Title: Development of peptide-conjugated morpholino oligomers as pan-arenavirus inhibitors

Authors: Benjamin W. Neuman¹, Lydia H. Bederka², David A. Stein³, Joey P. C. Ting⁴, Hong M. Moulton⁵, Michael J. Buchmeier²

Affiliations: ¹University of Reading, Reading, RG6 6AJ UK, ²The University of California, Irvine, CA, ³Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton, OR, ⁴Lilly Biotechnology Center, San Diego, CA, and ⁵Oregon State University, Corvallis, OR. ⁶To whom correspondence should be addressed.

Running head: Inhibition of Arenavirus infections with PPMO

Keywords: Arenavirus, LCMV, morpholino, antisense, antiviral

Abstract

Members of the Arenaviridae are a threat to public health and can cause meningitis and hemorrhagic fever, yet treatment options remain limited by a lack of effective antivirals. In this study, we found that peptide-conjugated phosphorodiamidate morpholino oligomers (PPMO) complementary to viral genomic RNA were effective in reducing arenavirus replication in cell cultures and in vivo. PPMO complementary to the Junín virus genome were designed to interfere with viral RNA synthesis, translation, or both. However, only PPMO designed to potentially interfere with translation were effective in reducing virus replication. PPMO complementary to sequence that is highly conserved across arenaviruses and located at the 5’-termini of both genomic segments were effective against Junín, Tacaribe, Pichinde and Lymphocytic Choriomeningitis arenavirus-infected cell cultures, and suppressed viral titers in the livers of LCMV-infected mice. These results suggest that arenavirus 5’-genomic-termini represent promising targets for pan-arenavirus antiviral therapeutic development.
Introduction

Most arenaviruses are carried by a single species of rodent host (reviewed in (5)). In these rodents, arenaviruses have a unique life cycle characterized by benign and persistent infection. Arenavirus infections of humans, however, may cause serious illness. Arenaviruses are typically divided into Old World viruses, which are prevalent in Africa, and New World viruses which are endemic to the Americas. *Lassa virus*, an Old World Arenavirus, is a significant public health problem, primarily in West Africa, causing 300,000–500,000 cases of Lassa fever and approximately 5,000 deaths annually (21). Several New World arenaviruses, including *Junín, Machupo* and *Guanarito*, can cause potentially deadly hemorrhagic fever syndromes in humans, and occur epidemiologically as outbreaks, usually in South America (5).

No highly effective antiviral therapeutic against arenavirus infection is approved for use in humans. Intravenous treatment with ribavirin early after the onset of Lassa fever is effective in reducing mortality (13), but ineffective in preventing neurological sequelae including deafness (16). The only approved prophylactic against arenavirus infection is an attenuated Candida strain of the New World *Junín virus*, which has been used to vaccinate people in endemic areas against Argentine Hemorrhagic Fever, but is generally unavailable elsewhere (15).

The unusual process of arenavirus gene expression may offer several opportunities for therapeutic intervention. All arenaviruses have a single stranded, ambisense RNA genome containing the two segments, ‘L’ and ‘S’, each of which encode two genes. The ‘L’ segment encodes polymerase (L), matrix (Z), and the ‘S’ segment nucleoprotein (NP) and glycoprotein (GPC). All arenaviruses contain near-identical complementary sequences at the 5’ and 3’-termini of each genomic segment (4).

Although the exact replication initiation mechanism has yet to be fully clarified, the ‘S’ segment requires a panhandle structure formed by these complementary 5’ and 3’ genomic termini for RNA synthesis (23). Studies using *Tacaribe virus* have provided evidence for a non-template nucleotide incorporation at the 5’ genomic terminus for viral RNA replication to yield a novel ‘prime and realign’ mechanism for replication initiation (11, 12, 26).
Arenavirus proteins are expressed from subgenomic mRNAs that contain a 5’-cap structure and untemplated nucleotides at the 5’ end, but lack 3’ poly-adenylation. It has been proposed that a highly stable intergenic RNA stem-loop structure, present within each transcript, functionally compensates for the lack of 3’ polyadenylation (17, 24). NP and L are expressed from subgenomic mRNA transcripts that are transcribed from full-length packaged virion RNA segments (vRNA) whereas Z and GP are expressed from subgenomic mRNA transcripts produced as nascent complements of full-length packaged virion RNA segments (vcRNA) (5).

