Hepatitis E Virus Infection and the Treatment

Changbo Qu
The studies presented in this thesis were performed at the Laboratory of Gastroenterology and Hepatology, Erasmus MC-University Medical Center Rotterdam, the Netherlands.

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Hepatitis E Virus Infection and the Treatment
Hepatitis E virus infecties en de behandeling

Thesis

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Chapter 1

General Introduction and Aim of the Thesis
1 Hepatitis E virus as an important cause of viral hepatitis

Hepatitis or liver inflammation is one of the most common liver diseases and imposes a heavy global health burden. The main causes vary depending on circumstances but mainly include infection, metabolism and autoimmunity-related factors. Viral infections including hepatitis A, B, C, D, and E virus (HAV, HBV, HCV, HDV, and HEV) are the leading causes. Among these hepatitis viruses, HEV accounts for the most dominant etiology of acute hepatitis worldwide.

HEV belongs to the Hepeviridae family and is a non-enveloped and positive-strand RNA virus of 32-34 nm in diameter. Its complete genome is 7.2 kb in length and includes three or four open reading frames (ORFs). ORF1 encodes non-structural proteins that are essential for viral replication, including a methyltransferase (MeT), a Y domain (Y), a papain-like cysteine protease (PCP), a proline-rich hinge domain, an X domain, an RNA helicase domain (Hel), and an RNA-dependent RNA polymerase (RdRp) domain [1]. ORF2 encodes the capsid protein which represents the predominant antigen targeted by the human host immune system. ORF3 protein has been reported to mediate release of virus from infected cells [2]. Recently, a novel ORF4 (nt 2835-3308) has been identified from HEV genotype 1 and was shown to drive HEV replication [3].

Several genotypes of HEV have been identified [4]. Genotypes 1 and 2 are obligate human pathogens transmitted by the fecal-oral route and have been associated with large outbreaks and epidemics in developing countries. Genotypes 3, 4, and 7 are responsible for sporadic cases of zoonotic hepatitis E, primarily in industrialized countries [5]. Generally, HEV infection is self-limiting and thus no specific antiviral treatments are required, irrespective of the genotype involved [6]. However, high mortality in pregnant women following HEV genotype 1 infection has been reported and HEV genotype 3 infection rapidly causes liver cirrhosis in immunocompromised populations. Therefore, HEV represents an emerging global health issue and needs to be comprehensively studied for the development of novel antiviral strategies, especially for these specific risk populations.
2 Hepatitis E virus and host interaction

The efficient replication of HEV requires various factors including the successful interfacing with host cell replication machinery, as well as its ability to overcome the host innate immune defenses. Accumulating evidence has indicated that mitochondria are involved in mediating these defenses.

Mitochondria are the organelles of eukaryotic cells responsible for the ATP production through the electron transfer chain (ETC). ETC is a highly active component of mitochondria and has been shown to actively interact with hepatitis viruses. For example, HBx protein has been shown to down-regulate the ETC activity. HCV replication inhibits ETC and results in the subsequent reduced production of ATP. By profiling the role of different ETC complexes, complex III was found to support HEV replication [7].

Besides their role in energy production, mitochondria play a vital role as scaffolds on which various antiviral signaling pathways are converged, and as thus they execute an important role in cell-autonomous innate immune signaling. There are two essential receptors including RIG-I and MDA5 that are capable of detecting viral double-stranded RNA in the cytoplasm. Upon recognition of viral RNA, RIG-I and MDA5 interact with mitochondrial antiviral-signaling protein (MAVS), resulting in the aggregation of MAVS molecules on mitochondria. These aggregated MAVS complexes then lead to the activation of transcription factors NF-kB and IRF 3/7, in turn leading to the production of various antiviral cytokines including type I IFNs. HEV infection has been found to upregulate the expression of both RIG-I and MDA5. Besides, unlike other hepatitis viruses (HAV, HBV, and HCV) which cleave MAVS from mitochondria, HEV specifically induces aggregation of MAVS in the outer membrane of mitochondria, suggesting a distinct interaction between HEV and MAVS-dependent innate immunity. Of note, an increasing body of evidence suggests that the mitochondrial metabolic pattern is highly associated with the regulation of innate immune response. However, the underlying mechanism is elusive and needs to be further investigated.
3 HEV prevention and treatment

3.1 HEV vaccination

As stated, an important risk group for HEV infection are patients taking immunosuppression, for instance, orthotopic organ transplantation recipients. In a subset of such patients, the dose-reduction of immunosuppressant leads to viral clearance, confirming the potential role of host immunity for combating HEV infection. The successful establishment of cell culture systems that recapitulate essential elements of the HEV life cycle promotes a better understanding of the biology of HEV and facilitate vaccine development. In a large phase III clinical trial in China, virus-like particles (VLP) HEV 239 vaccine (Hecolin®) which encompasses amino acids 368-606 of the HEV open reading frame 2 (ORF2) capsid protein from HEV genotype 1, showed high efficacy to prevent genotype 1 and 4 HEV infection. There is no clear evidence showing the efficacy of this vaccine against genotype 3 HEV. As mentioned above, HEV has traditionally been classified solely as a non-enveloped virus. Recent studies have identified HEV as a quasi-enveloped virus, a novel viral form that may help HEV to escape the host immune system, reshaping our understanding of vaccine design [8].

Figure 1. Genome organization of the hepatitis E virus. (Adapted from Kiyoshi Himmelsbach, et al. Emerging Microbes & Infections, 2018)
3.2 HEV detection

Serological detection such as enzyme-linked immunosorbent assays (ELISAs) are thought to work well for broad detection of previous or ongoing HEV infections due to the single serotype of HEV. However, it should be noted that the assays that measure HEV antibody concentrations varies considerably in sensitivity and are not standardized, complicating the interpretation of available serological detection methodology. In addition, data presented based solely on seropositivity are not conclusive and should be accompanied by corroborating evidence such as the detection of HEV RNA.

3.3 Treatment for HEV infection

Chronic HEV infections are defined as the HEV RNA persisting in the liver of immunosuppressed patients for at least three months. After this period, it is unlikely that patients achieve spontaneous viral clearance without therapeutic intervention. To date, there are no approved drugs available for HEV treatment. Ribavirin, an off-label treatment for HEV, is effective for chronic hepatitis E. However, treatment failure frequently occurs in a subset of patients [9]. Sofosbuvir (SOF), the direct-acting anti-hepatitis C virus (HCV) drug (targeting HCV RdRp; RNA-dependent RNA polymerase), has recently been reported to be a potential anti-HEV drug [10]. However, there is debate concerning the effectiveness of this drug against HEV, hampering its further development as novel anti-HEV therapy [11]. In parallel, a variety of preclinical compounds, including nucleoside and non-nucleoside antiviral agents [12], protein kinase-targeted compounds [13], inhibitors targeting mitochondrial metabolism [7], inhibitors of nucleotide synthesis [14] and natural compounds [15], are being investigated in experimental models. Drug repurposing allows rapid identification of new treatment from existing drugs that can dramatically speed up the clinical implementation. Thus, extensive efforts are needed to investigate the anti-HEV effect of the existing FDA-approved medications.
Aims of the Thesis

Hepatitis E provokes a tremendous burden of disease worldwide. It has become clear that the dynamic virus-host interactions determine the outcome of virus pathogenesis, irrespective of the genotypes. Thus improved understanding of this mutual interference may allow the development of novel antiviral therapies. This thesis aims to expand our understanding of virus-host interactions and to develop novel antiviral drugs.

Thesis Outline

In chapter 2, I first aim to give a comprehensive description of the interaction between mitochondria and hepatitis viruses. We conclude that unlike other hepatitis viruses, HEV specifically modulates the mitochondrial biology, which may explain the particularities with respect to HEV responses to IFN treatment. In chapter 3, I show that mitochondrial electron transport chain complex III supports HEV replication, providing a potential therapeutic target for HEV treatment. In chapter 4, a screening of a library containing over 1,000 FDA-approved drugs was performed. We have identified deptropine, a classical histamine H1 receptor antagonist used to treat asthmatic symptoms, as a potent inhibitor of HEV replication. In chapter 5, I show that a nucleoside analogue, 2'-C-methylcytidine, potently inhibits HEV in multiple cell lines. Mitochondria play a vital role in the mediation of innate immune response. In chapter 6, I discuss the recent progress on the studies of innate immunity during HEV infection. Since innate immunity plays a critical role in determining the clinical outcome of HEV infection, understanding of HEV-host innate immunity provides the basis for the development of effective antiviral treatment. In summary, our works describe the HEV-host interaction and provide novel antiviral strategies. This may revolutionize the current management of hepatitis E and represent a milestone in response to the global call towards the elimination of viral hepatitis.
Chapter 1

References


Chapter 2

Mitochondria in the biology, pathogenesis and treatment of hepatitis virus infections

Changbo Qu, Shaoshi Zhang, Yang Li, Yijin Wang, Maikel P. Peppelenbosch, Qiuwei Pan.
Summary

Hepatitis virus infections affect a large proportion of the global population. The host rapidly responds to viral infection by orchestrating a variety of cellular machineries, in particularly the mitochondrial compartment. Mitochondria actively regulate viral infections through the cellular innate immunity and metabolic reprogramming. In turn, hepatitis viruses are able to modulate the morphodynamics and functions of mitochondria, but the mode-of-actions are distinct with respect to different types of hepatitis viruses. The resulting mutual interactions between viruses and mitochondria partially explain the clinical presentation of viral hepatitis, influence the response to antiviral treatment and offer rational avenues for novel therapy.

KEYWORDS: mitochondria, hepatitis virus, pathogenesis, treatment
Key Points

- Mitochondrial dysfunction is common in viral hepatitis patients.
- All five major types of hepatitis viruses actively but differentially interact with the mitochondrial compartment and alter mitochondrial morphodynamics, mitochondrion-mediated innate immunity, and metabolism.
- The mutual interactions between hepatitis viruses and mitochondria orchestrate the pathogenesis, clinical outcome, and response to antiviral medication.
- The prominent role of mitochondria in cellular pathology of viral hepatitis offer opportunity for both combating infection and for the prevention of hepatitis-associated liver cancer.
Introduction

Hepatitis or liver inflammation is one of the most common liver diseases that imposes a heavy global health burden.\(^1\)\(^-\)\(^2\) Acute hepatitis is either self-resolving, or develops into chronic hepatitis and subsequently progresses to cirrhosis or hepatocellular carcinoma (HCC).\(^3\) The main etiologies include infection, metabolism and autoimmune-related causes. Viral infections including hepatitis A, B, C, D and E virus (HAV, HBV, HCV, HDV, and HEV) are the leading causes (Table 1).

Host cells rapidly respond to viral infection by orchestrating a variety of cellular machineries. In particular, the mitochondrial compartment appears important in this respect and responds in various ways, including by acting as scaffold on which the antiviral molecular machinery is built.\(^4\) Mitochondria antiviral-signaling protein (MAVS) acts as an adaptor for transcription and production of interferons (IFN), the most potent antiviral cytokines, in response to viral infection. Interestingly, different hepatitis viruses differentially interact with MAVS, resulting in enhancement or antagonism of host antiviral defense.\(^5\) In parallel, mitochondrial DNA (mtDNA) is able to elicit innate immune response through Toll-like receptor 9 (TLR9) and stimulator of interferon genes (STING) signaling.\(^6\) Finally, the release of citric acid cycle intermediates from the mitochondrial matrix into the cytosol following viral infection also regulates host innate immunity.\(^7\) Together, these mechanisms likely impact on the infection course, pathogenesis and the clinical outcome of IFN-α treatment in hepatitis virus infections.

The liver is a metabolic powerhouse, and accordingly hepatocytes contain abundant numbers of mitochondria to support the energy requirement associated with high metabolic activity.\(^8\) As double-membraned organelles, mitochondria are essential for energy production and cellular homeostasis. Viruses require energy and low molecular-weight precursors from the host to complete their life cycle, but on the other hand can modulate the host metabolic machineries.\(^9\) Hepatitis viruses are known to regulate the number, quality, and dynamics of mitochondria, resulting in altered mitochondrial morphology and function.\(^10\) Accordingly, morphological and functional alterations of mitochondria are commonly observed in liver tissues obtained from viral hepatitis patients.\(^11\)\(^-\)\(^13\)
Intriguingly, mitochondria serve as a hub mediating many cellular signaling pathway, including inflammatory responses that are prominent features of viral hepatitis. Adenosine 5'-triphosphate (ATP), the primary carrier of energy, plays pleiotropic roles in inflammation by acting as an extracellular signaling molecule. In normal physiology, ATP reaches the extracellular environment at low basal rate and this plays a role in cell-to-cell communication. However, inflammation is associated with increased release of ATP which in turn triggers inflammatory responses. Accordingly, decreased ATP levels facilitate HCV viron secretion and evasion of innate immunity. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), an activator of ATP production, has been shown to counteract both HCV and HEV infection. HBV infection results in a decrease of ATP levels in hepatocytes.

Several other metabolites from mitochondria, in particular citrate and succinate, are implicated in the pathological processes of viral hepatitis and cirrhosis. Given the complexity, whether it is a sequential or causal relationship between mitochondrial alteration and hepatitis remains unclear. In this review, we aim to in-depth decipher the multifaceted interactions of mitochondria with hepatitis virus infections, and emphasize the implications in understanding the pathogenesis and advancing therapeutic development.

1 MITOCHONDRIAL DYSFUNCTION IN VIRAL HEPATITIS PATIENTS

Mitochondrial dysfunction is associated with many common disorders. It is a prominent feature of liver cell injury, and is often manifested in patients with viral hepatitis. HBV and HCV infections are frequently reported to be accompanied by mitochondrial dysfunction. In patients, HCV infection results in morphological alteration of mitochondria, reduction in the copy number, and oxidative damage triggered mutations in the genome of mtDNA. Interestingly, mitochondrial abnormalities in HCV patients are in a genotype-dependent manner. Their frequency is higher in genotype 1b than genotype 2a/c or 3a infection, suggesting a greater intrinsic cytopathic effect of genotype 1b HCV. The current direct-acting antivirals are highly effective in inhibiting HCV infection.
Table 1. Features of hepatitis virus infections

<table>
<thead>
<tr>
<th></th>
<th>HAV</th>
<th>HBV</th>
<th>HCV</th>
<th>HDV</th>
<th>HEV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size (nm)</strong></td>
<td>27-32</td>
<td>42</td>
<td>55-62</td>
<td>36-43</td>
<td>27-34</td>
</tr>
<tr>
<td><strong>Genome</strong></td>
<td>+ssRNA</td>
<td>dsDNA</td>
<td>+ssRNA</td>
<td>-ssRNA</td>
<td>+ssRNA</td>
</tr>
<tr>
<td><strong>Incubation period (days)</strong></td>
<td>15-45</td>
<td>30-180</td>
<td>15-160</td>
<td>30-60</td>
<td>15-60</td>
</tr>
<tr>
<td><strong>Genome length (nt)</strong></td>
<td>7,500</td>
<td>3,200</td>
<td>9,600</td>
<td>1,700</td>
<td>7,200</td>
</tr>
<tr>
<td><strong>Envelope</strong></td>
<td>No/quasi-enveloped</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No/quasi-enveloped</td>
</tr>
<tr>
<td><strong>Transmission</strong></td>
<td>Fecal-oral</td>
<td>Blood and other body fluids</td>
<td>Blood</td>
<td>Blood and other body fluids</td>
<td>Fecal-oral</td>
</tr>
<tr>
<td><strong>Infection course</strong></td>
<td>Acute</td>
<td>Acute; Chronic</td>
<td>Acute; Chronic</td>
<td>Acute; Chronic</td>
<td>Acute; Chronic</td>
</tr>
<tr>
<td><strong>Severity of hepatitis</strong></td>
<td>±</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td><strong>Liver cancer development</strong></td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Not clear</td>
</tr>
<tr>
<td><strong>Vaccine</strong></td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes (in China only)</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>N/A</td>
<td>Yes</td>
<td>Yes</td>
<td>*No approved medication</td>
<td>*No approved medication</td>
</tr>
</tbody>
</table>

ss, single-stranded; ds, double-stranded; nt, nucleotide. N/A, not applicable. *For HDV, no FDA approved medication is available. Peg-IFN-α is the only recommended therapy, but the efficacy is unsatisfactory. For HEV, no FDA approved medication is available. Ribavirin has been used as off-label treatment with good efficacy.

However, whether mitochondria dysfunction persists in patients after HCV eradication remains an interesting question to be investigated. In HBV patients, a lower level of serum mtDNA content is related to an increased risk of HCC development, indicating that circulating mtDNA may be a potential non-invasive marker of HCC risk.26 Extensive mitochondrial gene dysregulation and global downregulation of mitochondrial function have been observed in HBV-specific CD8 T cells from patients with chronic infection. Treatment with mitochondria-targeted antioxidants restore antiviral activity of these exhausted HBV-specific CD8 T cells.27 Data regarding the mitochondrial status in hepatitis A and E patients remain limited, promoting for future research in this respect.
2 THE MUTUAL INTERACTIONS BETWEEN HEPATITIS VIRUSES AND MITOCHONDRIAL COMPARTMENTS

2.1 Apoptosis in the Pathogenesis of Viral Hepatitis

There is an accumulating evidence supporting the role of liver cell apoptosis in the pathogenesis of viral hepatitis.\(^{28}\) Although there are multiple modes of programmed cell death, pyroptosis and apoptosis cascades through the extrinsic and intrinsic pathways are the predominant forms for viral hepatitis.\(^{29}\) The extrinsic signaling is activated via the cell surface death receptors including TNFR1, TRAIL-R1 and Fas. The intrinsic pathway is mainly triggered by non-receptor stimuli, but characterized by the permeabilization of the outer mitochondrial membrane. This leads to the release of pro-apoptotic factors from the mitochondrial inter-membrane space into the cytosol.\(^{30}\) A recent study demonstrates that the extrinsic and intrinsic apoptotic pathways activate pannexin-1 to drive NLRP3 inflammasome assembly, which is involved in the pathogenesis of viral hepatitis.\(^{31, 32}\)

The numbers of apoptotic hepatocytes in chronic hepatitis B and C patients are found to be small but higher than that in healthy individuals.\(^ {33}\) It is now generally accepted that cytotoxic T lymphocytes mediate the immune clearance of hepatitis virus-infected hepatocytes. Immune-mediated apoptosis plays an important role in liver damage and pathogenesis.\(^ {34}\) However, the direct effects of hepatitis viruses on apoptosis have also been indicated. The role of the HBV X gene product (HBx) in hepatocyte apoptosis is multifaceted. Pro-apoptotic function of HBx has been reported in hepatocytes of transgenic mice,\(^ {35}\) whereas it has also been shown to block Fas-induced apoptosis in liver cells.\(^ {36}\) Similarly, HCV infection enhances susceptibility to Fas-mediated apoptosis.\(^ {37}\) whereas several HCV proteins (core, E1, E2, and NS proteins) haven been shown to inhibit TNF-α-mediated apoptosis.\(^ {38}\) Recently, HEV has been reported to induce hepatocyte apoptosis via mitochondrial pathway in mongolian gerbils.\(^ {39}\) However, the underlining interaction between apoptosis and HEV infection remains largely obscure.

