU (C). The luciferase activity was determined after 24 h, 48 h, and 72 h. Accordingly, Huh7 cells harboring infectious HEV also were treated with increasing doses of BQR (A), LFM (B), and 6-AU (C). HEV RNA was quantified by qRT-PCR after 48 h of treatment. Data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM from four to seven experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

conjunction, these results show that the depletion of the pyrimidine nucleotide pool is a powerful anti-HEV strategy.

Inhibitors of purine and pyrimidine synthesis provoke cellular antiviral immune responses through nucleotide depletion. We previously demonstrated that the IMPDH inhibitor MPA can induce the expression of ISGs to combat hepatitis C virus (HCV) infection, although the underlying mechanism remained unclear (19). ISGs are the ultimate antiviral effectors and generally are assumed to be induced solely through the action of antiviral cytokines, especially interferons. In HEV infection models, we observed that MPA as well as three other selected IMPDH inhibitors were able to induce the expression of a panel of antiviral ISGs (Fig. 9A), challenging this dogma. The induction of ISGs by IMPDH inhibitors was associated with purine nucleotide depletion, since

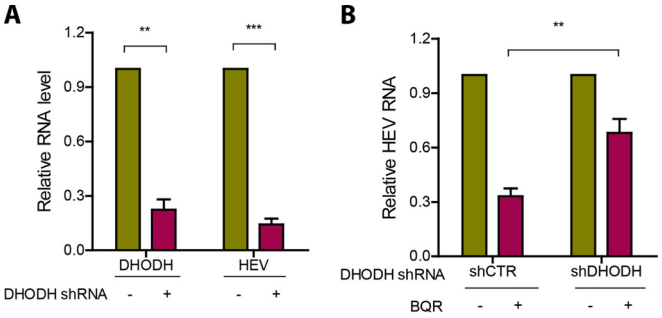

FIG 7 Anti-HEV activity by BQR can be attributed to the inhibition of its target, DHODH. (A) Huh7 cells were transduced with lentiviral shRNA to stably silent DHODH (DHODH shRNA positive). Huh7 cells transduced with lentiviral shRNA targeting GFP were used as a control (DHODH shRNA negative). DHODH knockdown was assessed by qRT-PCR ($n = 3$). DHODH knockdown resulted in the significant inhibition of HEV replication. HEV RNA levels were determined 72 h after HEV inoculation ($n = 6$). (B) DHODH knockdown abrogated the anti-HEV effect of BQR ($n = 7$). Data were normalized to cells without BQR treatment (green bar; set as 1). All data were normalized to two housekeeping genes and are presented relative to the control (shCTR) (set as 1) (means ± SEM). **, $P < 0.01$; ***, $P < 0.001$.

the supplementation of guanosine at least partly abrogated the induction of ISGs (Fig. 9B).

In parallel, we also investigated the effects of pyrimidine synthesis inhibitors. We employed an interferon response reporter in which Huh7 cells are stably integrated with an interferon-stimulated response element (ISRE)-driven luciferase gene that measures ISG transcription upon interferon stimulation. BQR potently induces luciferase activity in this reporter assay and triggers the expression of a panel of ISGs (Fig. 10A; also see Fig. S4 in the supplemental material). The supplementation of uridine completely abrogated these effects on ISG transcription (Fig. 10B; also see Fig. S4). Similar results also were observed with another pyrimidine synthesis inhibitor, 6-AU, targeting ODCase (Fig. 10C). Thus, both purine and pyrimidine synthesis pathways can interact with cellular antiviral immune responses, providing a rational explanation for their antiviral effects.

The induction of ISGs by nucleotide synthesis inhibitors is independent of the JAK-STAT machinery. Classically, ISGs are thought to be induced only by interferons through the activation of the JAK-STAT pathway. Briefly, the binding of interferons to their receptors leads to the activation of Janus activated kinase 1 (JAK1), resulting in the tyrosine phosphorylation of downstream substrates, including signal transducer and activator of transcription 1 (STAT1) and STAT2. The complex of STAT1-STAT2-IRF9 (IFN-regulatory factor 9) enters the nucleus and binds to the ISRE motifs in the target gene, subsequently regulating ISG transcription and mediating the innate antiviral immune response.