In an attempt to develop inhibitors of arenavirus infections, we designed antisense phosphorodiamidate morpholino oligomers (PMO) against several regions of viral genetic sequence considered important to arenavirus gene expression. PMO are single-strand nucleic acid analogs composed of uncharged phosphorodiamidate linkages connecting six-member morpholine ring subunits to which any nitrogenous base may be attached. PMO are uncharged, nuclease resistant compounds that bind their target RNA sequence via Watson-Crick base pairing and inhibit gene expression through steric blockade (30, 34). Conjugation of an arginine-rich cell-penetrating peptide to the 5’ end of a PMO, to produce peptide-PMO (PPMO), has been shown to greatly enhance cellular uptake (1, 10, 18). PPMO designed against the genetic material of numerous RNA viruses have shown considerable antiviral activity in vitro and in vivo (reviewed in (31)).

Here, PPMO activity was tested against four distantly related arenaviruses: LCMV, Pichinde (PICV), Tacaribe (TCRV) and Junín virus strain Candid#1 (JUNV). LCMV, an Old World arenavirus, is used as a model for the more dangerous Lassa virus; PICV is closely related to the North American hemorrhagic fever agent Whitewater Arroyo virus, and TCRV clusters phylogenetically with the South American hemorrhagic fever viruses Machupo and JUNV. Together these viruses cover the breadth of pathogenic human arenaviruses. We report that PPMO targeting the conserved 5’ terminal region of the viral genomic segments were effective at inhibiting viral protein expression and reducing viral titer in cell cultures and in LCMV-infected mice.

Materials and Methods
Cells and viruses

JUNV-Candid#1, LCMV-Arm53b, TCRV and PICV were grown in Vero-E6 cells as previously described (19). Vero-E6 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 5 mM L-glutamine and 10 mM HEPES buffer. For experiments using PPMO, cell cultures were maintained in serum-free VP-SFM (Invitrogen) supplemented with 1% penicillin/streptomycin and 5 mM L-glutamine. Arenaviruses were titrated by plaque assay on Vero-E6 cells as previously described for LCMV (6).

PPMO synthesis

PMO were synthesized as previously described (35) and covalently conjugated to arginine-rich cell-penetrating peptide of the composition (RXR)_4XB (in which X stands for 6-amino hexanoic acid and B stands for beta-alanine) as previously described (1), to produce PPMO. All PPMO were produced at AVI BioPharma Inc. (Corvallis, OR).

Bioinformatics

The viral RNA sequences used to design PPMO are shown in Table 1 and were derived from the following Genbank accession numbers: JUNV-Candid#1 (AY746353 and AY746354), PICV (NC_006439 and NC_006447), LCMV (NC_004291 and NC_004294) and TCRV (NC_004292 and NC_004293). Pathogenic JUNV sequences which were also used in PPMO design included strains Rumero (AY619640 and AY619641) and XJ13 (NC_005080 and NC_005081). BLAST-n (NCBI) was used to establish that these sequences were representative of their type species and ClustalW2 was used to align sequences.

Evaluation of PPMO toxicity to cell cultures and to mice

The cytotoxicity of PPMO was evaluated under conditions designed to mirror those of the antiviral assays described below. Briefly, Vero E6 cells were plated onto 6-well plates and incubated with VP-SFM (Invitrogen), supplemented with antibiotic-antimycotic (GIBCO) and L-glutamine. Increasing
Concentrations of PPMO were added to the cells and allowed to incubate for 24h. After 24h or 96h incubation, 200uL of 5 mg/ml MTT (Sigma) was added to each well and plates were allowed to incubate for an additional 40 minutes. Supernatant was removed, cells were solubilized with 1 ml DMSO and absorbance was measured at 560nm using the Victor plate reader (Perkin Elmer).

C57/Bl6 mice were used for all experiments. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed at The Scripps Research Institute (La Jolla, CA, USA). The mice were 6-8 weeks old at the beginning of the experiments. Mice were allowed food and water ad libitum throughout the studies, and all experimental procedures were pre-approved and performed according to the guidelines set by the SRI Animal Care and Use Committee. 

In vivo toxicity testing was performed by injecting uninfected male mice intraperitoneally with PPMO or sterile saline. Experimental animals were treated with one dose containing ~9 mg/kg TERM-L, TERM-S or TERM-L-REP PPMO per mouse per day, and treatment continued for five days. At the conclusion of the experiment, mice were humanely euthanized and sections of liver and kidney were stained with haematoxylin and eosin. Samples were randomized and re-labeled for blinded observation. Samples were examined using a light microscope for signs of chromatin degradation or other histopathologic changes.

