

Protection Against Lethal Marburg Virus Infection Mediated by Lipid Encapsulated Small Interfering RNA

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Background. Marburg virus (MARV) infection causes severe morbidity and mortality in humans and nonhuman primates. Currently, there are no licensed therapeutics available for treating MARV infection. Here, we present the in vitro development and in vivo evaluation of lipid-encapsulated small interfering RNA (siRNA) as a potential therapeutic for the treatment of MARV infection.

Methods. The activity of anti-MARV siRNAs was assessed using dual luciferase reporter assays followed by in vitro testing against live virus. Lead candidates were tested in lethal guinea pig models of 3 different MARV strains (Angola, Ci67, Ravn).

Results. Treatment resulted in 60%–100% survival of guinea pigs infected with MARV. Although treatment with siRNA targeting other MARV messenger RNA (mRNA) had a beneficial effect, targeting the MARV NP mRNA resulted in the highest survival rates. NP-718m siRNA in lipid nanoparticles provided 100% protection against MARV strains Angola and Ci67, and 60% against Ravn. A cocktail containing NP-718m and NP-143m provided 100% protection against MARV Ravn.

Conclusions. These data show protective efficacy against the most pathogenic Angola strain of MARV. Further development of the lipid nanoparticle technology has the potential to yield effective treatments for MARV infection.

Keywords. Marburg virus; filovirus; LNP; RNAi; siRNA; treatment; therapeutics; guinea pig.

Human viral hemorrhagic fevers are caused by 4 virus families, all of which are enveloped RNA viruses [1]. Hemorrhagic fever caused by the filoviruses Marburg virus (MARV) and Ebola virus exhibits the highest mortality rates, ranging from 23% to 90% in humans [2]. Filoviruses remain endemic to Central Africa and sporadic lethal outbreaks continue to occur [3–5]. Despite

the severity of disease caused by filoviruses, no licensed vaccine or therapeutic is currently available. Filoviruses have been the subjects of former biological weapons programs and have the potential for deliberate misuse. For these reasons, the filoviruses are categorized as Tier 1 select agents and Category A priority pathogens by several US government agencies.

MARV possess a single-stranded RNA genome of approximately 19 kb encoding 7 genes: NP (nucleoprotein), VP35 (polymerase cofactor), VP40 (matrix protein), GP (glycoprotein), VP30 (transcription activator), VP24 (secondary matrix protein), and an RNA-dependent RNA polymerase (L polymerase). These genes and their products represent targets for the development of therapeutic agents and vaccines. Current therapeutic strategies consist of passive antibody therapy, which has demonstrated protection of nonhuman primates (NHPs) against MARV-Ci67 using polyclonal NHP immunoglobulin G [6] and morpholino oligomers, which protected NHPs against MARV-Musoke [7]. It is unclear whether these

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therapeutic strategies could confer protection against MARV-Angola, which causes higher mortality rates in humans and a faster disease course in NHPs [8, 9].

RNA interference (RNAi) is a naturally occurring mechanism for the inhibition of gene expression. Small interfering RNA (siRNA) can inhibit the replication of several hemorrhagic fever viruses in vitro including dengue [10], MARV [11], Lassa [12], Junin [13], yellow fever [14], and Ebola virus [15]. The use of siRNA as a postexposure treatment presents numerous advantages including rapid design against new emerging agents, established large-scale manufacturing capability, and a defined mechanism of action that can be confirmed experimentally in vitro and in vivo. The main challenge in siRNA therapeutics is the requirement for safe and effective drug delivery vehicles that confer protection from nuclease-mediated siRNA degradation and facilitate siRNA uptake and delivery to target tissues and cells. Strategies explored to address this challenge include the use of modified nucleotide chemistries to improve pharmacologic properties, complexing siRNA with polycations or cyclodextrin polymers, and the encapsulation of siRNA in lipid-based carriers [16]. We have developed a platform directing efficient siRNA delivery through the use of novel amino-lipids that encapsulate siRNA within lipid nanoparticles (LNPs) [17, 18]. Importantly, we have successfully demonstrated that antiviral siRNA delivered by LNPs can confer complete protection to NHPs from lethal *Zaire ebolavirus* infection [19]. This result strongly suggests that LNP-mediated delivery of siRNA is an efficacious therapeutic strategy for filovirus infection.

To explore the use of siRNA-LNP therapeutics against other filoviruses, we describe here the design, screening, and in vivo efficacy of novel siRNA-LNP as postexposure treatment against MARV strains Angola, Ci67, and Ravn in newly developed lethal outbred guinea pig models of infection. We show for the first time 100% postexposure protection against the most pathogenic Angola strain of MARV in guinea pigs.

MATERIALS AND METHODS

Design and In Vitro Screening of siRNAs

MARV messenger RNA (mRNA) sequences for Angola 1379c (DQ447653.1), Ci67 (EF446132.1), Musoke (DQ217792.1), and Ravn (DQ447649.1) were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Sequences were aligned using ClustalW (<http://www.ebi.ac.uk/clustalw/>). siRNA were designed to target conserved sequence regions of MARV VP24, VP35, VP40, NP, and L polymerase (Lpol) genes manually or by using a conventional siRNA design algorithm [19]. We excluded siRNA with sequence homology to human mRNAs of 16 or more contiguous bases using BLAST (v.2.2.13) and eliminated siRNA that did not display 100% nucleotide sequence identity with at least 3 MARV strains (Angola, Ci67, and Musoke). The siRNAs were then 2'-O-methylated to abrogate

immune stimulatory activity as described previously [20]. Single-stranded RNA oligonucleotides were synthesized by Integrated DNA Technologies and annealed to form siRNA duplexes by denaturation at 95°C for 1 minute followed by 1 hour of incubation at 37°C. The siRNAs were then encapsulated within LNP as described [19].

