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# FDA-drug screening identifies deptropine inhibiting hepatitis E virus involving the NF-κB-RIPK1-caspase axis

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ABSTRACT

Hepatitis E virus (HEV) infection is the leading cause of acute hepatitis worldwide and can develop into chronic infection in immunocompromised patients, promoting the development of effective antiviral therapies. In this study, we performed a screening of a library containing over 1000 FDA-approved drugs. We have identified deptropine, a classical histamine H1 receptor antagonist used to treat asthmatic symptoms, as a potent inhibitor of HEV replication. The anti-HEV activity of deptropine appears dispensable of the histamine pathway, but requires the inhibition on nuclear factor-κB (NF-κB) activity. This further activates caspase mediated by receptor-interacting protein kinase 1 (RIPK1) to restrict HEV replication. Given deptropine being widely used in the clinic, our results warrant further evaluation of its anti-HEV efficacy in future clinical studies. Importantly, the discovery that NF-κB-RIPK1-caspase pathway interferes with HEV infection reveals new insight of HEV-host interactions.

## 1. Introduction

Acute liver inflammation is usually self-limiting upon elimination of the etiological agents. However, unresolved inflammation may lead to the development of chronic liver diseases and cancer. Hepatitis E virus (HEV) infection represents the most common cause for acute viral hepatitis (Crossan et al., 2014). It has caused many large hepatitis outbreaks, especially in resource-limited regions (Hakim et al., 2018). In the developing countries, the major clinical burden lies with the pregnant women population, which is usually caused by genotype 1 HEV. If no recovery from spontaneous clearance of the virus, these acutely infected patients will have high risk to develop liver failure and death. In western counties, chronic hepatitis E cases have been frequently reported in immunocompromised patients, in particular organ transplant recipients (Kamar et al., 2008). Genotype 3 and occasionally genotype 4 HEV are the main causes of chronic infection. For the infection in these specific populations including pregnant women and organ transplantation patients, safe and effective antiviral treatment is evidently required.

Besides supportive care, pegylated interferon alpha (PEG-IFNα) and ribavirin (Kamar et al., 2014) in particular have been explored in the clinic for treating some chronic HEV cases. Ribavirin is effective in general, but the failure of treatment has been frequently reported (Debing et al., 2016). However, the remaining challenges include that not all chronic HEV patients respond to ribavirin, the emerging of potential resistance strains and the substantial side effects that limit the applications in pregnant women, young children and elderly patients (Todt et al., 2016). Sofosbuvir, a clinically used direct-acting antiviral for treating hepatitis C virus (HCV) infection, has recently been proposed for treating HEV. However, mixed results were obtained from both experimental models and patients (Dao Thi et al., 2016; Kamar et al., 2017), and likely it is not very effective against HEV (Kamar et al., 2017). In parallel, various preclinical compounds, including nucleoside and non-nucleoside antiviral agents (Netzler et al., 2019), protein kinase-targeted compounds (Wang et al., 2017a), inhibitors targeting mitochondrial metabolism (Qu et al., 2019), inhibitors of nucleotide synthesis (Wang et al., 2016) and natural compounds (Todt et al., 2018), are being investigated in experimental models. Given the pathogenic nature and the specific populations affected by HEV, we hypothesize that repurposing currently used drugs represents an effective approach to develop antiviral treatment that can readily benefit the patients.

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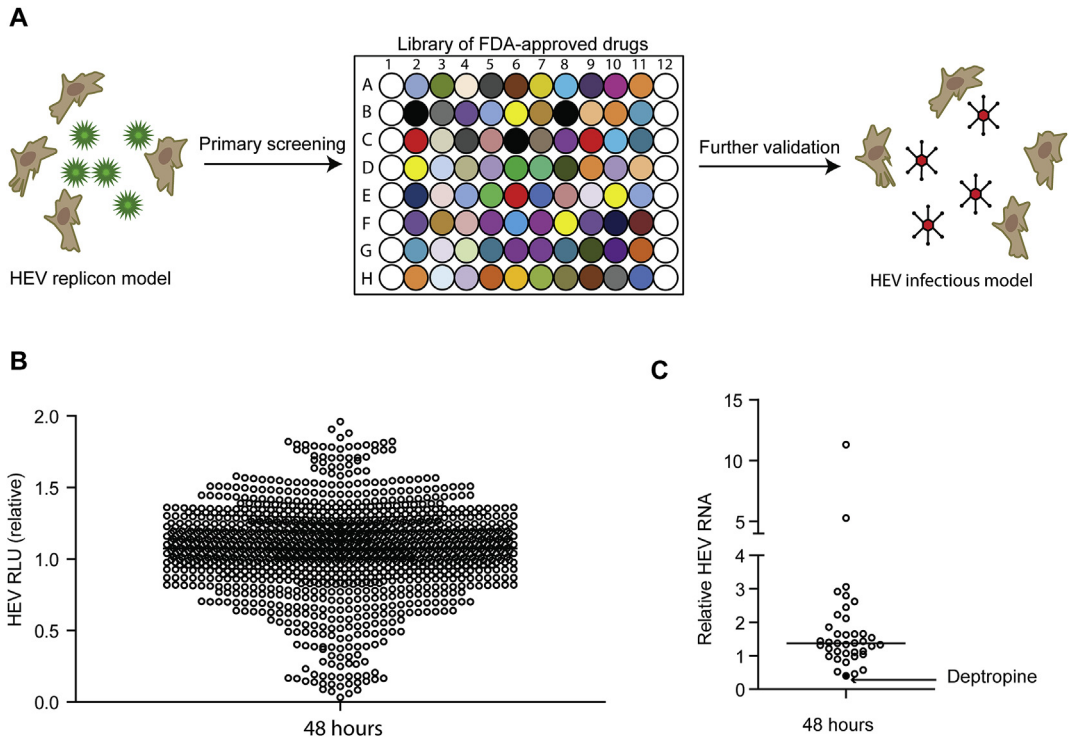


**Fig. 1.** Screening of a drug library identified deptropine as a potent inhibitor of HEV replication. (A) Schematic illustration of the screening strategy. The primary screening was conducted in the HEV replicon model, and the effects of selected primary hits was further evaluated in the HEV infectious model. (B) Huh7.5 cell based HEV replicon model was treated with 1280 compounds with 95% of FDA-approved drugs at 10 μM. The HEV-related Gaussia luciferase value (HEV RLU) was measured 48 h post-treatment. Points represent the relative HEV replication for each drug. (C) qRT-PCR analysis of HEV viral RNA level in Huh7.5 cell based HEV infectious model treated with the 38 selected drugs at 5 μM from the primary screening for 48 h. Data were normalized to the DMSO vehicle control (set as 1) and presented in dot plots. The black dot pointed by an arrow represents deptropine.