PPMO treatment and viral infection of cell cultures

Vero-E6 cells in T25 culture flasks were treated with PPMO in 2 ml VP-SFM and incubated for 3 h prior to inoculation. Cells were inoculated at an approximate MOI of 0.01. Inoculum was then added to the treatment medium, and removed after a 1h adsorption period. The cells were then rinsed once with phosphate-buffered saline and fresh VP-SFM containing the appropriate dose of PPMO was re-added. Cell supernatant and cell lysates were collected at various time points starting 24h post-inoculation. For cytoprotection assays, cells were fixed with neutral buffered formalin at 96h after inoculation, stained with crystal violet and evaluated using a light microscope.

Western immuno-blots
To obtain cell lysates, LCMV-infected Vero E6 monolayers were incubated for 10 minutes in 1× PBS/1mM EDTA, then harvested by centrifugation and triturated in 1% NP-40 lysis buffer (20mM Tris pH 7.6, 140mM NaCl, 5mM MgCl₂) containing a 1× protease inhibitor cocktail (RPI Corp). Protein concentration in cell lysates was determined using a standard BCA kit following the manufacturer’s protocol. Samples were separated using 12% Tris-Glycine gels under reducing conditions and proteins were transferred to nitrocellulose membranes. Membranes were blocked with buffer containing 5% milk protein, probed overnight with monoclonal antibodies targeting either the GP2 region of the LCMV glycoprotein (mAb 83.6) or LCMV nucleoprotein (mAb 1.1-3) (7), stripped with 0.2M NaOH and re-probed for actin (Millipore). Protein complexes were detected using an alkaline phosphatase detection system and visualized using the BioRad ChemiDoc XRS system.

Viral RNA quantitation with qRT-PCR with GAPDH control method

Total RNA was extracted at 24h post-infection using Trizol as per manufacturer’s protocol (Invitrogen). RNA samples were treated with DNaseI (New England Biolabs) according to the manufacturer’s protocol. cDNA synthesis, using GoScript Reverse transcription system (Promega) was performed using LCMV Armstrong NP-specific or monkey GAPDH-specific forward primers (NP forward: CGAAGCTTCCCTGGTCATTTC, NP reverse: CAGTTATAGGTGCTCTTCCGC; GAPDH forward: AGTCAACGGATTGTGCCTTCCGC; GAPDH reverse: GGGTGGAATCATACTGGAAAC). Cercopithecus-specific GAPDH primers for use with Vero-E6 cells were modified from published primers (14). qRT-PCR was performed using the Applied Biosystems 7300 Real Time PCR system platform with 250ng template cDNA, 12.5uL 2× Maxima SYBR Green/ROX qPCR master mix (Fermentas) and 0.2uM each forward and reverse gene-specific primers in 25uL reaction volumes. Melt curve analysis was performed to confirm PCR product specificity. qRT-PCR analysis was performed by the comparative CT method (28). The fold change (FC) in NP cDNA relative to the GAPDH control was determined using the equation FC = \text{-}2^{\Delta C_T} where \Delta C_T = (C_T NP - C_T GAPDH) infection with PPMO treatment - (C_T NP - C_T GAPDH) infection without PPMO treatment.
In vivo antiviral efficacy testing

Mice were administered 16 or 24 nmol (equivalent to ~6 or 9 mg/kg, respectively) of PPMO in water, or with sterile saline via intraperitoneal (IP) injection at 3h before IP inoculation with 1000 plaque-forming units of LCMV-Arm53b. Similar PPMO or saline treatments were repeated every 24h for 3 days. Samples of liver and spleen were collected 4d after inoculation, weighed and then homogenized in phosphate-buffered saline to release virus. Tissue homogenates were titrated by plaque assay as described above. Statistical analysis was performed using InStat (Graphpad, San Diego CA).

Results

PPMO design

PPMO were designed based on the Candid#1 vaccine strain of JUNV. All PPMO sequences and their arenavirus genomic complements are defined in Table 1. TERM-L and TERM-S were designed to differ slightly to permit exploration of small differences between the termini of the JUNV L and S RNA segments. TERM-L-REP includes an additional nucleotide in order to hybridize more efficiently with non-templated residues that are commonly found at the 5’-ends of some intracellular viral RNA species (5, 11, 12, 25). Five “TERM” PPMO were designed to disrupt interactions at the various genomic termini, but only TERM-L-REP, TERM-L and TERM-S target the 5’-terminus of viral mRNA species, and are therefore more potentially capable of interference with pre-initiation events in the translation process than are the other PPMO in this study. Four PPMO are specific to the translation initiation sites for the four JUNV mRNAs (AUG-GP, AUG-NP, AUG-Z and AUG-L).