To assess whether the induction of ISGs by nucleotide synthesis inhibitors also occurs via this classical pathway, we blocked the JAK-STAT cascade by employing the pharmacological JAK inhibitors JAK inhibitor 1 and CP-690550, which conceivably were identified to conceivably impair the expression of ISGs triggered by IFN-α (see Fig. S5 in the supplemental material). Surprisingly, the induction of ISGs as well as the anti-HEV effects of these inhibitors were not affected (Fig. 11). These results revealed that targeting nucleotide synthesis provokes ISG induction via a noncanonical mechanism that is independent of classical interferon signaling.

DISCUSSION

Nucleotides are key components involved in host cell metabolism and virus infection. Most of the inhibitors targeting *de novo* nucleotide biosynthesis have been well characterized by many studies, and their efficacy in inhibiting nucleotide synthesis has been thoroughly demonstrated (16, 17, 24–31). Based on these findings, we profiled and established the effects and mechanism of action of inhibiting *de novo* nucleotide biosynthesis on HEV replication. Unexpectedly, targeting the early steps of the purine nucleotide synthesis pathway (before the primary purine IMP formed) leads to the enhancement of HEV replication, whereas targeting a later step (IMPDH enzyme) results in potent antiviral activity against HEV, an effect apparently relating to purine nucleotide depletion. The inhibition of the pyrimidine nucleotide synthesis pathway also inhibits HEV replication. Mechanistically, these effects are related to an unconventional interaction with cell-autonomous antiviral immunity dependent on the very strong induction of antiviral ISGs.

It is counterintuitive that targeting the upstream enzymes of the purine pathway (before IMP formed) by pharmacological inhibitors facilitates HEV replication, but the specificity became evident from silencing genes encoding the enzymes involved. Supplementation with exogenous purine nucleotides (adenosine or guanosine) in culture medium in the presence of these purine synthesis inhibitors was not capable of abrogating the stimulation of HEV replication, suggesting these proviral effects only partly relate to the nucleotide synthesis pathway (see Fig. S6A to C in the supplemental material). It is worth noting that targeting the early stage of purine synthesis results in the depletion of the ATP and/or GTP pool. Cellular energy metabolism mediated by ATP might be important for the host cells to defend against virus infection (32, 33). Therefore, insufficient ATP levels might facilitate HEV infection by escaping from host cellular immunity. However, how the ATP levels regulate virus infection deserves further investigation. Similarly, a previous study reported proviral activity by nucleotide biosynthesis inhibitors LFM and FK778 in the hepatitis B virus model, although these two compounds generally are antiviral against other viruses (17). Thus, the question of whether the pro-HEV effects of targeting the early steps of the purine pathway are specific to this virus or a general phenomenon in virus biology remains unanswered.

IMPDH, as a target for antiviral drug development for a broad spectrum of viruses, has been widely investigated. We previously demonstrated that the IMPDH inhibitors ribavirin and MPA inhibit HEV replication *in vitro* (18, 20). This study further validated this notion by showing the anti-HEV potential of 23 specifically designed IMPDH inhibitors. The levels of efficacy of 23 IMPDH inhibitors on HEV infection were consistent to various degrees, which might be due to their different abilities and variable affinities in inhibiting IMPDH1 and IMPDH2. As a competitive IMPDH inhibitor, ribavirin has been used in the clinic to treat chronic hepatitis C for decades. However, ribavirin monotherapy has a barely detectable effect on HCV viral load reduction (34), but when combined with IFN-α it doubles the response rate compared to that with IFN-α alone (35). In contrast, ribavirin monotherapy as an off-label treatment appears very effective for treating chronic HEV infection, in that viral clearance was observed in the majority of the patients as reported by a recent large retrospective multicenter study (36), although prospective randomized trials