In this study, we screened a library of FDA-approved drugs. We identified deptropine, a traditional histamine receptor H1 antagonist used to treat asthmatic symptoms (de Vries et al., 2006; Schirm et al., 2002), as a potent inhibitor against HEV. Mechanistically, the anti-HEV activity of deptropine is dispensable of the histamine pathway, but requires the crosstalk between nuclear factor-κB (NF-κB) and caspase activity regulated by receptor-interacting protein kinase 1 (RIPK1). These results bears potential implications for the real-world treatment of HEV patients from developing to developed countries with acute or chronic infection.

## 2. Materials and methods

### 2.1. Drug screening

A commercially available library of FDA-approved drugs, the Prestwick chemical library (http://www.prestwickchemical.com/), was used. The screening was performed in a 96-well plate format with 10,000 cells in each well. Compounds were solubilized at 1 mM in dimethyl sulfoxide (DMSO) and all compounds were diluted in culture media for a final concentration of 10 μM during screening.

### 2.2. Cell culture models

Multiple cell lines including human hepatoma Huh7.5 cell line, Hep3B cell line and human embryonic kidney epithelial HEK 293T cell line were kindly provided from Department of Viroscience, Erasmus Medical Center. These cell lines were cultured with Dulbecco's modified Eagle medium (DMEM) (Lonza Biowhittaker, Verviers, Belgium) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS) (Hyclone, Lonan, Utah), 100 IU/mL penicillin, 100 μg/mL streptomycin. Mouse embryonic fibroblast (MEF) WT and NF-κB −/− cells were generated by Dr. A. Hoffmann (Signaling Systems Lab, Los Angeles, CA), and were grown in DMEM with 10% FCS, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 20 mM HEPES as described previously (Xu et al., 2017).

For genotype 3 HEV models, Huh7.5, Hep3B, and 293T cell lines were electroporated with the subgenomic HEV RNA (p6 clone) coupled with a Gaussia luciferase reporter gene to establish the replication model (subgenomic replicon). Electroporation of the full-length p6 HEV genome was used to establish the infectious model, and more details have been described previously (Wang et al., 2017b). MEF cells were seeded in 96 well plate ($1 \times 10^4$ per well) and were inoculated with cell culture-produced HEV particles ($1.5 \times 10^6$ HEV RNA copies/ml) for 48 h before being subjected to qRT-PCR analysis.

For HEV genotype 1 replicon model, Huh7.5 cells were electroporated with Sar 55/S17/luc HEV RNA, and viral replication was detected by Gaussia luciferase activity (Xu et al., 2017). For HCV replicon model, Huh7.5 cells were electroporated with the subgenomic HCV bicistronic replicon (I389/NS3–3V/LucUbiNeo-ET), and viral replication was monitored by measuring firefly luciferase activity (Pan et al., 2009). For rotavirus model, Caco 2 cells were inoculated with simian rotavirus SA11. NF-κB, AP-1 luciferase reporter cells were generated by transducing Huh7.5 cells with lentiviral vectors expressing the firefly luciferase gene under the control of the promoters containing the NF-κB, AP-1 motifs (Wang et al., 2017a), respectively (System Biosciences).

### 2.3. Statistical analysis

Statistical analysis was performed using the nonpaired, nonparametric test with Mann-Whitney test (GraphPad Prism version 5.01; GraphPad Software). P values $< 0.05$ were considered statistically significant. (See Supplemental Information for more detailed methods).

## 3. Results

### 3.1. Screening of a library of FDA-approved drugs identifies deptropine as a potent Anti-HEV agent

To identify potential anti-HEV drug candidates, we employed a library of 1280 compounds (95% are FDA-approved drugs). The initial screening was performed in a Huh7.5 cell-based genotype 3 HEV replicon model in which the 5' portion of HEV ORF2 was replaced with the in-frame secreted form of Gaussia luciferase. Thus, accumulation of luciferase indicates viral replication, whereas the absence of the ORF2, which encodes the capsid protein precludes the propagation of novel viral particles. This model was treated with each compound at a concentration of 10 μM and the DMSO vehicle control for 48 h (Fig. 1A). We found that some drugs inhibit but some promote, whereas the majority have no major effect on HEV replication-related luciferase activity (Fig. 1B). To exclude the possibility that the antiviral ability may be attributed to its cytotoxicity (Fig. S1), 38 drugs identified with more than 50% inhibition on HEV replication-related luciferase activity and less than 50% cytotoxicity were selected (Supplementary Table 1). Their antiviral effects were further investigated at lower concentration (5 μM) in the full-length HEV infectious model by quantifying the HEV RNA using qRT-PCR assay (Fig. 1C). In both models, deptropine showed the most potent anti-HEV effects and thus was subjected to further detailed study.

### 3.2. Deptropine inhibits HEV in multiple cell models

Since HEV can cause a wide range of extrahepatic manifestations, we further validated the antiviral effect in several other cell models, including hepatic and nonhepatic cell lines. In line with the results observed in Huh7.5 cell model (Fig. 2A), deptropine dose-dependently inhibited HEV replication in HEK293T (Fig. 2B) and Hep3B cells (Fig. S2A). Of note, in the Hep3B cell model, treatment with 5 μM deptropine potently inhibits HEV without affecting cell viability, excluding the antiviral effect of deptropine is through nonspecific cytotoxicity. The 50% inhibition and cytotoxicity (IC50 and CC50) concentrations of deptropine were 2.89 μM and 12.20 μM in Huh7.5 cells, and 0.84 μM and 18.64 μM in HEK293T cells. Interestingly, genotype 1 HEV replicon is more sensitive to deptropine with an IC50 of 0.49 μM in Huh7.5 cells (Fig. 2C). The anti-HEV effect of deptropine was further verified by the re-infection assay (Fig. S2E). In addition, we found that deptropine significantly reduced the secretion of HEV into the supernatant (Fig. S2F).

We next examined the effect of long-term treatment as described previously (Qu et al., 2017). Treatment with 5 μM deptropine dramatically reduced HEV viral RNA by 80% after 39 days, and this effect was further confirmed at ORF2 protein level (Fig. 2D). We further evaluated whether deptropine has a broad antiviral activity and found that deptropine potently inhibits HCV (Fig. S2C) but not rotavirus (Fig. S2D) replication.

### 3.3. Histamine H1 receptor is dispensable for the Anti-HEV activity of deptropine

Because deptropine is a well-characterized histamine H1 receptor antagonist, we investigated whether this receptor is involved in its anti-HEV effect. We found that the expression of the H1 receptor was hardly detectable in both Huh7.5 and 293T cells (Fig. S3A), which is consistent with a previous study showing that Huh7.5 cells are deficient of this receptor (Pietschmann, 2017). Diphenhydramine, another H1 antagonist (Fig. 3A), exerted no inhibitory effect on HEV in both Huh7.5 and 293T cells (Fig. 3B). Chlorcyclizine HCl (CCZ), an H1 antagonist that has been shown to block HCV entry (He et al., 2015), also could not inhibit HEV (Fig. 3C and Fig. S3B), or HCV replication in a subgenomic replicon model (Fig. S3B). These results collectively suggest that the inhibitory effect of deptropine on HEV replication is likely dispensable of H1 receptor-mediated pathway.