One additional PPMO, complementary to the loop region of the intergenic transcription terminator (IGS-S), was designed to potentially interfere with viral transcription. A negative control PPMO (RANDOM; identical to DSCR in (20) that was not complementary to JUNV was used to control for non-sequence-specific effects of the PPMO chemistry. Detailed PPMO complementarity information is given in Fig. S1. PPMO binding sites are presented schematically in Fig. 1a and the hypothetical antiviral mechanism of each PPMO is given in Fig. 1b.
**PPMO Cytotoxicity**

To determine the maximum concentration of PPMO that could be used in antiviral cell culture experiments without incurring loss of cell viability, we performed MTT cytotoxicity assays. After 24h of treatment, TERM-L-REP, TERM-L and TERM-S PPMO showed low impact on viability at concentrations up to 20 μM, and mild toxicity at 50 μM in Vero-E6 cells (Fig. 2a). Prolonged incubation with PPMO for 96h produced slightly stronger toxic effects (Fig. 2b). Similar results were obtained previously for PPMO in Vero or Vero-E6 cells (10, 20, 29).

**Antiviral Assays with PPMO in cell culture**

Our stock of JUNV-Candid#1 grew relatively poorly in Vero-E6 cells. We sequenced the S segment of our JUNV-Candid#1, which was found to match GenBank accession number AY746353. To facilitate antiviral testing, we selected a more rapidly growing variant of JUNV-Candid#1, produced by serial passage every 24h, instead of the more usual 48-72h, in Vero-E6 cells. After 17 passages, nucleotide sequencing results showed a mixture of wild-type sequence and mutant sequence, including a guanosine to adenosine change at position 590 (based on the numbering of AY746353).

After passage 20, virus was cultured from individual plaques and sequenced again. Passage 20 virus showed a homogeneous G590A sequence. This mutation encodes a change of alanine to threonine that introduces a potential N-linked glycosylation site at amino acid 168 of GPC, which is in the GP1 region. The mechanism by which the G590A change increases viral output is not clear. The virus from passage 20 grew to ~100-fold higher titer and induced cell fusion at neutral pH (Fig. 3), unlike the parental strain (data not shown).

We next evaluated the effectiveness of PPMO in protecting Vero-E6 cells from JUNV-mediated cell fusion and cytopathic effects such as rounding and detachment from the culture flask that are the basis for the arenavirus plaque assay. Images of Vero-E6 cell monolayers 96h after inoculation with LCMV, JUNV or mock-inoculation are shown in Fig. 3. Long-term TCRV infection, PICV infection and PPMO cytotoxicity (not shown) produced similar cytopathic effects to long-term LCMV infection. To assess the effectiveness of PPMO in preventing changes in cell adherence and
morphology, cells were pretreated with PPMO for 3h before inoculation at low multiplicity. Morphology and adherence were evaluated four days after inoculation, when virus titer typically peaks at between $10^6$ and $10^8$ PFU/ml. PPMO complementary to the 5’-terminus of the viral genomic RNA provided the highest overall protection against cytopathic effects caused by all four arenaviruses (Table 2). Of these, TERM-S PPMO was more consistently protective than TERM-L or TERM-L-REP, perhaps due to lower cytotoxicity (Fig. 2). JUNV-specific AUG-GP, AUG-L and AUG-Z PPMO provided less protection against JUNV and did not protect against heterologous arenaviruses. The difference in effectiveness of terminus- versus AUG-binding PPMO was most striking in JUNV, where PPMO directed at translation of GPC and NP did not significantly reduce virus-mediated cell fusion, but TERM-L, TERM-L-REP and TERM-S PPMO treatment did. Randomized control, JUNV-specific IGS and AUG-NP PPMO and both pan-arenavirus 3’TERM PPMO were ineffective against all viruses.

We also compared the growth of four arenaviruses in cells that were treated with PPMO for 3h before inoculation. As in the cytoprotection assay above, all three PPMO targeted to the genomic 5’-terminus were highly effective against each arenavirus, AUG PPMO showed moderate JUNV-specific effects, and all other PPMO were ineffective (Table 3). The efficacy of TERM-L and TERM-L-REP PPMO was similar at the 20 μM treatment dose (Table 3) and for LCMV both PPMO were similarly effective over a 20-fold dosage range (Fig. 4).