Cytochrome c, an essential component of the electron transport chain (ETC) transferring electrons from complex III to IV, plays a key role in the early events of mitochondria-mediated apoptosis. Serum cytochrome c has been suggested as a
potential new marker for fulminant hepatitis in patients. During apoptosis, cytochrome c is released from mitochondrial intermembrane space to induce caspase activation. HCV could induce, whereas HEV can block the release of cytochrome c from mitochondria to cytosol (Figure 1). The possible correlation between the amount of serum cytochrome c and the severity of hepatitis may be interesting to be further explored as the potential relevance to diagnosis. Besides cytochrome c, mutual interactions between caspase activation and viral infection have also been observed. Several viruses express proteins which could be cleaved by the caspase protease, resulting in inhibition of apoptosis. For example, HCV infection induces caspase activation to cleave the viral nonstructural protein 5A, which subsequently translocates to nucleus to enhance the transcription of several NF-κB target genes to inhibit apoptosis. HEV ORF2 has been found to have different forms and could translocate to the cell nucleus. However, whether ORF2 is cleaved by the host protease and whether it regulates apoptotic pathway remain to be further studied. Taken together, apoptosis is likely an important mechanism in pathogenesis of viral hepatitis. Hepatitis viruses can modulate apoptotic pathways at various levels. Thus, detection and quantification of particular apoptosis-related molecules may be explored as potential biomarkers for disease diagnosis in viral hepatitis patients.

2.2 MAVS and mtDNA-mediated Innate Immune Response

The early and non-specific detection of hepatitis viruses is generally through the recognition by Pathogen-Associated Molecular Patterns (PAMP) as the innate immunity sensors. This leads to the activation of downstream IFN signal pathway and subsequent production of the ultimate antiviral effectors, interferon-stimulated gene (ISG). MAVS, acting as an adaptor for transcription and production of IFN, shows specific interactions with different hepatitis viruses. HAV and HCV provoke a blockade in cell-autonomous IFN production by inducing proteolytic release of a part of the extra-mitochondrial domain of MAVS. This is clinically supported by the presence of cleaved MAVS in the liver biopsies of HCV- but not HBV-infected patients. The HCV protease NS3/4A cleaves MAVS off the mitochondria, whereas HAV uses a stable, catalytically active polyprotein processing intermediate to target MAVS for proteolysis. Instead of directly provoking MAVS proteolysis, HEV induces MAVS to form “prion-like” polymers, resulting in type III IFN response
(Figure 1). The sequestering of MAVS in morphologically altered mitochondria may explain the relatively poor response to IFN treatment in the clinical management of HEV compared to that in HCV-infected patients.\textsuperscript{5} Thus, exploring drugs preventing aggregation of MAVS on the outer membrane of mitochondria could be potentially used as a combination with IFN to enhance the anti-HEV efficacy. HBV infection is another case altogether, and investigation of liver biopsies from chronic HBV patients indicates the absence of activated innate immune response.\textsuperscript{54} Thus, HBV is likely invisible to pattern recognition receptors, and the role of MAVS may not be prominent.

Because mtDNA contains remnants of bacterial nucleic acid sequences and is methylated in a different way from nuclear DNA, it resembles non-self DNA and is thus more prone to be degraded after transferring to cytosol, leading to the activation of innate immune system.\textsuperscript{55} mtDNA-mediated immune activation involves TLR9 and STING, which contributes to the clearance of invading pathogens and provokes inflammasome activation, interleukin-1 production and pyroptosis.\textsuperscript{56, 57} Due to bidirectional transcription, mtDNA is capable of generating overlapped transcripts. These formed long double-stranded RNA structures engage in MDA5-mediated antiviral signaling to trigger a type I IFN response.\textsuperscript{58} In clinic, IFN treatment in HCV patients significantly decreases the frequency of mtDNA mutations in hepatocytes and increases the mtDNA copy numbers in peripheral leukocytes.\textsuperscript{12, 59} Moreover, mtDNA was reported to mediate IFN response.\textsuperscript{60} Even though hepatocytes contain hundreds of copies of mtDNA, it is possible that the combination of mtDNA deletions and point mutations, together with mtDNA strand breaks by increased ROS, could reach a threshold sufficient to induce mitochondrial dysfunction, contributing to the pathogenesis of viral hepatitis. Very recently, it has been reported that new mtDNA synthesis can activate the NLRP3 inflammasome.\textsuperscript{61} As described, activation of NLRP3 inflammasome is closely related to the pathogenesis of chronic liver diseases, including viral hepatitis.\textsuperscript{32}
Figure 1. The mutual interactions of the mitochondrial compartment with hepatitis viruses and the consequences on the infections. Hepatitis viruses differentially modulate MAVS signaling. HAV and HCV cleave, while HEV induces MAVS aggregation. These interactions with MAVS result in enhancement or antagonism of innate immune response. Hepatitis viruses either induce or block the MPTP opening, regulating the release of mitochondrial contents such as mtDNA fragment or ATP, which then lead to antiviral defense. mtDNA that are not completely degraded are able to enter the endocytic pathway through mitochondria-derived vesicles, which engage Toll-like receptor 9 (TLR9) in lysosomes and lead to the activation of the NF-κB signaling and IFN production. Sustained apoptosis caused by hepatitis virus infection triggers damage of membrane integrity, resulting in the liberation of mitochondrial contents into the extracellular milieu.

2.3 Mitochondrial Morphodynamics in Response to Hepatitis Virus Infection

The mitochondrial life cycle entails frequent fusion (in which two mitochondria form a single organelle) and fission (the division of one mitochondria into two daughter organelles) events.62 These two opposing processes collaboratively control the number and size of mitochondria and maintain cell homeostasis. Mitofusin-1 (Mfn1), Mitofusin-2 (Mfn2) and optic atrophy 1 (Opa1) are the key regulators of fusion, whereas Dynamin-related protein 1 (Drp1) tightly modulates fission (Figure 2A).
main reason for continual mitochondrial fission and/or fusion is that it facilitates the
degradation of damaged organelles by mitophagy, which is regulated by Parkin and
Pink proteins. It promotes mitochondrial turnover and prevents accumulation of
dysfunctional mitochondria. HCV and HBV infections have been shown to promote
mitophagy. The role of mitophagy in other hepatitis viruses needs to be further
studied.

Upon infection, hepatitis viruses rearrange the intracellular microenvironment,
including the mitochondrial compartment. Mitochondrial fission has been frequently
observed in HBV and HCV infections. HCV promotes fission by inducing Drp1
phosphorylation. This correlates with oxidative stress, presenting as excessive lipid
peroxidation and deficiency of tissue hepatocellular antioxidant stores, which in turn
contributes to steatosis that is highly prevalent in HCV infection. In contrast, HEV
is able to trigger mitochondrial fusion to promote viral replication (Figure 2B).
Because mitochondrial fission is the initial step of mitophagy, the differential
regulation of mitochondrial morphodynamics by HEV compared to HCV may suggest
a negative regulation of mitophagy during its propagation.

The fission and fusion processes in hepatocytes is responsible for the exchange and
reallocation of mitochondrial contents including mtDNA. Inhibition of mitochondrial
fusion is related to mtDNA depletion. Importantly, the equilibrium between fission
and fusion is crucial for stabilizing mtDNA copy number and maintaining a healthy
liver function. Hence, modulation of mitochondrial morphodynamics could
potentially affects virus-induced liver dysfunction.

In addition, morphodynamics also regulates innate immunity by affecting the
distribution of MAVS on the mitochondrial outer membrane. As reorganization of
MAVS spatial distribution is a key event in IFN production in response to viral
infection, such spatial reorganization has important consequences. Mitochondrial
fusion promotes, whereas fission inhibits RIG-I-like Receptor (RLR) signaling.
Fibroblasts lacking of mitofusin proteins produce less IFN and pro-inflammatory
cytokines upon viral infection. Small molecules, such as mitochondrial division
inhibitor 1 (Mdivi1) which inhibits Drp1 activity, have been developed. Hence, the
effects of these agents on different hepatitis viruses are interesting be investigated.
2.4 The Role of Mitochondrial Electron Transport Chain

Mitochondrial ETC consists of a series of complexes that transfer electrons from donors to acceptors via redox coupled with the transfer of protons across a membrane. It is the site for oxidative phosphorylation and generation of ATP. Mitochondrial morphodynamics can regulate the respiratory rate. Fused mitochondria enhance, whereas mitochondrial fission decrease the respiratory.

Figure 2. Mitochondrial morphodynamics is differentially regulated by hepatitis viruses to modulate innate immune response. (A). The mitochondrial life cycle entails frequent fusion and fission events. Mitofusin-1 (Mfn1), Mitofusin-2 (Mfn2) and optic atrophy 1 (Opa1) are the key regulators of fusion, whereas Dynamin-related protein 1 (Drp1) and mitochondrial fission 1 protein (Fis1) modulate fission.
HBV and HCV induce fission, whereas HEV triggers fusion. (B). Immunofluorescence staining of human liver cells infected with HEV showing the induction of mitochondrial fusion. HEV capsid protein (red; anti-ORF2), mitochondria (green; anti-HSP60) and DAPI (blue). Cells were visualized with 63 x oil immersion lens at identical settings.

Thus changing the dynamics of mitochondrial fission and fusion influences mitochondrial function and constitutes an evident target for viruses to corrupt mitochondria-mediated innate immunity. It has been demonstrated that hepatitis viruses actively interact with the ETC. HBx protein has been shown to down-regulate the ETC activity.\(^{77}\) HCV replication inhibits ETC and subsequent the production of ATP.\(^{78}\) By profiling the role of different ETC complexes, complex III was found to support HEV replication.\(^{19}\)

During cellular respiration, byproducts like reactive oxygen species (ROS) are produced under stressed condition.\(^{79}\) Increased ROS production is associated with liver injury and the pathogenesis of viral hepatitis.\(^{80}\) Furthermore, ROS production are involved in various cellular signaling pathways, including those mediating immune responses. ROS can induce aggregation of MAVS on mitochondrial outer membrane to initiate IFN response. Cells with reduced ETC activity are impaired with production of IFNs and proinflammatory cytokines during viral infection.\(^{81}\) In contrast, increased ROS production counteracts HCV replication.\(^{82}\) Thus the ETC emerges as a primary target for viral infection, although hepatitis viruses likely target its functionality indirectly, for instance by modifying mitochondrial morphodynamics.

### 2.5 Mitochondrial Permeability Transition Pore and Hepatitis Viruses

Mitochondria actively communicate with the cytosol and nuclear compartments. The signals involved are mediated through proteins located on the mitochondrial membrane, including the mitochondrial permeability transition pore (MPTP). Mitochondrial contents can escape from the mitochondrial matrix during MPTP opening.\(^{83}\), \(^{84}\) The products related to the action of ETC, such as ATP and cytochrome c, are transferred through MPTP to cytosol to exert biological functions. MPTP is composed of voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane, the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane, and cyclophilin D (CypD) as its regulator in the matrix.
Hepatitis viruses have various interactions with MPTP. HBx protein has been shown to co-localize with VDAC, leading to alteration of mitochondrial transmembrane potential. The 68-117 region of HBx interacts with mitochondria, and is necessary for membrane permeabilization. HEV ORF3 protein sustains high levels of oligomeric VDAC to preserve mitochondrial potential and membrane integrity, thereby protecting infected cells from mitochondrial depolarization and death. HBV and HCV core proteins provoke MPTP opening; whereas HEV prevents such an event. In line with this, the MPTP inhibitor cyclosporine A (CsA) inhibits HBV and HCV, whereas promotes HEV replication. As highlighted the importance of mtDNA in innate immunity, mtDNA fragments in fact are also released through MPTP. Thus, targeting MPTP opening represents a potential antiviral strategy.

3 THE IMPACT OF MITOCHONDRIAL METABOLITES

Metabolites produced from the mitochondrial tricarboxylic acid (TCA) cycle, including citrate, succinate, fumarate and acetyl-CoA are important regulators of signaling transduction when released from the mitochondria. Citrate synthase and succinate dehydrogenase are up-regulated in HBV-infected cells, leading to elevation of the corresponding metabolites such as fumarate and succinate. Succinate has been recognized as an emerging signal transducer to activate inflammatory pathways. An example is the increase in antigen-presenting capacity of dendritic cells if cytosolic succinate levels increase. Thus, it is rational to assume that such molecules may modulate innate immunity in hepatocytes as well. HCV infection has been related to elevated level of acetyl-CoA, a metabolite that participates in many biochemical reactions in protein, carbohydrate and lipid metabolism. It has been widely recognized that acetyl-CoA contributes to lysine acetylation by donating its acetyl. Lysine modification controls many aspects of protein function and provides an obvious mechanism as to how acetyl-CoA can influence cellular function. HBV replication is regulated by the acetylation status of the cccDNA-bound H3/H4 histones. Acetylation of retinoic acid-inducible gene I (RIG-I) regulates its antiviral functions, and RIG-I is essential in sensing HAV, HBV, HCV and HEV infections. Importantly, adequate cytosolic acetyl-CoA level is required for interferon-γ (IFNγ) production. Other metabolites can inhibit inflammatory
responses. For example, lactate acts through the lactate receptor to reduce hepatitis in mouse models.\textsuperscript{104} There is an increase in lactate production in HCV-infected cells, probably because the corruption of mitochondrial function provokes increased dependency in the hepatocyte on glycolysis to support its energy needs.\textsuperscript{105} In apparent agreement, targeting mitochondrial metabolism has been proposed to prevent chronic neuroinflammation.\textsuperscript{106} This may bear implications for treating neurologic diseases caused by HEV infection.\textsuperscript{107}

4 IMPLICATIONS IN THERAPEUTIC DEVELOPMENT

IFN-\(\alpha\) has been used in the clinic for decades to treat chronic HBV and HCV infections. The effects of IFN on viral replication have been linked to mitochondrial functions\textsuperscript{108} but conversely, mitochondria regulate antiviral IFN responses via MAVS or the production of ROS. The development of direct-acting antivirals (DAA), in particular the nucleoside/nucleotide analogues, constitutes a landmark in advancing the treatment for viral hepatitis.\textsuperscript{109} Nucleoside/nucleotide analogues can efficiently inhibit viral replication by inhibition of the viral polymerase activity.\textsuperscript{110} However, these drugs may exert off-target effects by inhibition of mitochondrial DNA polymerase, resulting in a reduction of mtDNA copy number, although a minor reduction may not present a clinically apparent phenotype.\textsuperscript{111, 112} Fialuridine, a nucleoside analogue investigated for treating HBV infection, has caused five deaths from liver failure associated with lactic acidosis, and two required liver transplantation.\textsuperscript{113} The toxicity is primary due to damaging mitochondria, particularly in nerves, liver, skeletal, and cardiac muscle, as these tissues are abundant with mitochondria.\textsuperscript{114} Thus, the degree of causing these side-effects is detrimental whether this class of drugs can be further developed into clinic, even though the antiviral effect may be very promising.

Despite the launch of various antiviral drugs, new therapeutics remains required for eliminating viral hepatitis. Unlike HCV, the persistence of cccDNA prevents cure but only inhibits viral replication in HBV patients.\textsuperscript{115} For HEV, besides supportive care and off-label treatment with ribavirin or IFN-\(\alpha\) for some cases, there is no proven antiviral medication available. Mitochondria represent as a viable target for new therapeutic development. As mitochondrial dysfunction is widely present in HBV
patients, treatment with mitochondria-targeted antioxidants mitoquinone (MitoQ) and the piperidine-nitroxide MitoTempo has been shown to restore the antiviral activity of HBV-specific CD8 T cells.\textsuperscript{27} MitoQ is based on the delivery of a potent anti-oxidant with targeted lipophilic cations that leads to accumulation up to several-hundred fold in mitochondria. It has been extensively studied and demonstrated safety in humans.\textsuperscript{23, 116, 117} Because increased oxidative stress and subsequent mitochondrial damage are the key mechanisms causing pathogenesis in viral hepatitis, treatment with MitoQ has been shown to decrease liver damage in HCV patients.\textsuperscript{116} It has also been shown to attenuate liver fibrosis in mice.\textsuperscript{118}

The mitochondrial ETC complexes have long been recognized as antiviral target.\textsuperscript{119} The complex I inhibitor, metformin, has been shown to inhibit HBV and HCV infections in experimental models,\textsuperscript{120, 121} although the effects in patients remain unclear. Complex III sustains HEV replication and can be targeted by pharmacological inhibitors to inhibit viral replication in experimental models, but requires further clinical validation.\textsuperscript{19}

Lastly, mitochondria-mediated apoptosis is essential in the pathogenesis of viral hepatitis, however, no optimal drug has been identified to prevent or treat liver injury. In this respect, the most promisors are mitochondria-targeted antioxidants or caspase inhibitors, but require further investigation.

