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Letter

The guanosine nucleotide analog bemnifosbuvir inhibits hepatitis E virus infection in cell and organoid models

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Dear Editor,

Hepatitis E virus (HEV), a single-stranded positive-sense RNA virus, is a leading cause of acute viral hepatitis worldwide (Li et al., 2020). Generally, HEV infection is asymptomatic or self-limiting in healthy individuals, but can cause severe complications in specific populations. Infection with genotype (GT) 3 HEV in immunocompromised transplant patients is associated with chronic hepatitis (Kamar et al., 2008). Infection with GT1 HEV in pregnant women can cause severe maternal and fetal complications, resulting in high mortality in mother and child (Hakim et al., 2017).

Currently, mono- or combined administration of ribavirin and pegylated interferon-alpha (IFN-α) has been explored as off-label treatment mainly for chronic HEV infection in the clinic (Ma et al., 2022). Generally, ribavirin treatment can achieve sustained clearance of the viral RNA in most patients. However, the emerging clinical mutants appear to have low sensitivity to ribavirin with a possible association with treatment failure (Todt et al., 2016). Moreover, ribavirin is well-known to be contraindicated in pregnant women due to its teratogenicity (Roberts et al., 2010). IFN-α administration in a small cohort of chronic hepatitis E patients has been shown to induce HEV viral clearance, but the considerable adverse side-effects and the risk of triggering acute rejection in organ transplant patients caution against its application (Ma et al., 2022). Therefore, there is an urgent need for further developing new therapeutics against HEV infection.

Bemnifosbuvir (AT-527) is an orally available double prodrug of a guanosine nucleotide analog (Supplementary Fig. S1). It was originally developed for the treatment of hepatitis C virus (HCV) infection by inhibiting the viral RNA-dependent RNA polymerase (RdRp) (Berliba et al., 2019; Good et al., 2020). In addition, bemnifosbuvir has demonstrated inhibitory activity against SARS-CoV-2 and has therefore been investigated as a potential treatment for COVID-19 (Good et al., 2021; Shannon et al., 2022).

In this study, we investigated the effects of bemnifosbuvir on HEV infection (Supplementary Methods). Of note, our initial discovery was included in a patent application filed in July 2024 and made publicly available in January 2025 (Pan et al., 2024), whereas here we present the comprehensive findings. We first examined the effects of AT-527 on viral replication in human hepatoma Huh7 cells, harboring a genotype 3 (GT3) HEV subgenomic replicon (the p6 clone) coupled with a luciferase reporter (Shukla et al., 2012). We found that the compound AT-527 dose-dependently inhibited viral replication-regulated luciferase activity at 24, 48 and 72 hours after treatment (Fig. 1A). Inhibitory potency increased progressively over time, reaching a half maximal inhibitory concentration (IC_{50}) of 6.98 μM after 72 hours of treatment (Fig. 1B). Importantly, minimal cytotoxicity was detected even at the highest concentration tested (100 μM), preventing accurate determination of the half maximum cytotoxic concentration (CC_{50}; >100 μM) and calculation of the selectivity index. Overall, the large separation between the IC_{50} and CC_{50} values highlights the favorable safety of AT-527 and a wide therapeutic window (Fig. 1B). As a benchmark, ribavirin was evaluated under identical experimental conditions and demonstrated significant but substantially weaker inhibition of HEV replication, with an IC_{50} value of 86.15 μM after 72 hours of treatment (Fig. 1C and D). The inhibitory activity of AT-527 on viral replication was also demonstrated in Huh7 cells harboring a GT1 subgenomic replicon (Supplementary Fig. S2).

