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Inhibition of Hepatitis E Virus Infection by Peptide-Conjugated Morpholino Oligomers

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ABSTRACT

Hepatitis E virus (HEV) infection is a cause of hepatitis in humans worldwide. 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 designed and evaluated antisense peptide-conjugated morpholino oligomers (PPMO) as potential HEV-specific antiviral compounds. Two genetically-distinct strains of human HEV, genotype 1 Sar55 and genotype 3 Kernow C1, which cause acute and chronic hepatitis, respectively, were used to evaluate PPMO inhibition of viral replication in liver cells. Four anti-HEV PPMOs tested led to a significant reduction in the levels of viral RNA and HEV capsid protein as well as luciferase yield from Sar55 replicons in S10-3 liver cells, indicating an effective inhibition of HEV replication. PPMO HP1, which targets the ORF1 translation initiation region, 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, potent and effective, suggesting that further exploration of HP1 as a potential HEV-specific antiviral agent is warranted.

Keywords: hepatitis E virus, morpholino oligomers, antisense, PPMO, antiviral.

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, where it causes both sporadic cases and epidemic outbreaks. Sporadic HEV infections with disease consequences also occur in non-endemic regions (Kamar et al., 2014; Khuroo, 2011). In pregnant women, HEV infection can lead to fulminant hepatitis that has a mortality rate of up to 30% (Jameel, 1999; Kumar et al., 2013). The World Health Organization (WHO) estimates that there are over 3 million acute cases of hepatitis E and over 56,600 deaths annually (WHO, 2014). Hepatitis E is now a recognized 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). More recently, chronic and persistent HEV infections have been reported in increasing numbers of immunocompromised individuals in industrialized countries, including organ transplant recipients and leukemia, lymphoma and HIV/AIDS patients (Kamar et al., 2014). Chronic hepatitis E has now become a significant clinical problem, warranting an effective antiviral drug, especially for management of HEV infection in immunosuppressed individuals (Kamar et al., 2014).

The HEV genome is approximately 7.2 kb in length and consists of three open reading frames (ORFs) (Tam et al., 1991). ORF1 encodes a polyprotein which is supposed to be cleaved to produce 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). A single bicistronic RNA was found to encode both ORF2 and ORF3,

which start from two closely spaced initiation codons in different reading frames (Graff et al., 2006).

HEV strains are highly diverse in sequence and those strains infecting humans are classified into four major genotypes (genotypes 1-4) (Lu et al., 2006). All four genotypes of human HEV belong to the genus *Orthohepevirus*. HEV genotypes 1 and 2 are restricted to humans with no known animal reservoir, whereas genotypes 3 and 4 are known to be zoonotic, and infect several animal species in addition to humans (Ahmad et al., 2011; Meng, 2010). The identification of new HEV strains has prompted a recent proposal from the HEV Study Group of ICTV to reorganize the family *Hepeviridae* in order to accommodate a more elaborate taxonomy (Smith et al., 2014).

There is no specific anti-HEV drugs though it has been over two decades since the sequence of the first full-length HEV genome was published (Tam et al., 1991). Off-label use of ribavirin and pegylated interferon for treatment of acute 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 safety and efficacy concerns with respect to those options. Ribavirin belongs to the FDA Pregnancy Risk Category X and is not recommended for use by pregnant women. Thus, there is a pressing need for the development of a specific anti-HEV therapeutic, especially for treating immunocompromised patients and for chronic infections.

Phosphorodiamidate morpholino oligomers (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), facilitate

delivery into cells (Abes et al., 2006; Summerton, 1999). PPMOs are water soluble and enter cells readily.

In this study, PPMOs were tested for their ability to inhibit HEV replication in liver cells. Several PPMOs demonstrated potent inhibition of HEV genotype 1 strain replication. Notably, PPMO HP1 also effectively inhibited infection 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 PPMOs (Stein, 2008), the PPMOs for this study were designed to target genomic sequence of HEV Sar55 strain. PPMO HP1 and HP2 are complementary to the 5'end of genomic and subgenomic RNA, respectively (Fig. 1 and Table 1). HP3U is complementary to sequence in the terminal region of the 3' UTR. HPN3 is reverse complement to HP1 and was intended to interfere with the synthesis of 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-Fl) was used in the PPMO uptake assay. PPMOs 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 were done 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.