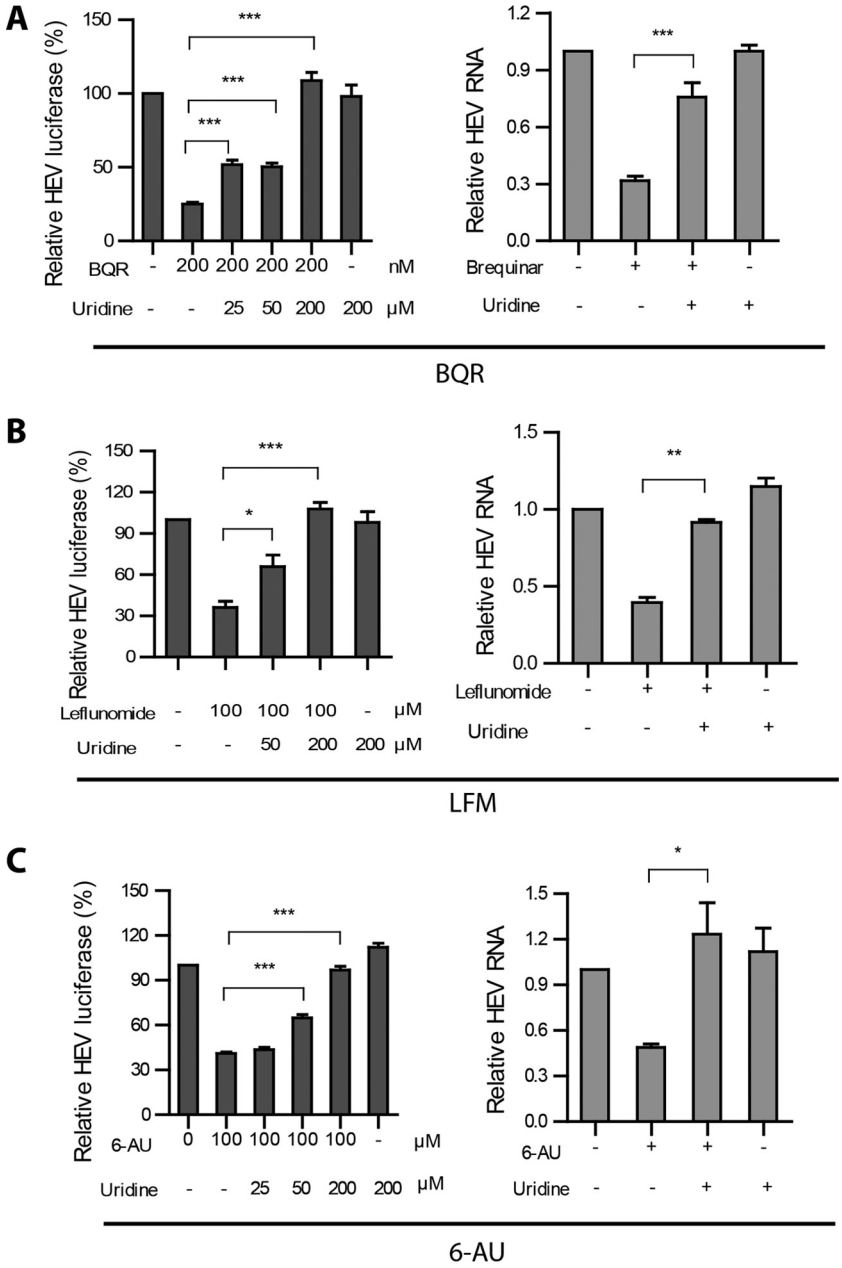

FIG 8 Uridine reverses the anti-HEV activity mediated by pyrimidine inhibition. The Huh7 subgenomic HEV replicon was incubated with BQR (A), LFM (B), and 6-AU (C) and supplemented with increasing doses of uridine. After 72 h, luciferase activity was determined. Accordingly, Huh7 cells harboring full-length HEV RNA were treated with BQR (A), LFM (B), and 6-AU (C) and supplemented with 200 μM uridine. HEV viral RNA was assessed by qRT-PCR 48 h after treatment. Data were normalized to two housekeeping genes and are presented relative to the control (CTR) (set as 1). Data represent means ± SEM from four to seven experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

still are required to confirm the findings. In addition to IMPDH inhibition, ribavirin also possesses pleiotropic biological properties, including immunomodulation, inhibition of gene translation, interaction with viral RNA-dependent RNA polymerase (RdRp), and mutation of virus (37–39). Thus, the exact anti-HEV mechanism used by ribavirin remains to be further elucidated, but the present study provides evidence that the answers lie in its relation to nucleotide biosynthesis.

