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Inhibition of hepatitis E virus replication by peptide-conjugated morpholino oligomers

Yuchen Nan [a], Zexu Ma [a], Harilakshmi Kannan [a,1], David A. Stein [c], Patrick I. Iversen [d], Xiang-Jin Meng [e], Yan-Jin Zhang [a,b,*]

[a] VA-MD College of Veterinary Medicine, University of Maryland, College Park, MD, United States
[b] Maryland Pathogen Research Institute, University of Maryland, College Park, MD, United States
[c] Department of Biomedical Science, Oregon State University, Corvallis, OR, United States
[d] Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, United States
[e] Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, United States

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ABSTRACT

Hepatitis E virus (HEV) infection is a cause of hepatitis in humans worldwide and has been associated with a case-fatality rate of up to 30% in pregnant women. Recently, persistent and chronic HEV infections have been recognized as a serious clinical problem, especially in immunocompromised individuals. To date, there are no FDA-approved HEV-specific antiviral drugs. In this study, we evaluated antisense peptide-conjugated morpholino oligomers (PPMO) designed against HEV genomic sequences as potential HEV-specific antiviral compounds. Two genetically-distinct strains of human HEV, genotype 1 Sar55 and genotype 3 Kernow-C1, isolated from patients with acute and chronic hepatitis, respectively, were used to evaluate inhibition of viral replication by PPMO in liver cells. The anti-HEV PPMO produced a significant reduction in the levels of HEV RNA and capsid protein, indicating effective inhibition of HEV replication. PPMO HP1, which targets a highly conserved sequence in the start site region of ORF1, was also effective against the genotype 3 Kernow-C1 strain in stably-infected HepG2/C3A liver cells. The antiviral activity observed was specific, dose-responsive and potent, suggesting that further exploration of PPMO HP1 as a potential HEV-specific antiviral agent is warranted.

1. Introduction

Hepatitis E virus (HEV) is a single-stranded positive-sense RNA virus in the family *Hepeviridae* (Emerson et al., 2004). HEV is an etiologic agent of acute hepatitis in humans, and is endemic to various tropical and subtropical regions of the world. In pregnant women, infection with HEV genotype 1 can lead to fulminant hepatitis having a case-fatality rate of up to 30% (Jameel, 1999; Kumar et al., 2013). Hepatitis E is now recognized as a zoonotic disease, and strains of HEV from pig, chicken, mongoose, rabbit, rat, ferret, bat, fish and deer have been genetically characterized (Haqshenas et al., 2001; Li et al., 2005; Meng, 2011; Meng et al., 1997). Over the past several years, chronic and persistent symptomatic HEV infections have been reported in increasing numbers of immunocompromised individuals, including organ transplant recipients and leukemia, lymphoma and HIV/AIDS patients, in industrialized countries (Kamar et al., 2014a).

The HEV genome is approximately 7.2 kb in length and consists of three open reading frames (ORFs) (Tam et al., 1991). ORF1 encodes all the putative nonstructural proteins involved in HEV replication. ORF2 encodes the capsid protein, the major structural protein in the HEV virion. ORF3 encodes a multi-functional phosphoprotein that is essential for establishing HEV infection in macaques and pigs (Graff et al., 2005; Huang et al., 2007). HEV strains are highly diverse in sequence and those strains infecting humans are classified into four major genotypes in the genus *Orthohepevirus* (Lu et al., 2006; Smith et al., 2014). HEV genotype 1 and 2 are restricted to humans and have no known animal reservoir, whereas genotype 3 and 4 infect several animal species in addition to humans and are known to be zoonotic (Ahmad et al., 2011; Meng, 2010).

Although it has been over two decades since the sequence of a full-length HEV genome was first published (Tam et al., 1991), there are as yet no FDA-approved HEV-specific drugs. Off-label use of ribavirin and pegylated interferon for treatment of acute

* Corresponding author at: VA-MD College of Veterinary Medicine, University of Maryland, College Park, MD, United States.
E-mail address: zhangyj@umd.edu (Y.-J. Zhang).
[1] Present address: Merck & Co., Inc., West Point, PA, United States.

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and chronic hepatitis E patients has been reported (Gerolami et al., 2011; Kamar et al., 2010; Mallet et al., 2010; Wedemeyer et al., 2012), but there are side effects and efficacy concerns with respect to those options (Kamar et al., 2014b; Pischke et al., 2013). Ribavirin belongs to the FDA Pregnancy Risk Category X and is not recommended for use in pregnant women. Interferon cannot be used in most transplant patients. Thus, there is a pressing need for the development of a HEV-specific antiviral therapeutic agent, especially for treating severe infections in pregnant women and chronic infections in immunocompromised patients.

In this study, phosphorodiamidate morpholino oligomers (PMO) were tested for their ability to inhibit HEV replication in liver cells. PMO are nuclease-resistant single-stranded DNA analogs containing a backbone of morpholine rings and phosphorodiamidate linkages (Summerton, 1999). PMO bind to mRNA by Watson–Crick base pairing and can interfere with translation through steric blockade of the AUG-translation start site region. Conjugation of PMO to an arginine-rich cell penetrating peptide, yielding peptide-conjugated PMO (PPMO), facilitates delivery into cells (Abes et al., 2006; Summerton, 1999). PPMO are water soluble and enter cells readily. This study demonstrates that several anti-HEV PPMO display potent inhibition of HEV genotype 1 replication. Notably, PPMO HP1, which targets the start site region of HEV ORF1, also effectively inhibited replication of genotype 3 Kernow-C1 strain in liver cells.