Next, we tested the effects in Huh7 cells harboring a GT3 infectious (p6) clone (Shukla et al., 2012). Consistently, AT-527 significantly inhibited HEV replication at viral RNA levels after 48 hours of treatment (Fig. 1E), with an IC_{50} value of 0.297 μM (Fig. 1F). Again, under identical experimental conditions, ribavirin exerted significant but weaker inhibitory effects against HEV (Fig. 1E), with an IC_{50} value of 7.206 μM (Fig. 1F). The anti-HEV effects in this model were further validated by Western blotting (Fig. 1G) and immunofluorescence detecting HEV

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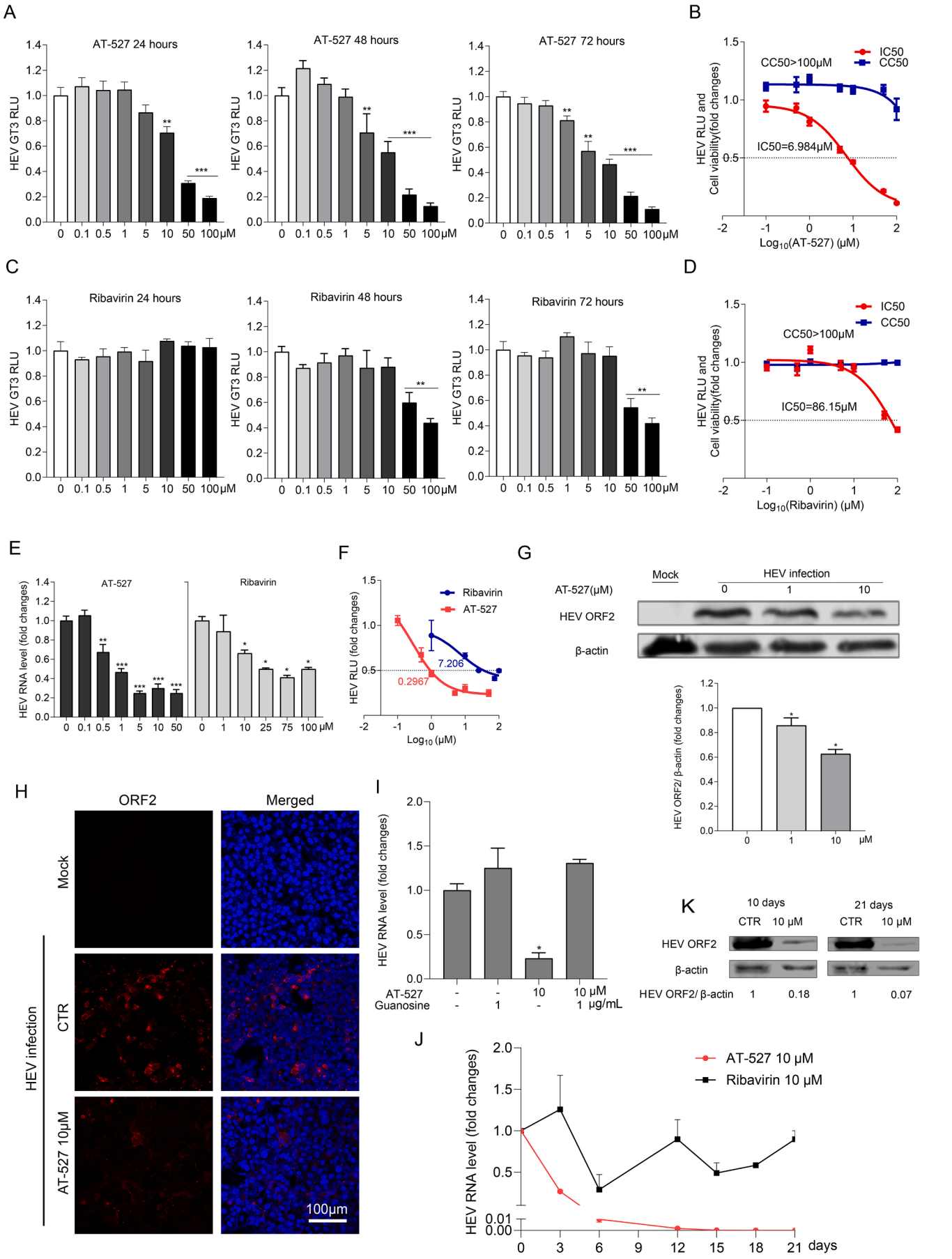