The siRNA activity against MARV VP24, VP35, VP40, NP, and Lpol mRNAs were confirmed in vitro using a dual luciferase reporter assay assessing reduction in the expression of the respective viral transgenes cloned into the psiCHECK2 vector (Promega) in HepG2 cells (ATCC No. HB-8065) grown in minimum essential medium (Gibco) supplemented media. Gene synthesis and subcloning into psiCHECK2 was conducted by GenScript Inc. HepG2 cells were reverse-transfected with Lipofectamine 2000 (Invitrogen) complexes containing 0.75 µg of psiCHECK2 plasmid and LNP (20–0.8 nM) in 96-well plates. LNPs containing siRNA targeting Renilla luciferase (Rluc) or a nonspecific target (ApoB) were used as positive and negative controls, respectively. Cells were lysed 48 hours after transfection. Luminescence was detected using the Dual Luciferase Reporter Assay kit according to the manufacturer's protocol (Promega) using a Berthold luminometer (Berthold Detection Systems). The Renilla luciferase signal (reflecting target transgene expression) was normalized to the Firefly luciferase signal and expressed as percentage of gene expression relative to a plasmid-only control.

In Vitro Antiviral Efficacy

To confirm the efficacy of lead siRNA in LNPs, it was tested in vitro against live MARV-Angola. The work was conducted at the Galveston National Laboratory (GNL) under BSL-4 biocontainment. HepG2 cells were seeded into 24-well plates and incubated at 37°C/5% CO₂ for 24 hours prior to LNP treatment (10 nM or 100 nM). After 24 hours' incubation, cells were infected at a multiplicity of infection (MOI) of 0.5 or 0.1 for 1 hour, after which the wells were washed with phosphate-buffered saline (PBS) and fresh media was added. The cells were then incubated for an additional 48 hours prior to the collection of supernatants for plaque assay.

In Vivo Immune Stimulation Assays

A mouse study was performed in accordance with the Canadian Council on Animal Care guidelines following approval by the local Animal Care and Use Committee at Tekmira Pharmaceuticals Corporation, in compliance with the animal welfare act and other federal statutes and regulations relating to animals and experiments involving animals, and the Guide for the Care and Use of Laboratory Animals, National Research Council. Six- to 8-week-old female CD1 ICR mice (n = 36; 4 per group) were subjected to a 1-week quarantine and acclimation period before study start. LNPs (5 mg/kg) were administered by bolus intravenous injection in the lateral tail vein in 0.2 mL PBS. Plasma and livers were harvested 4 hours after injection of LNPs.

Plasma cytokine measurements and liver interferon (IFN)-induced protein with tetratricopeptide repeats (IFIT1) mRNA analysis were performed as described previously [20]. Clinical signs and organ weights were monitored and no differences from the PBS control-treated group were observed.

Antiviral Efficacy in Guinea Pigs

We recently developed outbred Hartley strain guinea pig models for MARV strains Angola, Ci67, and Ravn by serial passaging of virus isolated from infected livers and/or spleens. The guinea pig-adapted MARV-Angola was developed by 4 passages in Hartley guinea pigs; the guinea pig-adapted MARV-Ci67 by 2 passages in strain 13 guinea pigs and 3 passages in Hartley guinea pigs; and the guinea pig-adapted MARV-Ravn by 2 passages in strain 13 guinea pigs and 1 passage in Hartley guinea pigs. The resulting adapted strains give rise to high plasma viremia (up to 10^7 plaque-forming units [PFU]/mL) and are uniformly lethal with death occurring 6–9 days after challenge for MARV-Angola and 8–14 days after challenge for both MARV-Ci67 and MARV-Ravn. Animal studies were completed under biosafety level (BSL) 4 biocontainment at the GNL and were approved by the University of Texas Medical Branch Institutional Laboratory Animal Care and Use Committee (IACUC) in accordance with state and federal statutes and regulations relating to experiments involving animals and the Institutional Biosafety Committee. Female Hartley guinea pigs (351–400 g) were purchased from Charles River Laboratories and subsequently quarantined and acclimatized for 1 week prior to MARV challenge. The siRNA duplexes were synthesized by Integrated DNA Technologies. Lipid encapsulation of the siRNAs was performed at Tekmira Pharmaceuticals as described [19]. Individual animals were infected with approximately 1000 PFU of guinea pig-adapted MARV-Angola, MARV-Ci67, or MARV-Ravn by intraperitoneal injection. Approximately 1 hour after viral inoculation, LNPs containing single or a 1:1 cocktail of 2'-O-methylated siRNA (0.5 mg/kg total) was administered by bolus retro-orbital injection and subsequent doses were given daily for a total of 7 doses. Control animals were treated with LNP carrying a nonspecific siRNA (Luc-mod) or remained untreated. Survival of the animals was followed for 28 days, after which survivors were killed at the study endpoint per IACUC protocol.

Statistical Analysis

Kaplan–Meier survival curves were plotted using GraphPad Prism software for each in vivo study. Differences between treatment groups and the control group were analyzed for statistical significance using the log-rank test ($P < .00833$ when adjusted for multiple comparisons using Bonferroni correction) and Fisher exact test (1-sided; $P < .05$).

Virus Titration by Plaque Assay

MARV titration was performed by conventional plaque assay on Vero E6 cells from cell culture supernatants from in vitro

efficacy experiments or from plasma collected from guinea pigs at day 7 postinfection, as described elsewhere [21, 22]. In brief, increasing 10-fold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (200 μ L). The limit of detection was 25 PFU/mL.

