Marburg virus infection in nonhuman primates: Therapeutic treatment by lipid-encapsulated siRNA


Marburg virus (MARV) and the closely related filovirus Ebola virus cause severe and often fatal hemorrhagic fever (HF) in humans and nonhuman primates with mortality rates up to 90%. There are no vaccines or drugs approved for human use, and no postexposure treatment has completely protected nonhuman primates against MARV-Angola, the strain associated with the highest rate of mortality in naturally occurring human outbreaks. Studies performed with other MARV strains assessed candidate treatments at times shortly after virus exposure, before signs of disease are detectable. We assessed the efficacy of lipid nanoparticle (LNP) delivery of anti-MARV nucleoprotein (NP)–targeting small interfering RNA (siRNA) at several time points after virus exposure, including after the onset of detectable disease in a uniformly lethal nonhuman primate model of MARV-Angola HF. Twenty-one rhesus monkeys were challenged with a lethal dose of MARV-Angola. Sixteen of these animals were treated with LNP containing anti-MARV NP siRNA beginning at 30 to 45 min, 1 day, 2 days, or 3 days after virus challenge. All 16 macaques that received LNP-encapsulated anti-MARV NP siRNA survived infection, whereas the untreated or mock-treated control subjects succumbed to disease between days 7 and 9 after infection. These results represent the successful demonstration of therapeutic anti–MARV-Angola efficacy in nonhuman primates and highlight the substantial impact of an LNP-delivered siRNA therapeutic as a countermeasure against this highly lethal human disease.

INTRODUCTION

For more than 35 years, the filoviruses Marburg virus (MARV) and Ebola virus (EBOV) have been associated with periodic episodes of hemorrhagic fever (HF) in Africa that produce severe disease in infected patients (1). Mortality rates in outbreaks have ranged from 23 to 90% depending on the strain or species of filovirus. Filoviruses have been the subjects of former biological weapons programs and have the potential for deliberate misuse. In addition, there have been two recent imported cases of MARV HF to Europe (2) and the United States (3), further increasing concern regarding the public health threat posed by these deadly viruses. For these reasons, and because there are no licensed countermeasures, the filoviruses are categorized as Tier 1 select agents and Category A priority pathogens by several U.S. government agencies.

MARV particles contain a 19-kb noninfectious single-stranded RNA genome that encodes seven structural proteins. The genome shows the following characteristic gene order: nucleoprotein (NP), virion protein 35 (VP35), VP40, glycoprotein, VP30, VP24, polymerase L protein (1). Five of these proteins are associated with the viral genomic RNA in the ribonucleoprotein complex: NP, VP24, VP30, VP35, and the L protein (4). The L and VP35 proteins together comprise the polymerase complex that is responsible for transcribing and replicating the MARV genome. The L protein provides the RNA-dependent RNA polymerase activity of the complex. These seven genes and their products represent targets for the development of therapeutic agents and vaccines.

Conventional clinical trials with viruses such as MARV and EBOV are not practical. To address the development of countermeasures for exotic pathogens such as filoviruses, the U.S. Food and Drug Administration (FDA) implemented the Animal Efficacy Rule in 2002 (5). This rule specifically applies to the development of countermeasures when human efficacy studies are not possible or ethical. Briefly, this rule permits the evaluation of vaccines or therapeutics using data generated from studies performed in animal models that faithfully recapitulate human disease. In the case of filoviruses, nonhuman primates (NHPs) are considered the most relevant animal model (1).