Extensive complementarity between a PPMO and its target sequence was necessary but not sufficient for antiviral activity. All highly effective PPMO in the cell culture studies here had at least 19 contiguous nucleotide matches with at least one target site on the vRNA or vcRNA of one genomic segment. However, less effective AUG PPMO and the ineffective IGS and 3TERM PPMO also contained no mismatches with JUNV RNA, highlighting the importance of target site selection in PPMO efficacy (Fig. S1). Several ineffective PPMO increased viral titers slightly (Table 3). These results suggest that while antisense PPMO may inhibit virus growth, treatment with ineffective PPMO may enhance virus growth, as reported previously (2, 8, 20, 22).
PPMO inhibition of viral protein synthesis

To investigate the mechanism of PPMO antiviral effects, we analyzed the expression of LCMV GP-C and NP in cells treated with PPMO before infection. Treatment with 2 μM TERM-L or TERM-L-REP PPMO reduced expression of viral proteins NP and GP to below the threshold of detection (Fig. 5a-d) and also reduced LCMV growth by ~1000-fold (Fig. 4). The highest PPMO concentrations tested also decreased the expression of actin. These experiments demonstrate that PPMO complementary to the genomic 5’-terminus specifically reduced viral protein expression, but also that the selectivity window between effective and cytotoxic concentrations is not wide.

To determine if PPMO-mediated inhibition of viral protein expression could also be observed when using a multiplicity of infection higher than the 1 PFU/cell used in the experiment shown in Fig. 5a-d, cells were treated with the same PPMO concentrations as in Fig. 5a-d, and inoculated at 5 PFU per cell. All inoculation doses tested resulted in the same pattern of inhibition of viral protein expression as seen in cells inoculated with 1 PFU/cell (data not shown), indicating that low micromolar doses of PPMO were capable to suppress the growth of high multiplicities of virus.

To investigate mechanism of antiviral action, we next measured the effects of an effective PPMO (TERM-L) on NP RNA and protein levels. PPMO treatment caused a dose-dependent reduction in intracellular RNA levels, using a qRT-PCR methodology which would detect positive-sense NP from mRNA and vRNA, and appeared to inhibit RNA- more than protein-accumulation (Fig. 5e-g). After 24h treatment with 1 micromolar TERM-L, NP RNA levels were reduced 10-fold while protein levels were reduced only two-fold (Fig. 5g).

PPMO efficacy in vivo

As a prelude to antiviral testing in a mouse model of arenavirus infection, a dose equivalent to approximately 9 mg/kg PPMO was administered intraperitoneally once per day for seven consecutive days to uninfected mice. The amount of PPMO used for treatment in vivo was based on published studies of PPMO efficacy and toxicity in mice using IP administration (8, 9, 32, 33). After five
treatments, mice were sacrificed and the liver and kidney were examined for signs of toxicity. No gross histopathological differences were detected between control and PPMO-treated mice (data not shown), nor did weight loss occur in PPMO- or mock-treated mice.

We next evaluated the effectiveness of TERM-L, TERM-S and TERM-L-REP PPMO during acute LCMV infection in mice. Mice were administered ~6 mg/kg/day PPMO or sterile saline via IP injection at three hours before IP inoculation with LCMV, and treatments were repeated daily for 3 days after infection. To obtain an indicator of PPMO efficacy, we evaluated viral load in the spleen and liver at 4 days post infection. Treatment with TERM-L-REP, TERM-L, TERM-S PPMO significantly reduced viral load per gram of tissue in both the liver and spleen compared to the combined RANDOM-treated and saline-treated controls (Fig. 6a). We repeated the experiment using a higher dose of ~9 mg PPMO/kg/day. The higher dose resulted in a small decrease in spleen LCMV titer, but virus titer reduction was most prominent in the liver, where titers were reduced by about tenfold (Fig. 6b). The reduction in virus load in either the spleen or liver after high-dose TERM-L PPMO treatment was statistically significant compared to pooled saline and RANDOM PPMO treatments (ANOVA; P < 0.05 for spleen and P < 0.01 for liver). However, high-dose TERM-L treatment compared to RANDOM PPMO treatment alone in either tissue did not quite achieve statistical significance. Together these results demonstrate that short-term administration of antisense terminus-binding PPMO can reduce arenavirus growth in an animal model.

Discussion

In this proof of concept study, PPMO complementary to the conserved 5’-termini of arenavirus genome segments were effective against a broad range of arenavirus infections in cell cultures, and in vivo suppressed LCMV growth in the liver and spleen, the major sites of viral replication during the acute phase of LCMV infection (reviewed in (5)). LCMV grows to high titers in mice, allowing logistically uncomplicated experimentation in the natural host animal. The disadvantage is that LCMV, like all other known arenaviruses, characteristically produces only mild disease in its natural host. The pathogenesis of arenavirus hemorrhagic fevers is complex and is not fully reproduced in
animal models. The results of this study represent an initial indication of antiviral efficacy by PPMO against an arenavirus in vivo. The reduction in the accumulation of arenavirus protein and RNA shown here in vitro suggest a mechanism whereby PPMO treatment could suppress virus growth and enable more rapid viral clearance during an in vivo infection. However, much further investigation will be needed to determine whether PPMO could be useful in treating arenavirus infections in humans.