5 CONCLUDING REMARKS

Liver cells are enriched in mitochondria that support the unique features of hepatic metabolism but also orchestrate cell-autonomous antiviral immunity upon viral infection. Mitochondrial dysfunction commonly occurs in viral hepatitis patients. This associates with the disease progression from acute, chronic infection to cancer development. Hepatitis viruses actively interact with the mitochondrial compartment at various levels, including regulation of mitochondrial morphodynamics, innate immune response, bioenergetics and metabolism. The mode-of-actions of these interactions may differ among the five major types of hepatitis viruses, but are essential for understanding the pathogenesis, clinical outcome and treatment response in viral hepatitis patients.
The prominent role of mitochondria in contributing to pathology has provided opportunities for therapeutic development against viral hepatitis and prevention of liver cancer development. Several mitochondrial-related or targeted agents have been used in the clinic or tested in clinical trials, including the complex I inhibitor metformin, the MPTP inhibitor CsA, the NAD⁺ precursor nicotinamide mononucleotide, the mitochondria-targeted protective compounds MitoQ and Bendavia, and the antioxidant coenzyme Q₁₀. However, the development and application of mitochondria-related therapies remain at their infancy (see Outstanding Questions). We propose to enhance the therapeutic development by identifying and repurposing the existing FDA-approved medications with mitochondria-targeted properties. On the other hand, dietary and herbal supplements¹²² and other new approaches¹²³,¹²⁴ shall also be explored for their potential to modulate or restore the mitochondrial function.

Conflict of interest statement

The authors declare that they have no competing interests.

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Chapter 3

Mitochondrial electron transport chain complex III sustains hepatitis E virus replication and represents an antiviral target

Summary

Hepatitis E virus (HEV) infection has emerged as a global health issue. However, no approved medication is available and the infection biology remains largely elusive. Electron transport chain (ETC), a key component of the mitochondria, is the main site that produces Adenosine triphosphate (ATP) and Reactive oxygen species (ROS). By profiling the role of the different complexes of the mitochondrial ETC, we found that pharmacological inhibition of complex III, a well-defined drug target for the treatment of malaria and pneumocystis pneumonia, potently restricts HEV replication. This effect demonstrated in our HEV models is equivalent to the anti-HEV potency of ribavirin, a widely used off-label treatment for chronic hepatitis E patients. Mechanistically, we found that this effect is independent of ATP production, ROS level and pyridine depletion. By using pharmacological inhibitors and genetic approaches, we identified that mitochondrial permeability transition pore (MPTP), a newly identified component of ETC, provides basal defense against HEV infection. Interestingly, HEV interferes with the pore opening of MPTP. Furthermore, inhibition of MPTP attenuates the anti-HEV effect of complex III inhibitors, suggesting that MPTP mediates the antiviral effects of these inhibitors. These findings have revealed new insights on HEV-host interactions and provided viable anti-HEV targets for therapeutic development.

KEY WORDS: Hepatitis E ∙ mitochondria ∙ ETC ∙ MPTP ∙ antiviral target

ABBREVIATIONS: AMA, antimycin A; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; ATP, adenosine triphosphate; BKA, bongkrekic acid; CsA, cyclosporin A; CypD, cyclophilin D; COX I, cytochrome c oxidase I; COX II, cytochrome c oxidase II; COX IV, cytochrome c oxidase IV; CYTB, cytochrome b; EB, ethidium bromide; ETC, electron transport chain; HEV, hepatitis E virus; HCV, hepatitis C virus; H2O2, hydrogen peroxide; MAVS, mitochondrial antiviral-signaling protein; MYXO, myxothiazol; MPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; OLM, oligomycin A; OCR, oxygen consumption rate; ORF, open reading frame; ROT, rotenone; ROS, reactive oxygen species; VE, vitamin E acetate.
Introduction

Hepatitis E virus (HEV) infection is the major cause of acute viral hepatitis globally. Although the infection is asymptomatic and self-limiting in healthy populations, it often causes high mortality in pregnant women and chronic hepatitis in organ transplantation recipients (1). More recently, extrahepatic manifestations of HEV infection in particular neurologic disorders have been widely reported (2). Although ribavirin is effective as off-label treatment for some cases of chronic hepatitis E, a substantial proportion of patients are not eligible or do not tolerate the treatment. Furthermore, development of resistance mutations may result in failure of viral clearance (3, 4). Further study of HEV-host interactions is essential for understanding the pathogenesis, revealing novel antiviral targets and developing new anti-HEV therapies (5).

Mitochondria are unique double-membraned organelles present in all eukaryotic organisms. The numbers of mitochondria in a cell vary widely, but liver cells are rich in these organelles, containing more than 2,000 mitochondria per cell (6). Dysfunctions of mitochondrial are implicated in various liver diseases, in particular viral hepatitis. Acting as a powerhouse to generate energy, it plays essential roles in physiology to sustain life. Electron transport chain (ETC), a key component of mitochondria, is the main site of production of Adenosine triphosphate (ATP) and Reactive oxygen species (ROS). The ETC is located in the inner mitochondrial membrane and consists of four multi-subunit enzyme complexes (complexes I to IV). Electrons harvested from NADH through complex I or FDAH2 through complex II are received by ubiquinol. Ubiquinol subsequently transfers the electrons to cytochrome c through complex III and is oxidized to ubiquinone, which is required for the de novo biosynthesis of pyrimidines by dihydroorotate dehydrogenase (7). Cytochrome c then passes the electrons to complex IV, which uses the electrons and hydrogen ions to reduce molecular oxygen to H₂O (8, 9). During this process, the high energy of the electron is converted to the electrochemical proton gradient across the inner membrane, which drives the synthesis of ATP by the ATP-synthase. Meanwhile, leakage of electrons from the ETC leads to the production of ROS.

Accumulating evidence has shown that mitochondria serves as a signaling hub for the innate immune response and facilitate downstream signaling leading to interferon
synthesis. On the other hand, virus strategically alters mitochondrial function to influence the energy production, metabolism and immune signaling (10). In respect to defending pathogen invasion and maintaining homeostasis, mitochondria constantly communicate with cytosol to initiate biological events. Mitochondrial permeability transition pore (MPTP), a newly identified component of ETC (11, 12), is a nonselective channel that facilitates the exchange of molecules between the mitochondrial matrix and cytoplasm. Most of the RNA viruses, including hepatitis C Virus (HCV) and HEV, develop solely in cytoplasm. HCV has been shown to trigger mitochondrial permeability transition to establish chronic liver disease (13). HEV has been shown to protect cells from mitochondria depolarization (14). However, whether MPTP is involved in this process remains unknown. In this study, we aim to investigate the role of the mitochondrial ETC in HEV infection and to explore the potential of therapeutic targeting.

MATERIALS AND METHODS

Reagents and antibodies
Rotenone (ROT), 2-Thenoyltrifluoroacetone (TTFA), antimycin A (AMA), myxothiazol (MYXO), potassium cyanide (KCN), oligomycin A (OLM), ribavirin, vitamin E acetate (VE), ethidium bromide (EB), 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), uridine, cytidine, cyclosporin A (CsA) and bongkrekic acid (BKA) were purchased from Sigma-Aldrich. Hydrogen peroxide (H$_2$O$_2$) was purchased from Merck Millipore. The mouse monoclonal antibody against cyclophilin D (CypD) was purchased from Santa Cruz Biotechnology. The HEV-specific antibody against open reading frame 2 (ORF2) was purchased from EMD Millipore.

Cell culture and establishment of ETC-deficient cell culture model
Human hepatoma Huh7.5 cell line, Hep3B cell line and human glioblastoma U87 cell line were kindly provided from Department of Viroscience, Erasmus Medical Center. Huh7.5, Hep3B and U87 cells 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. ETC-deficient cell culture model was established as follows: cells harboring the p6 infectious clone were cultured in presence of medium EB (50
ng/ml), pyruvate (100 μg/ml), and uridine (50 μg/ml) for 96 h, as previously described (15, 16).

HEV cell culture models
A plasmid construct containing subgenomic HEV sequence coupled with a Gaussia luciferase reporter gene (p6-Luc) and a construct containing the full-length HEV genome (Kernow-C1 p6 clone, GenBank Accession Number JQ679013) was used to generate HEV genomic RNA by using the Ambion mMESSAGE mMACHINE® in vitro RNA transcription Kit (Life Technologies Corporation) (17, 18). Cells were electroporated with p6-Luc subgenomic HEV RNA or p6 full-length HEV RNA to generate replication luciferase and infectious models, respectively (2). For the HEV genotype 1 replicon model, viral RNA was generated from a Sar55/S17/luc-encoding plasmid. Huh7.5 cells were electroporated with Sar55/S17/luc HEV RNA to generate a genotype 1 replicon model (19).

ATP production measurement
The ATP content in cultured cells was measured using ATP Bioluminescence Assay Kit HS II according to the manufacturer’s instructions (Roche Life Science). In brief, cells were harvested and suspended in dilution buffer at a concentration of $10^5$ per ml. The same volume of cell lysis reagent was added to the above cell suspension and incubate at 15 °C for 5 min and extra 2 min at 100 °C. Then, it was centrifuged at 10000 × g for 60 s and the supernatant was transferred to a fresh tube. 50 μl of the above supernatant was mixed with luciferase reagent and was subjected to luciferase measurement using LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany). The supernatant was kept on ice until measurement.
Flow cytometry analysis

Intracellular ROS was measured by using CM-H₂DCFDA according to the manufacturer's instructions (Thermo Fisher). In brief, cells were seeded into 12-well with 5000 cells per well. After treatment with indicated reagents, cells were harvested and re-suspended in pre-warmed PBS containing 10 μM CM-H₂DCFDA and incubated at 37 °C for 20 min. After incubation, cells were re-suspended and returned to pre-warmed growth medium. Then, the cells were subjected to flow cytometry analysis with excitation of 488 nm and emission of 530 nm.

MPTP assay was performed as the manufacturer's instructions (Biovision). Briefly, cells were harvested and re-suspended using pre-warmed MPTP Wash Buffer at a final concentration of 10⁶ cells per ml. The cell suspension was then incubated with indicated reagents at 37 °C for 15 min, and centrifuged at 1000 × g for 5 min to pellet cells to remove excess staining and quenching reagents. Cells were then re-suspended in 1 ml of MPTP Wash Buffer and were subjected to flow cytometry analysis using a flow cytometer with 488 nm excitation filter. Cell suspension was kept on ice and analyzed within 1 h.

Western-blot assay

Whole cell lysates were heated at 95 °C for 8 min, followed by loading onto a 15% sodium dodecyl sulfate polyacrylamide gel and separating by electrophoresis. After separated in 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen). Subsequently, the membrane was blocked for 1 h at room temperature followed by incubation with mouse anti-CypD (1:500) antibody overnight at 4 °C. Membrane was washed 3 times followed by incubation for 1.5 h with an anti-mouse peroxidase–conjugated secondary antibody (1:10000). After washing 3 times, protein bands were detected with Odyssey 3.0 Infrared Imaging System.

Quantification of HEV replication

For the HEV luciferase model (p6-Luc), the activity of secreted Gaussia luciferase in the cell culture medium was measured using BioLux Gaussia Luciferase Flex Assay Kit (New England Biolabs, Ipswich, MA), as quantification of viral replication. Luciferase activity were quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany). All presented HEV luciferase values were
normalized by MTT assay to exclude the effect of cell toxicity on HEV replication and the MTT results were shown in Supplemental Figure 1. For the p6 infectious model, SYBR Green-based quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify genomic RNA. All the other RNA levels in this study were also quantified by qRT-PCR assay and the primer sequences were provided as below: the HEV primer sequences were 5'-ATCGGCGAGAAGTGGTTTTTAC-3' (sense) and 5'-CCGTGGCTATAAAGTGCTCT-3' (antisense); the GAPDH primer sequences were 5'-GTCTCCTCTGACTTCAAAGCG-3' (sense) and 5'-ACCACCTGTGCTGTAGCCAA-3' (antisense); the CYTB primer sequences were 5'-CCCTAACAAACTAGAGC-3' (sense) and 5'-TCTGCGCTAGTAGCCTCCT-3' (anti-sense); the COX I primer sequences were 5'-CCTGACTGCGTATTAG-3' (sense) and 5'-GATAGGATGTTTCATGTG-3' (antisense); the COX II primer sequences were 5'-CATCCCTACGCATCCTTTAC-3' (sense) and 5'-GGTCTACACAGATTTCC-3' (antisense); the COX IV primer sequences were 5'-CAGAGGGCAGCTGAGAGG-3' (sense) and 5'-TCATGTCCAGCTCCTTTG-3' (antisense); the CypD primer sequences were 5'-CGACTTACAAACACATTGCC-3' (sense) and 5'-GGTGTTAGCAGACAAAGCC-3' (antisense).

**MTT assay**

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

**Gene knockdown by lentiviral vectors**

The pLKO.1 based shRNA lentiviral vectors (Erasmus Center for Biomics) targeting CypD (shCypD) was used to knockdown CypD gene expression and scrambled control vector (shSCR) was used as control. Lentiviral pseudoparticles were generated as described previously (18). After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. To obtain stable gene knockdown cell line, cells were transduced with shRNA lentiviral particles for 3 days and selected by puromycin (Sigma-Aldrich, Zwijndrecht, the Netherlands) at a concentration of 2.5
μg/ml. The shRNA sequence was: CypD, CCGGGCTCTCTAAAGAGTGGGAGGACATCTCGAGATGTCCTCCACTCTTTAGAGCTTTTTG.

**Oxygen consumption rate measurement**

Mitochondrial respiration was measured using the High Resolution Oxygraph (OROBOROS; Oxygraph-2k, Innsbruck, Austria). Respiration was measured at 37 °C in 2 ml chambers, and the cells were suspended in HT (Hepes Tris)-buffer. HT buffer contained 4.2 mM KCl, 132 mM NaCl, 10 mM HEPES, 1.2 mM MgCl₂, and 1 mM CaCl₂, and was adjusted to pH = 7.4 with Tris. Equilibration with the ambient air was achieved when the oxygen concentration in each chamber reached a constant level. After calibrating the chambers for volume, cells were introduced to each chamber to measure the mitochondrial basal respiration. After 10 min stabilization of the oxygen flux, the basal respiration was measured for 10 min. The corresponding average oxygen consumption was calculated using Dat-Lab5 software (Oroboros, instrument, Innsbruck, Austria).

**Statistical analysis**

Statistical analysis was performed using the nonpaired, nonparametric test with Mann-Whitney test and one-way ANOVA with Bonferroni post-test (GraphPad Prism version 5.01; GraphPad Software). P values < 0.05 were considered statistically significant.

**RESULTS**

**Profiling ETC inhibitors identified an essential role of complex III in sustaining HEV replication**

To study the potential role of ETC in HEV infection, we profiled the effects of targeting different complexes of ETC by pharmacological inhibitors. The Huh7.5 liver cell line and the U87 neuronal cell line were used to model HEV infection (2). We transfected Huh7.5 and U87 cells with an HEV subgenomic replicon (p6 luciferase model) and treated the cells with various inhibitors targeting complex I, II, III or IV, respectively. In the Huh7.5-p6 luciferase model, 24 h treatment with complex III inhibitors, AMA and MYXO, significantly inhibited HEV replication (Fig. 1A) and these
effects were further enhanced after 48 h treatment (Fig. 1B). Similar results were observed in U87 cells harboring the HEV subgenomic replicon (Fig. 1D). We next evaluated this effect in both cell lines harboring the full-length infectious HEV genome (the p6 infectious model). Consistently, 48 h treatment resulted in significant inhibition of viral RNA determined by qRT-PCR (Fig. 1E and F). The anti-HEV effect was further confirmed by western blotting at viral protein level (Fig. 1G). Of note, the complex II inhibitor TTFA exerted significant inhibitory effect with high concentration after 48 h treatment in the Huh7.5 HEV subgenomic replicon cells (Fig. 1B). However, this effect was not seen in other models. Encouragingly, we found that the complex III inhibitors at 0.1 μM showed comparable inhibitory effect on HEV replication as compared to ribavirin at 100 μM (Fig. 1H). Besides, complex III inhibitors also showed strong inhibition of HEV in Hep3B cell model (Fig. 1I) and effectively restricted genotype 1 HEV-related luciferase activity in Huh7.5-based genotype 1 replicon model, Sar55/S17/luc (Fig. 1J). Taken together, complex III is essential in supporting HEV replication and can be targeted by pharmacological inhibitors to inhibit viral replication.
Figure 1. Mitochondrial complex III inhibitors specifically inhibit HEV replication. Analysis of HEV-related Gaussia luciferase activity in Huh7.5-p6 luciferase model treated with indicated concentrations of different complex inhibitors for 24 h (A) and 48 h (B) and in U87-p6 luciferase model treated with indicated concentrations of different complex inhibitors for 24 h (C) and 48 h (D). qRT-PCR analysis of HEV viral RNA level in Huh7.5 (E) and U87 (F) cell-based p6 infectious models treated with indicated concentrations of different complex inhibitors for 48 h. The above data are means ± SD of four independent experiments and presented relative to the CTR (CTR, set as 1); CTR means the DMSO treatment control. Data were normalized to housekeeping genes, GAPDH. *P < 0.05; **P < 0.01; ***P < 0.001. (G) Western-blot analysis of HEV ORF2 expression in Huh7.5-p6 infectious model after treatment with indicated concentrations of different complex inhibitors for 48 h (CTR means non-treatment control; 0.1 μM ROT; 100 μM TTFA; 0.1 μM AMA; 0.1 μM MYXO; 1000 μM KCN). (H) qRT-PCR analysis of HEV viral RNA level in Huh7.5 cell-based p6 luciferase model treated with indicated concentrations of ribavirin. (I) Analysis of HEV-related Gaussia luciferase activity in Hep3B-p6 luciferase model treated with indicated concentrations of complex III inhibitors for 24 h. (J) Analysis of genotype 1 HEV (Sar55/S17/luc) viral replication-related Gaussia luciferase activity in Huh7.5-Sar/S17/luc model treated with indicated concentrations of complex III inhibitors for 48 h. The above data are means ± SD of four independent experiments and presented relative to the CTR (CTR, set as 1); CTR means the DMSO treatment control. Data were normalized to housekeeping genes, GAPDH. *P < 0.05; **P < 0.01; ***P < 0.001.
The anti-HEV effects of complex III inhibition require the integrity of ETC

To further validate the specificity, we depleted ETC by using EB and then tested the HEV RNA level (Fig. 2A). EB is widely used to establish ETC-deficient cell model by depleting mitochondrial DNA (mtDNA) (15, 20). Cytochrome c oxidase I (COX I), cytochrome c oxidase II (COX II) and cytochrome b (CYTB) are encoded by mtDNA (21). Cytochrome c oxidase IV (COX IV) is encoded by nuclear gene and was used as a control (22). We found that, compared with Huh7 cells, U87 cells are more sensitive to EB treatment and showed less toxicity when treated with complex inhibitors (Supplemental Figure. 1I, J and K). Thus, U87 cells were employed to establish ETC-deficient cell model. After 4 d treatment, the mRNA level of COX I, COX II and CYTB showed remarkable reduction with unchanged mRNA level of COX IV (Fig. 2B). These data demonstrated the successful establishment of ETC-deficient cell model. Interestingly, EB treatment has no effect on HEV RNA level (Fig. 2C). We then tested the anti-HEV effect of AMA and MYXO in this model. Interestingly, lack of ETC strongly attenuated the anti-HEV effect of AMA and MYXO as determined by qRT-PCR (Fig. 2D, E, F and G). These data suggest that ETC is required for complex III inhibitors to restrict HEV replication.
Figure 2. ETC deficiency reverses the anti-HEV effects of complex III inhibitors. (A) U87-p6 infectious cells were treated with EB to establish ETC-deficient cell model and was maintained in a medium supplemented with uridine and pyruvate to complement the metabolic deficiency connected with the defective ETC. (B) mRNA levels of COX I, COX II, COX IV and CYTB in EB-treated or untreated group were measured by qRT-PCR. The data are means ± SD of four independent experiments and presented relative to EB-untreated group; Data were normalized to housekeeping genes, GAPDH. *P < 0.05; **P < 0.01; ***P < 0.001. (C) U87-p6 infectious cells were treated with EB for indicated time before measurement of HEV RNA. EB-treated or untreated group were incubated with 0.1 μM AMA for 2 d before measurement of HEV luciferase activity (D) and HEV RNA (E). EB-treated or untreated group were incubated with 0.1 μM MYXO for 2 d before measurement of HEV luciferase activity (F) and HEV RNA (G). Date in EB-untreated group are presented relative to EB-untreated CTR (CTR, set as 1). Date in EB-treated group are presented relative to EB-treated CTR (CTR, set as 1). The above data are means ± SD of four independent experiments. Data were normalized to housekeeping genes, GAPDH. *P < 0.05; **P < 0.01; ***P < 0.001.