Since we have detected the expression of other histamine receptors to some extent, we performed a competition assay by co-incubating deptropine with histamine in the replicon model to assess the possible involvement of these receptors. We found that histamine has no effect on either HEV replication or deptropine-induced inhibition of HEV replication. Surprisingly, in the infectious model, histamine was able to inhibit HEV and slightly attenuated the anti-HEV effect of deptropine (Fig. 3D). Taken together, these results indicate that deptropine potently inhibits HEV replication independent of the histamine signaling, whereas we do not fully exclude that the histamine signaling may interfere with the other steps of HEV life cycle, such as viral entry. Because benztropine, a chemically similar drug with deptropine (Cui et al., 2017), has been shown to exert its effect through alpha-7 nicotinic receptor (α7 receptor) and expression of this receptor has been reported in hepatocytes (Wu et al., 2015). This provoked us to investigate the possible role of α7 receptor in the anti-HEV effect of deptropine. However, we found that the expression of this receptor is very low in Huh7.5 cells (Fig. S3C). In addition, α-bungarotoxin, the alpha-7 nicotinic receptor antagonist, has no effect on HEV RNA level and the anti-HEV effect of deptropine is not affected by the alpha-7 nicotinic receptor agonist nicotine (Fig. S3C), suggesting a α7 receptor-independent antiviral mechanism of deptropine.

### 3.4. Deptropine inhibits HEV in an NF-κB-dependent manner

NF-κB plays a central role in promoting the expression of genes involved in inflammation and is activated in various viral infections (Tai et al., 2000). Moreover, the H1 antagonists are shown to inhibit NF-κB activity (Roumestan et al., 2008). We thus sought to investigate the potential role of NF-κB signaling in the deptropine-mediated anti-HEV effect. A lentiviral vector expressing the firefly luciferase gene under control of the NF-κB promoter was transduced into Huh7.5 cells. We found that inoculation of HEV particles significantly activates the NF-κB transcription activity, which correlates with enhanced viral replication (Fig. 4A). In contrast to the potent NF-κB activator TNFα, treatment with deptropine significantly inhibited the NF-κB transcription activity (Fig. 4B). Supplementation with TNFα significantly attenuated the inhibitory effects of deptropine on NF-κB and anti-HEV activity (Fig. 4C), suggesting the possible involvement of NF-κB in the anti-HEV action of deptropine. To further evaluate the role of NF-κB on HEV replication, we tested additional compounds targeting NF-κB activity. The NF-κB activators, including betulinic acid and calcimycin, significantly inhibit HEV replication, whereas BAY11-7085 has no effect on HEV replication (Fig. S5).

To further confirm the involvement of NF-κB in the antiviral effect of deptropine, wild type (WT), NF-κB knockout (NF-κB−/−) mouse embryonic fibroblasts (MEF) cells were used (Zhou et al., 2015). An decreased HEV RNA was observed in the NF-κB −/− MEF cells incubated with culture medium containing HEV particles compared with its wild type, suggesting a supportive role of NF-κB for HEV replication. Importantly, the anti-HEV effect of deptropine was completely abolished in the NF-κB−/− MEF cells (Fig. 4D), demonstrating the requirement of NF-κB for the anti-HEV action of deptropine.

Activator protein 1 (AP-1) transcription activity is closely related to NF-κB activity and has also been reported to be upregulated upon viral infection. We found that HEV slightly increased AP-1 activity (Fig. S4A) and deptropine could inhibit AP-1 activity (Fig. S4B). Moreover, associated with the obtained results that histamine could partially reverse the antiviral effect of deptropine in the HEV infectious model (Fig. 3D), the inhibitory effects of either AP-1 or NF-κB transcriptional activity by deptropine was also partially attenuated after adding histamine (Fig. S4C).