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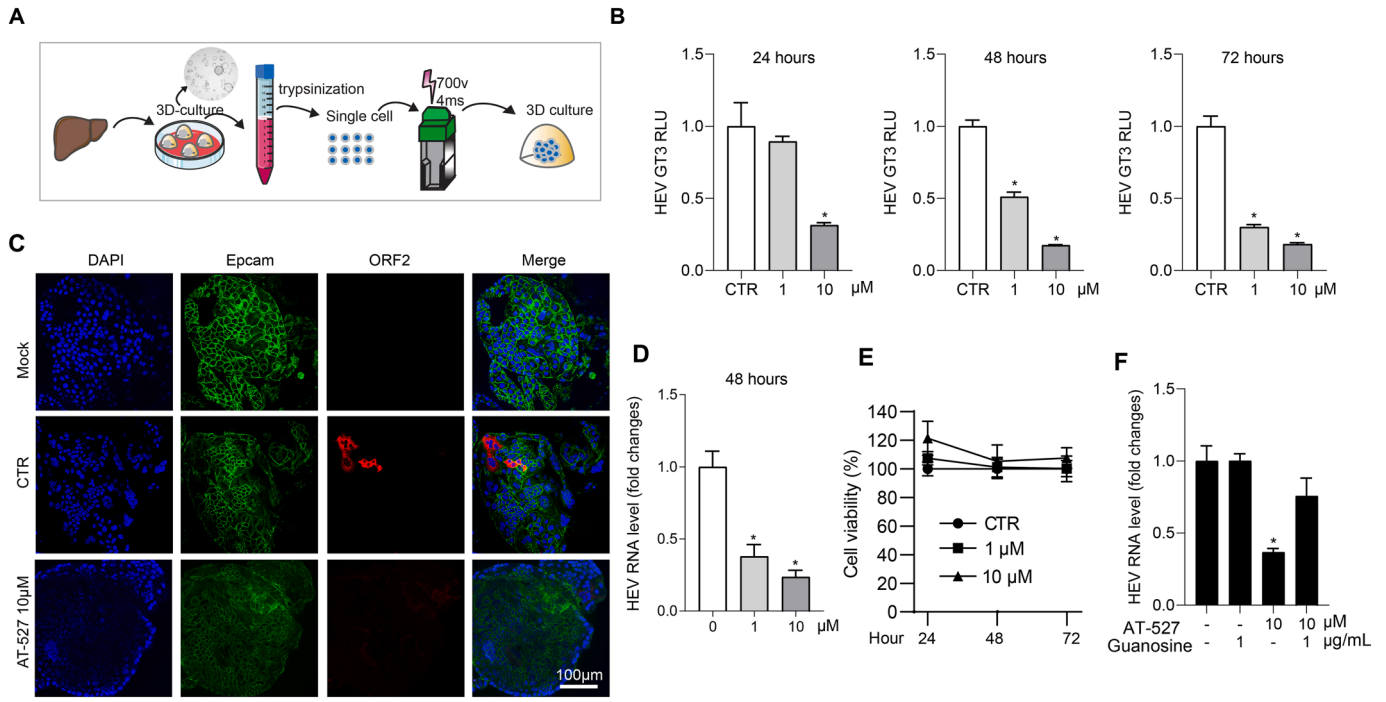

Fig. 2. AT-527 inhibited HEV replication in human liver-derived organoids. **A** Schematic representation of the experimental procedure. **B** The effects of AT-527 treatment for 24, 48 or 72 hours on viral replication-related luciferase units (RLU) in organoids harboring GT3 subgenomic replicon. The untreated group serves as control (CTR) (set as 1) (n = 4). **C** Immunofluorescence analysis of viral ORF2-encoded capsid protein after treatment with 10 μM AT-527 for 48 hours in organoids. Uninfected organoids incubated with the anti-HEV capsid protein antibody serve as mock. HEV infected but untreated organoids serve as the positive control. DAPI (blue) and Epcam (green) were applied to visualize nuclei and cytomembrane. **D** Organoids harboring the infectious HEV clone were treated with indicated concentrations of AT-527 for 48 hours (1 and 10 μM; n = 4). The effects on viral RNA were quantified by qRT-PCR. **E** The cytotoxicity was assessed by AlamarBlue assay in organoids treated with AT-527 for 24, 48 and 72 hours (1 and 10 μM; n = 6). **F** HEV RNA in organoids harboring infectious HEV was quantified by qRT-PCR after 48 hours of treatment with 10 μM AT-527 by adding 1 μg/mL exogenous guanosine (n = 4). Scale bar, 100 μm. 40 × oil immersion objective. Data are presented as means ± SEM. *P < 0.05. Mann-Whitney test.