The anti-HEV effect of complex III inhibition is independent of ATP and ROS production, or pyrimidine depletion

Up to 90% of ATP is produced in ETC. AMA and MYXO are well-known complex III inhibitors that specifically block the transportation of electron from cytochrome b to c, resulting in loss of intracellular ATP (15, 23, 24). We further calculated the energy cost for each step of the HEV life cycle (Fig. 3A; See details in Supporting Information), using the method as previously described for influenza virus (25). The energy consumption of HEV is lower than influenza virus; whereas influenza virus only consumes 1% of the cell energy budget. This suggests that HEV may not sensitive to the change of intracellular ATP abundance.

Dramatic reduction of oxygen consumption rate (OCR) was observed in Huh7.5 cell treated with AMA and MYXO (Fig. 3B and C). To investigate whether ATP production is involved in the anti-HEV effect, we co-incubated AMA or MYXO with AICAR, an 5’ AMP-activated protein kinase activator that is capable of accumulating ATP. AICAR effectively increased ATP level and significantly reversed the reduction of ATP caused by AMA and MYXO in Huh7.5 cells (Fig. 3D). However, AICAR neither increase the HEV RNA level nor attenuate the anti-HEV effects of AMA or MYXO (Fig. 3E). To further confirm this, we treated the Huh7.5 cells with OLM which binds to ATP synthase and inhibits ATP production. OLM showed comparable inhibition of ATP production as AMA and MYXO, but not on HEV RNA level (Fig. 3F and G).
Figure. 3. Inhibition of HEV by complex III inhibitors is independent of ATP level reduction. (A) Energetic cost of each step of HEV life cycle. 1, Cell entry ($<10^3$ ATP); 2, Intracellular transportation ($<10^3$ ATP); 3, Uncoating of capsid; 4, Translation (from parental genome); 5, Transcription ($<3\times10^5$ ATP); 6, Translation ($\sim1.1\times10^7$ ATP); 7, Budding ($\sim3.9\times10^6$ ATP). Oxygen consumption rate of Huh7.5 cells in absence and presence 0.1 μM AMA (B) and 0.1 μM MYXO (C) at indicated time point. (D) Huh7.5 cells were treated with 200 μM AICAR, 0.1 μM AMA, or 0.1 μM MYXO, alone or in combination for 3 h. After treatment, the cell lysates were subjected to ATP assay. (E) Huh7.5-p6 infectious model were treated with 200 μM AICAR, 0.1 μM AMA, or 0.1 μM MYXO, alone or in combination for 48 h before subjected to qRT-PCR analysis of HEV RNA. (F) Huh7.5 cells were treated with 0.1 μM OLM for 3 h. After treatment, the cell lysates were subjected to ATP assay. (G) Huh7.5-p6 infectious model were treated with 0.1 μM OLM for 48 h before qRT-PCR analysis of HEV RNA. The above data are means ± SD of four independent experiments and presented relative to the CTR (CTR, set as 1); CTR means the DMSO treatment control. Data were normalized to housekeeping genes, GAPDH. **P < 0.01; ***P < 0.001.

The complex III is also the major site to produce ROS. ROS has been implicated in innate immune response against pathogen infections (26). Inhibition of complex III has been shown to increase the cellular ROS level. Both AMA and MYXO increase the ROS production. H2O2 induced remarkable ROS production serving as a positive control (Fig. 4A, B and C). To investigate the potential role of ROS in the anti-HEV activity of AMA and MYXO, vitamin E was added to scavenge ROS (Fig. 4A, B and C). However, the anti-HEV effects of AMA and MYXO were not affected by supplementation of vitamin E (Fig. 4D and E). Thus, the anti-HEV effects of AMA and MYXO are likely independent of ATP and ROS production.

Strong inhibition of pyrimidine biosynthesis has been observed after treatment with AMA or MYXO (20, 27). We have previously shown that inhibition of pyrimidine pathway exerts potent anti-HEV effect (7). Besides, 2CMC, a cytidine nucleotide analogue, also has remarkable anti-HEV effect (28). However, supplementation of uridine or cytidine did not reverse the anti-HEV effects of AMA or MYXO, excluding pyrimidine depletion as a principal mechanism of their anti-HEV effects (Fig. 4F, G, H and I).
Figure. 4. Inhibition of HEV by complex III inhibitors is independent of ROS production. (A, B and C) Flow cytometry analysis of ROS level in Huh7.5 cells treated as indicated for 3 h. 100 μM VE was added 1 h before treatment with 0.1 μM AMA, 0.1 μM MYXO or 100 μM H2O2; Control means the non-treatment group. Co-incubation of 100 μM VE with 0.1 μM AMA or 0.1 μM MYXO for 48 h before measurement of HEV luciferase activity (D) or HEV RNA (E). The data are means ± SD of four independent experiments; CTR means the DMSO treatment control (CTR, set as 1). Data were normalized to housekeeping genes, GAPDH. **P < 0.01; ***P < 0.001. (F and G) Huh7.5-p6 luciferase model was treated with 0.1 μM MYXO, 0.1 μM AMA, uridine (20 μM or 200 μM) or cytidine (20 μM or 200 μM), alone or in combination for 48 h before measurement of HEV luciferase activity. (H and I) Huh7.5-p6 infectious model was treated with 0.1 μM MYXO, 0.1 μM AMA, uridine (20 μM or 200 μM) or cytidine (20 μM or 200 μM), alone or in combination for 48 h before measurement of HEV RNA by qRT-PCR assay. The above data are means ± SD of four independent experiments and presented relative to the CTR (CTR, set as 1); CTR means the DMSO treatment control. Data were normalized to housekeeping genes, GAPDH. **P < 0.01; ***P < 0.001.

HEV blocks MPTP opening and genetic silencing of the MPTP regulator enhances viral replication

MPTP, a newly identified component of ETC, is a non-selective channel through which mitochondria constantly exchange metabolites with the cytoplasm (11, 12). Opening of MPTP may lead to leakage of ETC respiratory substrates to cytoplasm (29). We investigated whether HEV interacts with the pore opening. CoCl2 quenching assay has been widely used to assess the opening of MPTP (30-32). In Huh7.5 cells, in the absence of CoCl2 and ionomycin, MPTP dye is presented in the cytosol as well as in the mitochondria (Fig. 5B), resulting in a bright signal compared to the negative control without treatment of MPTP dye (Fig. 5A). In the presence of CoCl2 alone, MPTP dye in the mitochondria emits fluorescence, but the cytosolic fluorescence is reduced by CoCl2 quenching (Fig. 5C). Opening of MPTP induced by ionomycin leads to further reduction of mitochondrial fluorescence signal in comparison to the cells treated with CoCl2 only (Fig. 5D). Interestingly, in the p6 infectious model, ionomycin-induced reduction of fluorescence was significantly reversed (Fig. 5E and F), indicating that HEV is able to block the MPTP pore opening. Genetic ablation of CypD in hepatocytes and neurons has been shown to reduce MPTP opening and effective in correcting pathological conditions that are involved in mitochondrial dysfunction, oxidative stress or cell necrosis (29, 32). To test this effect on HEV, we silenced CypD expression in Huh7.5 and U87 based p6 infectious models, which is confirmed by qRT-PCR and western-blot assays (Fig. 5G, H and I).
This resulted in $2.0 \pm 0.27$-fold (Mean ± SEM, n=4, P<0.05) and $2.9 \pm 0.14$-fold (Mean ± SEM, n=4, P<0.05) increase of HEV RNA levels, respectively (Fig. 5J and K). These data indicate a basal defense role of MPTP against HEV infection.

**Figure. 5.** HEV Blocks MPTP opening and genetic silencing of the MPTP regulator enhances viral replication. Flow cytometry analysis of MPTP opening in Huh7.5 cells (A, B, C, D) and in Huh7.5-p6 infectious cells (E) by measuring retained fluorescence after treatment with indicated molecules for 15 min. (F) Mean retained fluorescence from four independent experiments ± SEM. The data are presented relative to the CTR (CTR, set as 1); CTR means the PBS treatment control. **P < 0.01. (G) Western-blots analysis of CypD expression in Huh7.5 and U87 based p6 infectious cells transduced with lentiviral shRNA vector targeting CypD (shCypD) or scrambled control (shSCR). The CypD expression level (H and I) and HEV viral RNA level (J and K) were analyzed by qRT-PCR in stable CypD knockdown or scramble control cells. The data are means ± SEM of four independent experiments and presented relative to shSCR (shSCR, set as 1). Data were normalized to housekeeping genes, GAPDH. **P < 0.01; ***P < 0.001.

**MPTP mediates the anti-HEV action of the complex III inhibitors**

We next examined whether the anti-HEV effect of inhibition of complex III is dependent of MPTP. CsA potently prevents MPTP opening through binding and
inhibiting CypD (11). CsA alone significantly increased the HEV RNA level (Fig. 6A), which is consistent with our previous findings (18). Importantly, adding CsA significantly reversed the anti-HEV effects of AMA and MYXO (Fig. 6B and C). This result was further confirmed by adding another MPTP inhibitor, BKA, which favors the closed conformation of adenine nucleotide translocase (Fig. 6A). Thus, the anti-HEV effects of complex III inhibitors require MPTP opening.
Figure 6. AMA and MYXO inhibit HEV through MPTP. (A) Huh7.5-p6 infectious model was treated with MPTP inhibitors (5 μg/ml CsA and 50 μM BKA). HEV RNA was analyzed by qRT-PCR assay. The data are means ± SD of four independent experiments and presented relative to DMSO treatment control. Data were normalized to housekeeping genes, GAPDH. ***P < 0.001. (B and C) Co-incubation of 0.1 μM AMA, 0.1 μM MYXO, 5 μg/ml CsA and 50 μM BKA, alone or in combination for 48 h. HEV RNA was analyzed by qRT-PCR assay. The data are means ± SD of four independent experiments. Date in solely AMA or MYXO treated group were presented relative to non-treatment group (non-treatment group, set as 1). Date in combination group of AMA with CsA or BKA were presented relative to CsA or BKA solely treated group, respectively (CsA or BKA solely treated group, set as 1). Date in combination group of MYXO with CsA or BKA were presented relative to CsA or BKA solely treated group, respectively (CsA or BKA solely treated group, set as 1). Data were normalized to housekeeping genes, GAPDH. *P < 0.05; **P < 0.01; ***P < 0.001. (D) Summary diagram. HEV, BKA, and CsA inhibit MPTP opening to block the release of metabolites from mitochondrial matrix to cytosol. CypD is located in mitochondrial matrix and specifically targeted by CsA. Inhibition of complex III by AMA and MYXO leads to increase of ROS level, decrease of ATP level and inhibition of pyrimidine synthesis. UQ, ubiquinone; UQH₂, ubiquinol.

DISCUSSION

The liver is a metabolically active organ and contains abundant numbers of mitochondria. Mitochondria are the major energy source for the cell and act as central hub for multiple signal transduction, including dictating the immune response (33). Metabolites from mitochondria such as succinate and citrate are engaged in the process related to immunity and inflammation, which is essential to maintain liver homeostasis and prevent pathogen invasion (34, 35). Alteration in mitochondrial metabolic states has been associated with various liver diseases including chronic hepatitis C (36). However, the interactions of mitochondrial metabolism and HEV remain largely unexplored.

Viruses highly rely on their host for energy production, reproduction and survival. Meanwhile, the virus strategically interferes with the host metabolism to establish persistent infection. Hepatitis B virus infection has been shown to affect hepatic metabolic responses, including glucose, lipid, nucleic acid, bile acid and vitamin metabolism (37). HCV leads to increased expression of many glycolytic enzymes and change of intracellular ATP level during replication (38, 39). In turn, the host has developed various ways to combat viral infection. In this study, we roughly calculated ATP consumption of the HEV life cycle, and the total energy cost is lower than that of
influenza virus. Of note, influenza viral infection only costs 1% of the total energetic budget of eukaryotic cell (25). This suggests that HEV consumes extremely low percentage of the host energy budget which may explain why the reduction of intracellular ATP is not responsible for the anti-HEV effect of the complex III inhibitors. Release of mitochondrial ROS into the cytoplasm leads to activation of transcription factors, specifically nuclear factor-κB (NF-κB) and hypoxia-inducible factor 1α (HIF1α), which coordinate the function of cells during virus infection (33). Importantly, ROS has been shown to be involved in oligomerization of mitochondrial antiviral-signaling protein (MAVS), an adaptor for transcription and production of interferons, by remodeling the mitochondrial outer membrane property (40). Interestingly, HEV has been recently shown to be able to induce MAVS oligomerization, suggesting a potential role of mitochondrial signaling in HEV infection (41). Most of the ATP and ROS are produced from the ETC which is closely linked to the mitochondrial metabolic state. The ETC consists of series of enzyme complexes (I, II, III and IV). The important role of mitochondrial ETC in viral infection has been demonstrated by using ETC inhibitors and uncouplers (42). The hepatitis B virus-X protein has been shown to down regulate ETC complex activity. Expression of the HCV polyprotein inhibited complex I activity (43, 44). These findings suggest the specific role of different complexes in the setting of particular viral infection. Complex III has diverse biological functions (15, 20). For example, CD4+ T cells with a deficient ETC complex III fail to induce the translocation of nuclear factor to the nucleus, leading to decreased transcription of IL-2 mRNA. Loss of mitochondrial complex III results in impaired antigen-driven T cell responses in vivo (33). Of note, targeting complex III is the mode of action of a currently used antimalarial drug (45). In this study, we found that inhibition of complex III inhibits HEV replication. One of the feature that differentiates complex III from other ETC complexes is the Q cycle by which complex III moves protons and transfers electrons to cytochrome c (46). Inhibition of Qi site by MYXO showed comparable inhibitory effect on HEV with inhibition of Q0 site by AMA at the same concentration. We also found that deficiency of ETC remarkably abolishes the anti-HEV effect of these inhibitors. However, the anti-HEV effect is independent of ATP production, ROS level and pyridine depletion. HEV completes its life cycle solely in cytosol which is separated from ETC by the mitochondrial inner membrane. MPTP is a non-selective channel that allows the communication of molecules between the mitochondrial matrix to the cytosol to
convey their signals. Most recently, MPTP has been identified as a vital component of ETC (11, 12). Inhibition of CypD, a MPTP channel regulator, decreases the channel opening (47). HCV has been reported to open the MPTP to facilitate its replication (29). In this study, we have shown that silencing of CypD promotes HEV replication. This is further demonstrated by adding MPTP inhibitors, CsA and BKA. Consistent with our previous study (18), both inhibitors increase HEV replication. In contrast, CsA inhibits HCV replication. Whether these opposing effects are attributed to the specific role of MPTP on these two viruses remains an intriguing question to be further investigated.

Recent evidence showed that the HEV ORF3 protein could form an ion channel in the plasma membrane to facilitate viral replication (48). Moreover, it has been shown that ORF3 could prevent the release of cytochrome c (14). In line with these findings, we have demonstrated that HEV could robustly block the MPTP opening. MPTP is involved in several neurological diseases and is a potential therapeutic target for neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (49). Interestingly, HEV has recently been associated with several types of neurological diseases. Thus, it is intriguing to examine whether HEV-associated neurological diseases are related to its effect on MPTP.

In summary, we have identified mitochondrial ETC complex III in sustaining HEV infection through MPTP. Pharmacological inhibition of complex III effectively inhibits HEV replication. Because therapeutic targeting of complex III has been widely explored for treating different diseases, repurposing or optimizing these existing FDA-approved or upcoming drugs represents a viable option for therapeutic development against HEV infection.

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C. Qu and Q. Pan wrote the paper; S. Zhang and N. Raat contributed new reagents or analytic tools.

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Chapter 4

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 1,000 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.

Key words: HEV, NF-κB signaling, RIPK1, Caspase, Deptropine
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 [1]. It has caused many large hepatitis outbreaks, especially in resource-limited regions [2]. 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 [3]. 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 [4] in particular have been explored in the clinic for treating some chronic HEV cases. Ribavirin is effective in general, but failure of treatment has been frequently reported [5]. 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 [6]. 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 [7, 8], and likely it is not very effective against HEV [8]. In parallel, various preclinical compounds, including nucleoside and non-nucleoside antiviral agents [9], protein kinase-targeted compounds [10], inhibitors targeting mitochondrial metabolism [11], inhibitors of nucleotide synthesis [12] and natural compounds [13], 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.
In this study, we screened a library of FDA-approved drugs. We identified deptyropine, a traditional histamine receptor H1 antagonist used to treat asthmatic symptoms [14, 15], as a potent inhibitor against HEV. Mechanistically, the anti-HEV activity of deptyropine 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.