Although there are no approved vaccines or postexposure treatment modalities available for preventing or managing filovirus infections, remarkable progress has been made over the last decade in developing candidate preventive vaccines that can protect NHPs against MARV and EBOV (1, 6, 7). Progress in developing antiviral drugs and other postexposure interventions has been much slower, although recent studies have shown substantial promise. Recombinant vesicular stomatitis virus–based vaccines, monoclonal antibodies, polyclonal antibodies, phosphorodiamidate morpholino oligomers, and small interfering RNA (siRNA) have all been shown to confer complete protection of NHPs against lethal EBOV challenge when administered within 48 hours of exposure before viremia is first detected (8–14). In addition, coadministration of adenovirus–vected interferon-α with a pool of anti-EBOV monoclonal antibodies conferred complete protection to rhesus macaques when administered at day 3 after infection when viremia was first detected (15). Survival in the EBOV NHP models with antibody-based approaches appears
Fig. 1. Treatment with LNP-encapsulated NP-718m siRNA protects NHPs against MARV-Angola up to 72 hours after infection. (A) Survival of animals infected with 1000 PFU of MARV-Angola and then treated with NP-718m–LNP at 30 to 45 min (n = 4), 24 hours (n = 4), 48 hours (n = 4), or 72 hours (n = 4) after infection. Untreated and Luc LNP–treated animals were used as negative controls (n = 5). NP-718m–LNP significantly protected NHPs against MARV-Angola infection (100% survival, four of four animals surviving for each group), whereas untreated animals and animals administered Luc LNP succumbed (*P = 0.0286, Fisher’s exact test). Numbers in parentheses beside each group in the legend indicate the number of surviving animals/total number of animals in that group. (B) NP-718m–LNP treatment effectively reduces serum viremia. Plasma samples taken from untreated, negative control Luc LNP–administered, and NP-718m–LNP–treated animals were assessed by plaque assay for infectious viral particles at various time points after infection. NP-718m–LNP treatment significantly reduced infectious peak viral load in infected animals by 5 to 7 log_{10} PFU/ml compared to control animals when administered at 30 to 45 min, 24 hours, 48 hours, and 72 hours after infection (*P = 0.00497, two-way analysis of variance (ANOVA), with Bonferroni correction for pairwise comparisons). Data are group means ± SD. The lower limit of detection is 15 PFU/ml. (C) NP-718m–LNP treatment significantly abrogates serum viral RNA. Viral RNA was quantified using quantitative RT-PCR (qRT-PCR) in serum samples taken from untreated, negative control Luc LNP–administered, and NP-718m–LNP–treated animals at various time points after infection. Animals treated with NP-718m–LNP at 30 to 45 min, 24 hours, 48 hours, and 72 hours after infection with MARV-Angola had lower peak levels [5 to 11 log_{10} Genome equivalents (GEq)/ml] of viral RNA compared to control animals (*P = 0.0497 (day 3), ***P < 0.0001 (day 6), two-way ANOVA, with Bonferroni correction for pairwise comparisons). Data are group means ± SD. The lower limit of quantitation is 1 × 10^{5} GEq/ml. (D) Viral RNA levels in tissues from NP-718m–LNP–treated animals are reduced. RNA was extracted from various tissues taken at necropsy from untreated, control Luc LNP–administered, and NP-718m–LNP–treated animals infected with MARV-Angola, and quantified for viral RNA using qRT-PCR. Viral RNA levels in all assessed tissues from NP-718m–LNP–treated animals were 4 to 10 log_{10} GEq/g lower than peak viral RNA loads in infected tissues from untreated and negative control Luc LNP–administered animals (*P = 0.0457, two-way ANOVA, with Bonferroni correction for pairwise comparisons). ND, not detected; Ax LN, axillary lymph nodes; Ig LN, inguinal lymph nodes. Data are group means ± SEM. The lower limit of quantitation (LLOQ) is 1 × 10^{5} GEq/g. Tissues were taken on day 7 to day 9 for untreated animals, on day 8 for Luc LNP groups, and on day 28, the scheduled study end date for animals in treated groups, which all survived to study end.

To drop off markedly when treatment is initiated at day 3 after infection. Fewer studies have assessed postexposure treatment of MARV in NHPs. Recombinant vesicular stomatitis virus–based vaccines (16, 17), polyclonal NHP antibodies (11), phosphorodiamidate morpholino oligomers (10), and a broad-spectrum nucleoside analog (18) have all demonstrated potential in protecting NHPs against lethal MARV challenge when treatment was initiated within 48 hours of exposure, but no study has assessed efficacy when treatment was initiated at the onset of viremia or clinical signs of illness, that is, therapeutic treatment. Of equal importance is the strain of MARV used in these previous studies. Specifically, one MARV postexposure treatment study in NHPs (11) evaluated efficacy against the Ci67 strain associated with the initial MARV outbreak in Europe in 1967 that resulted in 7 fatalities among 31 cases (19). All other MARV postexposure treatment studies in NHPs evaluated efficacy against the Musoke strain. The Musoke strain was isolated from a nonfatal human case (20) and has been associated with a much more protracted disease course in NHPs, where animals typically succumb 10 to 13 days after exposure (10, 16–18). In contrast, the Angola strain was the causative agent of the largest outbreak of MARV HF thus far, with a mortality rate of 90% in more than 200 confirmed cases (21). Advanced development of any MARV countermeasure will need to demonstrate efficacy against MARV-Angola, the most pathogenic MARV strain, which also manifests as a more rapid disease course (7 to 9 days) in NHPs (1, 22).