As a noncompetitive IMPDH inhibitor, MPA has been used as an immunosuppressant to prevent allograft rejection following organ transplantation (40). Despite the opposing effects on HEV of inhibitors targeting early or later steps of the purine synthesis cascade, we demonstrated that the anti-HEV action of MPA was independent of those early-step enzymes (see Fig. S6D in the supplemental material). Interestingly, clinical evidence appears to support our experimental observation that the use of immunosuppressive treatments containing mycophenolate mofetil (the prodrug of MPA) leads to more frequent HEV clearance in heart transplant recipients (41). Nevertheless, because of limited patient numbers, we still do not have sufficient evidence to draw a solid

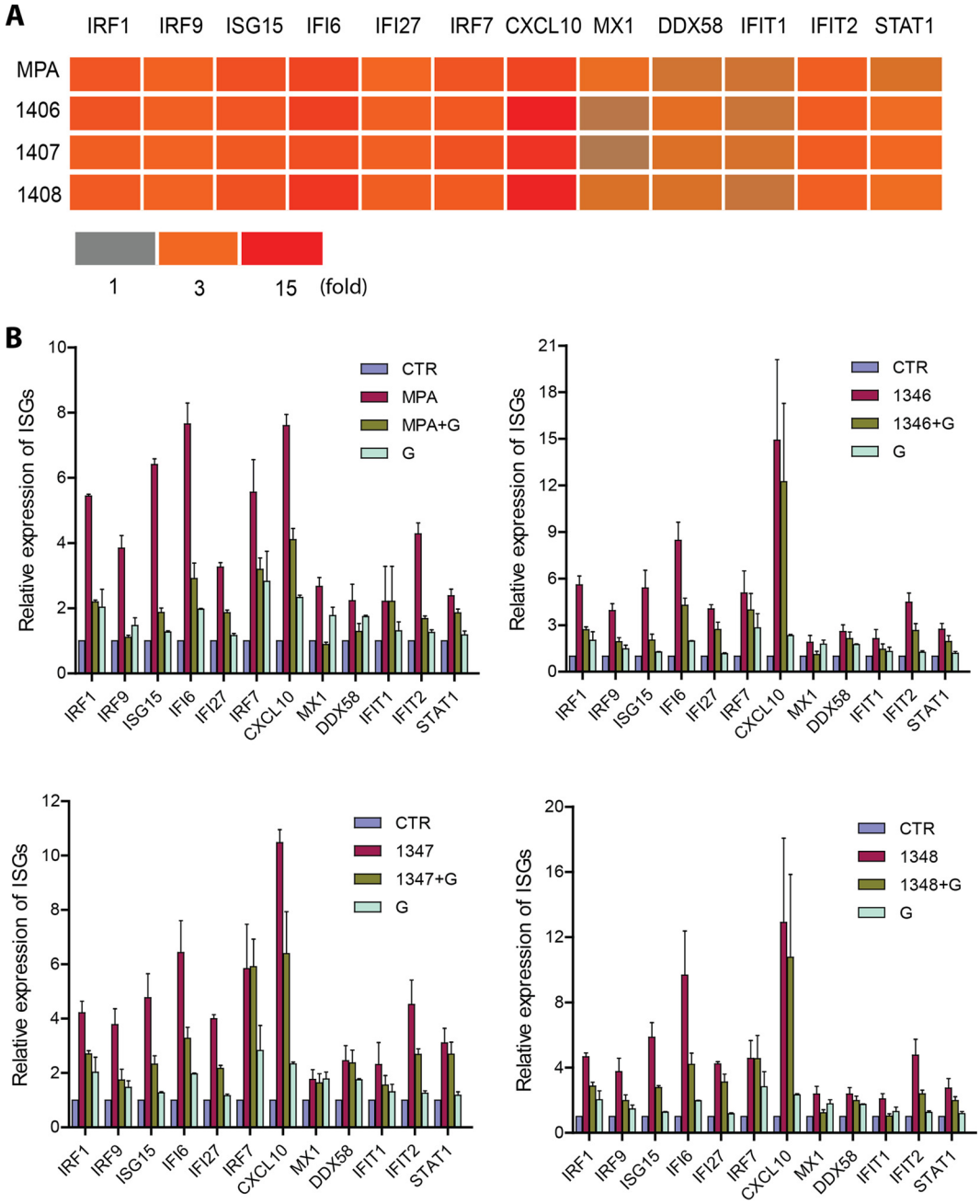

FIG 9 Inhibition of IMPDH stimulates ISG expression through purine nucleotide deprivation. (A) Huh7 cells infected with HEV were treated with MPA or 3 other IMPDH inhibitors (1346, 1347, and 1348). The expression of a panel of ISGs was determined by qRT-PCR after 48 h of treatment. Data were normalized to basal ISG expression without treatment (gray bar; set as 1). (B) Supplementation of guanosine abrogated the induction of ISGs by IMPDH inhibitors. The expression of ISGs was determined by qRT-PCR 48 h after treatment. Data were normalized to basal ISG expression without treatment (purple bar; set as 1). All data were normalized to two housekeeping genes and represent means ± SEM from four experiments.

conclusion regarding the *in vivo* effect of MPA. A recent cohort study reported the anti-HEV activity by ribavirin was not affected by MPA in patients, but they did not analyze the direct effect of MPA on HEV infection (42).