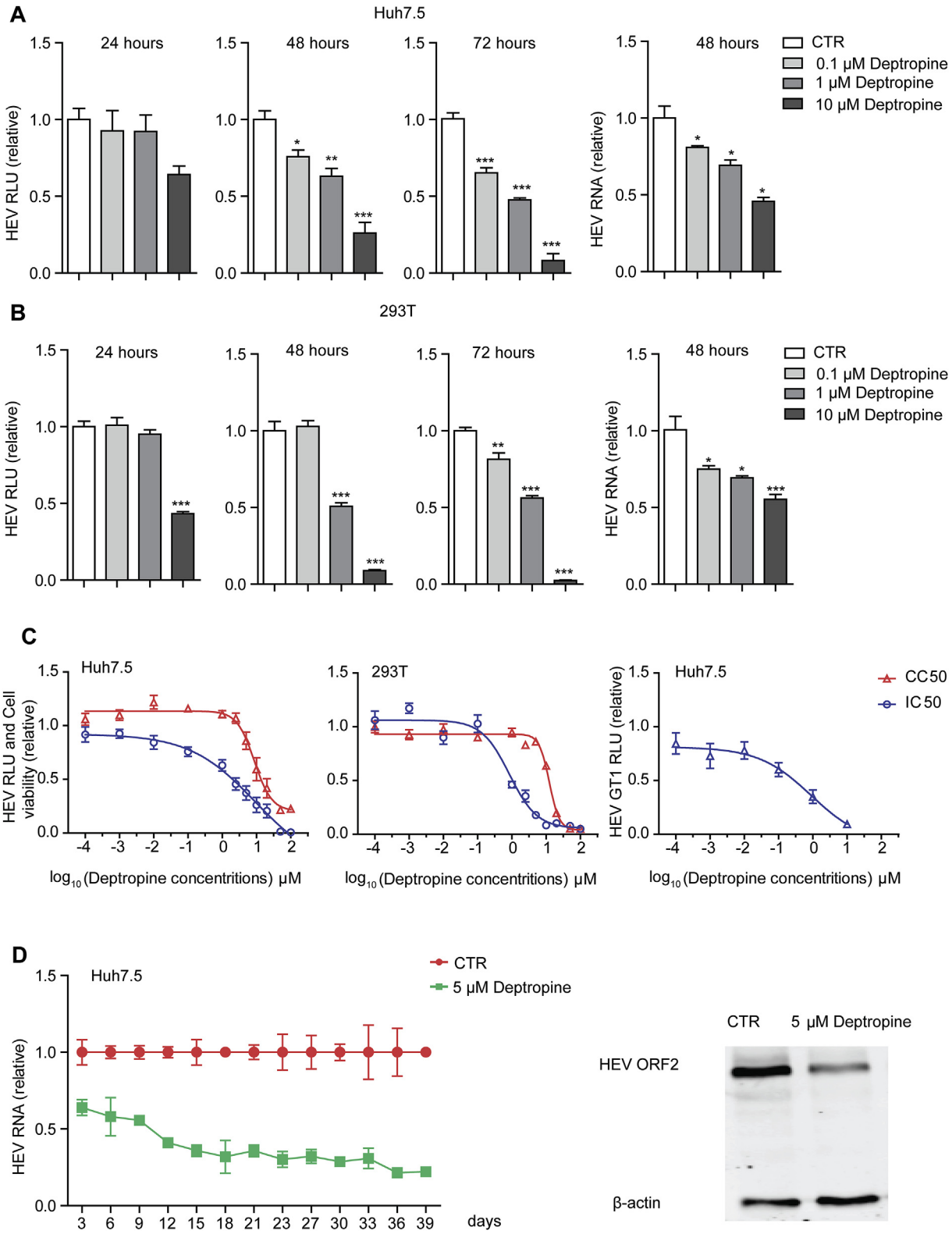


**Fig. 2.** Deptropine inhibits HEV replication in multiple cell models. (A) Huh7.5 cell based HEV replicon and infectious models were treated with indicated concentrations of deptropine for indicated time period before the measurement of HEV, and the untreated (CTR) group serve as control (set as 1) (n = 8). (B) 293T cell based HEV replicon and infectious models were treated with indicated concentrations of deptropine for indicated time period before the measurement of HEV, and the untreated (CTR) group serve as control (set as 1) (n = 8). (C) The 50% inhibitory concentration (IC50) and 50% cytotoxic concentration (CC50) of deptropine against HEV replication in indicated cell lines were calculated using GraphPad Prism 5 software (n = 4–8). The IC50 of deptropine against genotype 1 (GT1) HEV replication were calculated using GraphPad Prism 5 software (n = 5). (D) Treatment with 5 μM deptropine for 39 days retains anti-HEV effect in Huh7.5 cell based HEV infectious model. The untreated (CTR) group serve as control (set as 1) (n = 2). Western blot analysis of HEV capsid ORF2 protein levels in the Huh7.5 cell based HEV infectious model treated with deptropine for 39 days. Data are presented as means ± SD. (*P < 0.05; **P < 0.01; ***P < 0.001).

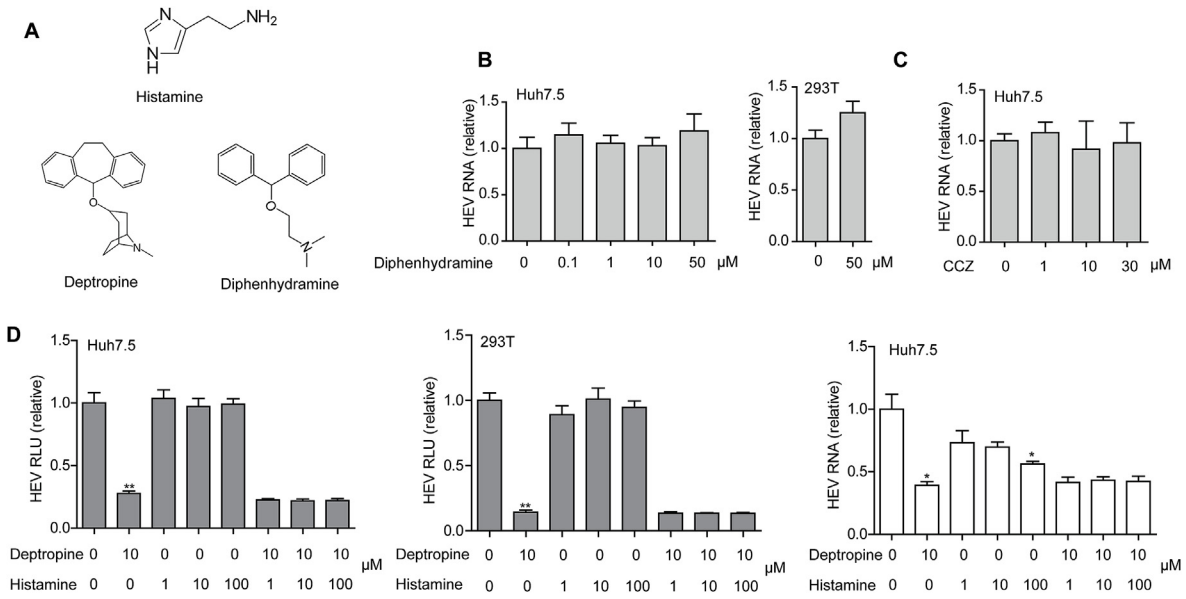


**Fig. 3.** The anti-HEV effect of deptropine is dispensable of the histamine pathway. (A) Chemical structures of the indicated compounds. (B) qRT-PCR analysis of HEV RNA level in the Huh7.5 and 293T cell based HEV infectious models treated with indicated concentrations of diphenhydramine for 48 h. The untreated group serve as control (set as 1) (n = 4). (C) qRT-PCR analysis of HEV RNA level in the Huh7.5 cell based HEV infectious model treated with indicated concentrations of chlorcyclizine HCl (CCZ) for 48 h. The untreated group serve as control (set as 1) (n = 6). (D) Co-incubation of indicated concentrations of deptropine and histamine in Huh7.5 and 293T for 48 h before testing the HEV-related luciferase. The untreated group serve as control (set as 1) (n = 5–6). Co-incubation of indicated concentration of deptropine and histamine in Huh7.5 cell based HEV infectious model for 48 h before measurement of HEV RNA by qRT-PCR assay. The untreated group serve as control (set as 1) (n = 4). The above data are means ± SD. (*P < 0.05; **P < 0.01).

### 3.5. Caspase activity is required for the Anti-HEV effect of deptropine

It has been reported that activation of NF-κB signaling negatively regulate apoptosis by increasing the expression of anti-apoptotic genes, such as survivin, which function mainly by binding to active caspase 3/7 (Moghoofei et al., 2019). We found that the loss of NF-κB signaling dramatically abolished the increase of caspase 3/7 activity induced by deptropine (Fig. 4D). Concurrently, the expression of survivin was significantly reduced after treatment with deptropine (Fig. 4D).

Caspase activity is well-recognized for regulating viral infection. We found that deptropine significantly elevated cellular caspase 3 and 7 activity (Fig. 5A), accompanied with a low level of apoptosis induction (Fig. S6A). Interestingly, HEV infection further augments the induction of caspase activity by deptropine (Fig. 5A). Importantly, co-incubation with Z-VAD-FMK, a general caspase inhibitor, attenuated the caspase induction and anti-HEV activity of deptropine. Similar results were observed in the combination of TNFα with Z-VAD-FMK (Fig. 5B). Moreover, in line with the results of histamine on HEV (Fig. 3D), treatment with histamine for 48 h significantly increased the caspase activity and decreased the activation of caspase induced by deptropine (Fig. 5C). These data suggest the requirement of caspase activity for the anti-HEV effect of deptropine.