MATERIALS AND METHODS

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 10000 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.

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 [16].

For genotype 3 HEV models, Huh7.5, Hep3B, and 293T cell lines were electroporated with the subgenomic HEV sequence (p6 clone) coupled with a Gaussia luciferase reporter gene (replicon model) and a construct containing the full-length HEV genome (infectious model), as described previously [17]. For HEV
genotype 1 replicon model, Huh7.5 cells were electroporated with Sar55/S17/luc HEV RNA, and viral replication was detected by Gaussia luciferase activity [16]. 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 [18]. For rotavirus model, Caco2 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 related promoter, respectively.

**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).
RESULTS

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 hours (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 and less than 50% cytotoxicity were selected (Supplementary Table 1). Their antiviral effect 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.
Figure 1. Screening of a drug library identified depropine 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 1,280 compounds with 95% of FDA-approved drugs at 10 μM. The HEV-related Gaussia luciferase value (HEV RLU) was measured 48 hours 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 hours. Data were normalized to the DMSO vehicle control (set as 1) and presented in dot plots. The black dot represents depropine.
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 (Figure 2A), deptropine dose-dependently inhibited HEV replication in HEK293T (Figure 2B) and Hep3B cells (Supplementary Figure 2A). 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 (Figure 2C).

We next examined the effect of long-term treatment. 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 (Figure 2D). We further evaluated whether deptropine has a broad antiviral activity and found that deptropine potently inhibits HCV (Supplementary Figure 2C) but not rotavirus (Supplementary Figure 2D) replication.
Figure 2

A

24 hours 48 hours 72 hours

HEV RLU (relative)

Huh7.5

293T

48 hours

HEV RNA (relative)

0.0

1.0

2.0

CTR

0.1 μM De tropine

1 μM De tropine

10 μM De tropine

B

24 hours 48 hours 72 hours

HEV RLU (relative)

48 hours

HEV RNA (relative)

0.0

1.0

2.0

CTR

0.1 μM De tropine

1 μM De tropine

10 μM De tropine

C

Huh7.5

293T

HEV RLU (relative)

log₂(De tropine concentrations) μM

HEV RNA (relative)

log₂(De tropine concentrations) μM

log₂(De tropine concentrations) μM

CC50

IC50

D

Huh7.5

HEV RNA (relative)

days

CTR

5 μM De tropine

HEV ORF2

β-actin
Figure 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).

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 (Supplementary Figure 3A), which is consistent with a previous study showing that Huh7.5 cells are deficient of this receptor [19]. Diphenhydramine, another H1 antagonist (Figure 3A), exerted no inhibitory effect on HEV in both Huh7.5 and 293T cells (Figure 3B). Chlorcyclizine HCl (CCZ), an H1 antagonist that has been shown to block HCV entry [20], also could not inhibit HEV (Figure 3C and Supplementary Figure 3B), or HCV replication in a subgenomic replicon model (Supplementary Figure 3B). 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 (Figure 3D). Taken together, these results indicate that deptropine potently inhibits HEV replication.
independent of the histamine signaling, whereas we do not fully exclude 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 [21], has been shown to exert its effect through alpha-7 nicotinic receptor (α7 receptor) and expression of this receptor has been validated in hepatocytes [22]. 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 (Supplementary Figure 3C). 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 (Supplementary Figure 3C), suggesting a α7 receptor-independent antiviral mechanism ofdeptropine.
Figure 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 hours. 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 hours. 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 hours 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 hours 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).
Deptropine Inhibits HEV in a 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 [23]. Moreover, the H1 antagonists are shown to inhibit NF-κB activity [24]. 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 (Figure 4A). In contrast to the potent NF-κB activator TNFα, treatment with deptropine significantly inhibited the NF-κB transcription activity (Figure 4B). Supplementation with TNFα significantly attenuated the inhibitory effects of deptropine on NF-κB and anti-HEV activity (Figure 4C), suggesting the possible involvement of NF-κB in the anti-HEV action of deptropine.

To further confirm this, wild type (WT), NF-κB knockout (NF-κB-/-) mouse embryonic fibroblasts (MEF) cells were used. 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 (Figure 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 (Supplementary Figure 4A) and deptropine could inhibit AP-1 activity (Supplementary Figure 4B). Moreover, associated with the obtained results that histamine could partially reverse the antiviral effect of deptropine in the HEV infectious model (Figure 3D), the inhibitory effects of either AP-1 or NF-κB transcriptional activity by deptropine was also partially attenuated after adding histamine (Supplementary Figure 4C).
Figure 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 hours 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 hours before testing NF-κB luciferase value and for 48 hours 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 hours 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 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 hours 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).

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 [25]. We found that the loss of NF-κB signaling dramatically abolished the increase of caspase 3/7 activity induced by deptropine (Figure 4D). Concurrently, the expression of survivin was significantly reduced after treatment with deptropine (Figure 4D).

Caspase activity is well-recognized for regulating viral infection. We found that deptropine significantly elevated cellular caspase 3 and 7 activity (Figure 5A), accompanied with a low level of apoptosis induction (Supplementary Figure 5A). Interestingly, HEV infection further augments the induction of caspase activity by deptropine (Figure 5A). Importantly, co-incubation with Z-VAD-FMK, a general caspase inhibitor, attenuated the caspase induction and anti-HEV activity by deptropine. Similar results were observed in the combination of TNFα with Z-VAD-FMK (Figure 5B). Moreover, in line with the results of histamine on HEV (Figure 3D), treatment with histamine for 48 hours significantly increased the caspase activity and decreased the activation of caspase induced by deptropine (Figure 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 (Figure 5C), and this is accompanied by the increase of HEV RNA when deptropine was added in combination with caspase 3 inhibitor (Figure 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 (Supplementary Figure 5B), 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 (Supplementary Figure 5C). Taken together, these data suggest that the basal caspase activity may support HEV replication; however the extra caspase activation contributes to the anti-HEV ability of deptropine.
Figure 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 hours 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 hours 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 hours 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 hours 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 hours 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).

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 [26]. We examined the potential involvement of RIPK1. We found that gene silencing of RIPK1 significantly inhibited HEV replication (Figure 6A and 6B). Importantly, loss of RIPK1 completely blocked the anti-HEV effect of deptropine (Figure 6C). The kinase activity of RIPK1 is essential in promoting apoptosis [27]. Consistently, we found that deptropine robustly induced phosphorylation of RIPK1 and the phospho-PIPK1 is remarkably decreased in the presence of caspase 3 inhibitor (Figure 6D). These results indicate that the kinase activity of RIPK1 is essential in caspase-mediated anti-HEV effect of deptropine (Figure 5D).

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 (Figure 7A), but synergistic effect with ribavirin (Figure 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 (Supplementary Figure 6A). Although it has been reported that IFNα is able to induce caspase activation [28], no increased caspase 3 and 7 activity was observed after treatment with IFNα for 48 hours in Huh7.5 cells (Supplementary Figure 6B). Furthermore, the anti-HEV effect of IFNα was not affected in cells lacking RIPK1 (Supplementary Figure 6C), which is distinct from the mechanism of deptropine.
Figure 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 hours 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 hours. The levels of indicated proteins were analyzed by western blot assay.
Figure 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).
**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 [29]. 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 [4]. 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 [30]. In this study, we found that supplementation with histamine has no effect 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 [31] and HCV by H1 antagonists [31]. However, the antiviral effect of H1 antagonist seems not require the H1 receptor [20].

It has been reported that H1 antagonist could regulate the transcription factor NF-κB and caspase activity [32]. 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 [33]. 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 [34, 35]. For hepatic viruses, HCV was reported to increase NF-κB and AP-1 activation, which was further augmented by the presence of hepatitis B virus X protein [36]. 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 [37]. Consistent with a recent study [38], we found that the presence of HEV ORF2 protein in cell nucleus (Supplementary Figure 4D). 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 [39]. Thus, the interactions of HEV with NF-κB multifaceted, requiring further clarification.

There are mutual interactions between caspase activation and viral infection [40]. Several viruses express proteins targeted by the caspase protease and cleavage of these protease results in inhibition of apoptosis [41, 42]. HCV infection induces caspase activation to cleave the viral nonstructural protein 5A, which subsequently translocates to nucleus to enhance the transcription of several NF-κB target genes to inhibit apoptosis [43]. However, some viruses can benefit from caspase activation. Influenza A virus and papillomaviruses require caspase activity for efficient replication [44, 45]. Recent evidence indicate that HEV infection is associated with caspase activation [46]. 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 [47]. Consistently, our recent study has shown that HEV is able to block ionomycin-induced MPTP opening [11], which is a vital process to activate caspase. In this study, we found that blocking of the excessive caspase activation induced by depropine 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 [48, 49]. 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 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 viral hepatitis by 2030.

Notes

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

Conflict of interest statement. The authors declare that they have no competing interests.
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Supplemental Information

Reagents and Antibodies

Dextropine, diphenhydramine, chlorcyclizine HCl, nicotine, histamine, TNFα, ribavirin, and IFNα were bought from Sigma-Aldrich. Caspase 1, 3, 8 and 9 inhibitors, and Z-VAD-FMK were brought from Santa Cruz Biotechnology. α-bungarotoxin was purchased from Abcam. The antibody against receptor-interacting protein kinase 1 (RIPK1) was purchased from R&D Systems. The antibody against Phospho-RIPK1 (Ser166) was purchased from Cell Signaling Technology, the Netherlands. The HEV-specific antibody against open reading frame 2 (ORF2) was purchased from Millipore (MAB8002).

Apoptosis Measurement

Apoptosis was measured using the Annexin V/FITC apoptosis detection kit from BD (BD Pharmingen™). Cells (1 × 10⁵/well) were treated with indicated drugs for 48 hours and harvested by centrifugation at 1,000 rpm. Cells were then resuspended in binding buffer and stained with Alexa Fluor 488 Annexin V and PI at room temperature for 15 mins. Detection of apoptosis was performed by FACS and the results were analyzed by FlowJo software. The scatter plot was divided into four quadrants: the left lower quadrant represents viable cells; the left upper quadrant represents necrotic cells; the right lower quadrant represents early apoptotic cells; and the right upper quadrant represents late apoptotic cells.

Caspase 3/7 Activity Measurement

Caspase 3/7 activity was measured according to the manufacturer’s instructions (Caspase-Glo® 3/7 Assay Systems, Promega). In brief, Huh7.5 cells (1 × 10⁴/well) were seeded into 96 well, with a total volume of 100 μL per well. After treatment with indicated drugs for 48 hours, 100 μL of Caspase-Glo® 3/7 Reagent was added into each well and incubated at 37 °C for 1 hour. After incubation, cells were subjected to luciferase detection assay.

Confocal laser electroscope assay

Cells grown on coverslips were fixed with 4% (w/v) paraformaldehyde (PFA) for 10 mins at room temperature. After washing with PBS for three times, cells were
permeabilized with 0.1% (v/v) Triton X-100 for 10 mins and washed three times with PBS. Cells were then incubated with primary anti-HEV ORF2 (1:200) antibody at 4°C overnight. Subsequently, samples were incubated with 1:1000 dilutions of anti-mouse-Alexa Fluor® 488-conjugate antibody (Cell Signaling Technology) for 1 hour at room temperature. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Images were detected using confocal electroscope.

**Luciferase Quantification Assay**

For Gaussia luciferase, the secreted luciferase activity in the cell culture medium was measured by BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs). For firefly luciferase, luciferin potassium salt (100 mM; Sigma) was added to cells and incubated for 10 mins at 37 ºC. The luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG Lab Tech, Offenburg, Germany). All HEV-related luciferase values were normalized by MTT assay to exclude the effect of cell toxicity on the luciferase activity.

**Western Blot Assay**

Whole cell lysates were heated at 95 ºC for 8 minutes, followed by loading onto a 15% sodium dodecyl sulfate polyacrylamide gel and separating by electrophoresis. After separated in 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen). Subsequently, the membrane was blocked with blocking buffer (Li-COR, Lincoln, USA) mixed with PBS containing 0.05% Tween 20 (PBST) in ratio of 1:1 for 1 h at room temperature, followed by incubation with mouse RIPK1 (1 μg/mL) or rabbit phospho-RIPK1 (1:800) antibody overnight at 4 ºC. Membrane was washed 3 times followed by incubation for 1.5 hours with an anti-mouse peroxidase–conjugated secondary antibody (1:10000). After washing 3 times, protein bands were detected with Odyssey 3.0 Infrared Imaging System.

**Quantitative Real-time Polymerase Chain Reaction**

RNA was extracted with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified by using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared using the cDNA Synthesis Kit (TAKARA BIO INC). The cDNA was amplified for 50 cycles and quantified with a SYBR-Green-based real-time PCR
(Applied Biosystems) according to the manufacture’s instruction. The primer sequences were provided as below: the HEV primer sequences were 5’-ATCGGCCAGAAAGTTGGTTTTTAC-3’ (sense) and 5’-CCGTGGCTATAACTGTGGTCT-3’ (antisense); the GAPDH primer sequences were 5’-GTCTCCTCTGACTTCAACAGCG-3’ (sense) and 5’-ACCACCCTGGTTGCTGTAGCCAA-3’ (antisense); the survivin primer sequences were 5’-CCACTGAGAACGAGCCAGACTT-3’ (sense) and 5’-GTATTACAGGCAGTAAGCCACC-3’ (antisense); the RIPK1 primer sequences were 5’-TATCCAGTGCTGAGACCAAC-3’ (sense) and 5’-GTAGGCTCCAATCTGAATGCCAG-3’ (antisense); the SA11 Rotavirus primer sequences were 5’-TGGTTAAACGCAGGATCGGA-3’ (sense) and 5’-AACCTTTCCGCGTCTGGTAG-3’ (antisense).

**MTT assay**

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

**Gene Knockdown by Lentiviral Vectors**

The pLKO.1 based shRNA lentiviral vectors (Erasmus Center for Biomics) targeting RIPK1 (shRIPK1) was used to knockdown RIPK1 gene expression and scrambled control vector (shCTR) was used as control. Lentiviral pseudoparticles were generated as described previously [1]. After pilot study, the shRNA vectors exerting optimal gene knockdown were selected. To obtain stable gene knockdown cell line, cells were transduced with shRNA lentiviral particles for 3 days and selected by puromycin (Sigma-Aldrich, Zwijndrecht, the Netherlands) at a concentration of 2.5 μg/mL. The shRNA sequence was: shRIPK1 No.1, CCGGCCCTTTGATAATGACTTCCACTCGAGTGGAAGTCATTATCAACAAGGTTT TT; shRIPK1 No.2, CCGGCCCTTTTCAACAGAGGAGGAAACTCGAGTTTTCTCCTCTCTCTGTTTGATTTT
### Supplementary Table 1.

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From the primary screening, 38 drugs identified with more than 50% inhibition on HEV replication-related luciferase activity and less than 50% cytotoxicity (by MTT assay) were selected for further validation in HEV infectious model.
Figure S1. Huh7.5 cells were treated with drugs for 48 hours before being subjected to MTT assay. Points represent the relative MTT value for each drug.
Figure S2. Evaluation of the antiviral effect of deptropine.

(A) Hep3B cell based HEV replicon model was treated with indicated concentrations of deptropine for indicated time period before the measurement of HEV luciferase. The untreated (CTR) group serve as control (set as 1) \((n = 4)\). (B) Hep3B cells were treated with indicated concentrations of deptropine. MTT assay was performed after 72 hours \((n = 4)\). (C) HCV replicon model was treated with 10 μM deptropine for indicated time period before measurement of HCV-related luciferase \((n = 4)\). (D) Caco2 cells harboring SA11 rotavirus were treated with deptropine for 48 hours before qRT-PCR analysis of virus RNA \((n = 6)\). The above data are means ± SD. (*\(P < 0.05\); **\(P <0.01\)).
Figure S3. (A) The basal expression levels of histamine receptors were evaluated by qRT-PCR. (B) Huh7.5 cell based HEV replicon model and Huh7.5 cell based HCV replicon model were treated with Chlorcyclizine HCl (CCZ) for indicated time period before the measurement of virus-related luciferase (n = 4). Huh7.5 cells were treated with indicated concentration of CCZ for 72 hours before being subjected to MTT assay to evaluate the cell viability (n = 6). (C) The basal expression levels of alpha-7 nicotinic receptor were evaluated by qRT-PCR. Huh7.5 cell based HEV infectious model was treated with indicated concentration of α-bungarotoxin for 48 hours before measurement of HEV RNA by qRT-PCR assay (n = 6). Huh7.5 cell based HEV replicon model was treated with indicated concentration of compounds for 48 hours before measurement of HEV luciferase (n = 5). The above data are means ± SD. (**P < 0.001).
Figure S4. (A) The Huh7.5 cell based AP-1 reporter model 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 AP-1 luciferase was measured at indicated time point (n = 4). (B) The Huh7.5 cell based AP-1 reporter model was treated with indicated agents for 24 hours before testing the AP-1 luciferase value. The untreated group serves as control (set as 1) (n = 6). (C) The Huh7.5 cell based AP-1 reporter model and NF-κB reporter model were treated with indicated agents for 24 hours before testing the luciferase value. Data in deproidine-treated group was presented relative to the un-treated control group (set as 1). Data in combination group of deproidine with histamine was presented relative to histamine-solely treated group (set as 1) (n = 6-8). The above data are means ± SD. (***P < 0.001). (D) Immunofluorescence staining of Huh7.5 cells harboring HEV RNA. HEV capsid protein (green; anti-ORF2) and DAPI (blue). Cells were visualized with 63 x oil immersion lens at identical settings.
Figure S5. (A) Flow cytometry analysis of apoptosis in Huh7.5 cells treated with indicated agents for 48 hours. (B) Huh7.5 cell based HEV infectious model was treated with indicated concentration of caspase inhibitors for 48 hours before measurement of HEV RNA by qRT-PCR assay (n = 4). (C) HCV replicon model was treated with indicated agents for indicated time period before measurement of HCV-related luciferase. Data in deptropine-solely treated group was presented relative to the untreated control group (set as 1). Data in combination group of deptropine with caspase 3 inhibitor or Z-VAD-FMK were presented relative to caspase inhibitor or Z-VAD-FMK-solely treated group, respectively (set as 1) (n = 4). The above data are means ± SD. (*P < 0.05; ***P < 0.001).
Figure S6. (A) The Huh7.5 cell based NF-κB reporter model were treated with indicated agents for 24 hours before testing luciferase value (n = 4). (B) The Huh7.5 cells were treated with indicated agents for 48 hours before the measurement of caspase 3 and 7 activity (n = 6). (C) Huh7.5 based HEV infectious model transduced with lentiviral shRNA vector targeting RIPK1 (shRIPK1) or scrambled control (shCTR). shRIPK1 or shCTR cells were incubated with indicated concentration of IFNα for 48 hours before measurement of HEV RNA. Data in shCTR group were presented relative to the untreated shCTR group (set as 1). Data in shRIPK1 group was presented relative to the un-treated shRIPK1 group (set as 1). (n = 3). The data are means ± SD. (*P < 0.05; ***P < 0.001).