We tested PPMO sequences that had been designed to interfere with viral RNA synthesis, viral translation, or both. The PPMO TERM, 3’TERM and IGS-S target sites are expected to be important primarily to viral RNA synthesis, as they represent RNA that is not directly involved with translation. These three PPMO did not reduce viral titer or prevent virus-induced cytopathic effects. PPMO AUG-GP, AUG-NP, AUG-Z, AUG-L were designed to interfere with viral translation by binding to sequence spanning the start site of each viral open reading frame, and they also showed moderate or low activity. However, PPMO designed to target sequence present in the 5’ terminal region of all positive-sense viral RNA species (TERM-L-REP, TERM-L and TERM-S) and thought to participate both in translation of mRNA and RNA synthesis events had consistent antiviral effects in cell cultures and in vivo. Studies on a variety of RNA viruses have identified the 5’ genomic terminus of positive-strand RNA viruses, as well as sequence in the 5’ UTR and surrounding the AUG translation start codon of viral mRNA, as frequently productive target regions for antiviral PPMO (reviewed in (31)). In those studies, as in the study here, it has generally been concluded that the most likely mechanism of action for the PPMO producing antiviral effects is through interference in events involved in the pre-initiation or initiation of translation, although we cannot rule out interruption of the terminal heteroduplex or direct binding of PPMO to the L polymerase as potential antiviral mechanisms. It is not immediately clear why translation should be a more susceptible process to inhibition by PPMO than the interactions involved in viral RNA synthesis. Further investigation is needed to determine in more detail how the presence of a PPMO hybridized to viral mRNA inhibits events of translation.

In this study we found that under some conditions, high doses of PPMO can produce a mildly pro-viral state inside the cell. PPMO-mediated enhancement of RNA virus growth has been reported
previously for alphaviruses (22), coronaviruses (8, 20) and Japanese encephalitis virus (2), suggesting that this effect may be operative in RNA virus-infected cells in general. This effect may be related to the small but reproducible cell growth-enhancing effect which we observed at intermediate PPMO concentrations (Fig. 2). Further investigation is needed to understand the mechanism of PPMO-mediated enhancement of viral growth.

Treatment with siRNA has also shown efficacy against arenavirus infection of cell cultures (3, 27), and it appears that sequence-specific approaches to arenavirus drug development may warrant further development. For PPMO, future investigation will likely include further evaluation of efficacy in vivo against arenaviruses of importance to public health, generation and characterization of resistant virus, as well as efforts to improve the toxicity profile of PPMO.

Acknowledgments

This work was supported by the Pacific Southwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases (AI-065359) and the California Center for Antiviral Drug Discovery (UCOP-MRPI-143226). LHB was supported by the National Science Foundation (DGE-0638751). The authors wish to thank Joseph Klaus and Radin Aur for technical assistance, and the Chemistry Department at AVI BioPharma, Corvallis, OR for PPMO production.
Table 1. PPMO sequences used in this study.

<table>
<thead>
<tr>
<th>PPMO</th>
<th>5’- Sequence -3’ Positiona Strandb</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TERM-L</td>
<td>R-CGCCTAGGATCCCCGGTGCG 1 - 20</td>
<td>L vRNA</td>
</tr>
<tr>
<td>TERM-L-REP</td>
<td>R-GGCCCTAGGATCCCCGGTGCGC 1 - 20d</td>
<td>L vRNA</td>
</tr>
<tr>
<td>TERM-S</td>
<td>R-GCCTAGGATCCCCCTGTGCGC 1 - 19d</td>
<td>S vRNA</td>
</tr>
<tr>
<td>3’TERM-L</td>
<td>R-GCCACCGGGGGATCTAGGCG 7095-7114</td>
<td>L vRNA</td>
</tr>
<tr>
<td>3’TERM-S</td>
<td>R-GCCACAGTGGATCTAGGCA 3394-3413</td>
<td>S vRNA</td>
</tr>
<tr>
<td>AUG-GP</td>
<td>R-TAATGAACTGCCCCATGTGC 81-101</td>
<td>S vRNA</td>
</tr>
<tr>
<td>AUG-NP</td>
<td>R-ATGCCAGAAGTTCTGGTGATT 3327-3348</td>
<td>S vcRNA</td>
</tr>
<tr>
<td>AUG-L</td>
<td>R-GATTCCTCCATGCTCAAGTGC 7074-7094</td>
<td>L vcRNA</td>
</tr>
<tr>
<td>RANDOM</td>
<td>R-AGTCTCGACTTGCTACCTCA --</td>
<td>--</td>
</tr>
</tbody>
</table>

aNucleotide positions on vcRNA are numbered as the corresponding bases of the vRNA based on GenBank #AY746353 and #AY746354
bSegment L or S, followed by the sense that would match the target sequence
cAll sequences have a 5’-conjugated (RXR)4 XB peptide which mediates delivery
dThese PPMO include one additional 3’-terminal nucleotide that can base pair with the non-templated 5’-G residue that is found on intracellular viral RNA template strands