Virus mRNA Quantitation by Quantitative Reverse Transcription Polymerase Chain Reaction

Whole cell (HepG2) lysates from MARV-Angola infected cells were incubated with 1 mL of Trizol (Invitrogen) for 15 minutes with rocking every 5 minutes, and total RNA isolation was performed according to manufacturer instructions. Isolated RNA was quantified using a NanoDrop 2000 (Thermo Scientific). Two micrograms of total RNA was used to isolate mRNA using a Dynabead mRNA purification kit (Invitrogen). The mRNAs (30 ng) were used in a 1-step quantitative reverse transcription polymerase chain reaction (qRT-PCR) using primers and FAM-labeled probes for each MARV-Angola mRNA in question (VP35, VP40, or L). Primers and a VIC probe for human β -actin were used to normalize each viral mRNA to the amount of human β -actin mRNA present in each mRNA sample. FAM and VIC signals were recorded for each sample, and the Bio-Rad CFX manager software was used to collect the normalized expression of MARV-Angola mRNA compared to human β -actin mRNA.

RESULTS

siRNA Design and In Vitro Screening

The genus *Marburgvirus* consists of only 1 species, *Lake Victoria marburgvirus* [2], divided into 2 lineages. One of these lineages comprises a number of strains including Angola, Ci67, Musoke, Ozolins, and Popp, which exhibit genomic sequence differences of only 0%–7.4%, whereas the second lineage comprising the Ravn strain shows greater sequence disparity (21% when compared to strains in the other lineage). The broad range of sequence variation among the different strains confounds the design of broad-spectrum siRNA therapeutics; however, the NP and VP24 genes show a higher percentage of conserved contiguous bases that are more amenable to siRNA design. The objective of our design strategy was to obtain siRNA with broad spectrum activity against 3 (Angola, Musoke, and Ci67) or 4 (Angola, Musoke, Ci67, and Ravn) different MARV strains.

Target mRNA sequences from MARV strains were aligned using ClustalW, and conserved regions were identified. Using the MARV-Angola mRNA sequence for each target as a template, we designed a panel of siRNAs against VP24, VP35, NP, and L polymerase, which was then triaged based on nucleotide complementarity between the target mRNA sequence and the 19 nucleotide core sequence of the antisense strand of the

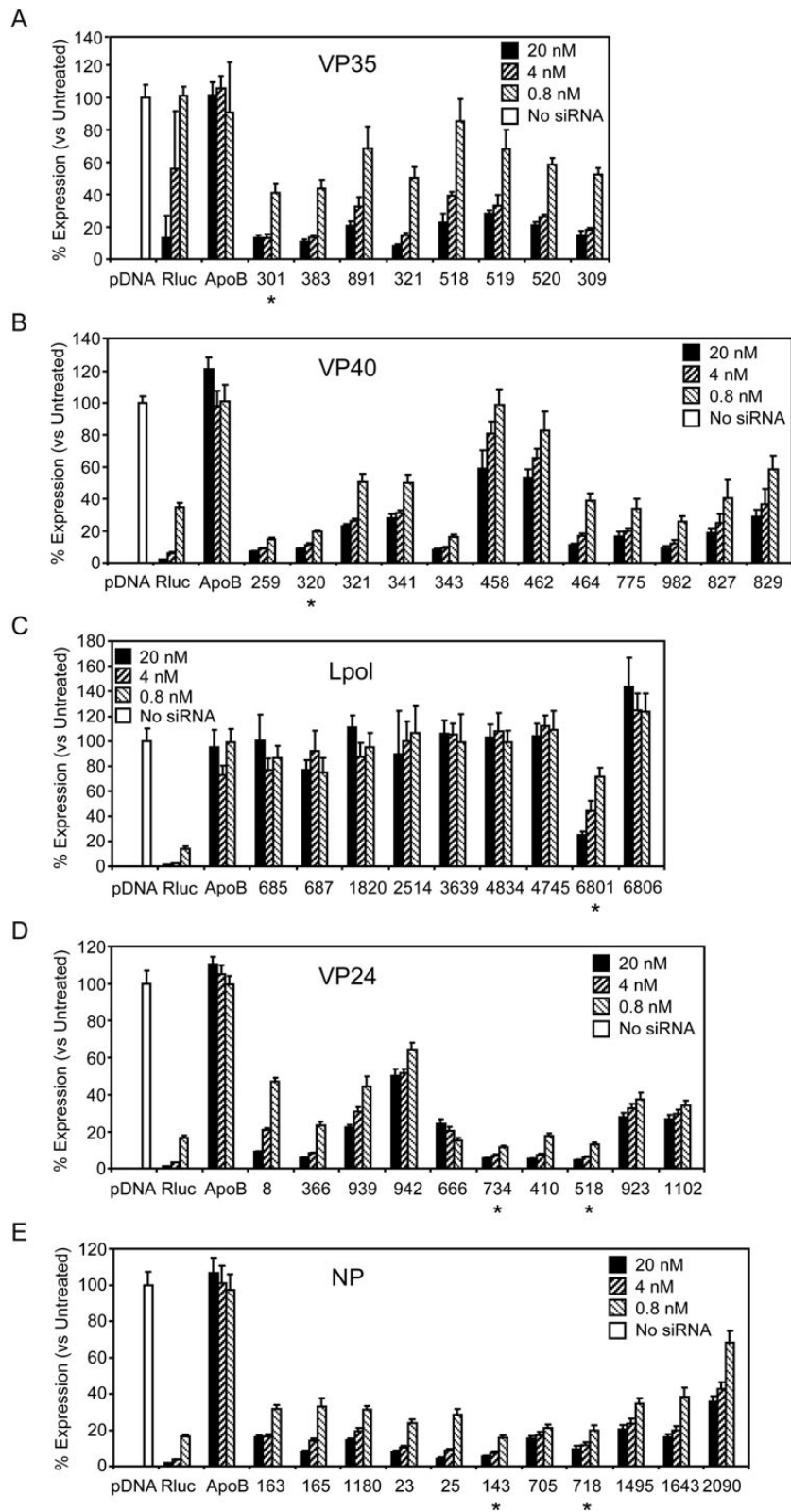


Figure 1. Small interfering RNAs (siRNAs) targeting Marburg virus (MARV) genes display activity in a dual luciferase reporter assay. HepG2 cells were reverse-transfected with plasmid containing the MARV target genes VP35 (A), VP40 (B), L polymerase (C), VP24 (D), and NP (E), and 0.8–20 nM of lipid nanoparticles containing siRNAs (numbered). Cells were lysed 48 hours later for sequential measurement of Renilla luciferase (fused to MARV target transgene expression) and Firefly luciferase signals. The Renilla luciferase signal was normalized to the Firefly luciferase signal and expressed as the percentage of gene expression relative to a plasmid-only control (pDNA) assigned a value of 100%. An siRNA against Renilla luciferase (Rluc) and an siRNA against ApoB were used as positive and negative controls, respectively. Asterisks denote lead siRNAs selected for further efficacy evaluation. Data represent the mean±SD (n = 3) of experimental results.