REFERENCE

Chapter 5

Nucleoside analogue 2’-C-methylcytidine inhibits hepatitis E virus replication but antagonizes ribavirin

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ABSTRACT
Hepatitis E virus (HEV) infection has emerged as a global health issue; whereas no approved medication is available. The nucleoside analogue 2'-C-methylcytidine (2CMC), a viral polymerase inhibitor, has been shown to inhibit the infection of a variety of viruses, including hepatitis C virus (HCV). Here, we report that 2CMC significantly inhibits the replication of HEV in a subgenomic replication and a full-length infectious models. Importantly, long-term treatment with 2CMC did not attenuate its antiviral potency, indicating a high barrier to drug resistance development. However, the combination of 2CMC with ribavirin, an off-label treatment for HEV, exerts antagonistic effects. Our results indicate that 2CMC serves as a potential antiviral drug against HEV infection.

Key words: 2'-C-methylcytidine, hepatitis E virus, replication, antiviral drug, nucleoside analogue
INTRODUCTION

Hepatitis E virus (HEV) is a single-stranded, positive-sense RNA virus, and its genome consists of three open reading frames (ORFs). ORF1 encodes a polyprotein that has all the nonstructural proteins needed for HEV replication. ORF2 encodes the capsid protein of the HEV virion. ORF3 encodes a small multifunctional protein with a molecular mass of 13kDa [11]. HEV was initially thought to only cause acute infection confined only to developing countries. However, over the last decade, hepatitis E cases are frequently reported in developed countries, and have been recognized mainly as autochthonous cases rather than an imported disease [11, 12]. Generally, HEV infection is self-limiting and asymptomatic with a consequence of low mortality rate; whereas it can cause high mortality in pregnant women. However, in immunocompromised patients receiving organ transplantation, more than 60% of HEV-infected patients will develop chronic disease and quickly progress towards severe liver complications such as fibrosis and cirrhosis [20]. Besides hepatitis, this virus has been associated with a broad range of extra-hepatic manifestations, in particular renal and neurological injuries [14, 21]. Therefore, the development of specific antiviral drugs for HEV infection is urgently required.

Nucleoside analogues have been used in clinic for almost 50 years and represent as cornerstones for treating patients with cancer or viral infection. Ribavirin (RBV) has been used as an off-label antiviral drug showing high efficacy in many chronic HEV patients, but HEV mutations associated with ribavirin treatment failure have been reported [4, 7]. Sofosbuvir (SOF), a potent direct-acting agent (DAA) against hepatitis C virus (HCV) [2], has been recently suggested to inhibit HEV replication in cell culture and exert an additive effect when combined with ribavirin [4]. However, other in vitro and clinical studies have demonstrated that sofosbuvir is not very effective against HEV infection [8, 18, 19], suggesting that this drug might not a promising candidate for the treatment of chronic HEV patients.

2'-C-methylcytidine (2CMC) was initially identified as a competitive inhibitor of the HCV RNA-dependent RNA polymerase (RdRp). Besides HCV, it has been shown to inhibit the replication of a variety of other viruses (e.g. dengue virus and norovirus) [13, 15]. It also has been reported to inhibit cutthroat trout virus, a non-pathogenic fish virus, which is remarkable similar to HEV [6]. In this study, we have demonstrated that 2CMC efficiently inhibit HEV replication, thus serves as a potential candidate for anti-HEV drug development.
MATERIALS AND METHODS

Reagents and Antibodies

2CMC, RBV, Guanosine triphosphate (GTP) and Cytidine 5'-Triphosphate (CTP) were purchased from Sigma-Aldrich, and were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO). The HEV-specific antibody was purchased from EMD Millipore (MAB8002).

HEV Cell Culture Models

Multiple cell lines were employed in this study, including human hepatoma cell line (Huh7 and PLC/PRF/5), human embryonic kidney cell line (HEK293), human primary glioblastoma cell line (U87), human fetal lung fibroblast cell line (MRC5). Huh7 and U87 cell lines were kindly provided by Professor Bart Haagmans from Department of Viroscience, Erasmus Medical Center. Human embryonic kidney 293 cell line, PLC/PRF/5 and MRC5 were originally obtained from ATCC (www.atcc.org). These cells were cultured in Dulbecco’s modified Eagle medium (Lonza Biowhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For the full-length HEV model, a plasmid construct containing the full-length HEV genome (Kernow-C1 p6 clone; GenBank Accession Number JQ679013) was employed to generate HEV genomic RNA by using the Ambion mMessage mMachine in vitro RNA transcription Kit (Thermo Fisher Scientific Life Sciences) [16]. Huh7, PLC/PRF/5, HEK293, U87 and MRC5 cells were electroporated with full-length HEV genome RNA to generate consecutive HEV-infected cell models (Huh7-p6, PLC/PRF/5-p6, HEK293-p6, U87-p6 and MRC5-p6).

To generate the subgenomic (p6-Luc) HEV model, a plasmid construct containing subgenomic HEV was used. This plasmid has an HEV sequence in which the 5’ portion of HEV ORF2 was replaced with the in-frame Gaussia princeps luciferase reporter gene [16]. Huh7, U87 and HEK293 cells were electroporated with HEV subgenomic RNA to generate HEV subgenomic models (Huh7-p6-Luc, U87-p6-Luc, and HEK293-p6-Luc). To normalize non-specific effects of 2CMC on the luciferase signal, Huh7 cells stably expressing a non-secreted firefly luciferase under control of the human phosphoglycerate kinase (PGK) promoter (PGK-Luc) were used [18]. In addition, Huh7 cells harboring a subgenomic HCV bicistronic replicon (I389/NS3-3V/LucUbiNeo-ET) (Huh7-HCV-Luc) were used as positive control of antiviral activity.
Quantification of HEV Replication

For Gaussia luciferase, the secreted luciferase activity in the cell culture medium was measured by BioLux® Gaussia Luciferase Flex Assay Kit (New England Biolabs). Gaussia luciferase activity was quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany). For the full-length infectious models (HEV-p6), intracellular viral RNA was quantified. RNA was isolated with a Machery-Nucleo Spin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (TAKARA BIO INC). HEV RNA level was quantified with a SYBR Green-based real-time PCR (Applied Biosystems® SYBR® Green PCR Master Mix, Life technologies, CA, USA) according to the manufacturer’s instructions. PCR steps consisted of a 10 min holding stage (95 °C) followed by 40 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was considered as reference gene to normalize gene expression. Relative gene expression was normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT_{\text{sample}} - \Delta CT_{\text{control}}$). The HEV primer sequences were as followed: HEV-F 5'-ATTGGCCAGAAGTTGGTTTTCAC-3'; HEV-R 5'-CCGTGGCTATAATTGTGGTCT-3'; GAPDH-F 5'-TGTCATCCACCCCAATGTATC-3'; GAPDH-R 5'-CTCGATGCGCTGACTACCTT-3'.

**MTT assay**

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

**Long-term treatment assay**

For the long-term treatment assay of subgenomic model (Huh7-p6-luc), the cells were seeded into 96 wells with 5000 cells per well. The cells of CTR or 2CMC treatment groups were passaged and seeded with the same cell numbers every 3 days (d), meanwhile maintaining the cell incubated with vehicle (non-treatment) or 2CMC (10 µM) respectively throughout the entire incubation period. For the long-term treatment assay of infectious model (Huh7-p6), the cells were seeded into 48 wells
with \(2 \times 10^4\) cells per well. The cells of CTR or 2CMC treatment groups were passaged and seeded with the same cell numbers every 3 days, meanwhile maintaining the cell incubated with vehicle (non-treatment) or 2CMC (10 µM) respectively throughout the entire incubation period.

**Western blot assay**
Cultured cells were lysed in Laemmli sample buffer containing 0.1 M DTT and heated 5 mins at 95 °C, followed by loading onto a 10 % sodium dodecyl sulfate polyacrylamide gel and separation by electrophoresis. After 90 mins running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride membrane (Invitrogen) for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with a mixture of 2.5 ml blocking buffer (Odyssey) and 2.5 ml phosphate-buffered saline containing 0.05% Tween 20. It was followed by overnight incubation with anti-HEV capsid protein primary antibodies (1:1000) at 4 °C. The membrane was then washed 3 times followed by incubation for 1 h with goat anti-mouse IRDye-conjugated secondary antibody (Li-COR Biosciences, Lincoln, USA) (1:5000). After washing 3 times, protein bands were detected with the Odyssey 3.0 Infrared Imaging System.

**IC50 and CC50 calculation**
50% inhibition concentration (IC50) value and 50% cytotoxic concentration (CC50) were calculated based on model \(Y = \frac{Bottom + (Top - Bottom)}{1 + 10^{((\text{LogIC50 - X)})*\text{HillSlope})}\) by using GraphPad Prism 5 software (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA, USA).

**Statistical Analysis**
Statistical analysis was performed using the nonpaired, nonparametric test with Mann-Whitney test and one-way ANOVA with Tukey’s Multiple Comparison post-test (GraphPad Prism version 5.01; GraphPad Software). P values <0.05 were considered statistically significant.
RESULTS

In this study, the potential anti-HEV effect of 2CMC was investigated in HEV replication models with concentrations ranging from 0.1 μM to 10 μM. We demonstrated that 2CMC significantly reduced HEV-driven luciferase activity, and the anti-HEV activity was even comparable with its anti-HCV effect at the concentration of 10 μM (Fig. 1A). IC50 value of 2CMC against HEV replication was 1.64 μM, CC50 of 2CMC to Huh7 cells was 111.2 μM and selectivity index (SI, CC50/IC50) was 67.8 (Fig. 1B). The anti-HEV effect of 2CMC was further confirmed in the full-length (Kernow-C1, p6) infectious model of HEV genotype 3 by both RT-PCR assay (Fig. 1C) and western blot assay (Fig. 1D).
Figure 1. 2CMC exerts potent anti-HEV effect. (A) Huh7-p6-Luc cells and Huh7-HCV-Luc cells were treated with indicated concentrations of 2CMC for 24h, 48h, or 72h, and the untreated (CTR) group serves as control. Luciferase value was measured at indicated time points. Data are means ± SEM of four independent experiments; CTR means the non-treatment control. *P < 0.05; **P < 0.01; ***P < 0.001. (B) Huh7-p6-Luc cells were treated with 10 μM 2CMC for 48 h. 50% inhibition concentration (IC50) and 50% cytotoxic concentration (CC50) of 2CMC against HEV replication were calculated using GraphPad Prism 5 software. (C) Huh7-p6 cells were treated with indicated concentrations of 2CMC for 48 h. RT-PCR analysis of HEV RNA or Cell viability analysis were performed. Data are means ± SEM of four independent experiments; CTR means the non-treatment control. Abs 490 means absorption at 490 nm. ***P < 0.001. (D) Immunoblot analysis of HEV ORF2 protein level in Huh7 cell based HEV infectious cell model (Huh7-p6) treated with 2CMC (10 µM) for 48 h. Data are means ± SEM of four independent experiments; CTR means the non-treatment control. *P < 0.05. (E) Hepatic and extra-hepatic cells were treated with indicated concentrations of 2CMC for 48 h. RT-PCR analysis of HEV RNA was performed. Data are means ± SEM of four independent experiments; CTR means the non-treatment control. (F) HEK293T-p6-luc and U87-p6-luc cells were treated with indicated concentrations of 2CMC for 48 h and then were subjected to luciferase activity analysis. Data are means ± SEM of three independent experiments; CTR means the non-treatment control. *P < 0.05. (G) Indicated cells were treated with 2CMC for 48 h and then the cells were subjected to cell viability analysis using MTT assay.

Since HEV related extra-hepatic manifestations have been reported [12], we extended our study to some other hepatic and extra-hepatic cell lines. HEV infectious or replication models were established in HEK293, PLC/PRF/5, MRC5 and U87 cells. The anti-HEV potential of 2CMC in these cell lines was tested. In line with the results observed in Huh7-based HEV replication and infectious models, we observed similar anti-HEV effect of 2CMC in all these cell models without affecting the cell viability (Fig. 1E to G). Drug resistance is a main factor that limits the effectiveness of the antiviral treatment. To characterize 2CMC in this respect, we performed experiments in which both HEV replication and infectious models were constantly exposed to 2CMC (10 µM). Interestingly, 2CMC retained its anti-HEV activity in both models even after long-term exposure (Fig. 2A and B). Furthermore, the negative control retained high levels of luciferase activity after long-term incubation with 2CMC, excluding the loss of cell viability during the experimental period (Fig. 2C). Taken together, 2CMC displays a high barrier for drug resistance development.
Chapter 5

Figure 2. 2CMC retains anti-HEV effect in Huh7-p6-luc and Huh7-p6 models after long-term treatment. (A) Treatment of 2CMC in the Huh7-p6-luc model for 27 days. The cells were passaged every 3 days, and were incubated with vehicle (non-treatment) or 2CMC (10 μM) throughout the entire period. Data are means ± SEM of four independent experiments; CTR means the non-treatment control. (B) Treatment of 2CMC in the Huh7-p6 model for 15 days. The cells were passaged every 3 days, and were incubated with vehicle (non-treatment) or 2CMC (10 μM) throughout the entire period. Data are means ± SEM of four independent experiments. (C) Treatment of 2CMC in the Huh7-p6-luc model for 27 days. The absolute luciferase values of Huh7-p6-luc cells are measured at indicated time points.

Theoretically, nucleoside/nucleotide analogs serve as potential direct-acting antivirals because they bind to the viral RNA polymerase active site to block viral replication. To evaluate the inhibitory specificity of 2CMC against HEV replication, we performed a competition assay employing the substrate cytidine triphosphate (CTP) as an analogous competitor of 2CMC. Our results indicated that the CTP dose-dependently reversed the inhibitory effects of 2CMC on HEV replication activity. In contrast, guanosine triphosphate (GTP) exerted no effect, implying the inhibitory specificity of 2CMC against HEV replication (Fig. 3A and B).
Figure 3. Combination of CTP and GTP with 2CMC in Huh7-p6-luc model (A) and Huh7-p6 (B) cell models. The cells were treated with 2CMC, CTP or GTP, alone or in combination for 72 h before measurement of luciferase activity. Data are means ± SEM of four to six independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

Another nucleoside analogue, ribavirin, has been used as an off-label treatment for HEV infection in clinic. Thus, its combination with 2CMC was tested related to their anti-HEV effects. Interestingly, a moderate antagonistic effect (-36.93 µM² %) was observed, implying a similar antiviral mechanism they employed (Fig. 4A and B).
Figure 4. 2CMC antagonizes ribavirin in the Huh7-p6-luc model. (A) The Huh7-p6-Luc cells were treated with 2CMC and ribavirin, alone or in combination, for 72 h before analysis of luciferase activity. Untreated group serves as control. (B) The combinatory effect of 2CMC and ribavirin on HEV replication was analyzed using the mathematical model MacSynergy. 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 of the two compounds. Data are means ± SEM of three independent experiments.

Discussion
Nucleoside analogue has been used against a variety of viruses due to its broad spectrum of antiviral effects and its high barrier to drug resistance development. Ribavirin, an guanosine analogue, is the choice for treating most of the chronic HEV patients. However, treatment failure has been observed in some cases. Sofosbuvir, a prodrug of a uridine nucleoside analogue that is very effective against HCV, has been recently investigated for its anti-HEV potency. However, debates have been sparked regarding its potency against HEV [9, 17]. 2CMC, a cytidine nucleoside
analogue, has been shown to inhibit the infection of a variety of viruses, including HCV and HIV [5]. In this study, we have demonstrated that 2CMC potently inhibits HEV replication in different cell models, even though a slight difference between these different cell models (Fig 1A and C). The possible explanation is that these models recapitulate the different steps of the HEV life cycle. The full-length infectious clone (Huh7-p6) models the entire cycle of HEV infection; whereas the subgenomic model (Huh7-p6-luc) only mimics viral replication due to lacking of ORF2 and ORF3.

Encouragingly, the anti-HEV activity was even comparable with its anti-HCV effect at particular concentrations. More importantly, in the long-term treatment experiment, 2CMC displays a high barrier to resistance development. Furthermore, we have extensively demonstrated that it is a specific anti-HEV effect, but not due to cytotoxicity. It has been suggested that after absorbed into the cells, 2CMC is converted to its 5′-triphosphates (2CMC-CTP) which serves as active molecule that compete with natural substrate CTP. Consistently, our results have demonstrated that CTP but not GTP reverses the anti-HEV effect of 2CMC, revealing a potential mechanism-of-action of 2CMC against HEV.

Since ribavirin has been widely used to treat chronic HEV patients, a combined therapy of ribavirin with 2CMC might be envisaged. To test this, the combinatorial effects of both drugs were investigated. Unexpectedly, an antagonistic effect was observed. These findings are in agreement with the earlier observation of the combinatorial effects of ribavirin and 2CMC on HCV and HIV [3].