The PPMO uptake assay was performed in uninfected S10-3 cells. PPMO CP1-Fl was added to the medium at a final concentration of 8 μM and incubated at 37°C for 4 h. The medium was removed and the cells were rinsed with PBS pH7.2. Fluorescence microscopy was conducted 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 as previously described (Nan et al., 2014a; Nan et al., 2014b). For PPMO treatment of the S10-3 cells, cell culture supernatant was discarded 5 hours after RNA transfection and the cells then rinsed twice with Opti-MEM. PPMOs suspended in 0.5 mL Opti-MEM were then added to the cell monolayer. Four hours after PPMO treatment, 1 mL DMEM with 10% FBS was added to each well. The cells were then cultured at 34.5□C for 7 days prior to further analysis for viral protein or RNA. Luciferase activity from pSK-E2-Luc in the cells 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). IFA with chimpanzee anti-HEV antibody was conducted to confirm the virus replication. Subsequently, the Kernow-infected cells were seeded into 12-well plates. PPMO was then added to the HepG2/C3A cells in fresh medium once every two days for 6 days (3 treatments total). The cells were maintained at 37□C and harvested for protein and RNA analysis one day after the final PPMO treatment.

2.4. Cell viability assay. Viability of S10-3 cells after PPMO treatment was determined with CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Briefly, S10-3 cells were treated with the PPMO as described above and lysed 48 h later with 1X reporter lysis buffer. CellTiter-

Glo Reagent was mixed with the lysate at 1:1 ratio in a 96-well plate and luminescence signal was measured.

2.5. Immunofluorescence assay (IFA). IFA and confocal fluorescence microscopy were carried out as reported previously with chimpanzee antibody against the HEV capsid protein (Nan et al., 2014b).

2.6. Western blot analysis. Cells were lysed in Laemmli sample buffer. Total protein was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described previously (Patel et al., 2010; Zhang et al., 2007). An anti-HEV ORF2 monoclonal antibody (EMD Millipore, Billerica, MA) was used at dilution of 1:1000. The Quantity One Program (Version 4.6) and a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA) were used for digital signal acquisition and densitometry analyses. β-tubulin was also detected as a protein load control.

2.7. Reverse transcription and real-time PCR (RT-qPCR). RNA isolation, reverse transcription and real-time PCR were performed as previously described (Nan et al., 2012; Nan et al., 2014c). For the detection of HEV-specific RNA, HEV specific reverse primer (Sar55-R3, CAGAATCCACGCAGACCTTA) was used in reverse transcription. Primers Sar55-F3 (TGAGTTTGATTCCACCCAGA) and Sar55-R3 were used for real-time PCR on Sar55 cDNA. For absolute quantification of HEV RNA, the pSK-E2 (Sar55) plasmid served as the template to establish standard curve.

2.8. Statistical analysis. The significant differences of luciferase level or HEV RNA copies between the groups of cells in the presence or absence of PPMO were assessed by Student t-tests. A two tailed P-value of less than 0.05 was considered significant.

3. RESULTS

3.1. PPMOs inhibit target mRNA translation in cell-free luciferase reporter assay. To validate binding of the PPMOs to their respective target sequences, each PPMO was tested against RNA containing the PPMO target region upstream of and in frame with luciferase coding sequence. PPMOs were added at various concentrations to cell-free translation reactions containing in vitro transcribed RNA from each reporter plasmid. Compared with CP1, each HEV-targeted PPMO reduced luciferase signal significantly (Fig. 2). PPMO HP1 produced a 99% reduction at 100 nM (Fig. 2). Similarly, PPMO HP2, and HP3U reduced luciferase expression by around 90% at 200 nM (Fig. 2). All PPMOs behaved in a dose-dependent manner with HP1 producing the most potent inhibition.

3.2. PPMOs inhibit HEV replication in S10-3 liver cells. We next conducted a PPMO uptake assay in uninfected S10-3 cells with PPMO CP1-Fl. Highly efficient uptake of the CP1-Fl was observed, as indicated by the presence of green fluorescence signal present in all cells (Fig. 3A). Having established that PPMO enter S10-3 cells effectively, we next tested whether the anti-HEV PPMO were able to inhibit HEV replication. S10-3 cells were transfected with full-length Sar55 RNA, then treated with 16 μM PPMO. PPMO HP1, HP2, HP3U and HPN3 produced marked reduction of capsid protein expression, indicating inhibition of HEV replication, while CP1 had minimal effect (Fig. 3B). The results indicate that the four HEV-targeted PPMOs generated specific inhibition of HEV replication.

We also tested whether the PPMO produced cytotoxicity to S10-3 cells, as an impact on cell viability could produce non-specific inhibition of viral replication. When the cells were

treated with 16 μM PPMO HP1 under the same conditions as the antiviral assays above, no impact on cell viability was observed by the cell viability assay (data not shown).