In a previous study, we identified an siRNA targeting the MARV NSP (designated “NP-718m”) that, when encapsulated in lipid nanoparticles (LNPs), inhibited the replication of MARV in vitro and displayed broad-spectrum activity against three different MARV strains in infected guinea pigs, with complete protection against MARV-Angola infection (23). Here, we assessed the utility of using NP-718m–LNP as a therapeutic intervention in a uniformly lethal rhesus macaque model of MARV-Angola infection.
**RESULTS**

**NP-718m–LNP treatment results in 100% survival of MARV-Angola–infected NHPs when treatment is initiated at the onset of viremia**

A total of 21 rhesus macaques were challenged with a uniformly lethal dose of MARV-Angola. Initiation of NP-718m–LNP treatment occurred at 30 to 45 min, 24 hours, 48 hours, and 72 hours after infection, and comprised seven daily bolus intravenous doses. Infection and nontargeting controls consisted of no treatment and administration of LNP containing siRNA targeting firefly luciferase (Luc) LNP, respectively. All animals given NP-718m–LNP survived MARV-Angola challenge, whereas control untreated and Luc LNP–treated animals succumbed on days 8 and 9 after infection (Fig. 1A). NP-718m–LNP treatment–associated survival was found to be statistically significant ($P = 0.0286$, Fisher’s exact test).

**Viremia and viral RNA load are effectively reduced upon NP-718m–LNP treatment**

NP-718m–LNP treatment at 30 to 45 min, 24 hours, 48 hours, and 72 hours after infection reduced peak viremia as measured by plaque assay by 5 to 7 log$_{10}$ plaque-forming units (PFU)/ml when compared to control untreated and Luc LNP–administered animals (Fig. 1B), and this was statistically significant (**$P < 0.0001$, two-way ANOVA for days 6 and 8 with Bonferroni correction for pairwise comparisons**). Treatment with NP-718m–LNP reduced viral RNA load in blood by a range of 4 to 10 log$_{10}$ units by day 6 after infection when compared to untreated animals (Fig. 1C; a decrease of 4 log$_{10}$ units when comparing the 72-hour group mean, a decrease of 6 log$_{10}$ units for the 24-hour group mean, and a decrease of 10 log$_{10}$ units for the 30- to 45-min and 48-hour group means). NP-718m–LNP treatment also decreased viral RNA load in tissues by an equivalent range when treatment group means were compared to the untreated group mean (Fig. 1D; a decrease of 10 log$_{10}$ units for all treatment groups except the 30- to 45-min group in the liver and a decrease of 4 log$_{10}$ units for the 30- to 45-min group in the kidney). Two of four animals were viremic by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) on day 3 before treatment in the 72-hour group (a third and different animal was also viremic by infectivity assay), indicating that NP-718m–LNP treatment is effective when treatment is initiated at this trigger to treat. The liver is a primary target organ for MARV (1) that is associated with high viral loads as seen in control animals (Fig. 1D). In agreement with the reduction seen in vivo, NP-718m–LNP treatment in HepG2 cells (a human hepatocyte model) and subsequent 5′-RACE (rapid amplification of complementary DNA ends) PCR confirmed RNA interference (RNAi) as the mechanism of action for viral reduction (fig. S1).

**NP-718m–LNP treatment ameliorates clinical and pathological disease associated with MARV HF**

Clinical illness associated with MARV-Angola infection was much less severe in NP-718m–LNP–treated animals than in untreated controls or Luc–treated controls (Fig. 2, A and B, and fig. S2; see Table 1 and tables S1 and S2 for individual animal data). Serum samples from all infected animals were assessed for liver-associated function markers such as ALT and AST. These markers markedly increased during MARV HF. NP-718m–LNP treatment was able to protect against liver damage induced by MARV-Angola (Fig. 2, A and B). Coagulopathy is a hallmark feature of filoviral HF (1). NP-718m–LNP treatment was able to protect against HF–associated coagulopathy including prolonged PT and APTT normally observed in the course of MARV HF (Fig. 2, C and D; see Table 1 for individual animal data). Examination of tissue sections showed various degrees of lesions and MARV antigen in the five control animals consistent with historical controls (22, 24) (Fig. 3, A, B, E, and F). No lesions or clear immunoreactivity for MARV antigen was detected in tissue sections of any NP-718m–LNP–treated animal that survived challenge (Fig. 3, C, D, G, and H). This ability of