Table 2. Quantitation of the effects of PPMO on changes in cellular adherence and morphology in arenavirus-infected cells.

<table>
<thead>
<tr>
<th>PPMO</th>
<th>JUNV %Adherent</th>
<th>TCRV %Nuclei in Syncytia</th>
<th>PICV %Adherent</th>
<th>LCMV %Adherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>100 ± 13c</td>
<td>0 ± 0</td>
<td>100 ± 13</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>Untreated</td>
<td>+ 64 ± 11</td>
<td>16 ± 5</td>
<td>50 ± 19</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>TERM-L</td>
<td>+ 108 ± 7</td>
<td>0 ± 0</td>
<td>115 ± 17</td>
<td>53 ± 17</td>
</tr>
<tr>
<td>TERM-L-REP</td>
<td>+ 97 ± 12</td>
<td>1 ± 2</td>
<td>108 ± 5</td>
<td>71 ± 19</td>
</tr>
<tr>
<td>TERM-S</td>
<td>+ 114 ± 16</td>
<td>1 ± 1</td>
<td>108 ± 12</td>
<td>100 ± 17</td>
</tr>
<tr>
<td>3’TERM-L</td>
<td>++ nt</td>
<td>nt</td>
<td>nt</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>3’TERM-S</td>
<td>++ nt</td>
<td>nt</td>
<td>nt</td>
<td>90 ± 18</td>
</tr>
<tr>
<td>AUG-GP</td>
<td>+ 56 ± 28</td>
<td>11 ± 8</td>
<td>47 ± 21</td>
<td>48 ± 14</td>
</tr>
<tr>
<td>AUG-NP</td>
<td>+ nt</td>
<td>nt</td>
<td>nt</td>
<td>78 ± 19</td>
</tr>
<tr>
<td>AUG-Z</td>
<td>+ 99 ± 21</td>
<td>9 ± 3</td>
<td>53 ± 13</td>
<td>34 ± 14</td>
</tr>
<tr>
<td>AUG-L</td>
<td>+ nt</td>
<td>nt</td>
<td>nt</td>
<td>48 ± 17</td>
</tr>
<tr>
<td>IGS-S</td>
<td>+ nt</td>
<td>nt</td>
<td>nt</td>
<td>62 ± 14</td>
</tr>
<tr>
<td>RANDOM</td>
<td>+ 47 ± 23</td>
<td>15 ± 16</td>
<td>62 ± 17</td>
<td>60 ± 22</td>
</tr>
</tbody>
</table>

aVero-E6 Cells were pre-treated with 20 μM PPMO for 3h, inoculated at low multiplicity in the presence of PPMO, rinsed with saline to remove inoculum and maintained in medium containing PPMO
bInfected

cMean number ± standard deviation of adherent cells per microscope field from four representative image fields, expressed as a percentage relative to the mean of uninfected untreated control cultures
dNot tested
Table 3. Effects of 20 μM PPMO treatment on virus titera.