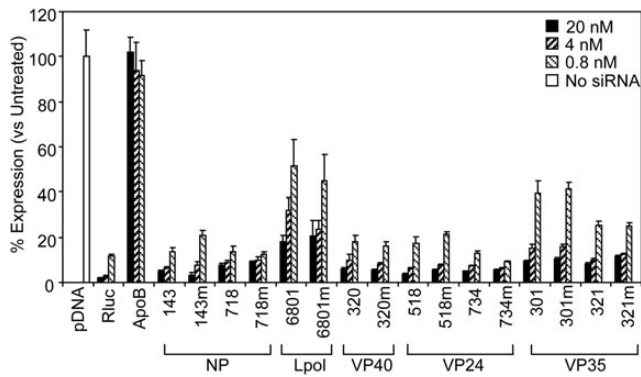


Figure 2. 2'-O-methylated small interfering RNAs (siRNAs) targeting Marburg virus (MARV) genes retain activity in a dual luciferase reporter assay. HepG2 cells were reverse-transfected with plasmid containing the MARV target genes VP35, VP40, L polymerase, VP24, and NP, and 0.8–20 nM of lipid nanoparticles. Cells were lysed 48 hours later for sequential measurement of Renilla luciferase (fused to the MARV target transgene) and Firefly luciferase signals. The Renilla luciferase signal was normalized to the Firefly luciferase signal and expressed as the percentage of gene expression relative to a plasmid-only control (pDNA) assigned a value of 100%. Lead native and 2'-O-methylated (designated "m") siRNAs are compared. An siRNA against Renilla luciferase (Rluc) and an siRNA against ApoB were used as positive and negative controls, respectively. Data represent the mean±SD (n = 3) of experimental results.

siRNA. We down-selected siRNAs that did not have 100% sequence complementarity with at least 3 of the virus strains under consideration. siRNA were screened in a dual luciferase reporter assay against the relevant viral mRNA target on HepG2 cells (Figure 1). Two siRNAs targeting VP35 mRNA (301 and 321; Figure 1A), 2 siRNAs targeting VP40 (259 and 320; Figure 1B), 1 siRNA targeting Lpol (6801; Figure 1C), 1 siRNA targeting VP24 (518; Figure 1D), and 1 siRNA targeting NP (718; Figure 1E) were selected as lead candidates based on their potency (assessed by their ability to provide the highest mRNA knockdown at the lowest dose) and their antisense sequence complementarity with MARV strains Musoke, Angola, and Ci67 mRNAs. VP35-520, VP40-827, VP24-734, and NP-143 were chosen using additional criteria of antisense sequence complementarity with MARV-Ravn mRNAs.

Modification of siRNAs for In Vivo Applications

To minimize the inherent immune stimulatory properties of native siRNA sequences [23, 24], lead MARV siRNAs were synthesized incorporating select 2'-O-methyl chemical modifications in both strands (Supplementary Table 1) as described previously [20]. The activity of both nonmodified and modified lead MARV siRNAs was compared in a dual luciferase reporter assay to confirm that their activity was not compromised by 2'-O-methylation (Figure 2).

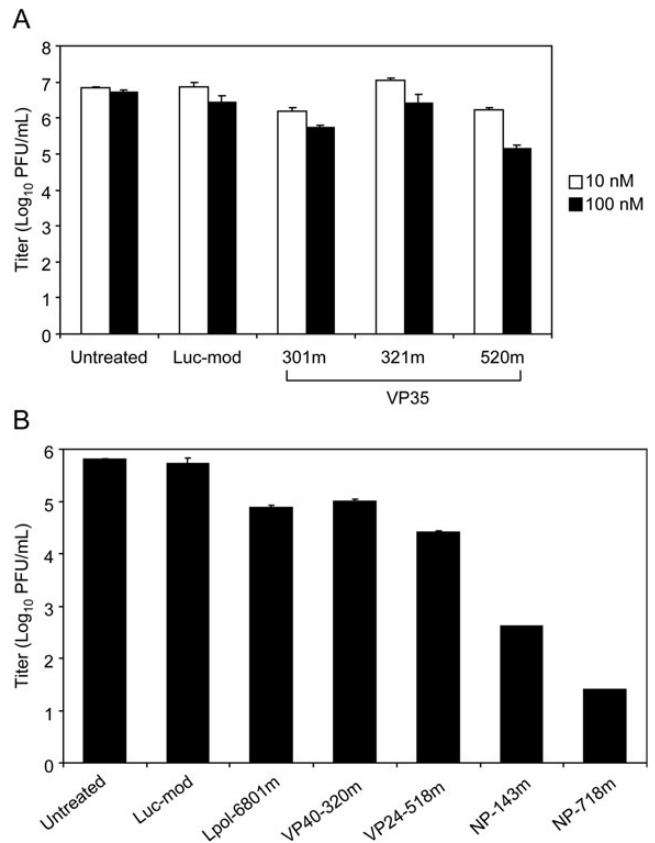


Figure 3. 2'-O-methylated small interfering RNA (siRNA) lipid nanoparticle (LNP) treatment of HepG2 cells results in the reduction of Marburg virus (MARV) titers in infected cells. HepG2 cells were treated with LNPs targeting individual viral messenger RNAs (A, VP35; B, VP40, L polymerase, VP24, and NP) and Luc control LNPs or left untreated and infected with MARV (Angola strain). Cell supernatants were harvested and serially diluted, then plated on top of Vero76 cells. As a negative control, cells were left untreated or were treated with siRNA targeting luciferase (Luc-mod). Data represent the mean±SD (n = 3) of experimental results. Abbreviation: PFU, plaque-forming unit.