3.3. PPMO treatment generates dose-dependent inhibition of HEV replication. Next, an HEV replicon system containing a luciferase reporter (pSK-E2-Luc) was used to further verify the antiviral effect of selected PPMOs. In the pSK-E2-Luc replicon, insertion of luciferase coding sequence into HEV ORF2/3 region disrupts ORF2 and ORF3 expression but provides a quantitative means to measure translation of subgenomic viral RNA (Graff et al., 2006). Cells were transiently transfected with pSK-E2-Luc and treated with HP1, HP3U and HPN3 PPMO. Luciferase yields in cells treated with the 16 μM PPMO were significantly lower than that in mock-treated cells (Fig. 4A).

Further evaluations showed that PPMO HP1, HPN3 and HP3U generated dose-dependent reductions of luciferase expression (Fig. 4B). Luciferase expression in the cells treated with HP1 at 2, 4, and 8 μM was reduced by 53%, 94%, and 99%, respectively, compared to that of mock-treated control. PPMO HPN3 reduced luciferase expression by 40%, 90% and 99%, when used at 2, 4, and 8 μM respectively. PPMO HP3U at 2, 4, and 8 μM reduced the luciferase expression by 78%, 86% and 92%, respectively.

Of the three PPMOs tested above in two cell-based systems, HP1 produced the most potent inhibition of HEV replication. To further evaluate HP1 in S10-3 cells, we measured inhibition of virus replication by immuno-blot detection of the HEV capsid protein. Cells receiving HP1 treatment at 2, 4, and 8 μM had relative capsid protein at 0.5, 0.07 and 0.04-fold, respectively, of cells treated with CP1, as indicated by densitometry analysis of the Western blots (Fig. 4C).

We next tested if PPMO treatment reduced the level of HEV RNA production. Cells were transfected with Sar55 RNA and treated with 8 μM HP1. HEV RNA present in the supernatant of cell cultures was detected by RT-qPCR at seven days post transfection. The HP1 treatment led to reduction of HEV RNA from 2.8 x 10^6 copies to less than 3.1 x 10^4 copies per mL (Fig. 4D). The results were consistent with those of capsid protein detection and the luciferase reporter assay (pSK-E2-Luc) described above.

3.4. PPMO HP1 inhibits HEV genotype 3 Kernow C1 replication. Kernow C1, a genotype 3 HEV strain, has been successfully adapted to propagate in HepG2/C3A cells (Shukla et al., 2011). Since Kernow C1 replication does not cause cytopathic effect, we established HepG2/C3A cells stably infected with the Kernow C1 virus that can be passaged multiple rounds. Active replication of HEV Kernow C1 in HepG2/C3A cells was confirmed by both IFA and Western blotting (Fig. 5A and B). Sequence alignment of the genotype 1 Sar55 and genotype 3 Kernow C1 revealed that the target sequence of PPMO HP1 is 100% conserved , while there are 4 nt mismatches between Kernow C1 and Sar55 strains at the HP3U target site. So we tested PPMO HP1 in Kernow-infected HepG2/C3A cells. HP1 reduced the capsid protein level to 0.3-fold that of untreated cells (Fig. 5C). Evaluation of capsid protein expression showed that HP1 inhibition of Kernow C1 replication was dose-dependent (Fig. 5D).

Taken together, the data from experiments using two HEV genotypes and three different cell-based systems showed PPMO HP1 to be an effective inhibitor of HEV replication.

4. DISCUSSION

Our results demonstrate that PPMO targeting HEV RNA can inhibit virus replication effectively. Inhibition of HEV replication in cells was demonstrated by reductions in both viral RNA and capsid protein levels. PPMO HP1, which targets the ORF1 translation initiation region, demonstrated the most potent inhibition of virus replication in each of the experimental systems used in this study. HP1 effectively inhibited the replication of genotype 1 Sar55 strain as well as established infections of genotype 3 Kernow C1 strain. The HP1 target site is perfectly conserved between the Sar55 and Kernow genomes, and highly conserved across the four HEV genotypes that infect humans (data not shown). The overall efficacy of PPMO HP1 in this study suggests it may be an HEV inhibitor with antiviral activity across multiple HEV genotypes.

PPMO HPN3 and HP3U were able to inhibit the Sar55 replication in a dose-dependent manner. The target sites of HP3U and HPN3 are in the terminal region of the 3' ends of HEV genomic plus-strand and replicative-intermediate minus-strand, respectively, where the HEV RNA-dependent RNA polymerase (RdRp) is expected to associate during RNA synthesis. We speculate that those two PPMOs may obstruct access of the RdRp to the respective RNAs, thereby interfering with viral RNA synthesis.