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**Fig. 2. Clinical pathology and clinical scoring in MARV-infected cynomolgus monkeys. NP-718m–LNP treatment effectively abrogates clinical signs of MARV-Angola infection when administered 30 to 45 min, 24 hours, 48 hours, or 72 hours after infection. MARV-mediated liver dysfunction either is completely prevented in NP-718m–LNP–treated animals ($n = 4$ per treatment group) or manifests transiently with delayed onset and reduced severity. (A) ALT activity. (B) AST activity. Data are group means ± SD. (C and D) Retention of (C) prothrombin time (PT) [$P = 0.0011$ (30–45 min Tx delay), $P = 0.0011$ (24 h Tx delay), $P = 0.0004$ (48 h Tx delay), $P < 0.0001$ (72 h Tx delay), two-way ANOVA, with Bonferroni correction for pairwise comparisons] and (D) activated partial thromboplastin time (APTT) [$P = 0.0193$ (1 h Tx delay), $P = 0.0034$ (24 h Tx delay), $P = 0.0226$ (48 h Tx delay), $P = 0.0176$ (72 h Tx delay), two-way ANOVA, with Bonferroni correction for pairwise comparisons] indicates protection against HF coagulopathy. Data are group means ± SEM. (E) Clinical scores for each individual within each group after MARV challenge.
Table 1. Clinical description and outcome of MARV-Angola–challenged NHPs. Days after MARV challenge are in parentheses. Fever is defined as a temperature more than 2.5°C over baseline or at least 1.5°C over baseline and ≥39.7°C. Mild rash: focal areas of petechiae covering less than 10% of the skin; moderate rash: areas of petechiae covering between 10 and 40% of the skin; severe rash: areas of petechiae and/or ecchymosis covering more than 40% of the skin. Lymphopenia and thrombocytopenia are defined as a ≥35% drop in the number of lymphocytes and platelets, respectively. Leukocytosis and granulocytosis are defined as a ≥35% increase in the number of white blood cells. Hypoalbuminemia is defined as a ≥35% decrease in the level of albumin. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; GGT, γ-glutamyltransferase; CRE, creatine; CRP, C-reactive protein.

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NP-718m–LNP to protect against MARV HF is also supported by the relative lack of change in clinical scores of treated animals from baseline scores (Fig. 2E). These data collectively suggest that LNP delivery of NP-718m siRNA mediates effective and potent postexposure treatment of MARV-Angola infection.

**DISCUSSION**

Human case fatality rates have ranged from 23% for some strains of MARV to as high as 90% for MARV-Angola infection (1, 19, 21). The increasingly frequent outbreaks of filoviral HF in Africa, evidenced by the current rapidly spreading outbreak in Guinea, Liberia, and Sierra Leone (25, 26), and the potential use of filoviruses as biological weapons illustrate the clear and present danger that filoviruses present to human health. Hence, the development of effective countermeasures against these pathogens is a critical need. The ability of a countermeasure to provide therapeutic treatment (that is, upon initial clinical signs and diagnosis) is a decisive threshold by which efficacy can be measured. Previous studies in NHPs examined countermeasures against MARV infection at times before any animals were viremic or showed any evidence of clinical illness. The goal of this study was to determine whether it was possible to protect animals against a lethal MARV-Angola challenge when treatment was started at the time of initial diagnosis. We have now demonstrated that NP-718m–LNP treatment completely protects rhesus monkeys against lethal MARV-Angola HF when treatment begins even up to 3 days after infection, at a stage when animals are viremic and demonstrate the first clinical signs of disease. The significance of delaying treatment until 72 hours after infection reflects the recognition that this is the earliest time point at which diagnosis by viral RNA can be detected, a critical step in the context of outbreak control. The ability to rapidly and accurately diagnose filovirus outbreaks has increased markedly in recent years as seen in the current outbreak in West Africa (25, 26). This substantial improvement in diagnostics provides the opportunity for siRNA-based therapies to be used at early stages of an outbreak. In particular, treatment of high-risk exposures and known contacts of cases should play a pivotal role in slowing and containing outbreaks.