In Vitro Efficacy

To assess the antiviral efficacy of 2'-O-methylated siRNA in LNP, HepG2 cells were treated for 24 hours and then infected with MARV-Angola. Limited antiviral efficacy was observed for all 3 VP35 siRNAs (Figure 3A). To verify that our siRNAs mediated specific VP35 viral mRNA reduction in vitro, we quantified viral mRNA from these cells by qRT-PCR (normalized to human β -actin; Figure 4). Although specific viral mRNA knockdown was observed, VP35-targeting LNPs were not selected for subsequent in vivo evaluation due to poor in vitro antiviral activity. LNPs targeting MARV Lpol, VP24, and VP40 mRNA exhibited antiviral efficacy, displaying up to 1 log₁₀ reduction in PFUs per milliliter (Figure 3B). LNPs targeting MARV NP mRNA exhibited significantly higher antiviral efficacy, resulting in 4 and 3 log₁₀ PFU/mL reductions with NP-718m and NP-143m, respectively, when compared to

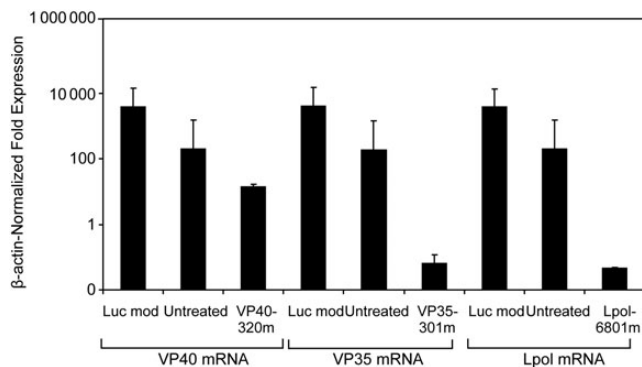


Figure 4. Treatment of infected cells with 2'-O-methylated small interfering RNA (siRNA) lipid nanoparticles (LNPs) downregulates viral messenger RNA (mRNA) expression. HepG2 cells were treated with LNPs targeting individual viral mRNAs or Luc control LNPs or were left untreated and 24 hours later infected with Marburg virus (Angola strain). Cells were lysed and viral mRNA levels were quantified by real-time polymerase chain reaction and normalized to human β -actin. Data represent the mean \pm SD (n = 3) of experimental results.

controls (Figure 3B). Lead siRNAs targeting MARV Lpol, VP24, VP40, and NP were chosen for in vivo immune stimulation testing prior to selection for in vivo protective efficacy studies.

In Vivo Immune Stimulation

Lead-modified siRNAs (Lpol-6801m, VP40-320m, VP24-518m, NP-143m, and NP-718m) were assessed for their ability to induce an immune response in ICR mice. A nonmodified luciferase-targeting (Luc) siRNA and a corresponding 2'-O-methylated siRNA (Luc-mod) were used as positive and

negative controls for immune stimulation, respectively (Supplementary Table 1). The Luc-positive control induced significant IFN- α , IFN- β , tumor necrosis factor α , and interleukin 6 cytokine induction in plasma and IFIT1 mRNA in liver at 4 hours posttreatment, whereas none of the other LNPs showed increases above the level of the Luc-mod negative control (Table 1). These results confirmed that the lead 2'-O-methylated siRNAs did not possess significant immune stimulatory ability.

In Vivo Activity

Our initial experiment in guinea pigs was designed to test lead siRNA LNPs against MARV-Angola, the virus strain that has been responsible for the largest and deadliest human MARV outbreak to date [8]. Animals treated with the nontargeting Luc-mod siRNA showed clinical signs indicative of MARV infection with 0% (0/5) survival and fatalities occurring in a time window between days 6 and 8 after virus challenge (Figure 5A, Supplementary Table 2A). All animals treated with VP40-320m, VP24-734m, and VP24-518m siRNA showed clinical signs of MARV infection with 0% (0/5) survival and fatalities occurring in a time window between days 8 and 11. A survival advantage was shown for animals treated with Lpol-6801m (20% survival [1/5]; clinical signs in all animals) and NP (NP-143m: 40% survival [2/5], clinical signs in all; NP-718m: 100% survival [4/4], clinical signs in 1 animal only). A single fatality (cannibalized by cage mates) occurred at day 15 in the NP-718m-treated group, but this animal was removed from the survival analysis as this animal appeared stunted prior to infection (Figure 5B). Survival differences were statistically significant for all comparisons between treated and Luc-control groups ($P < .01$; log-rank test with Bonferroni correction for

Table 1. Plasma Cytokine and Liver IFIT1 Messenger RNA Levels From Female ICR Mice (n = 4) 4 Hours After Treatment

Treatment	Cytokines, pg/mL of Plasma								Mean Fold Increase in IFIT1 mRNA Induction Relative to PBS (Normalized to GAPDH mRNA)	
	IFN- α		IFN- β		IL-6		TNF- α			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PBS	77	60	25	4	ND	ND	5	5	1	0.3
Luc	3822	2531	684	460	426	220	249	185	248.7	56.5
Luc mod	82	73	25	1	ND	ND	11	13	1.4	0.9
Lpol-6801m	49	4	24	2	ND	ND	ND	ND	1.3	0.6
VP40-320m	84	67	27	3	ND	ND	ND	ND	1.4	0.5
VP24-518m	46	1	24	1	ND	ND	ND	ND	1.8	0.8
VP24-734m	84	68	26	1	ND	ND	2	4	1.1	0.4
NP-143 m	47	6	30	5	ND	ND	5	8	1.5	0.4
NP-718 m	53	12	27	2	ND	ND	12	24	4.6	4.3