Antisense PMOs are currently in clinical trials, including a treatment for Duchenne muscular dystrophy in humans (Anthony et al., 2012; Mendell et al., 2013). PPMOs have also been used in a clinical trial, albeit in an ex-vivo model (Moulton, 2013). PPMOs have been documented as effective against numerous types of viral infections of the liver in experimental animal models. Importantly, upon systemic administration, PPMOs distribute to liver tissue, remains pharmacologically viable, and has been effective at reducing viral titers (Amantana et al.,

2007; Burrer et al., 2007; Paessler et al., 2008). These qualities, along with the efficacy against HEV replication in cultured cells that we observed in this study, suggest PPMO should be considered for further development as an inhibitor of HEV infections. Further evaluation and development of anti-HEV PPMOs will require *in vivo* investigation, and the pig model infected with genotype 3 HEV appears to be suitable (Meng et al., 1998).

In summary, our results indicate that PPMOs can be effective antiviral compounds against HEV infection. PPMO HP1 has potent activity against strains of HEV from two different genotypes, including an established infection of HepG2/C3A cells with Kernow strain. The results suggest that HP1 is a promising candidate for further development as a broad HEV-specific antiviral compound.

5. ACKNOWLEDGEMENT

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TABLES

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

Name	PPMO sequence (5′ to 3′)	Target site in HEV genome (position)[b]
HP1	GGGCCTCCATGGCATCGACC	ORF1 translation initiation region (18-37)
HP2	CATGGGCGCAGCAAAAGACA	ORF2 translation initiation region (5116-5135)
HP3	TTCATTCCACCCGACACAGA	ORF3 translation initiation region (5091-5110)
HP3U	GCGCGAAACGCAGAAAAGAG	Terminal region of 3' UTR (7169-7188)
HPN3	GGTCGATGCCATGGAGGCCC	3' terminal region of negative sense RNA[c]
CP1	GATATACACAACACCCAATT	None

a. PPMOs 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 HEV prototype of the genus *Orthohepevirus*.

c. HPN3 sequence is the reverse complement to HP1.

FIGURE LEGENDS

Fig.1. **Schematic illustration of HEV genome, subgenomic RNA, ORFs, and PPMO target locations**. The arrows in the PPMOs indicate their 5' to 3' orientation in relation to the HEV RNA genome.

Fig. 2. **Cell-free luciferase reporter assay**. PPMOs were added to *in vitro* translation reactions containing RNA transcribed from reporter constructs that include PPMO target sequences upstream of and in-frame with firefly luciferase coding sequence. Reactions treated with PPMO CP1 served as a negative control. Luciferase activity in the presence of the various PPMO is graphed as the relative percentage of untreated control reactions, set as 100%. The average of three tests is shown and the error bars represent variation among the experiments. ** indicates significant differences from CP1 at corresponding concentrations ($P < 0.01$).

Fig. 3. **PPMOs enter S10-3 liver cells and inhibit HEV replication. A**. PPMO uptake assay in S10-3 cells. Fluorescein-conjugated CP1 was added to S10-3 cells and incubated for 4 h before fluorescence microscopy. Green fluorescence indicates uptake of PPMO. The image on the right shows the same field of cells under bright field illumination. **B**. Immunofluorescence assay of S10-3 cells infected with HEV. Cells were transfected with Sar55 RNA transcribed from pSK-E2, treated with indicated PPMO (16 μM) 5 hours later, and fixed for IFA at 7 days post-transfection. In each panel, the left image shows HEV-positive cells detected with IFA, using HEV-specific antibody, and the right image shows same field with cell nuclei stained by DAPI.

Fig. 4. **Dose-dependent inhibition of HEV replication by PPMOs**. **A**. Luciferase assay of S10-3 cells transfected with Sar55 RNA from HEV replicon pSK-E2-Luc. Cells were transfected with the viral RNA, treated with 16 μM PPMO 5 h later, and harvested for luciferase assay at 7

days post-transfection. Relative percentages of luciferase activity are shown in comparison with mock-treated S10-3 cells. Error bars represent variation among three repeat experiments. ** indicates significant difference from the mock-treated cells ($P < 0.01$). **B**. Dose-dependent inhibition of HEV replication by PPMOs, using same experimental scheme as in A above. **C**. Treatment of S10-3 cells with PPMO HP1 inhibits HEV capsid protein production in a dose-dependent manner. Cells were transfected with HEV RNA from pSK-E2, treated with PPMO HP1 5 h later and harvested 7 days later for Western blotting. **D**. HEV RNA present in culture supernatant of S10-3 cells detected by RT-qPCR. Cells were transfected with Sar55 RNA, and treated 8 µM PPMO HP1 5 h later. The cell culture supernatant was harvested 7 days post-transfection. The Y-axis indicates HEV RNA copies per mL supernatant.