The three inhibitors used in our study that interfere with pyrimidine synthesis have been described in many previous studies (16, 29–31). Adding to the previous knowledge that pyrimidine synthesis inhibitors, such as BQR and LFM, have broad antiviral activity against a spectrum of viruses (16, 23, 43), we now report their potent anti-HEV activity. Both BQR and LFM are immunosuppressive agents, although whether the mechanism of action is solely via pyrimidine inhibition remains controversial and unclear (44–46). The efficacy of BQR against graft rejection has been investigated extensively in preclinical models (47–49), and LFM has been proposed as an off-label immunosuppressive therapy in bone marrow (11) and renal (50) transplantation. In addition, DHODH inhibitors have been explored as treatments of other diseases, including malaria, autoimmune and inflammatory dis-

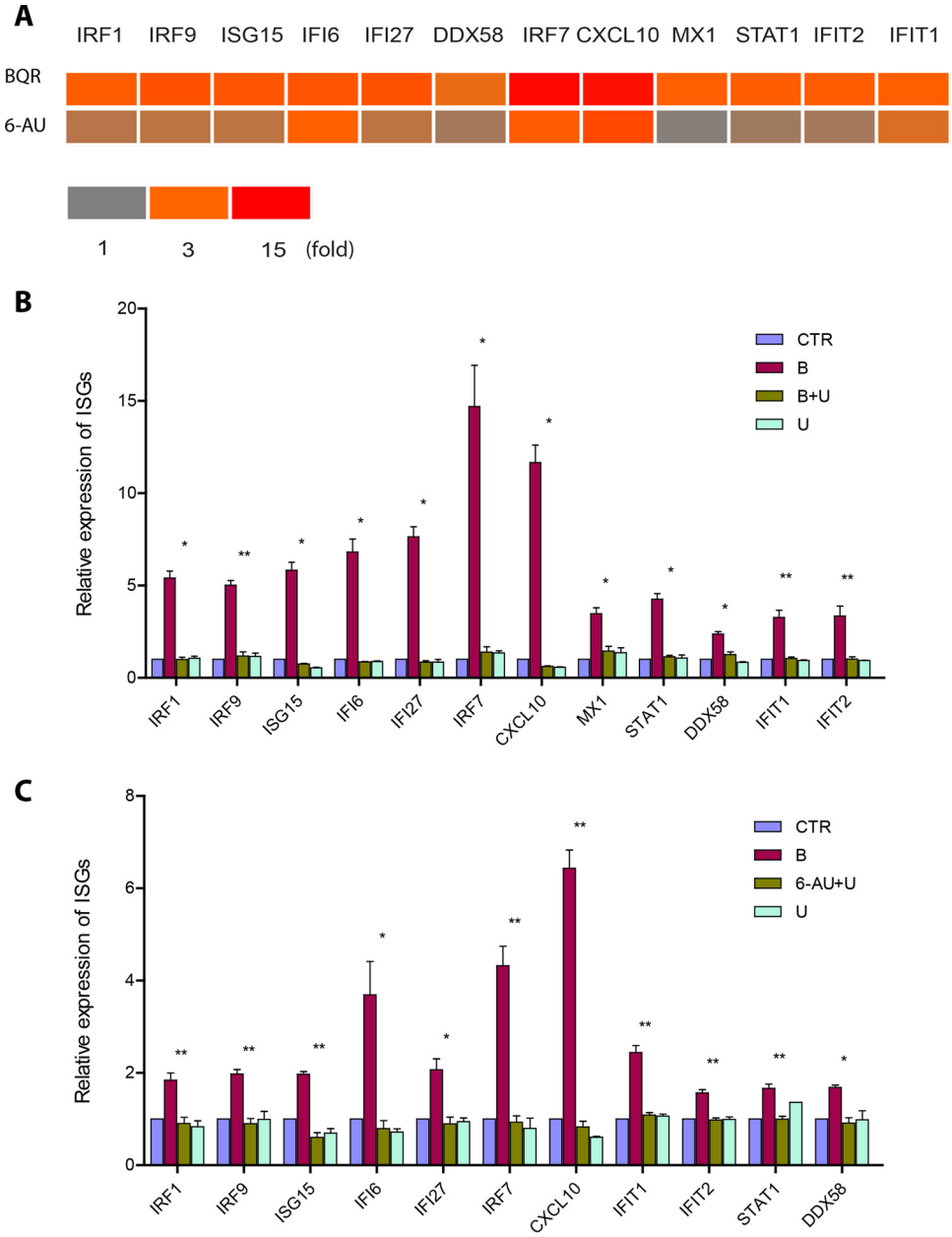

FIG 10 Inhibition of pyrimidine synthesis stimulates ISG expression through pyrimidine nucleotide depletion. (A) Huh7 cells infected with HEV were treated with BQR or 6-AU. After 48 h, the expression of a panel of ISGs was determined by qRT-PCR. Data were normalized to basal ISG expression without treatment (gray bar; set as 1). (B) Supplementation of uridine completely abrogated the induction of ISGs by BQR (B) or 6-AU (C).The expression of ISGs was determined by qRT-PCR at 48 h after treatment. Data were normalized to basal ISG expression without treatment (purple bar; set as 1). All data were normalized to two housekeeping genes and represent means ± SEM from five experiments. *, $P < 0.05$; **, $P < 0.01$.

eases, cancer, rheumatoid arthritis, and psoriasis (51–55). Given the bifunctional antiviral and immunosuppressive effects of BQR and LFM, these regimens may hold the potential to treat HEV-infected organ recipients.

Interestingly, nucleotide synthesis interacts with cellular antiviral immune responses. Here, we demonstrated a direct effect of the depletion of nucleotide pools on the transcription of antiviral ISGs. ISGs are antiviral effectors that are thought to be induced by interferons only. Although hundreds of ISGS have been identified, recent functional studies of individual ISGs surprisingly have found that only a small subset of ISGs actually have potent or broad antiviral activities; these ISGs include IRF1, DDX58, and IRF7 (56, 57). It is these antiviral ISGs that are induced in our HEV models upon treatment with nucleotide synthesis inhibitors. Consistent with this, previous studies in HCV models reported that the induction of IRF1 or IRF7 was associated with the antiviral activity of MPA (19) or ribavirin (58), respectively. Furthermore, the antiviral activity of inhibitors of pyrimidine biosynthesis against measles virus, chikungunya virus, and West Nile virus also was associated with the induction of ISGs (23).