Abbreviations: GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IFIT, Interferon-induced protein with tetratricopeptide repeats 1; IFN, interferon; IL, interleukin; mRNA, messenger RNA; ND, not detected; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

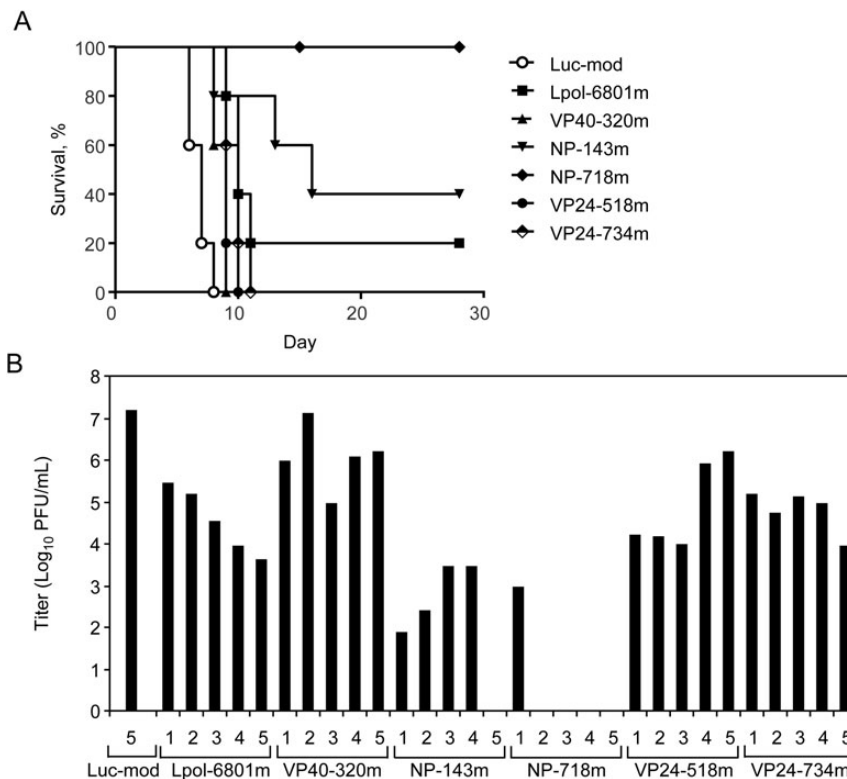


Figure 5. 2'-O-methylated small interfering RNAs (siRNAs) in lipid nanoparticles (LNPs) confer protection to guinea pigs against Marburg virus (MARV) Angola challenge. Survival (A) and plasma viremia (B) of Hartley strain guinea pigs after challenge with guinea pig-adapted MARV-Angola. Animals (n = 5 per group) were treated 1 hour after viral challenge, then treated daily for 6 additional consecutive days with LNPs containing 0.5 mg/kg of each one of the siRNAs directed against the MARV L polymerase, VP24, VP40, or NP messenger RNAs. An siRNA against Luciferase (Luc-mod) was used as a negative control. Viremia data from animal 4 in the Luc-mod group were not available. All other animals in the control group died from viral infection before collection.

multiple comparisons) with the exception of the VP40-320m treatment. Only treatment with NP-718m was statistically significant ($P < .01$) by the Fisher exact test. Comparisons between treatment groups and untreated historical controls (n = 10; Supplementary Figure 1) show that treatment with NP-718m was statistically significant (log-rank test, $P < .00833$ and Fisher exact test, $P < .01$). Treatment with NP-143m was also found to be statistically significant ($P < .00833$; log-rank test).

To determine whether fatalities were due to MARV-Angola infection, we assessed the viral load in plasma samples collected from individual animals at day 7 (Figure 5B). High viremia ($>7 \log_{10}$ PFU/mL) was detected from the single control (Luc-mod-treated) animal alive on day 7. Survivors in Lpol and NP treatment groups showed lower viremia than nonsurvivors in the Luc-mod control, and VP24 and VP40 treatment groups.

Encouraged by the data from the MARV-Angola challenge, we were interested in whether we could achieve protection across a broader spectrum of MARV lineages. To achieve this, we combined the 2 lead MARV NP siRNA (NP-143m and NP-718m) in 1 LNP for the treatment of animals and then challenged outbred guinea pigs with MARV-Ci67 or MARV-Ravn

(Figure 6). The NP cocktail LNP was tested in parallel with NP-143m and NP-718m LNPs against MARV-Ci67 (Figure 6A) or MARV-Ravn (Figure 6C) in the lethal outbred guinea pig challenge model. Control animals in these studies were not treated with LNPs and all succumbed to viral infection between days 8 and 12 while showing clinical signs consistent with MARV infection. Survival results in the MARV-Ci67-infected animals corroborated the results seen with MARV-Angola, showing that NP-718m (100% survival [5/5]) is more efficacious than NP-143m (60% survival [3/5]). Survival in the MARV NP cocktail-treated group suggested a dilutive effect of mixing both siRNAs (75% survival [3/4]), where the 3 surviving animals showed clinical signs of virus infection at days 8 and 9 but subsequently recovered (Figure 6A). However, given the small number of animals that were assessed, it is difficult to conclude whether the cocktail treatment was less efficacious (no statistical significance by log-rank test). Survival differences were statistically significant for all comparisons between individual siRNA-treated and untreated groups ($P < .0083$; log-rank test). Only treatment with NP-718m was statistically significant ($P < .01$) by the Fisher exact test. High overall virus levels were