<table>
<thead>
<tr>
<th>PPMO</th>
<th>JUNV</th>
<th>PICV</th>
<th>TCRV</th>
<th>LCMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0 ± 0.11</td>
<td>0.00 ± 0.12</td>
<td>0.00 ± 0.06</td>
<td>0.00 ± 0.07</td>
</tr>
<tr>
<td>TERM-L</td>
<td>3.50 ± 0.08</td>
<td>6.42 ± 0.11</td>
<td>&gt;6.9</td>
<td>2.46 ± 0.20</td>
</tr>
<tr>
<td>TERM-L-REP</td>
<td>2.09 ± 0.03</td>
<td>6.47 ± 0.19</td>
<td>&gt;6.9</td>
<td>4.38 ± 0.18</td>
</tr>
<tr>
<td>TERM-S</td>
<td>2.16 ± 0.14</td>
<td>6.13 ± 0.20</td>
<td>&gt;6.9</td>
<td>3.00 ± 0.04</td>
</tr>
<tr>
<td>3'TERM-L</td>
<td>-0.07 ± 0.14</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>3'TERM-S</td>
<td>-0.23 ± 0.03</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>AUG-GP</td>
<td>0.70 ± 0.04</td>
<td>-0.10 ± 0.04</td>
<td>-0.14 ± 0.03</td>
<td>-0.35 ± 0.03</td>
</tr>
<tr>
<td>AUG-NP</td>
<td>ntd</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>AUG-Z</td>
<td>0.97 ± 0.08</td>
<td>0.05 ± 0.07</td>
<td>-0.27 ± 0.04</td>
<td>-0.38 ± 0.05</td>
</tr>
<tr>
<td>AUG-L</td>
<td>0.93 ± 0.18</td>
<td>nt</td>
<td>nt</td>
<td>-0.29 ± 0.01</td>
</tr>
<tr>
<td>IGS-S</td>
<td>-0.75 ± 0.09</td>
<td>nt</td>
<td>nt</td>
<td>0.23 ± 0.07</td>
</tr>
<tr>
<td>RANDOM</td>
<td>-0.87 ± 0.08</td>
<td>-0.05 ± 0.07</td>
<td>-0.10 ± 0.02</td>
<td>-0.33 ± 0.03</td>
</tr>
</tbody>
</table>

*Cells were treated with PPMO for 3h before inoculation at low multiplicity.

*Log decrease in virus titer relative to untreated controls 48h after inoculation ± standard deviation.

*Negative values indicate that titer increased following treatment.

*Not tested.
Figure 1. Schematic representation of PPMO target sites on both arenavirus genomic RNA segments. (A) PPMO are shown in red and genes are labeled in the 5' to 3' orientation. Additional potential binding sites for TERM-L, TERM-S, TERM-L-REP, 3'TERM-L and 3'TERM-S PPMO at the viral genomic termini (black and white rectangles) are not shown on this diagram, but are listed in Supplementary Fig. S1. (B) Hypothetical mechanisms of action for each PPMO are indicated. Abbreviations: GR genomic replication, T subgenomic mRNA transcription.

Figure 2. Effect of PPMO on Vero E6 cell viability. MTT cytotoxicity assays were performed using uninfected Vero E6 cells incubated for 24h (A) or 96h (B) with increasing concentrations of indicated PPMO before incubation with MTT. After MTT treatment for 40 minutes, cells were solubilized with DMSO and insoluble compound formation was determined photometrically at 560nm. Viability is charted relative to mock-treated cells, set at 1.0. Error bars denote standard deviations calculated from three replicate assays.

Figure 3. Effect of arenaviruses on cellular adhesion and morphology. Cells that remained adherent after three saline washes and crystal violet staining are shown to illustrate changes in cellular adherence 48h after mock infection (left panel) or infection with LCMV (middle panel). An example of cell fusion induced by modified JUNV is shown in the right panel. Arrows point to clusters of nuclei in the center of each syncytium.

Figure 4. Antiviral effects of PPMO treatment in cell culture. Dose-inhibition curves for LCMV-infected cultures treated with indicated PPMO. Error bars indicate standard deviation from three replicate samples.

Figure 5. Reduction of LCMV protein and RNA expression following PPMO treatment. Vero-E6 cells were pretreated with TERM-L PPMO (A and B) or TERM-L-REP PPMO (C and D) 3h before inoculation with LCMV. Cell lysates were harvested for protein analysis 24h (A and C) and...
48h (B and D) after inoculation. Protein from purified virions appears in the leftmost lane of each panel (V). Quantitative RT-PCR was performed to measure the amount of RNA in which the NP gene was in the positive sense, which would include both NP mRNA and S segment vcRNA. Levels of NP RNA relative to GAPDH mRNA controls are expressed as a percentage ± standard deviation from three replicate samples relative to infected untreated control NP RNA levels 24h after inoculation (E). NP, GPC and GP2 protein levels were measured by densitometry analysis of western blots including those shown in panel A, and expressed as percentage ± standard error relative to infected untreated control protein levels (F). Panel G compares relative NP and NP RNA levels 24h after inoculation.

Figure 6. Antiviral efficacy of PPMO against LCMV in mice. Mice were administered sterile saline or ~6 mg/kg (A) or ~9 mg/kg (B) PPMO by intraperitoneal (IP) injection at 3 h before inoculation with LCMV, and daily for 3 days thereafter. Tissues were collected 96h after inoculation. Virus titers were determined by plaque assay and normalized to tissue weight. The dotted line indicates the threshold of detection in our plaque assays.
References


Figure 1.

Figure 2.

Figure 3.
Figure 4.

Figure 5.

Figure 6.