Fig. 5. Inhibition of HEV Kernow C1 virus replication in HepG2/C3A cells. **A**. IFA of Kernow C1-infected HepG2/C3A cells. The left image shows HEV-positive cells, and the right image shows the same field of cells stained with DAPI. **B**. Western blotting detection of HEV capsid protein in Kernow C1-infected HepG2/C3A cells. **C**. PPMO-mediated inhibition of Kernow C1 virus replication. Cells were treated with 16 µM PPMO in fresh medium every two days for six days, then harvested one day after the final treatment. Relative levels of HEV capsid protein production in PPMO-treated cells are shown in comparison with non-treated cells. **D**. Dose-dependent inhibition of Kernow C1 capsid production by HP1. The cells were treated with PPMO HP1, as in C above.

Figure 1

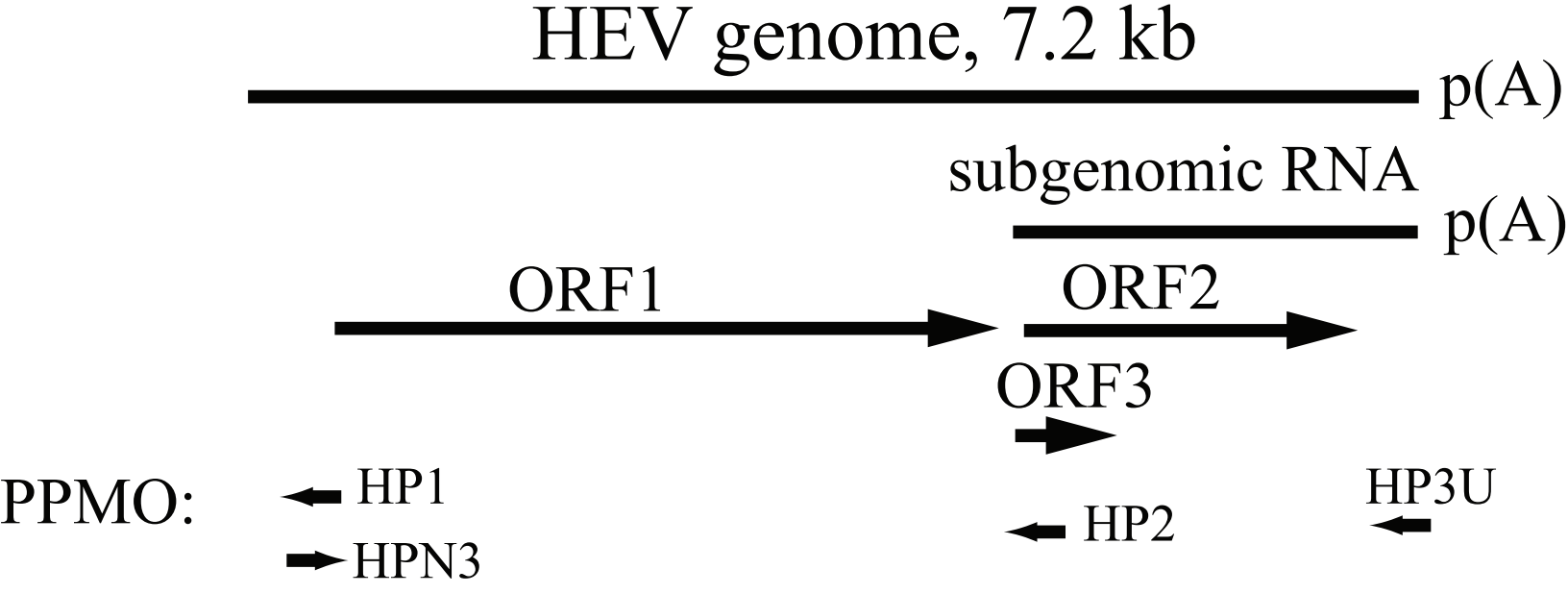

HEV genome, 7.2 kb
p(A)
subgenomic RNA
p(A)
ORF1
ORF2
ORF3
PPMO:
HP1
HPN3
HP2
HP3U