These studies are also the first to report 100% postexposure protection of NHPs from the MARV-Angola strain, a strain documented to cause a much more rapid disease course in NHPs than other MARVs (1). However, for MARV-Angola, this situation is reversed; rhesus monkeys succumb slightly earlier than cynomolgus macaques and are the more robust challenge for modeling postexposure treatment studies. In brief, with all experimental conditions being equal, using the same MARV-Angola seed stock, five rhesus macaques succumbed on days 7, 8, 8, 8, and 9, respectively, whereas six cynomolgus macaques all succumbed on day 9. Thus, the mean time to death for rhesus macaques is 8.0 ± 0.40 days after infection (n = 5) versus 9.0 ± 0 days after infection (n = 6) for cynomolgus macaques, which is statistically significant (P = 0.0067, Student’s t test).

In addition to extending the window of treatment, an important aspect of this study that distinguishes it from past filovirus studies using siRNA-based approaches (9) is that the anti-MARV siRNAs in the current study were designed to target conserved sequence regions of all major strains of the MARV NP. This is important because there is substantial nucleotide divergence of greater than 21% between some MARV strains, in particular among prototype strains and those in the Ravn lineage (1). Notably, previous studies have shown that

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vaccines that protect against prototype MARV strains do not always confer protection against the Ravn strain (27). In addition, all strains of MARV are endemic to the same regions of Africa and have even been identified during the same MARV outbreak (28). The NP-718m–LNP targets regions conserved among all major MARV strains including Ravn, has completely protected guinea pigs against three different strains of MARV including Ravn (23), and as shown here has provided complete protection to NHPs against the most pathogenic strain of MARV, MARV-Angola, in the most robust and relevant animal model. Thus, whereas most siRNA-based approaches are specific to a certain strain or species of virus, the approach used here targets all known strains of MARV.

NP-718m–LNP completely protects NHPs when administered even up to 72 hours after infection, at a time when initial clinical signs begin to appear. Although protection was demonstrated at the earliest time of detection of viremia, whether it will be an effective therapy at more advanced stages of illness remains to be determined. Other caveats for this interpretation of the results include the small group numbers (four or lower) in the studies described here, potential neutralizing effects that would interfere with the plaque assay, and the limitation of the sensitivity of the plaque and qRT-PCR assays for infectious viral particle and viral RNA detection, respectively. Although no viremia or viral RNA was detected in the 24-hour treatment delay animals, this was likely the consequence of the highly effective NP-718m–LNP treatment, and not because of a lack of infection, because the control animal in this study, which was infected in parallel to these treated animals, succumbed to MARV HF. NP-718m siRNA has been chemically modified and has been previously shown to display no immunostimulatory activity (23).

Together, the results presented here strongly support the further development of NP-718m–LNP as a therapeutic treatment for MARV infection in humans. Its success against the strain of MARV responsible for the largest and most lethal outbreak in history represents a substantial advance in countermeasure development, particularly when coupled with the complete protection seen when treatment is administered at the onset of viremia and clinical disease. The studies described here not only represent a proof of principle of the utility of an NP-718m–LNP in the treatment of MARV HF but are also obligate precurors to, and will inform the design of, the necessary animal studies that would be conducted to support licensure under the FDA Animal Rule. In future studies, the efficacy at time points greater than 72 hours after infection, as well as the benefits of a cocktail approach to enhance filoviral broad-spectrum activity, needs to be explored.

MATERIALS AND METHODS

Study design
Twenty-one healthy adult rhesus macaques (Macaca mulatta) of Chinese origin (4 to 8 kg) were used to conduct four separate studies (4 treated animals and 1 or 2 controls per study), where the time between virus challenge and initiation of treatment was increased from one study to the next. Performance of each study required complete protection of all treated animals in the previous study. Animals were randomized with Microsoft Excel into treatment or control groups. Experimental groups were treated with NP-718m–LNP beginning either at 30 to 45 min or at 24, 48, or 72 hours after MARV-Angola challenge. Treatments were given daily for 6 days after the initial treatment. The control animals were treated with nonspecific siRNA or were not treated. A number of parameters were monitored during the course of the study including survival, clinical observations, hematology, serum biochemistry, blood coagulation, viremia and viral load in tissues by qRT-PCR and plaque assay, and tissue pathology. The overall objective of the study as a whole was to assess survival rates with all other measurements being considered secondary objectives. This study was not blinded.