ORF2 protein (Fig. 1H). Next, we performed a re-infection assay, by inoculating naïve Huh7 cells with HEV particles harvested from infected cells treated with AT-527 for 48 hours. We observed substantial attenuation of secondary infection, demonstrated by immunostaining of ORF2 protein (Supplementary Fig. S3A) and qRT-PCR quantification of viral RNA (Supplementary Fig. S3B). Notably, treatment with AT-511, the free base form of AT-527 (Supplementary Fig. S1), also resulted in a pronounced reduction of HEV RNA levels (Supplementary Fig. S4). Supporting the mechanism-of-action, exogenous supplementation of guanosine largely reversed the anti-HEV activity of AT-527 (Fig. 1I).

Maintaining antiviral potency during long-term treatment is particularly important for managing chronic HEV infection in immunocompromised populations. To address this, we first evaluated the effects of AT-527 in Huh7 cells harboring a GT3 replicon over a 7-day period. Treatment with 1 μM and 10 μM AT-527 progressively reduced viral replication, as indicated by decreased luciferase activity (Supplementary Fig. S5A). This sustained inhibitory effect was even more pronounced against GT3 infectious virus, resulting in over 99% reduction of viral RNA between days 6 and 21 of treatment, whereas ribavirin showed fluctuating efficacy (Fig. 1J). At the protein level, AT-527 markedly suppressed HEV ORF2 expression at days 10 and 21, as determined by Western blotting (Fig. 1K). A similar long-term inhibitory effect was observed in the GT1 replicon, although to a lesser extent (Supplementary Fig. S5B).

We have previously established human liver organoid-based HEV models as illustrated (Fig. 2A), which are suitable for antiviral drug assessment (Li et al., 2022; Liu et al., 2025). Treatment with 1 μM and 10 μM of AT-527 significantly inhibited HEV replication in organoids harboring the GT1 replicon (Supplementary Fig. S6) and GT3 replicon (Fig. 2B). The inhibitory effects were also confirmed in organoids harboring the GT3 infectious clone, shown by immunostaining of ORF2 protein (Fig. 2C) and qRT-PCR quantification of viral RNA (Fig. 2D). No

Fig. 1. AT-527 inhibited HEV replication in Huh7-based cell models. **A** The effects of AT-527 treatment for 24, 48 or 72 hours on HEV replication-related luciferase units (RLU) in Huh7 cells harboring the GT3 replicon of the p6 clone. The untreated group serves as control (CTR) (set as 1) (n = 9). **B** The 50% inhibitory concentration (IC_{50}; n = 9) and 50% cytotoxic concentration (CC_{50}; n = 5–6) were calculated using GraphPad Prism software. **C**, **D** The effects of ribavirin were tested under the identical experimental conditions (n = 5), and IC_{50} and CC_{50} values were calculated accordingly. **E** Huh7 cells harboring the infectious HEV (p6) clone were treated with indicated concentrations of AT-527 (n = 6–8) or ribavirin (n = 4) for 48 hours. The effects on viral RNA were quantified by qRT-PCR, and **F** the IC_{50} values were calculated accordingly. **G** Western blot analysis and quantification of HEV capsid protein level in infected Huh7 cells treated with AT-527 for 48 hours. The uninfected group (mock) serves as the negative control, whereas the infected but untreated group was set as 1 (n = 4). **H** Immunofluorescence analysis of viral ORF2-encoded capsid protein (red) in Huh7 cells treated with 10 μM AT-527 for 48 hours. Huh7 cells incubated with the anti-HEV capsid protein antibody serve as mock. HEV infected Huh7 cells untreated and incubated with the anti-HEV capsid protein antibody serve as the positive control. DAPI (blue) was applied to visualize nuclei. **I** HEV RNA in Huh7 cells harboring infectious HEV was quantified by qRT-PCR after 48 hours of treatment with 10 μM AT-527 by adding 1 μg/mL exogenous guanosine (n = 4). **J** The effects of 21 days of treatment with AT-527 or ribavirin in Huh7 cells harboring infectious GT3 HEV were measured by qRT-PCR quantification of viral RNA. The untreated (CTR) group serves as control (set as 1) (n = 4). **K** Western blot analysis of HEV ORF2-encoded capsid protein was performed on 10 days and 21 days of treatment, the infected but untreated group was set as 1. Scale bar, 100 μm; images were acquired using a 40 × oil-immersion objective. The results are shown as means ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Mann-Whitney test.