Figure 2

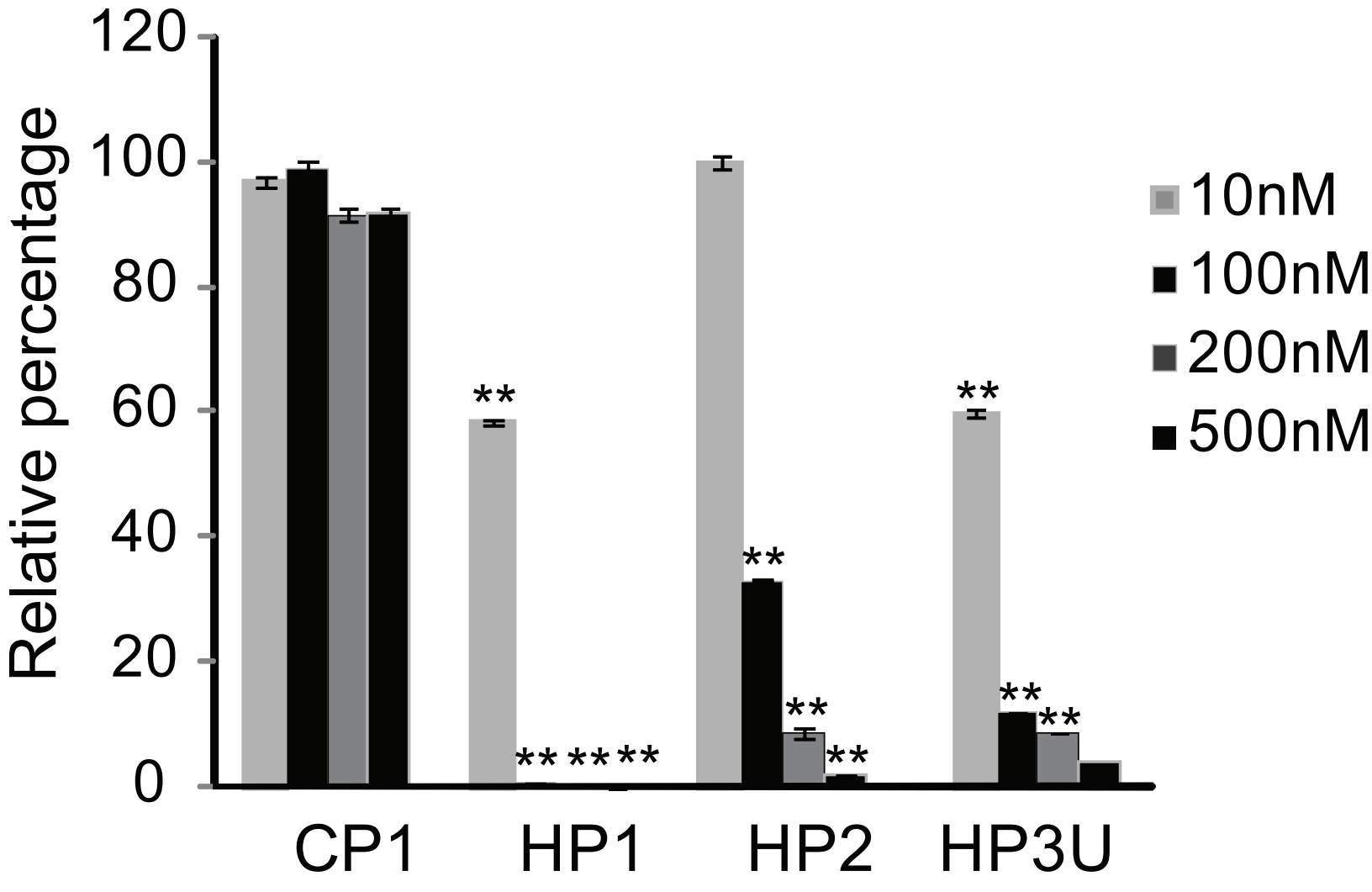
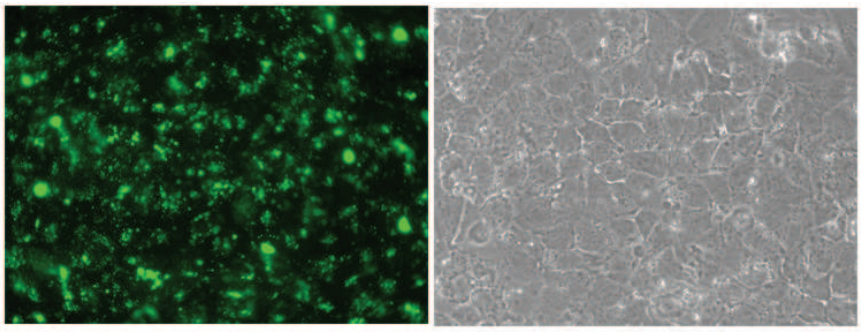

120
100
80
60
40
20
0
Relative percentage
10nM
100nM
200nM
500nM
**
**
**
**
**
**
**
**
CP1
HP1
HP2
HP3U