Of note, the potential adverse effects of 2CMC should be carefully evaluated in future studies. The clinical applications of nucleoside analogues have been limited in some cases due to the off target effects. Mitochondrial DNA polymerase is an important off target for many nucleoside analogues. It has been reported that the nucleoside analogue containing 2-C-methyl (2-CM) could reduce mitochondrial transcription and oxidative phosphorylation, resulting in dysfunction of cell metabolism [1, 10]. Therefore, efforts for the future anti-HEV drug development are proposed to focus on the design of less toxic agents based on the main chemical structure of 2CMC.
In conclusion, 2CMC exerts potent anti-HEV effects in well-established cell culture models, and serves as a potential backbone for anti-HEV drug design. To achieve better efficacy and less side effects, future research is still required for drug optimization based on the chemical structure of 2CMC.

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Compliance with ethical standards
This article does not contain any studies with human participants or animals performed by any of the authors and is in compliance with ethical standards for research.

Conflict of interest
The authors declare no conflict of interest.
References


Chapter 6

The interplay between host innate immunity and hepatitis E virus

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Abstract: Hepatitis E virus (HEV) infection represents an emerging global health issue, whereas the clinical outcomes vary dramatically among different populations. The host innate immune system provides a first-line defense against the infection, but the dysregulation may attribute to severe pathogenesis. A growing body of evidence has indicated the active response of the host innate immunity to HEV infection both in experimental models and in patients. In turn, HEV has developed sophisticated strategies to counteract the host immune system. In this review, we aim to comprehensively decipher the processes of pathogen recognition, interferon and inflammatory responses, and the involvement of innate immune cells in HEV infection. We further discuss their implications in understanding the pathogenies and developing antiviral therapies.

Keywords: Innate immunity, Hepatitis E virus, Interferon, Inflammation
1. Introduction

Hepatitis E virus (HEV) represents an emerging zoonotic pathogen imposing a growing health burden [1]. Globally, it has been estimated that 2.3 billion of the population have encountered this virus, whereas the clinical outcomes vary dramatically among different populations [2]. In general, HEV infection is self-limiting, but can cause severe diseases in special patients. Acute infection in pregnant women bears high risk of triggering acute liver failure (ALF), leading to high mortality rate, and most of these patients are from resource-limited regions infected with the non-zoonotic genotype 1 HEV [3-5]. In European countries, chronic infection has been widely described in immunocompromised patients, in particular organ transplantation patients [6, 7]. Chronic infection is mainly caused by the zoonotic genotypes 3 [6] and 4 [8]. Evidently, the host immune responses are critical in determining the distinct outcomes of HEV infection among the different individuals [9].

The host immune system including innate and adaptive immunity essentially protects against pathogen invasions, but over-activation or dysregulation may cause pathogenesis [10]. As the first line of host defense, the innate immune system rapidly but non-specifically responds to viral infections. Upon sensing of the infection, the host produces a large spectrum of cytokines and chemokines, in particular the antiviral interferons (IFNs). IFNs trigger the transcription of hundreds of interferon-stimulated genes (ISGs), as the ultimate antiviral factors to combat the infection or further regulate immune response. The pro-inflammatory cytokines and chemokines induce inflammatory response and recruit other immune cells to coordinately orchestrate the antiviral status [10, 11].

The quantity and quality of host immune responses essentially determines the infection course of HEV and the clinical outcome in patients. The response of host innate immunity to HEV infection has been widely observed in experimental models and in patients. In turn, HEV has been reported to evolve host immunity through multiple sophisticated strategies. Here, we aim to in depth decipher the dynamic interplay between HEV and the host innate immunity.
2. Innate immune response to HEV infection

2.1 Recognition by pathogen-recognition receptors in HEV host cells

The innate immunity controls and restricts the spread of infection immediately after contacting with the pathogen. Innate immune system detects viral infection through the recognition of pathogen-associated molecular patterns (PAMPs) by pathogen-recognition receptors (PRRs). These PAMPs are specifically present in the pathogen or generated during infection [12, 13]. There are three distinct classes of PRRs in recognizing viruses, including Toll like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs). TLRs sense virus in endosomes, whereas RLRs and NLRs detect virus in cytoplasm.

TLRs are cell membrane-associated PRRs that are present on the cell surface or in the endosomes. They can recognize viral nucleic acids or other components to trigger the production of IFNs and other cytokines through different cascades [14-16]. TLRs are abundantly expressed in hepatocytes, the primary target cells of HEV. TLRs serve as principal elements of the hepatic immune system that defend infections in general. Both TLR3 and TLR7 signaling has been found to recognize hepatitis B (HBV) and C (HCV) virus infections to trigger innate antiviral response [17-20]. In cell culture models, overexpression of TLR3 inhibits HEV replication, and blocking the TBK1/IKKε complex, the downstream molecules of TLR3, enhances HEV replication [21]. TLR signaling is roughly divided into two distinct pathways based on the usage of either MyD88 or TRIF adaptor molecules. Suppression of MyD88 and TRIF inhibits TLR3-mediated IL-8 induction by HEV [22]. MyD88 deficient, but not TRIF, attenuates the induction of IL-6 and RANTES, suggesting that TLR2 and TLR4 are also involved in sensing HEV [22]. Interestingly, ORF3 of HEV augments poly (I:C)-induced TLR3 pathway, and enhances TBK1-mediated IFN-β expression [23]. In pregnant women infected with HEV, the expression levels of TLR2, TLR3, TLR4, and IFN-γ are significantly higher in those who recovered from acute infection compared to the ones developed ALF [24]. Consistently, down-regulation of TLR3 and TLR7 as well as TLR downstream molecules has been observed in another cohort of HEV infected pregnant women with ALF [3]. Thus, both in vitro and in vivo data support the prominent role of TLRs in recognizing HEV and triggering antiviral and inflammatory responses. RLRs include melanoma...
differentiation associated gene 5 (MDA5), retinoic acid-inducible gene (RIG-I) and laboratory of genetics and physiology gene 2 (LGP2), and all locate in cytoplasm [25, 26]. Short dsRNA and 5’-triphosphate RNA are ligands for RIG-I, while MDA5 mainly recognizes long dsRNA [25, 27]. Both MDA5 and RIG-I consist of two caspase activation recruitment domains (CARDs) at N-terminal, a helicase domain, and a C-terminal domain. Upon binding to foreign RNA, the CARD domain of MDA5 and RIG-I recruits mitochondrial antiviral signaling (MAVS, also known as IPS-1, VISA, or Cardif) [28-31] to activate IRF3/7 and NF-κB, leading to the production of IFNs and other cytokines. In hepatocytes, both sensors have been reported to recognize the different components of HBV and HCV to initiate IFN-related antiviral response [32-35]. However, whether MDA5 and RIG-I represent bone fide sensors for HEV remains to be clarified, although infection of HEV has been found to upregulate the expression of both genes. Earlier studies have suggested that MDA5 and RIG-I may recognize HEV RNA in human hepatic cells [21, 36]. However, a recent study has demonstrated that loss of MDA5 or/and RIG-I in mouse embryonic fibroblasts does not affect HEV RNA-induced IFN response [37]. Whether this discrepancy is attributed to the different cell types remains to be further investigated.

Figure 1. The interplay of the IFNs antiviral signaling and HEV. Upon infection, HEV is recognized by the host pattern-recognition receptors, including MDA5, RIG-I, TLR3 and TLR7. This triggers their
downstream cascades, including IFR3 and NF-κB, leading to the production of IFNs. Subsequently, IFN binds to the receptors and results in phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 form a complex with IRF9 (ISGF3). This complex translocates to nucleus and binds to IFN-stimulated response element (ISRE) to induce the transcription of antiviral IFN-stimulated genes (ISGs). RIG-I and MDA5 have been identified to inhibit HEV replication. On the other hand, HEV has evolved strategies to evade host IFN response. ORF1 inhibits phosphorylation of STAT1 and IRF3 as well as deubiquitination of RIG-I. ORF2 inhibits activation of NF-κB. ORF3 blocks phosphorylation of STAT1 and IRF3, but also has been reported to induce IFN production through increased activation of RIG-I.

Besides hepatitis, HEV infection is also associated with a broad spectrum of extra-hepatic manifestations in patients including neurological diseases [38-40] renal injuries [41], and acute pancreatitis [42, 43]. In experimental models, the direct infection of non-hepatic cell types including neurons, brain microvascular cells and kidney cells has been demonstrated [44-46]. In pregnant women, HEV can also result in severe placental diseases, and the direct infection has been recently demonstrated at the human maternal-fetal interface. Genotype 1 HEV efficiently replicates in the tissue explants of decidua basalis and fetal placenta and stromal cells, and importantly triggers the production of a panel of cytokines and chemokines [47]. Thus, the innate sensors in these non-hepatic cells are also likely able to recognize HEV, but require future research to further clarify.

2.2 Innate immune cells on HEV infection

Viral infection rapidly recruits innate immune cells such as natural killer (NK) and natural killer T (NKT) to the site of infection [48]. Upon recognition, NK cells secrete cytokines including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) to modulate antiviral immune responses [49]. NK and NKT cells constitute a major fraction of lymphocytes in the liver, and they are particularly important in the pathogenesis of viral hepatitis [50]. Both cell types play a critical role in the induction of liver cell injury during acute hepatitis B and C [51, 52]. It has been suggested that NK cells may also mediate immune response to HEV infection [53]. In patients with acute hepatitis E, the percentage of NK and NKT cells among peripheral blood mononuclear cell (PBMCs) are significantly lower, whereas the proportions of activated NK and NKT cells are higher compared with that in health controls [54]. This is consist with another report that HEV infection is particularly severe in pregnant women with a decrease in NK cell activity [55]. The decreased percentages
of NK and NKT cells in circulation may be explained by increased migration of these activated cells to the liver or apoptosis induction because of activation. However, CD56 (natural killer cells) and Granzyme B (activated NK cell marker) are significantly increased in biopsies from ALF patients caused by hepatitis E, compared with normal livers [56], and CD69 (activated NK cell marker) expression is increased in immunocompromised organ transplant patients acutely infected with HEV [57]. This might be associated with enhanced cytotoxic activity [58]. Mast cells constitute another major sensory arm of the innate immune system. In Mongolian gerbils, HEV infection increases the number but also the activation of mast cells in the liver and small intestine [59].

2.3 IFN and inflammatory responses to HEV infection

Upon recognition of PAMP, PRRs activate intracellular signaling cascades, ultimately trigger IFN and inflammatory responses. Among the three types of IFNs, type I and III IFNs activate the same pathway involving the signal transducer and activates of transcription 1 (STAT1) and STAT2 [60]. Phosphorylated STAT1 and STAT2 form a complex with IRF9, also known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates into nucleus, and binds to IFN-stimulated response elements (ISREs) to activate the transcription of hundreds of ISGs [61, 62]. ISGs are the genetic effectors coordinating antiviral and immunomodulatory activities [10, 11, 63].

In cell culture, inoculation with infectious HEV particles activates type III, but not type I IFN response with transcription of multiple ISGs. This requires MAVS or MDA5, and to a less extent RIG-I [64]. Because the low infectivity in vitro fails to adequately mimic the robust replication in patients. A subsequent study of delivering in vitro generated HEV genomic RNA into cells has demonstrated the robust induction of type I IFN response. This is independent of MDA5, RIG-I and MAVS, but requires the downstream transcription factors IRF3/7 [37]. As discussed, the role of MDA5 and RIG-I in sensing HEV remains not fully clear [21, 36, 37], but their anti-HEV activities as ISGs have been convincingly demonstrated. By profiling the known antiviral ISGs, MDA5, RIG-I and IRF1 have been identified as the most potent anti-HEV effectors [65]. RIG-I inhibits HEV replication by further induction of antiviral ISGs partially through the JAK-STAT cascade, but without requirement of IFN production [65]. IRF1 inhibits HEV replication through induction of the expression and phosphorylation of
STAT1. This triggers the transcription of many antiviral ISGs, but without the production of IFNs [66]. ISG15 is an IFN-induced ubiquitin-like protein with anti- or proviral functions depending on the context [67, 68]. HEV infection has been demonstrated to induce ISG15 expression both in cell culture and pig models [69]. Interestingly, loss of ISG15 has no major effect on HEV replication but sensitized the response to IFN treatment. This is probably related to the enhanced expression of other general antiviral factors, including PKR, MX1 and OAS1 [69].

Inflammatory response coordinated by the secretion of various cytokines and chemokines protects against infection but dysregulation may cause serious pathogenesis. Production of inflammatory cytokines upon HEV infection has been widely observed in cell culture [22], animal models [70, 71] and patients [24, 72]. In HEV infected pregnant compared with non-pregnant women, the levels of TNF-α, IL-6, and IFN-γ are significantly higher [72]. This is in line with the observation that ex vivo culturing of PBMCs from acute hepatitis E patients without ALF produces more IFN-γ and TNF-α, compared to that from patients developed ALF [24]. Collective evidence have indicated a central role of TNF-α and the NF-κB pathway in HEV triggered inflammatory response. In cell culture, TNF-α has been shown to moderately inhibit HEV replication. Interestingly, it can induce a subset of ISGs via the NF-κB cascade to synergize the anti-HEV effect of IFN-α [73].

2.4 The implications of innate immune response in patient outcome and therapeutic development

Exposure to HEV in healthy individuals usually results in asymptomatic infection. This likely reflects a strong host immune response against the infection, but such mechanisms are difficult to be captured and detailed studied. In asymptomatic blood donors, it has been shown the role of anti-HEV antibody development in viral clearance [74]. In an Egyptian cohort, cell-mediated immune response has been indicated in asymptomatic individuals [75]. In symptomatic acutely infected patients, it is apparent that the innate immunity essentially contributes to the self-limiting process.

During pregnancy, the maternal immune system is dramatically altered to protect the fetus [76]. This has been proposed as a key mechanism causing severe outcomes upon HEV infection, while much details remain unknown [77]. Differences in the
expression of TLRs, production of inflammatory cytokines, and the immune cell compartment have been observed comparing HEV infected pregnant women developed ALF to those without ALF or the non-pregnant population. Speculatively, this dysregulation may favor the infection and concurrently cause more severe liver damage, resulting in ALF and even death. In organ transplant patients, the use of immunosuppressive medication has been widely recognized as the key mechanism of cause chronic hepatitis E [78, 79]. These drugs primarily target the adaptive immune cells, in particular T cells, to prevent organ rejection. But they are also able to innate antiviral effectors, and this at least, in part, contributes to the chronic development [80].

As a potent antiviral cytokine, IFN-α has been approved for treating chronic hepatitis B and C for decades. In cell culture [81] and animal models [82], IFN-α exerts substantial anti-HEV activities. In chronic hepatitis E patients, several retrospective case series and case reports have documented an efficacy, but often associated with severe adverse events, including graft rejection and thrombocytopenia [81, 83]. Nevertheless, understanding the anti-HEV mechanisms of IFN response will open new scenarios for developing more specific antiviral strategies. For instance, a number of MDA5 or RIG-I agonists are at various phases of preclinical or clinical development for treating viral infections [84]. These agents may exert more potent but specific antiviral efficacy alleviating the concerns of side effects.

3. Viral strategies to counteract innate immune response

Despite the robust host response to HEV infection, the virus has developed many strategies to counteract and evade the defense mechanisms. Current knowledge mainly decipher the interactions between HEV viral proteins with the host innate immunity.

ORF1 encodes a non-structural polyprotein, including several functional regions (Met, Y, PCP, HVR, X, Hel and RdRp), which are essential for HEV replication. ORF1 has been shown to inhibit IFN-β expression induced by poly (I:C) transfection in cell culture [85]. This is mainly attributed to the Papain-like cysteine protease domain (PCP) and macro domain (X) that are responsible for inhibiting the activation of RIG-I and TBK-1 as well as the phosphorylation of IRF3 [85]. The cooperation of Methyltransferase (Met) and PCP has been shown to inhibit the ISRE promoter
activity and the expression of ISGs through inhibiting nuclear translocation and phosphorylation of STAT1 [86]. Interestingly, the X domain is able to inhibit the secretion of ferritin in cell culture [87]. As an acute-phase protein, ferritin is abundantly secreted in HEV infected patients associated with inflammatory response. Thus, the X domain has been proposed to attenuate host immune response by preventing ferritin secretion [87]. Finally, RNA-dependent RNA polymerase (RdRp) domain has been reported to interact with miRNA to facilitate HEV replication [88].

ORF2 encodes the capsid protein, which binds 5’ end of the genome, and is involved in viral encapsidation [89]. Three forms have been identified, including the infectious, glycosylated and cleaved ORF2 [90]. However, only the infectious ORF2 is assembled into viral particles [90], and mediates entry [91]. ORF2 has been shown to inhibit NF-κB activity by inhibiting IκBα ubiquitination [92]. The host transmembrane protein 134 (TMEM134) interacts with ORF2 to attenuate its inhibitory effect on NF-κB by ORF2 [93]. ORF2 can also impair the host apoptotic response to favor HEV infection [94].

ORF3 as a multifunctional protein has been proposed to create a favorable environment for HEV infection and pathogenesis [95-97]. In respect to the regulatory effects on innate immunity, it has been shown to inhibit STAT1 phosphorylation and ISGs expression upon HEV infection [98]. ORF3 of genotype 1 has been reported to downregulate tumor necrosis factor receptor 1-associated death domain protein (TRADD) expression and receptor-interacting protein kinase 1 (RIP1), thus inhibiting TLR3 mediated activation of NF-κB upon poly (I:C) treatment [99]. In line with this, ORF3 inhibits the expression of endogenous IFNα/β through inhibiting the expression of TLR3 and TLR7 [95]. ORF3 also inhibits LPS-induced cytokines and chemotactic factors [56]. It has been further demonstrated that ORF3 inhibits the activation of NF-κB, JAK-STAT and JNK-MAPK pathways induced by TNFα, IFN-γ and Anisomycin, respectively [95]. Counterintuitively, another study has demonstrated that ORF3 enhances IFN production upon poly (I:C) treatment through increased activation of RIG-I in a genotype-dependent manner [23]. Overall, ORF3 has been widely demonstrated to inhibit the IFN and inflammatory responses to shape a favorable cellular environment for HEV infection.
Recently, ORF4 has been found in genotype 1 HEV. It has been reported to interact with RNA-dependent RNA polymerase (RdRp) to facilitate HEV replication [100], but whether and how ORF4 regulates host immune response remains unknown and deserves to be explored.