2. Materials and methods

2.1. PPMO design and synthesis

Based on previous studies targeting viral RNAs with PPMO (Stein, 2008), the PPMO for this study were designed to target genomic sequence of the HEV Sar55 strain (GenBank Accession # AF444002). PPMO HP1 and HP2 are complementary to a sequence in the 5′ end region of genomic and subgenomic RNA, respectively (Fig. 1 and Table 1). HP3U is complementary to a sequence in the terminal region of the 3′ UTR. HPN3 is the reverse complement of HP1 and was intended to interfere with the synthesis of positive-sense genomic RNA. A nonsense-sequence PPMO CP1 (Zhang et al., 2007), having little agreement with HEV or human mRNA sequences, was used as a negative control PPMO. CP1 with fluorescein conjugated at its 3′ end (CP1-F) was used in the PPMO uptake assay. PPMO were synthesized with an arginine-rich cell-penetrating peptide (P7) conjugated at the 5'end at AVI BioPharma Inc. (Corvallis, OR) as previously described (Abes et al., 2006).

2.2. Cell-free translation

PPMO target sequences were cloned upstream of the luciferase gene in reporter vector pCiNeoLucr as previously described (Zhang et al., 2007). Briefly, oligomers of 30-nt in length containing the target sequence for PPMO HP1, HP2, and HP3U were each cloned upstream of luciferase coding sequence in pCiNeoLucr vector. The *in vitro* transcription and translation reactions were carried out as previously described (Zhang et al., 2008). Luminescence signal was measured with VICTOR3™ Multilabel Counter (Perkin-Elmer Life and Analytical Sciences, Wellesley, MA).

2.3. Cells, viruses and transfections

S10-3 cells, a subclone of Huh-7 hepatoma cells (Graff et al., 2006), and hepatoma cells HepG2/C3A (ATCC CRL-10741) were maintained in DMEM medium supplemented with 10% fetal bovine serum.

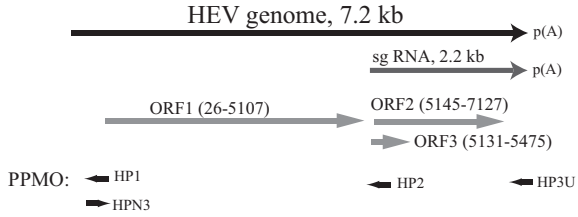

Fig. 1. Schematic illustration of HEV genome, subgenomic RNA (sg RNA), ORFs, and PPMO target locations. The numbers after ORFs indicate the nucleotide position of the beginning and end of the open reading frames (drawn in light gray arrows) within the HEV Sar55 genome (GenBank Accession # AF444002). The black arrows shown in the PPMO indicate their 5′–3′ orientation relative to the positive-sense HEV RNA genome.

Table 1
PPMO sequences and their target sites in HEV.[a]

Name	PPMO sequence (5′–3′)	Target site in HEV genome (position)[b]
HP1	GGGCCTCCATGGCATCGACC	Start site region of ORF1 (18–37)
HP2	CATGGGCGCAGCAAAAGACA	Start site region of ORF2 (5116–5135)
HP3U	GCGCGAAACGCAGAAAAGAG	Terminal region of 3′ UTR (7169–7188)
HPN3	GGTCGATGCCATGGAGGCCC	3′ terminal region of negative sense RNA[c]
CP1	GATATACACAACACCCAATT	None

[a] PPMO designed against HEV Sar55 strain (GenBank Accession # AF444002).
[b] Position of PPMO target sites in the genomic sequence of Burma isolate (GenBank Accession # M73218), the prototype of HEV genotype 1 in the genus *Orthohepevirus*.
[c] HPN3 sequence is the reverse complement of HP1.

The PPMO uptake assay was performed in uninfected S10-3 cells. Approximately 1.2×10^5 cells were seeded per well into 12-well plates, producing confluent monolayers in 24 h. PPMO CP1-F was added to the cell supernatant at a final concentration of 8 μM and the cells further incubated at 37 °C for 4 h. The medium was removed and the cells rinsed with PBS, before observation with fluorescence microscopy to assess PPMO uptake efficiency.

Transfection of S10-3 cells with HEV RNA *in vitro* transcribed from pSK-E2 (an infectious cDNA clone of HEV Sar55 strain) or pSK-E2-Luc (containing luciferase reporter) was performed using DMRIE-C reagent (Invitrogen, Grand Island, NY) as previously described (Nan et al., 2014a,b). For PPMO treatment of the S10-3 cells in 12-well plates, cell culture supernatant was discarded 5 h after RNA transfection and the cells were rinsed twice with Opti-MEM. PPMO suspended in 0.5 mL Opti-MEM was then added to the cell monolayers. Four hours after PPMO treatment, the PPMO solution was removed and 1 mL DMEM with 10% FBS was added to each well. The cells were then further incubated at 34.5 °C for 7 days prior to further analysis for viral protein, RNA or luciferase signal. Luciferase activity from pSK-E2-Luc was determined by using the Bright-Glo™ Luciferase Assay System (Promega, Madison, WI).

The HEV genotype 3 Kernow-C1 strain p6 was used to infect HepG2/C3A cells at a multiplicity of infection (MOI) of 1 (Shukla et al., 2011). Kernow-C1 replication does not cause cytopathic effect and infected HepG2/C3A cells were passaged five times to produce stably infected cells. Immunofluorescence assay (IFA) with chimpanzee anti-HEV antibody (a gift from Suzanne Emerson at the National Institutes of Health) was conducted to confirm virus replication. Subsequently, the Kernow-C1-infected cells were seeded into 12-well plates. PPMO was then added to the HepG2/C3A cells in fresh medium once every 2 days for 6 days (3 treatments total). The cells were maintained at 37 °C and harvested for analysis 1 day after the final PPMO treatment.