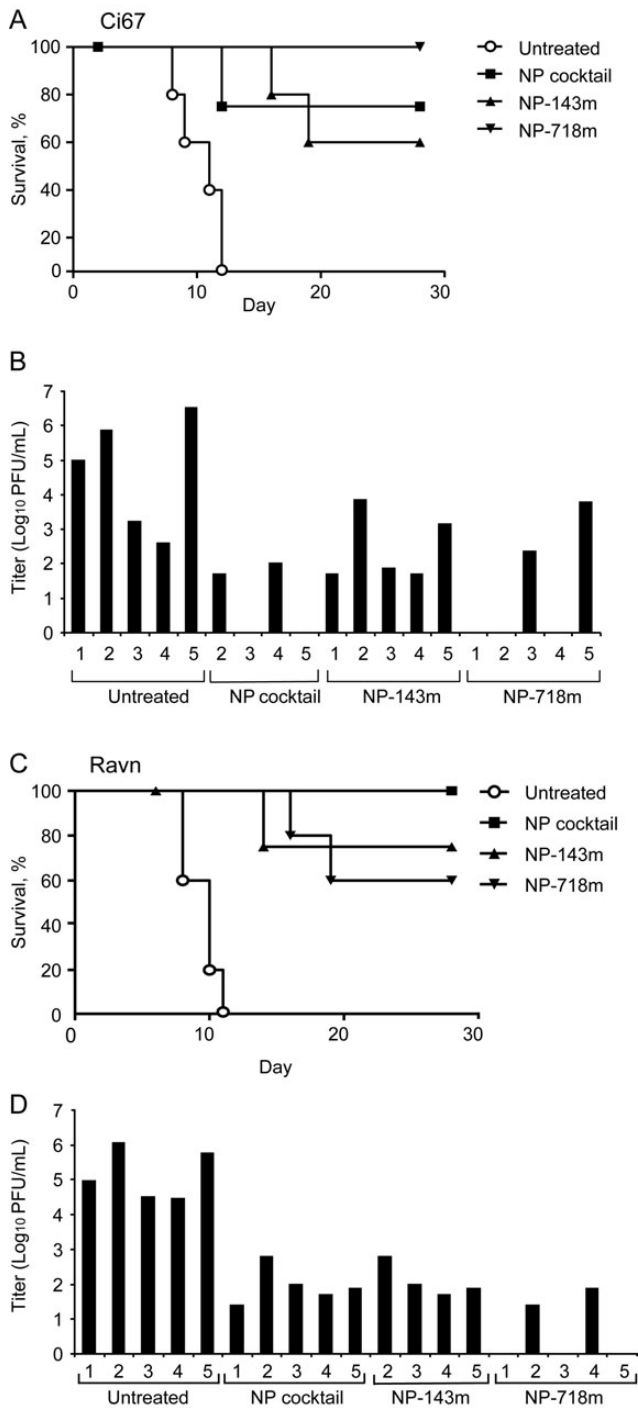


Figure 6. 2'-O-methylated small interfering RNAs (siRNAs) in lipid nanoparticles (LNPs) confer protection to guinea pigs against Marburg virus (MARV) Ci67 or MARV-Ravn challenge. Survival and viremia of MARV Ci67 (A and B) and MARV-Ravn-infected (C and D) Hartley strain guinea pigs after treatment with LNPs containing NP-targeting 2'-O-methylated NP siRNA. Animals (n = 5 per group) were treated 1 hour after viral challenge and treated daily for 6 consecutive days with LNPs containing 0.5 mg/kg of either individual siRNAs or a 1:1 cocktail of each one of the 2 siRNAs directed against NP messenger RNA. Viremia was not assessed for 2 animals (1 treated with NP cocktail [B] and 1 treated with NP-143 m [D]), as these died prior to collection for viremia analysis.

detected at day 7 in untreated animals, whereas they remained lower in treated animals (Figure 6B). When animals were infected with MARV-Ravn (Figure 6C), all untreated animals succumbed to virus infection within days 6 and 11 and showed clinical signs of disease (Supplementary Table 2C). Animals treated with NP-718m LNP achieved 60% (3/5) survival, whereas animals treated with NP-143m achieved 75% (3/4) survival. Interestingly, all animals in the NP cocktail-treated group survived, despite the presence of a low tolerance mismatch at position 9 in the NP-718m siRNA sequence against the NP mRNA sequence of MARV Ravn. Survival differences were statistically significant for all comparisons between treated and control groups ($P < .00833$; log-rank test). Only treatment with NP cocktail was statistically significant ($P < .01$) by the Fisher exact test. Comparison between treatment groups and untreated historical controls (n = 5 additional animals; Supplementary Figure 1), showed that survival differences were statistically significant for all comparisons between treated and untreated groups ($P < .001$; log-rank test and $P < .01$; Fisher exact test). Viral loads detected in control animals at day 7 with MARV-Ci67 or MARV-Ravn were lower than MARV-Angola, but otherwise the trends observed in the broad spectrum treatment study with MARV-Ci67 and MARV-Ravn, where control animals showed significantly higher viremia than NP drug-treated animals. It is unclear why certain animals that showed low or no viremia on day 7 subsequently died (Figure 6D).

DISCUSSION

Here we present the development and evaluation of lipid-encapsulated siRNA (LNP) as a potential therapeutic agent against a broad spectrum of MARVs. As a precursor to in vivo studies, RNAi activity of anti-MARV siRNA was initially assessed using dual luciferase reporter assays followed by in vitro testing against live virus. Lead candidates were tested in newly developed lethal outbred guinea pig models of MARV-Angola, MARV-Ci67, or MARV-Ravn infection, with MARV-Angola being the most lethal MARV strain to date [8]. Our data show that siRNA targeting MARV NP mRNA delivered by LNPs is a promising postexposure treatment strategy where treatment resulted in 60%–100% survival in guinea pigs infected with MARV strains Angola, Ci67, or Ravn. Although treatment with siRNA targeting other viral mRNA had a positive effect on survival of the animals, targeting the NP viral mRNA resulted in the highest survival rates.