Interestingly, we found that in the presence of caspase 3 inhibitor, treatment with deptropine even decreased the cellular caspase 3 and 7 activity (Fig. 5C), and this is accompanied by the increase of HEV RNA when deptropine was added in combination with caspase 3 inhibitor (Fig. 5D). Furthermore, treatment with other caspase inhibitors also robustly abolished the anti-HEV effect of deptropine, suggesting a general role of caspase-mediated anti-HEV effect of deptropine. Unexpectedly, we found that all the caspase inhibitors significantly reduced HEV RNA without affecting cell viability (Figs. S6B and S6D), indicating the basal caspase activity may support HEV replication. Lastly, unlike the effects on HEV, caspase inhibition barely reverse the effect of deptropine on HCV, suggesting a specific role of caspase mediated anti-HEV effect of deptropine (Fig. S6C). Taken together, these data suggest that the basal caspase activity may support HEV replication; however the deptropine-induced caspase activation may contribute to its anti-HEV effect.

### 3.6. Genetic silencing of RIPK1 restricts HEV replication and abolishes the Anti-HEV effect of deptropine

RIPK1 is positioned at the center of cell-fate 'decisions' by modulating the crosstalk between NF-κB and caspase activity (Oeckinghaus et al., 2011). We examined the potential involvement of RIPK1. We found that gene silencing of RIPK1 significantly inhibited HEV replication (Fig. 6A and B). Importantly, loss of RIPK1 completely blocked the anti-HEV effect of deptropine (Fig. 6C). The kinase activity of RIPK1 is essential in promoting apoptosis (Newton, 2015). Consistently, we found that deptropine robustly induced phosphorylation of RIPK1 and the phospho-PIPK1 is remarkably decreased in the presence of caspase 3 inhibitor (Fig. 6D). These results indicate that the kinase activity of RIPK1 is essential in caspase-mediated anti-HEV effect of deptropine (Fig. 5D).

### 3.7. Deptropine antagonizes the Anti-HEV effects of IFNα, but synergizes ribavirin

Since IFNα and ribavirin are clinically used for treating chronic HEV patients, we evaluated their combinatory effects with deptropine. We found that deptropine exhibited antagonistic effect with IFNα (Fig. 7A), but synergistic effect with ribavirin (Fig. 7B). To explore the potential mechanistic explanations, we evaluated their effects on NF-κB activity. Supportively, we found that IFNα but not ribavirin exerted strong inhibitory effect on NF-κB activity (Fig. S7A). Although it has been reported that IFNα is able to induce caspase activation (Apelbaum et al., 2013), no increased caspase 3 and 7 activity was observed after treatment with IFNα for 48 h in Huh7.5 cells (Fig. S7B). Furthermore, the anti-HEV effect of IFNα was not affected in cells lacking RIPK1 (Fig. S7C), which is distinct from the mechanism of deptropine.