Figure 3

A



B

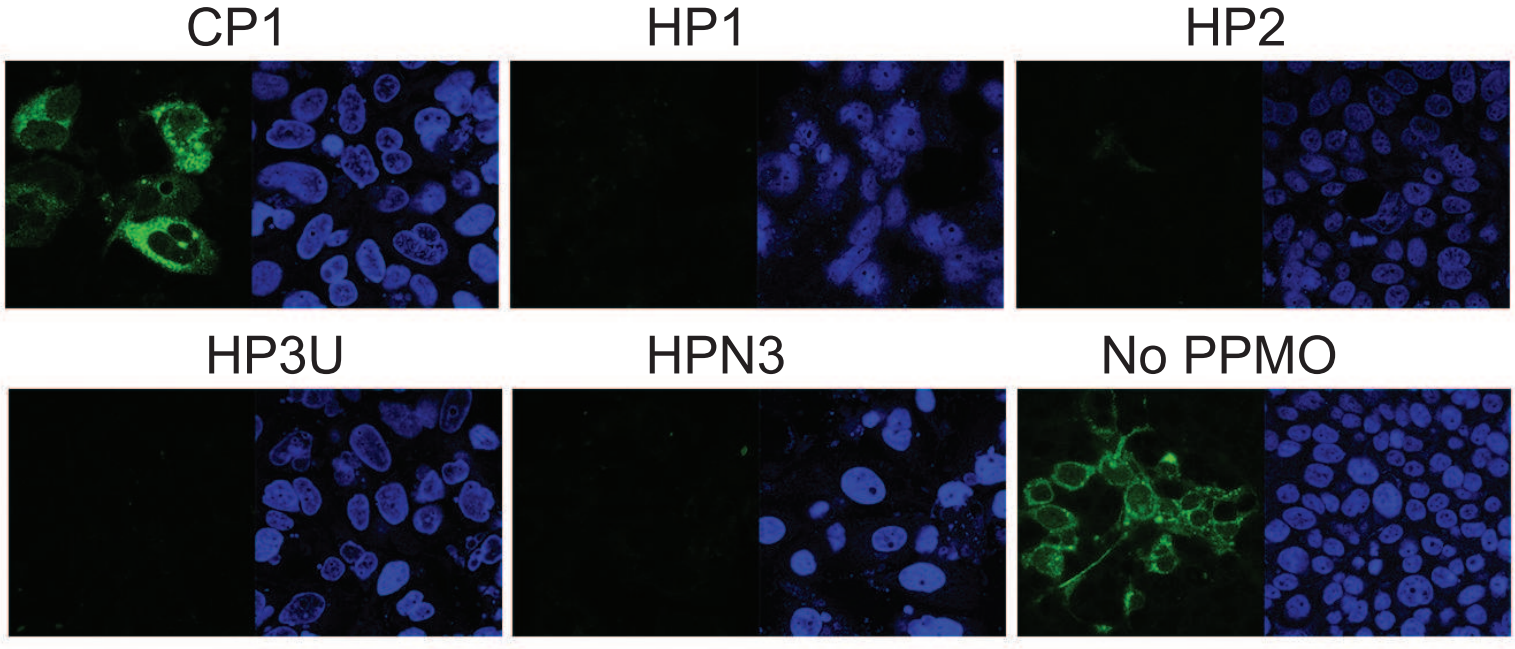


Figure 4

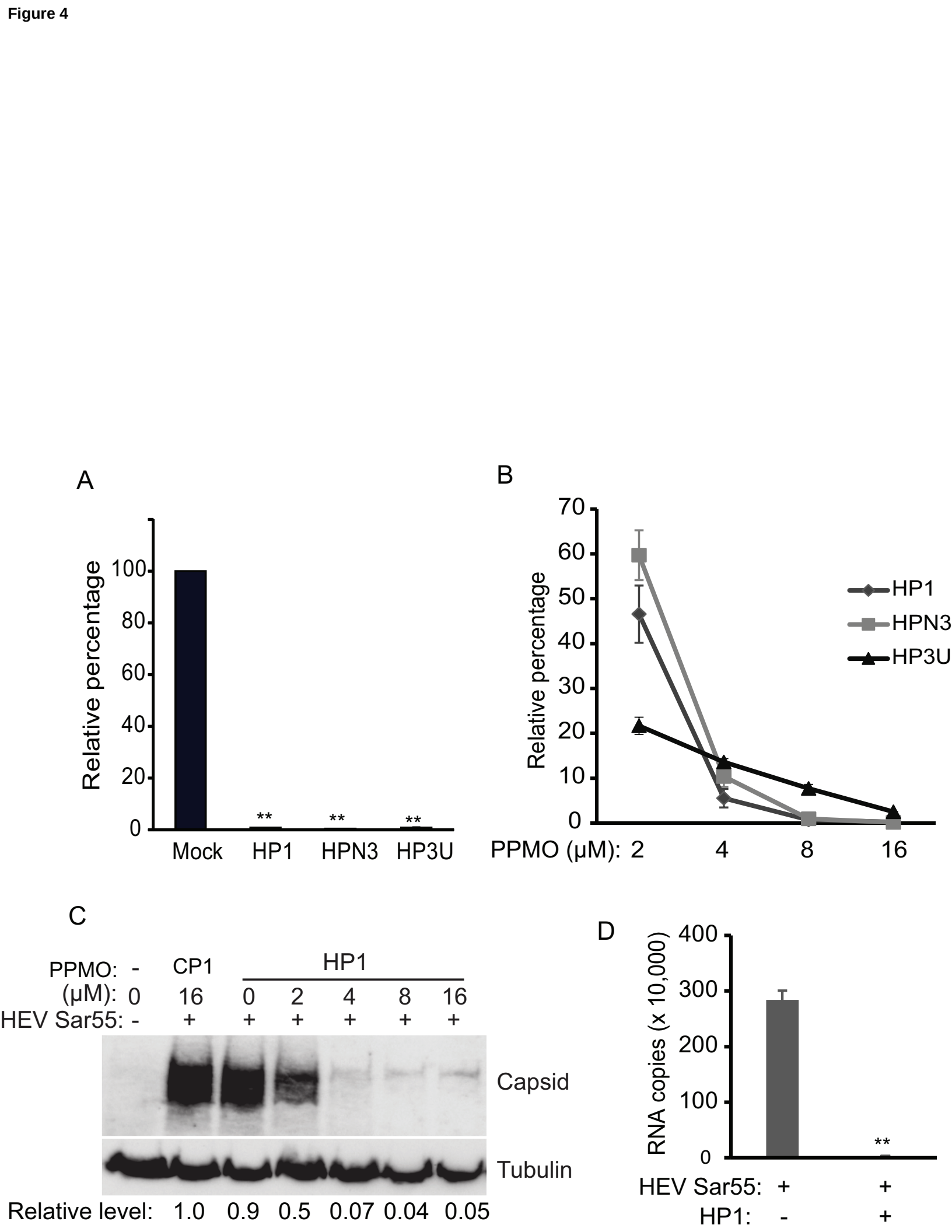
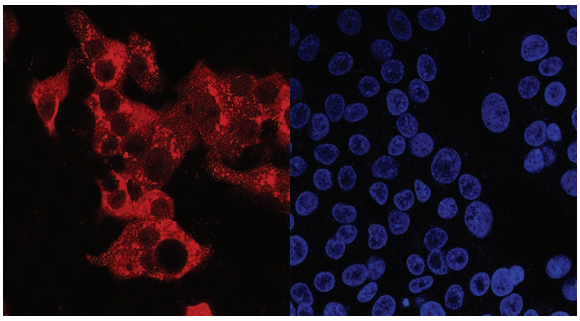

A
Relative percentage
100
80
60
40
20
0
**
**
**
Mock
HP1
HPN3
HP3U
B
Relative percentage
70
60
50
40
30
20
10
0
HP1
HPN3
HP3U
PPMO (μM): 2
4
8
16
C
PPMO: -
CP1
HP1
(μM): 0