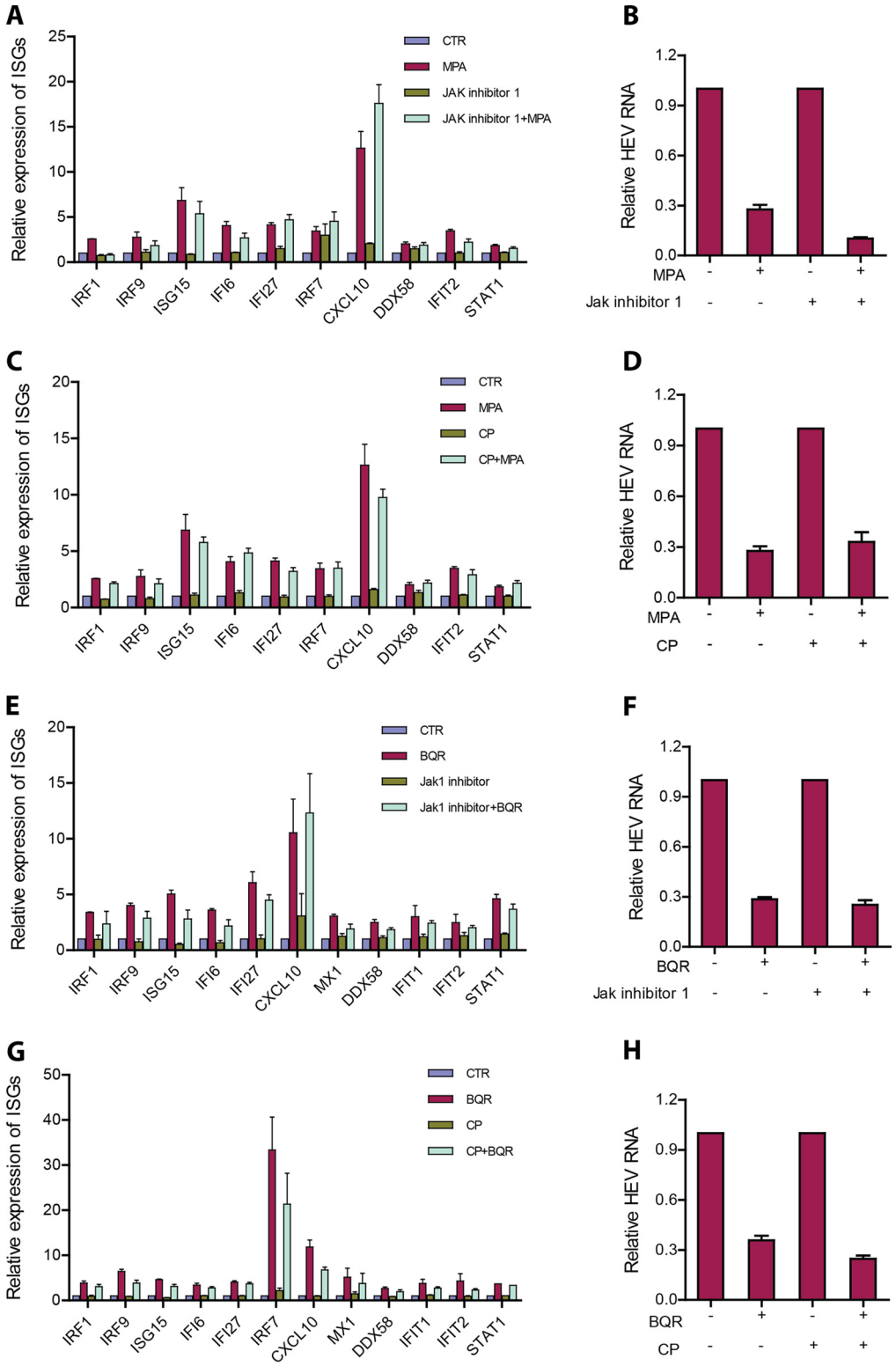

FIG 11 ISG induction and the anti-HEV activity triggered by nucleotide synthesis inhibitors are independent of the JAK-STAT signaling pathway. The induction of ISGs (A and C) and the anti-HEV effects (B and D) by MPA were quantified in the presence or absence of JAK inhibitor 1 (A and B) or CP-690550 (CP) (C and D). The induction of ISGs was normalized to basal ISG expression without MPA treatment (purple bar; set as 1). The relative HEV RNA levels were normalized to cells without treatment of MPA (set as 1). Similarly, the induction of ISGs (E and G) and the anti-HEV effects (F and H) mediated by BQR were quantified in the presence or absence of JAK inhibitor 1 (E and F) or CP (G and H). The induction of ISGs was normalized to basal ISG expression without BQR treatment (purple bar; set as 1). The relative HEV RNA levels were normalized to cells without BQR treatment (set as 1). Data were normalized to two housekeeping genes and represent means ± SEM from three or four experiments.

For now, the mechanistic details of inhibitors of nucleotide biosynthesis that can induce ISGs remain obscure. Classically, the transcription of ISGs is initiated from the binding of interferons to their receptors, which subsequently drives the activation of the JAK-STAT cascade (56). The inhibition of JAK1 in the phosphorylation STAT1, the key event of interferon signaling transduction, often results in the complete blockage of antiviral interferon responses (59). However, exceptions also exist, in that ISGs can be induced in the absence of JAK1 or STAT1 activation (60, 61). Here, we found that the induction of ISGs and the anti-HEV effects by nucleotide synthesis inhibitors are independent of the classical JAK-STAT cascade, suggesting the involvement of a noncanonical mechanism that is independent of interferons, and the identification of these mechanisms should have substantial value for our understanding of antiviral immunity.

In conclusion, selectively targeting host enzymes involved in *de novo* nucleotide biosynthesis potently inhibits HEV replication. Furthermore, nucleotide biosynthesis pathways interact with cellular immune responses, and all of the pharmacological inhibitors exerting anti-HEV activity are capable of triggering antiviral ISG transcription. Thus, targeting nucleotide biosynthesis represents a viable option for antiviral drug development against HEV.

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