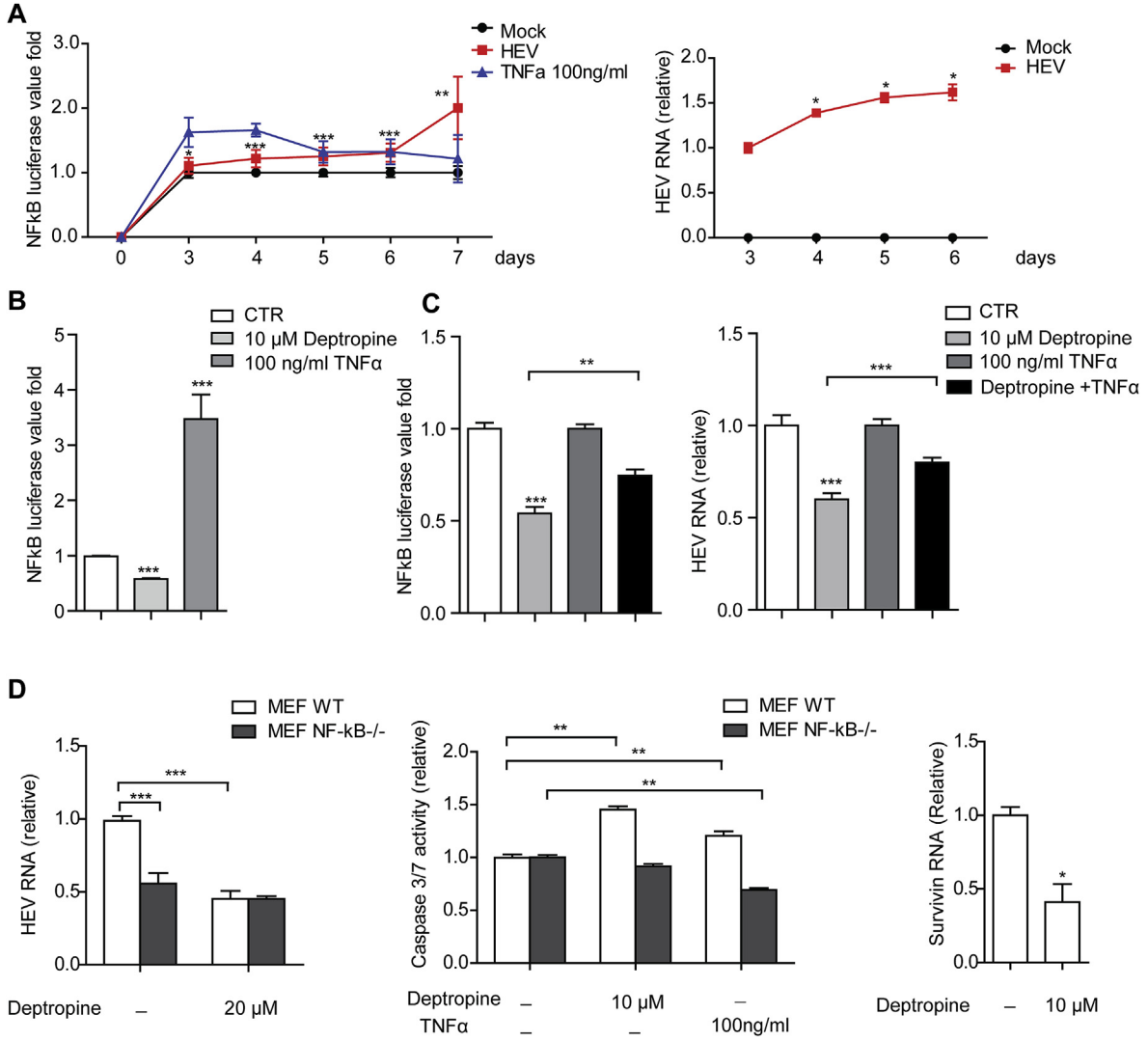


**Fig. 4.** The anti-HEV effect of deptropine requires its inhibition on NF-κB activity. (A) The Huh7.5 cell based NF-κB reporter model was inoculated with culture medium containing HEV particles. After 3 days inoculation, the medium was changed to normal cell culture medium without HEV particles. TNFα treatment serves as a positive control. The untreated mock group serves as control (set as 1). The NF-κB luciferase was measured at indicated time points (n = 8–12). qRT-PCR analysis of the HEV RNA level in the mock group or HEV-infected group at indicated time points. The HEV RNA tested on 4, 5, 6 days are relative to the HEV RNA on day 3 (set as 1) (n = 4). (B) The Huh7.5 cell based NF-κB reporter model was treated with indicated agents for 24 h before testing the NF-κB luciferase value. The untreated group serves as control (set as 1) (n = 6). (C) The Huh7.5 cell HEV infectious model was treated with indicated agents for 24 h before testing NF-κB luciferase value and for 48 h before testing HEV RNA level by qRT-PCR assay. Data in deptropine-treated group were presented relative to the untreated control group (set as 1). Data in the combination group of deptropine with TNFα were presented relative to TNFα-solely treated group (set as 1) (n = 6–8). (D) The wild type (WT) and NF-κB knockout (NF-κB −/−) mouse embryonic fibroblast (MEF) cells were treated with deptropine, and the HEV RNA level were measured by qRT-PCR assay after 48 h treatment. The untreated WT group serves as control (set as 1) (n = 8). The wild type (WT) and NF-κB knockout (NF-κB −/−) mouse embryonic fibroblasts (MEF) cells were inoculated with HEV particles ($1.5 \times 10^6$ HEV RNA copies/ml) and treated with indicated agents. Data in WT MEF was presented relative to the non-treated WT group (set as 1). Data in NF-κB −/− MEF were presented relative to the untreated NF-κB −/− MEF group (set as 1) (n = 6). Huh7.5 cells were treated with indicated concentrations of deptropine. The RNA level of Survivin was measured by qRT-PCR assay after 48 h treatment. The untreated group serves as control (set as 1) (n = 6). The above data are means ± SD. (*P < 0.05; **P < 0.01; ***P < 0.001).

## 4. Discussion

Ribavirin monotherapy or in combination with PEG-IFNα is effective for chronic hepatitis E. However, treatment failure frequently occurs in a subset of patients (Debing et al., 2014). More importantly, a large number of patients, especially acutely infected pregnant women, are not eligible for this treatment. Drug repurposing allows rapid identification of new treatment from existing drugs that can dramatically speed up the clinical implementation. For example, ribavirin was initially approved for treating HCV but now is used as off-label treatment for HEV (Kamar et al., 2014). Distinct from the empirical approach, recent development of high-throughput drug screening technology has enabled unbiased identification of potential treatment from a large set of compounds. For example, screening of the FDA-approved drug libraries has led to the discoveries of potential therapies for Ebola virus, HCV and Zika virus infections. Herein, by screening a library of FDA-approved drugs, we found that a histamine H1 receptor antagonist deptropine potently inhibits HEV.

Among the four histamine receptors (H1, H2, H3, and H4), H1 receptor is the main regulator of acute inflammation. Accordingly, most of the H1 antagonists function as inverse agonists, leading to the relief of inflammation. An increased release of histamine from hepatic mast cells has been reported in hepatitis (Francis and Meininger, 2010). In this study, we found that supplementation with histamine has no effect