cytotoxicity was observed in treated organoids (Fig. 2E). Moreover, exogenous supplementation of guanosine largely reversed the anti-HEV activity of AT-527 (Fig. 2F). It has been demonstrated that AT-527 is a specific inhibitor of the HCV RdRp (Good et al., 2020), and cryo-EM structures have confirmed binding of the active metabolite of AT-527 at the RdRp active site of SARS-CoV-2 (Shannon et al., 2022). Based on these findings, we speculate that the antiviral activity of AT-527 against HEV is likewise mediated through inhibition of the viral RdRp. This hypothesis is partly supported by our observation that supplementation with guanosine reverses the antiviral effects of AT-527, suggesting that AT-527 may function as a guanosine analog that competitively incorporates into the HEV RdRp and acts as a chain terminator during viral RNA synthesis.

The G1634R mutation in the GT3 HEV RdRp is potentially associated with ribavirin resistance in treated chronic hepatitis E patients (Todt et al., 2016). We thus introduced this variant into both Huh7 cells and liver organoids, and we found that AT-527 is capable of effectively inhibiting this variant in both models (Supplementary Fig. S7). In clinical practice, combining antiviral therapies is a common strategy to enhance efficacy and reduce the risk of drug resistance. Therefore, we assessed the effects of AT-527 in combination with ribavirin or IFN-α in Huh7 cells harboring the GT3 replicon. Both combinations exhibited synergistic anti-HEV activity, with the AT-527 and IFN-α combination achieving a higher synergy score compared to AT-527 with ribavirin (Supplementary Fig. S8). This difference may reflect the partial overlap in antiviral mechanisms between AT-527 and ribavirin as nucleotide analogs, whereas IFN-α provides a more complementary mode of action by activating host antiviral defense mechanisms.

Of note, this study has several limitations. First, our results are based on *in vitro* models, and *in vivo* validation will be essential. Second, nucleotide analogs have long been used to treat viral infections, but the potential emergence of resistance mutations is a well-recognized concern (Shannon and Canard, 2023). Therefore, the risk of drug-resistance development, particularly during long-term treatment, should be carefully evaluated for AT-527. Finally, although guanosine supplementation largely reversed the anti-HEV activity of bemnifosbuvir, its precise antiviral mechanism remains to be further elucidated.

In summary, we demonstrate that bemnifosbuvir potently inhibits HEV infection in human cell and organoid models. These findings support further investigation of repurposing bemnifosbuvir as a potential treatment for HEV infection. Importantly, AT-527 has already undergone extensive clinical testing in healthy volunteers and in patients with other viral infections (Berliba et al., 2019), demonstrating an excellent safety profile. This highlights the feasibility of advancing AT-527 toward clinical evaluation for treating HEV infection. Nevertheless, the relatively small number of HEV patients requiring treatment poses a challenge for further clinical development of AT-527, and collaborative efforts from multiple stakeholders may be needed to support this effort (Ma et al., 2022).

FOOTNOTES

Kuan Liu, Pengfei Li, and Qiuwei Pan are inventors of a patent application on this discovery filed on 5th, July, 2024. Part of this work is supported by a VIDI grant (number 91719300 to Q.P.) from the Netherlands Organisation for Health Research and Development (ZonMw), and a Young Investigator Grant from the Erasmus MC-University Medical Center (to P.L.). Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2026.05.010.

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