4. Conclusion and perspective

A growing body of evidence has revealed the active interactions between the host innate immunity and HEV infection. Upon infection, a spectrum of receptors or/sensors are able to sense HEV to induce IFN and inflammatory responses. This essentially establishes the antiviral states, but dysregulation may cause serious pathogenesis, such as in pregnant women and organ transplant patients. In turn, HEV has developed sophisticated strategies to counteract the host innate immune response mainly through the interactions with the viral proteins. Thus, the interplay between host immunity and the virus eventually deters the infection course and the clinical outcome.

There remains several knowledge gaps to be further investigated. Firstly, the exact receptors/sensors that recognize HEV and the subsequent cascades in initiating responses are not fully clear yet. Secondly, the mechanisms of how the dysregulation of immune and inflammatory responses cause pathogenesis remains largely elusive. Further understanding these insights shall help to reveal the detailed mechanisms of causing hepatic and extra-hepatic diseases, and to device therapeutic and interventional strategies to combat HEV infection.

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Chapter 7

Summary and Discussion
Historically, drug development mainly focuses on viral targets due to the perceived specificity of such an approach. However, there are advantages of targeting the host in this respect, including the creation of a high barrier to the development of viral resistance to treatment. In addition, such a strategy provides broad coverage of different genotypes/serotypes and expands the list of potential targets for antiviral medication.

HEV-host interaction

In chapter 2, I comprehensively reviewed the interaction between mitochondria and different hepatitis viruses. Unlike other hepatitis viruses which cleave MAVS on mitochondria, HEV infection leads to MAVS aggregation [1]. I have demonstrated that overexpression of MAVS significantly inhibits HEV replication (Fig. 1). In addition, the antiviral effect of MAVS seems not require interferon production, although increased secretion of interferon was observed upon MAVS overexpression (Fig. 2). The detail of the mechanisms involved needs to be further investigated and should provide a deep understanding of the interaction between HEV and mitochondria. HBV and HCV infection induce mitochondrial fission [2]; in contrast, HEV infection triggers mitochondrial fusion, in turn, promoting viral replication [3]. These findings illustrate that evolution has resulted in the development of multiple distinct strategies that allow HEV replication even in the presence of adequate immunity. In chapter 3, I investigated the potential of a novel host-specific target for new HEV treatment by investigating the role of the different electron transport chain (ETC) complexes for sustaining HEV infection. I identified that pharmacological inhibition of complex III strongly inhibits HEV replication. ETC Complex III is a well-defined drug target in the treatment of malaria and Pneumocystis pneumonia [4, 5]. Testing the anti-HEV effect of the existing drugs could speed up a clinical trial design for HEV treatment. We further demonstrated that the anti-HEV effect following inhibition of complex III is independent of ATP production and ROS levels, but relies on the mitochondrial permeability transition pore (MPTP) opening. MPTP opening is responsible for the transport of mitochondrial metabolites to the cytosol [6]. An increasing body of evidence shows that metabolites such as succinate and citrate, are implicated in immunity and inflammation [7]. Innate immunity serves as the first defensive line against pathogen invasion, including that of HEV. In Chapter 6, I reviewed the interplay between host innate immunity and hepatitis E virus infection. A variety of
studies in preclinical models as well as patient studies documents the pivotal role of innate immunity for successful resistance to HEV infection. In turn, HEV corrupts host immunity employing multiple sophisticated strategies. In hepatocytes, both RIG-I and MDA5 sensors recognize different components of HBV and HCV, initiating IFN-related antiviral responses. Whether MDA5 and RIG-I recognize HEV remains to be clarified, although infection of HEV has been found to upregulate the expression of both genes [8, 9, 10], suggesting that these sensor can interacts with components of HEV as well.

Fig. 1. (A) Overexpression of WT MAVS in Huh7.5 cells inhibited HEV replication. Overexpression of MAVS 70 in Huh7.5 cells (B) and PLC cells (D and E) inhibited HEV replication. (C) Overexpression of MAVS 70 slightly inhibited genotype 1 HEV.
Fig. 2. (A) Overexpression of MAVS 70 increased the IFN λ1 gene expression. (B and C) Huh7.5 cell-based ISRE and HCV replicon model were treated with medium collected from MAVS 70-overexpressed Huh7.5 cells. (D and E) The interferon receptor inhibitors (136R and B18R) slightly reversed the inhibition of HEV by MAVS70, but the effect is not significant.

**HEV treatment**

In **Chapter 5**, I reported that 2CMC efficiently restricts the replication of HEV in a subgenomic replication and full-length infectious models. Importantly, I showed that long-term treatment with 2CMC does not attenuate its antiviral potency, indicating a high barrier to drug resistance development. However, I found that the combination of 2CMC with ribavirin, an off-label treatment for HEV, exerts antagonistic effects, which is reminiscent of the earlier observation of a combinatory effect of ribavirin and 2CMC on HCV and HIV. I concluded that 2CMC serves as a potential backbone for anti-HEV drug design. Having said that, a substantial body of work still needs to be done to minimize the chances for unexpected drug-drug interaction. In **Chapter 4**, I performed a screening of a library containing over 1,000 FDA-approved drugs. Deptropine, a classical histamine H1 receptor antagonist used to treat asthmatic symptoms, was identified as a potent inhibitor of HEV replication. Furthermore, I demonstrated that the anti-HEV activity of deptropine is independent of the histamine
Chapter 7

pathway, but requires the inhibition of nuclear factor-κB (NF-κB) activity. In addition, the crosstalk between NF-κB and caspase activity mediated by receptor-interacting protein kinase 1 (RIPK1) jointly mediate the anti-HEV effect of deptropine. Given that deptropine is being widely used in the clinic, our results warrant further evaluation of its anti-HEV efficacy in future clinical studies. Of note, the implementation of these results in an actual clinical setting may revolutionize the current management of hepatitis E. In 2016, the World Health Assembly adopted the Global Health Sector Strategy on viral hepatitis with the explicit goal to eliminate viral hepatitis by 2030. I hope my findings will be considered a milestone in this quest of humanity if that goal is actually reached.

Overall conclusions and future perspectives

HEV infection represents the leading cause of acute viral hepatitis. Developing effective antiviral therapy requires substantial efforts from virologists, immunologists, clinicians, and many other experts. Our studies provided novel insights on HEV-host interactions and identify potential drugs and targets for HEV treatment. However, more efforts are needed in order to reduce the global burden of HEV infection, in particular for specific populations including pregnant women and organ transplantation patients.
References


Chapter 8

Nederlandse Samenvatting
Dutch Summary
Hepatitis staat voor leverontsteking. Het woord hepatitis is afkomstig uit het klassiek Grieks. In deze dode taal betekende Hepar lever terwijl itis ontsteking betekent. Hepatitis E wordt veroorzaakt door het zogenaamde hepatitis E virus. Dit is een ander virus dan betrokken is bij hepatitis A, B, C of D. Al deze vormen van hepatitis hebben een virale oorzaak en worden onder het paraplugbegrip virale hepatitis geschaard. Hepatitis E komt wereldwijd voor maar de ziekte presenteert op verschillende plekken anders, dit door de aanwezigheid van verschillende virusstammen in verschillende werelddelen. In Nederland komt ook Hepatitis E voor. In ons land lijken de ziekteverschijnselen van hepatitis E op die zoals die gezien worden bij hepatitis A. In het eerste hoofdstuk van dit proefschrift geef ik een inleiding met betrekking tot deze ziekte en leg ik uit waarom het van belang is om met name de biologie van de virus infectie te begrijpen als we tot een betere behandeling willen komen.

In het tweede hoofdstuk leg ik verder uit dat wereldwijd zijn RNA virussen zoals Hepatitis E virus (HEV) verantwoordelijk voor onbeschrijfelijk veel menselijk leed. Vaak is er geen behandeling of is deze onbetaalbaar voor de grote meerderheid der patiënten. In dit proefschrift heb ik als nieuwe aanpak met betrekking tot dit probleem een bibliotheek van door de Federal Drug Administration goedkeurde medicatie getest (wat betekent dat dergelijke medicijnen in principe zonder problemen in patiënten kunnen worden getest). Deze bibliotheek betreft medicijnen waarvan de patentbescherming is verlopen (wat betekent dat dergelijke medicijnen goedkoop zouden moeten zijn). Ik heb deze bibliotheek getest voor bio-activiteit tegen Hepatitis E virus replicatie. Dit was een belangrijk onderzoek omdat Hepatitis E de meest voorkomende vorm van Hepatitis is. De ziekte kan ongemeen dodelijk zijn voor zwangere vrouwen, en ook in Nederland zien we een alarmerende stijging van haar incidentie. Wereldwijd raken meer dan 20 miljoen mensen geïnfecteerd en 70.000 mensen overlijden ten gevolge van deze ziekte. In Nederland is er een flink aantal organa transplantatie patiënten besmet met HEV, wat kan leiden in deze patiënten tot een chronische HEV infectie. Op haar beurt kan dit weer uitmonden in snelle toename van de lever fibrose en cirrose in de ontvangers van donorlevers en uiteindelijk zelfs resulteren in het verlies van het getransplanteerde orgaan. De enige oplossing in dat geval is dan een nieuwe levertransplantatie, maar dit is duur en legt beslag op de toch al zo schaarse beschikbaarheid van donorlevers. Er is momenteel nog geen geregistreerde medicatie voor Hepatitis E wat nieuwe vormen van aanpak,
zoals beschreven in dit proefschrift urgent maakt. Dit hoofdstuk documenteert dus de rationale voor de andere studies in dit proefschrift.

Gedreven door deze beweegredenen heb ik in hoofdstuk 4 een bibliotheek verzameld van ongeveer 1000 stoffen die in het verleden goedgekeurd zijn voor gebruik in menselijke ziekte, maar waarvan de patentbescherming is verlopen. Omdat medicijnen vaak meerdere werkingen tegelijkertijd hebben, is het goed mogelijk dat er tussen deze 1000 stoffen medicijnen zitten die replicatie van of Hepatitis E. Ik heb daarom een high-throughput systeem opgezet waarin ik het effect van deze stoffen op virusvermenigvuldiging kan testen. Als ik interessante kandidaten vond, ging ik die vervolgens verder testen in een meer geavanceerd systeem (organoïden) waarin virale infectie kan worden nagebootst in niet-geïmmortaliseerde cellen (wat tot hogere kwaliteit data moet leiden in vergelijking met een meer conventionele aanpak). Ook had ik toegang tot geavanceerde proefdiermodellen in het Erasmus MC. Ik hoopt voor het eerst medicatie te vinden waarmee Rotavirus en Hepatitis E bestreden kan worden. Door te concentreren op bestaande medicatie kon een kostbaar traject met klinische proeven vermeden worden, wat hopelijk geleid heeft tot een sneller toepasbaar resultaat. Het gebruik van geneesmiddelen waarvan de patentbescherming is verlopen zal ook zorg dragen dat dergelijke medicatie binnen het economisch bereik van een groot deel van de mensheid zal liggen.

Eén van de beste antivirale kandidaten die ik geïdentificeerd heb is detropine. Ik hoop dat testen wat betreft haar effectiviteit kort na dit promotieonderzoek gedaan kunnen gaan worden. De ontdekking van deze nieuwe antivirale medicatie geeft hoop voor het behandelen en genezen van patiënten die een ernstige infectie met het rotavirus of HEV doormaken. Het unieke van mijn bevinding is dat het een bestaande “off-patent” medicijn betreft. Hierdoor zullen de behandelingen erg goedkoop en dus gangbaar voor zowel de ontwikkelde- als de ontwikkelingslanden. Daarnaast kan men zich voorstellen dat het aantal levertransplantaties ten gevolge van HEV infecties in orgaantransplantatie patiënten zal verminderen.

Daarnaast heb ik geprobeerd de biologie van het HEV met de gastheercel beter te begrijpen. Hier concentreerde ik mij op het zogenaamde mitochondrion, de energiefabriek van de cel (zie ook hoofdstuk 2 waarin ik naast de eerder genoemde aspecten met betrekking tot de noodzaak voor anti-HEV medicatie ook de mogelijke rol van dit organel in HEV infectie probeer te objectiveren). In de mitochondria speelt
zich de oxidatieve fosforylering of elektronentransportketen af die samen met de
citroenzuurcyclus in het midden van het metabolisme staat van alle levende wezens
die zuurstof kunnen gebruiken. Ik zag dat farmacologische remming van de
ademhalingsketen de vermenigvuldiging van het HEV aan banden legde, maar wel
good verenigbaar was met het leven (hoofdstuk 3). Dit effect stond los van de rol
die de ademhalingsketen in de energieproductie heeft. Ik kon dan ook concluderen
dat de biologie van het HEV virus afhankelijk is van de aanwezigheid van de
ademhalingsketen en dat haar remming een nieuwe invalshoek kan zijn het
ontwerpen van antivirale therapie.
Voor andere virussen is al aangetoond dat het nucleoside analoog 2'-C-
methylcytidine (2CMC) virale polymerases (nodig voor virale vermenigvuldiging) kan
remmen. In hoofdstuk 5 laat ik zien dat dit middel ook werkt tegen HEV. Dit geeft
dus weer een andere nieuwe ingang bij het ontwerpen van strategieën voor de
behandeling van Hepatitis E.
Het mooiste is natuurlijk als het immuunsysteem zelf de virale infectie kan opruimen.
Om rationele strategieën te ontwikkelen die dit zouden kunnen bewerkstelligen is het
natuurlijk wel nodig om de interactie tussen virus en immuunsysteem te begrijpen. Dit
onderzoek ik middels een literatuurstudie in hoofdstuk 6. Uit deze studie wordt
duidelijk dat met name eiwitten die worden afgeschreven onder invloed van antivirale
cytokines uit de interferonfamilie een krachtige anti-HEV werking kunnen hebben.
Deze eiwitten moeten dus gemobiliseerd worden bij het ontwikkelen van dergelijke
strategieën.
Een samenvatting en discussie van de gevonden feiten alsmede een idee over hoe
toekomstig onderzoek er uit zou moeten zien geef ik in hoofdstuk 7. Ik hoop in ieder
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Appendix

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PhD Portfolio
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Publications


5. **Changbo Qu**, Yunlong Li, Yang Li, Pengfei Li, Peifa Yu, Wenshi Wang, Peppelenbosch MP, Pan Q. MAVS inhibits hepatitis E virus replication likely dispensable of interferon-like response. Pre-submission.


7. Yang Li, **Changbo Qu**, Wenshi Wang, Peppelenbosch MP and Qiuwei Pan. MDA5 against enteric viruses through induction of interferon-like response partially via JAK-STAT cascade. Pre-submission.


10. Wenshi Wang, **Changbo Qu**, Yijin Wang, Buyun Ma, Shaoshi Zhang, Shan Wang, Wanlu Cao, Lei Xu, Jingmin Zhao, Maikel P. Peppelenbosch, Qiuwei Pan. Noncanonical activation of STAT3 by hepatitis E virus shapes intracellular microenvironment to facilitate viral replication. In preparation.
# PhD Portfolio

<table>
<thead>
<tr>
<th>Name of PhD Student</th>
<th>Changbo Qu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erasmus MC Department</td>
<td>Gastroenterology and Hepatology</td>
</tr>
<tr>
<td>PhD Period</td>
<td>October 2015 – October 2019</td>
</tr>
<tr>
<td>Promotor</td>
<td>Prof. dr. Maikel P. Peppelenbosch</td>
</tr>
<tr>
<td>Copromotor</td>
<td>Dr. Qiuwei Pan</td>
</tr>
</tbody>
</table>

## PhD training

### Seminars
- 2015-2019, Weekly MDL seminar program in experimental gastroenterology and hepatology (attending); (42 weeks/year) (ETCS, 9.0);
- 2015-2019, Weekly MDL seminar program in experimental gastroenterology and hepatology (presenting); (2 times/year) (ETCS, 4.6);
- 2015-2019, Weekly research group education (attending); (42 times/year) (ETCS, 2.25);
- 2015-2019, Weekly research group education (presenting); (8 times/year) (ETCS, 2.28).

### General Courses and Workshops
- 2019, Biomedical English writing course (ETCS, 2.0);
- 2018, the course on Stem Cells, Organoids and Regenerative Medicine (ETCS, 0.5);
- 2019, the advanced course on Applications in flow cytometry (ETCS, 0.5);
- 2015, Basic introduction course course SPSS (ETCS, 1.0);
- 2017, the Advanced Immunology Course (ETCS, 0.5);
- 2016, the course on Microbiomics (ETCS, 0.6);
- 2015, the course Biomedical Research Techniques (ETCS, 1.5);
- 2016, introduction to Graphpad Prism Vs.6 (ETCS, 0.3);
- 2016, Workshop on NCBI& other open source software (ETCS, 0.2)
Appendix

National and International Conferences

- 2019, 7th European Congress of Virology, Rotterdam, the Netherlands (Poster presentation)
- 2019, 1st Essen Hepatitis E symposium, Essen, Germany (Oral presentation)
- 2018, the International Liver Congress, Paris, France (e-Poster presentation)
- 2017, DIGESTIVE DISEASE DAYS, the Netherlands (Oral presentation)
- 2017, the International Liver Congress™, Amsterdam, the Netherlands (Oral presentation)

Academic Awards

- Young Investigator Travel Awards, ILC Full Bursary (€650), International Liver Congress™ 2017, Amsterdam, the Netherlands.
- China Scholarship Council (CSC) Scholarship (201509110121), 2015.
Curriculum Vitae

Changbo Qu was born on February 2, 1988, in Zhengzhou, Henan province, China. He grew up and attended middle school in his hometown. In 2007, he started his Bachelor’s study in Animal Science at Henan Agricultural University. In 2011, he started his master’s study in Animal Nutrition at Nanjing Agricultural University under the supervision of Prof. Tian Wang. His main project was to evaluate the safe doses of sodium dehydroacetate, a feed additive in piglets.

After he graduated from Nanjing Agricultural University (2014), he worked as an assistant researcher in the lab of Prof. Qiyu Diao at Chinese Academy of Agricultural Science (CAAS), Beijing, China. In 2015, with the support of China Scholarship Council, he got an opportunity to start his Ph.D. study at the Department of Gastroenterology and Hepatology of Erasmus Medical Center in the Netherlands. Under the supervision of Prof. dr. Maikel P.Peppelenbosch and Dr. Qiuwei Pan, he has studied drug screening for hepatitis E treatment.