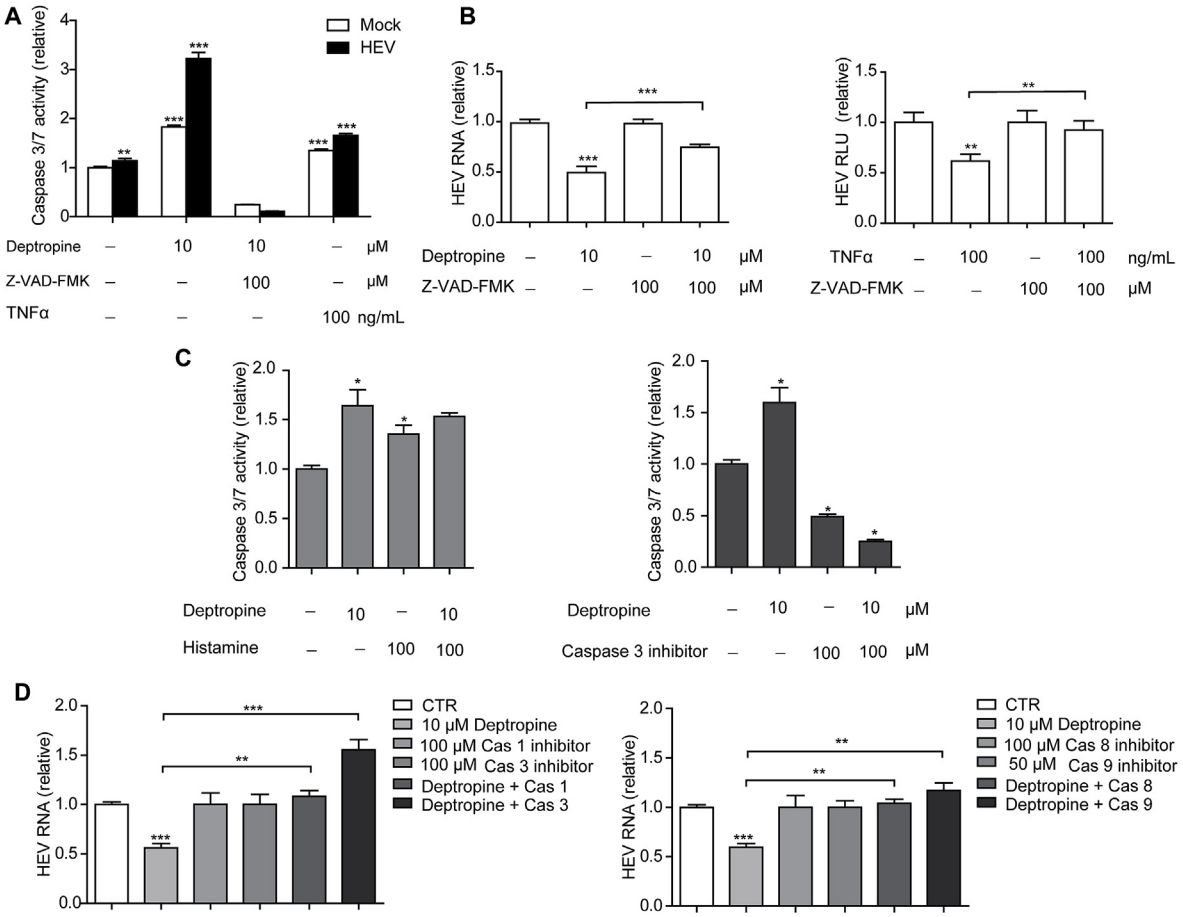


**Fig. 5.** Inhibition of caspase activity reverses the anti-HEV effect of deptropine. (A) Huh7.5 cell based HEV infectious model was treated with indicated agents for 48 h before subjected to caspase 3/7 activity analysis. The untreated mock group serves as control (set as 1) (n = 8). (B) Huh7.5 cell based HEV infectious and replicon models were treated with indicated agents, alone or in combination. After 48 h treatment, samples were subjected to qRT-PCR analysis. Data in deptropine or TNFα solely treated groups were presented relative to the untreated group (set as 1). Data in the combination group of deptropine or TNFα with Z-VAD-FMK were presented relative to Z-VAD-FMK solely treated group (set as 1) (n = 7–9). (C) Huh7.5 cells were treated with indicated agents for 48 h before being subjected to caspase 3/7 activity analysis. The untreated group serves as control (set as 1) (n = 4–5). (D) Huh7.5 cell based HEV infectious model was treated with indicated agents, alone or in combination. After 48 h treatment, samples were subjected to qRT-PCR analysis. Data in deptropine solely treated group were presented relative to the untreated group (set as 1). Data in combination group of deptropine with caspase 1 or caspase 3 were presented relative to caspase 1 or caspase 3 solely treated group (set as 1) (n = 6–9). Huh7.5 cell based HEV infectious model was treated with indicated agents, alone or in combination. After 48 h treatment, samples were subjected to qRT-PCR analysis. Data in deptropine solely treated group were presented relative to the untreated group (set as 1). Data in combination group of deptropine with caspase 8 or caspase 9 were presented relative to caspase 8 or caspase 9 solely treated group (set as 1) (n = 5–10). The above data are means ± SD. (*P < 0.05; **P < 0.01; ***P < 0.001).

on HEV replication in a subgenomic replicon that only mimics viral replication due to the lack of ORF2 capsid protein. However, histamine dose-dependently inhibits HEV infection in the infectious model with the full life-cycle, suggesting multiple interaction between HEV and histamine signaling. Previous studies have reported the inhibition of the entry of Ebola/Marburg virus (Cheng et al., 2015) and HCV by H1 antagonists (Cheng et al., 2015). However, the antiviral effect of H1 antagonist seems not require the H1 receptor (He et al., 2015).

It has been reported that H1 antagonist could regulate the transcription factor NF-κB and caspase activity (Haas et al., 2018). This coordinately modulates the inflammatory microenvironment, resulting in enhancement or antagonism of the host antiviral defense. Increased NF-κB activity induces the transcription of genes promoting cell survival (Schmitz et al., 2014). On the contrary, activation of caspases is thought to counteract the pro-survival role of NF-κB. Of note, RIPK1 actively modulates the crosstalk between caspase and NF-κB signaling. Viruses have developed sophisticated strategies to evade host defense. Dengue virus and influenza virus activate NF-κB to support their replication (Cassens et al., 2003; Nimmerjahn et al., 2004). For hepatitis viruses, HCV was reported to increase NF-kB and AP-1 activation, which was further augmented by the presence of hepatitis B virus X protein (Kanda et al., 2006). We found that inoculation of HEV particles increases NF-κB activity. In addition, we have demonstrated that the basal NF-κB activity serves as a supportive factor for HEV replication. In patients, liver biopsies from pregnant women infected with HEV who developed fulminant hepatic failure (FHF) have higher transcriptional activity of NF-kB compared to those from nonpregnant women or pregnant women infected HEV without development of FHF (Prusty et al., 2007). Consistent with a recent study (Lenggenhager et al., 2017), we found that the presence of HEV ORF2 protein in cell nucleus (Fig. S4D). We thus speculate the possibility that nuclear ORF2 may play a role in HEV-mediated regulation of NF-κB transcription. In contrast, HEV ORF3 protein has been reported to suppress TLR3-induced NF-κB activity (He et al., 2016). Thus, the interactions of HEV with NF-κB are multifaceted, requiring further clarification.

There are mutual interactions between caspase activation and viral infection (Saelens et al., 2001). Several viruses express proteins targeted by the caspase protease and cleavage of these proteases results in inhibition of apoptosis (Connolly and Fearnhead, 2017; Richard and Tulasne, 2012). HCV infection induces caspase activation to cleave the

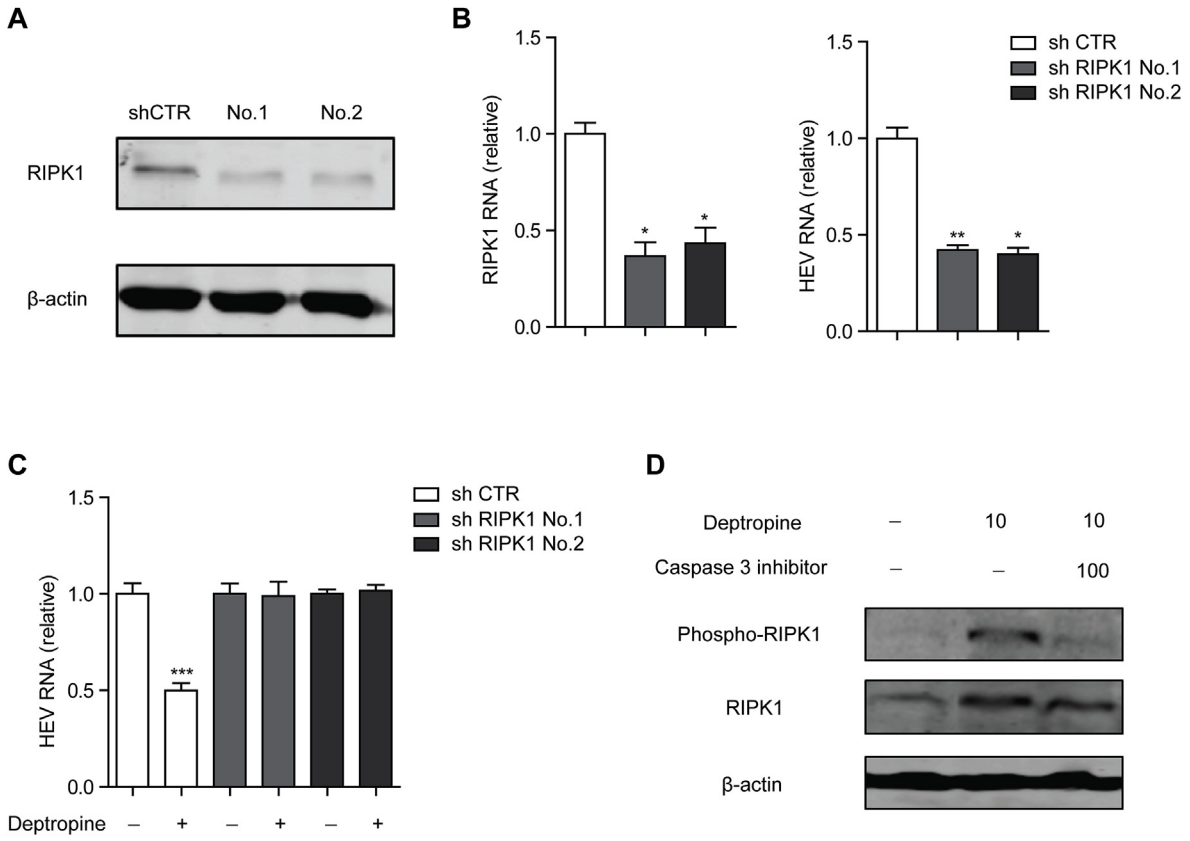


**Fig. 6.** The anti-HEV effect of deptropine is dependent of RIPK1. (A) Western blot analysis of RIPK1 expression in Huh7.5 based HEV infectious model transduced with different lentiviral shRNA vectors targeting RIPK1 (shRIPK1) or scrambled control (shCTR). (B) The RIPK1 expression level and HEV viral RNA level were analyzed by qRT-PCR in the stable RIPK1 knockdown or scramble control cells (n = 4–6). The data are means ± SD and presented relative to shCTR (shCTR, set as 1) (*P < 0.05; **P < 0.01). (C) shRIPK1 or shCTR cells were incubated with or without 10 μM deptropine for 48 h before measurement of HEV RNA. Data in shCTR group were presented relative to the untreated shCTR group (shCTR, set as 1). Data in shRIPK1 group were presented relative to the untreated shRIPK1 group (shRIPK1, set as 1) (n = 3–6). The data are means ± SD. (***P < 0.001). (D) Huh7.5 cells were treated with 10 μM deptropine and 100 μM caspase 3 inhibitor alone or in combination for 48 h. The levels of indicated proteins were analyzed by Western blot assay.