Until now, substantial protection against the most lethal MARV strain, Angola, has not yet been shown using any therapeutic candidate. Here, we show that MARV NP-718m siRNA in LNPs provided 100% protection against MARV-Ci67 and MARV-Angola. MARV NP-718m LNP is more efficacious

than NP-143m against strains where no nucleotide mismatches exist between the antisense strand and the target mRNA (strains Angola and Ci67). This difference in efficacy is absent when NP-718m is used as a single drug component to treat a MARV-Ravn infection (60% survival). In this case, a single nucleotide mismatch exists (U-G) in a low mismatch tolerance position (nucleotide 9 of the antisense strand) [25]. Despite this mismatch, NP-718m siRNA remained active against MARV-Ravn. Interestingly, in animals infected with MARV-Ravn, the cocktail of NP-718m and NP-143m siRNA in LNP provided 100% survival, whereas survival was lower using either of these siRNA alone.

Multivalent antiviral therapy, whereby multiple nucleic acid drug components have been used to target different viral mRNAs in the treatment of filovirus infections, has been used with some success [7, 19]. Here, we combined 2 different siRNAs in an attempt to provide broader-spectrum antiviral activity, and despite one of the siRNAs being less efficacious when used alone, we provided complete protection against MARV-Ravn. Future work will include evaluation of anti-MARV siRNA LNPs in NHPs.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. M. R., A. J., I. M., and T. W. G. claim intellectual property regarding RNA interference for the treatment of filovirus infections. R. U.-B., M. R., A. J., and I. M. are employees of Tekmira Pharmaceuticals. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

- Geisbert TW, Jahrling PB. Exotic emerging viral diseases: progress and challenges. *Nat Med* **2004**; 10:S110–21.
- Feldmann H, Sanchez A, Geisbert TW. Filoviridae: Marburg and Ebola viruses. In: Knipe DM, Howley PM, eds. *Fields virology*. 6th ed. Philadelphia: Lippincott Williams & Wilkins, **2013**:923–56.
- World Health Organization. Ebola haemorrhagic fever, Democratic Republic of the Congo. *Wkly Epidemiol Rec* **2012**; 87:338–9.
- World Health Organization. Ebola haemorrhagic fever, Uganda. *Wkly Epidemiol Rec* **2012**; 87:339.
- World Health Organization. Marburg haemorrhagic fever, Uganda. *Wkly Epidemiol Rec* **2012**; 87:437–8.
- Dye JM, Herbert AS, Kuehne AI, et al. Postexposure antibody prophylaxis protects nonhuman primates from filovirus disease. *Proc Natl Acad Sci U S A* **2012**; 109:5034–9.
- Warren TK, Warfield KL, Wells J, et al. Advanced antisense therapies for postexposure protection against lethal filovirus infections. *Nat Med* **2010**; 16:991–4.
- Towner JS, Khristova ML, Sealy TK, et al. Marburg virus genomics and association with a large hemorrhagic fever outbreak in Angola. *J Virol* **2006**; 80:6497–516.
- Geisbert TW, Daddario-DiCaprio KM, Geisbert JB, et al. Marburg virus Angola infection of rhesus macaques: pathogenesis and treatment with recombinant nematode anticoagulant protein c2. *J Infect Dis* **2007**; 196(suppl 2):S372–81.
- Caplen NJ, Zheng Z, Falgout B, Morgan RA. Inhibition of viral gene expression and replication in mosquito cells by dsRNA-triggered RNA interference. *Mol Ther* **2002**; 6:243–51.
- Fowler T, Bamberg S, Moller P, et al. Inhibition of Marburg virus protein expression and viral release by RNA interference. *J Gen Virol* **2005**; 86:1181–8.
- Muller S, Gunther S. Broad-spectrum antiviral activity of small interfering RNA targeting the conserved RNA termini of Lassa virus. *Antimicrob Agents Chemother* **2007**; 51:2215–8.
- Artuso MC, Ellenberg PC, Scolaro LA, Damonte EB, Garcia CC. Inhibition of Junin virus replication by small interfering RNAs. *Antiviral Res* **2009**; 84:31–7.
- Pacca CC, Severino AA, Mondini A, et al. RNA interference inhibits yellow fever virus replication in vitro and in vivo. *Virus Genes* **2009**; 38:224–31.
- Geisbert TW, Hensley LE, Kagan E, et al. Postexposure protection of guinea pigs against a lethal Ebola virus challenge is conferred by RNA interference. *J Infect Dis* **2006**; 193:1650–7.
- Gavrilov K, Saltzman WM. Therapeutic siRNA: principles, challenges, and strategies. *Yale J Biol Med* **2012**; 85:187–200.
- Jeffs LB, Palmer LR, Ambegia EG, Giesbrecht C, Ewanick S, MacLachlan I. A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA. *Pharm Res* **2005**; 22:362–72.
- Semple SC, Akinc A, Chen J, et al. Rational design of cationic lipids for siRNA delivery. *Nat Biotechnol* **2010**; 28:172–6.
- Geisbert TW, Lee AC, Robbins M, et al. Postexposure protection of non-human primates against a lethal Ebola virus challenge with RNA interference: a proof-of-concept study. *Lancet* **2010**; 375:1896–905.
- Judge AD, Robbins M, Tavakoli I, et al. Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. *J Clin Invest* **2009**; 119:661–73.
- Jones SM, Feldmann H, Stroher U, et al. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. *Nat Med* **2005**; 11:786–90.
- Daddario-DiCaprio KM, Geisbert TW, Stroher U, et al. Postexposure protection against Marburg haemorrhagic fever with recombinant vesicular stomatitis virus vectors in non-human primates: an efficacy assessment. *Lancet* **2006**; 367:1399–404.
- Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol* **2005**; 23:457–62.
- Robbins M, Judge A, MacLachlan I. siRNA and innate immunity. *Oligonucleotides* **2009**; 19:89–102.
- Ahmed F, Raghava GP. Designing of highly effective complementary and mismatch siRNAs for silencing a gene. *PLoS One* **2011**; 6:e23443.