16
0
2
4
8
16
HEV Sar55: -
+
+
+
+
+
+
Capsid
Tubulin
Relative level: 1.0 0.9 0.5 0.07 0.04 0.05
D
RNA copies (x 10,000)
400
300
200
100
0
**
HEV Sar55: +
+
HP1: -
+

Figure 5

A



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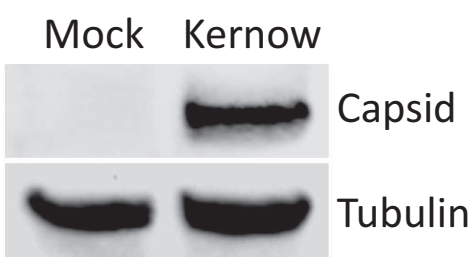
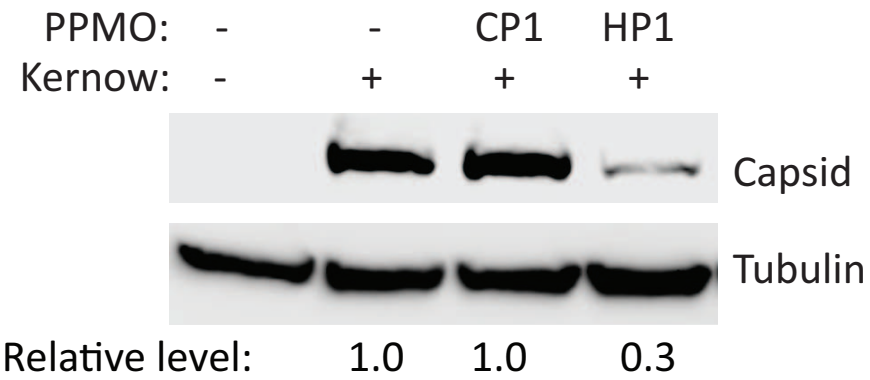


C



D