viral nonstructural protein 5A, which subsequently translocates to nucleus to enhance the transcription of several NF-κB target genes to inhibit apoptosis (Jiang et al., 2015). However, some viruses can benefit from caspase activation. Influenza A virus and papillomaviruses require caspase activity for efficient replication (Moody et al., 2007; Ozawa et al., 2007). Recent evidence indicate that HEV infection is associated with caspase activation (Yang et al., 2018). The release of cytochrome c from mitochondria is a key event to activate caspase. A previous study has reported that HEV prevents the release of cytochrome c induced by staurosporine, suggesting a negative regulation of caspase activation by HEV infection (Moin et al., 2007). Consistently, our recent study has shown that HEV is able to block ionomycin-induced MPTP opening (Qu et al., 2019), which is a vital process to activate caspase. In this study, we found that blocking of the excessive caspase activation induced by deptropine could reverse its anti-HEV effect. Counterintuitively, the basal caspase activity may support HEV replication as inhibition of caspase activity leads to reduction of HEV RNA.

RIPK1 is a key determinant of cell survival or death through its scaffold properties or kinase activity, respectively (Kondylis and Pasparakis, 2019; Lafont et al., 2018). This essentially involves the crosstalk between caspase and NF-κB signaling. We found that gene silencing of RIPK1 dramatically inhibits HEV replication. Importantly, loss of RIPK1 abolished the anti-HEV effect of deptropine. Interestingly, deptropine robustly induces RIPK1 phosphorylation and this effect can be inhibited by caspase inhibitor. These results have indicated the importance of RIPK1 in mediating the anti-HEV action of deptropine. However, the exact role of the scaffold property or/and kinase activity of RIPK1 in this process remains to be further clarified.

In summary, we have identified deptropine as a potent anti-HEV agent. The anti-HEV activity of deptropine involves the NF-κB-RIPK1-caspase axis, but the detailed working mechanisms remain to be further investigated. Given that deptropine is a safe and cheap drug that has been widely used to treat asthmatic symptoms in the clinic, it is warranted to evaluate its anti-HEV efficacy in future clinical studies. By implementation of these results in the clinical setting, this may revolutionize the current management of hepatitis E and represent as a milestone in response to the global call towards the elimination of hepatitis B and C by 2030.

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## Author contributions

C.Q., L.L., L.L., P.Y. and P.L. contributed to data acquisition; J.M.D and S.F.J.G contributed to key reagents, the study design and revised

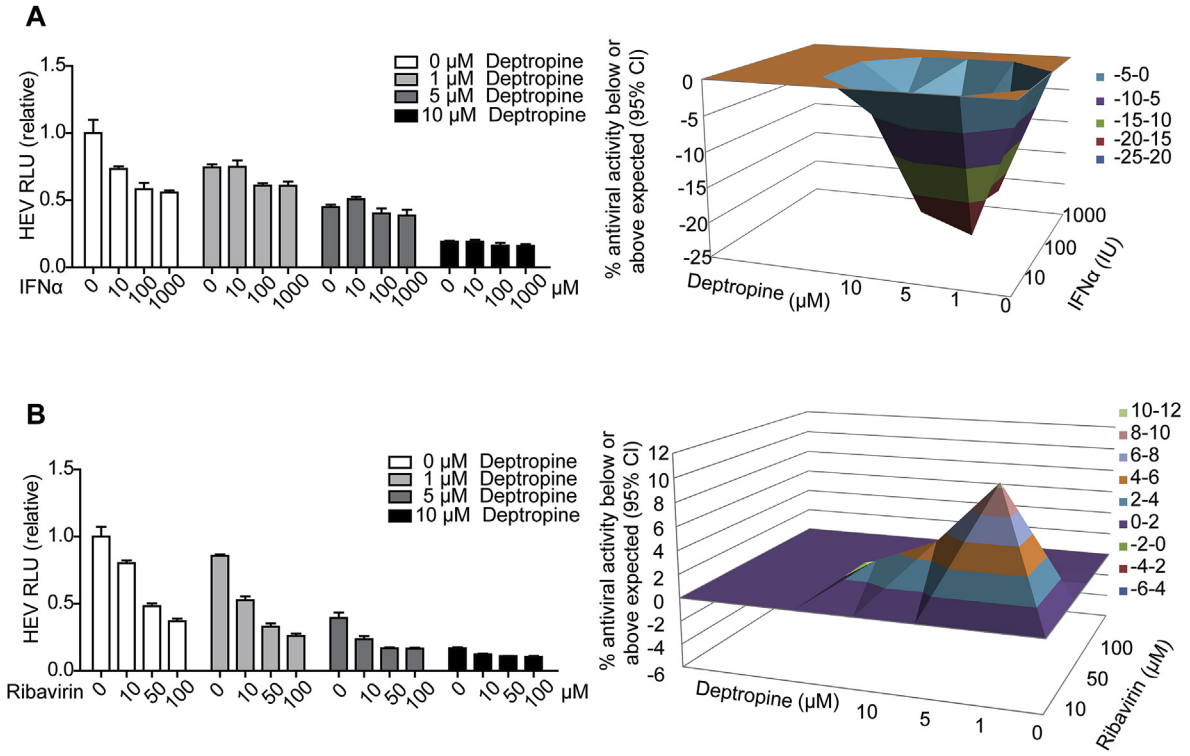


**Fig. 7.** The combination of deptropine with IFN-α and ribavirin results in antagonistic or synergistic effect against HEV. The antiviral effects of deptropine in combination with IFN-α (A) or ribavirin (B) was analyzed by the MacSynergyII model. The three-dimensional surface plot represents the differences (within 95% confidence interval) between actual experimental effects and theoretical additive effects of the combination at various concentrations (n = 4).

the manuscript, R.A.M. and M.P.P. contributed to the study design and revised the manuscript, C.Q. and Q. P. contributed to study concept, drafting of the manuscript, and obtaining funding.

## Conflicts of interest

The authors declare that they have no competing interests.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104588.

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