Fig. 3. Comparison of MARV pathology and antigen in representative tissues of rhesus monkeys either treated or not treated with NP-718m–LNP. (A) Liver, multifocal necrotizing hepatitis, sinusoidal leukocytosis, and eosinophilic cytoplasmic inclusion bodies in a MARV-infected control animal. (B) Liver, diffuse cytoplasmic immunolabeling (brown) of sinusoidal lining cells, Kupffer cells, and hepatocytes. (C and D) No overt lesions (C) or immunolabeling (D) in the liver of an NP-718m–LNP–treated animal. (E) Spleen, diffuse lymphoid depletion of the white pulp and fibrin deposition in the red pulp in a MARV-infected control animal. (F) Spleen, diffuse cytoplasmic immunolabeling of dendriform mononuclear cells in the red and white pulp of a MARV-infected control animal. (G and H) No overt lesions (G) or immunolabeling (H) in the spleen of an NP-718m–LNP–treated animal. All representative images taken at ×40 from treated animal 0807175 or control animal 0904099. Scale bars, 50 μm.
LNP encapsulation of siRNA
The design, chemical modification, and immunostimulation abrogation testing of NP-718m siRNA have been previously described (23). NP-718m siRNA (23) (synthesized by Integrated DNA Technologies) was encapsulated in LNP by the process of spontaneous vesicle formation as previously reported (29). The resulting LNP were dialyzed against phosphate-buffered saline and filter-sterilized through a 0.2-μm filter before use. Particle sizes were highly consistent, ranging from 78 to 79 nm for all studies, with low polydispersity values (0.04 to 0.09). High encapsulation efficiencies (97 to 98%) were obtained for material prepared for each of the four studies. LNP containing siRNA targeting Luc, a nonendogenous mRNA transcript not found in mammals, was included as a negative control for nontargeting LNP effects.

Animal challenge
All animals were inoculated intramuscularly with a target dose of 1000 PFU of MARV-Angola (actual dose of MARV was determined to be 1775 PFU for the 30- to 45-min delay to treatment study, 1250 PFU for the 24-hour delay to treatment study, 1100 PFU for the 48-hour delay to treatment study, and 1000 PFU for the 72-hour delay to treatment study). In the first study, NP-718m–LNP (total siRNA dose, 0.5 mg/kg) was administered to four macaques by bolus intravenous infusion 30 to 45 min after MARV-Angola challenge, whereas two control animals received no treatment. All treated animals were given a total of seven daily doses of NP-718m–LNP after MARV-Angola challenge. In the second study using five macaques, NP-718m–LNP (0.5 mg/kg) was administered to four macaques by bolus intravenous infusion 48 hours after MARV-Angola challenge, whereas the control animal received no treatment. The four animals received additional treatments of NP-718m–LNP on days 2, 3, 4, 5, 6, and 7 after MARV-Angola challenge. In the third study using five macaques, NP-718m–LNP (0.5 mg/kg) was administered to four macaques by bolus intravenous infusion 48 hours after MARV-Angola challenge, whereas the control animal received an equal dose of nontargeting control Luc LNP. Treated animals received additional treatments of NP-718m–LNP or the control Luc LNP on days 3, 4, 5, 6, 7, and 8 after MARV-Angola challenge. In the final study using five macaques, NP-718m–LNP (1.0 mg/kg) was administered to four macaques by bolus intravenous infusion 72 hours after MARV-Angola challenge, whereas the control animal received an equal dose of the nontargeting control Luc LNP. These animals received additional treatments of NP-718m–LNP or control Luc LNP on days 4, 5, 6, 7, 8, and 9 after MARV-Angola challenge. All 21 animals were given physical exams, and blood was collected at the time of challenge and on days 3, 6, 10, 14, 21, and 28 after MARV-Angola challenge. In addition, all animals were monitored daily and scored for disease progression with an internal filovirus scoring protocol approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee. The scoring changes measured from baseline included posture/activity level, attitude/behavior, food and water intake, weight, respiration, and disease manifestations such as visible rash, hemorrhage, ecchymosis, or flushed skin. A score of ≥9 indicated an animal met criteria for euthanasia.

Detection of viremia
RNA was isolated from whole blood with the Viral RNA Mini kit (Qiagen) using 100 µl of blood into 600 µl of buffer AVL. Primers/probe targeting the NP gene of MARV were used for qRT-PCR with the probe used here being 6-carboxyfluorescein (6FAM)–5′-CCCCATAAGGTACCCCTCTT-3′–6-carboxytetramethylrhodamine ( TAMRA) (Life Technologies) (23). MARV RNA was detected using the CFX96 detection system (Bio-Rad Laboratories) in One-Step Probe qRT-PCR kits (Qiagen) with the following cycle conditions: 50°C for 10 min, 95°C for 10 s, and 40 cycles of 95°C for 10 s and 59°C for 30 s. Threshold cycle (CT) values representing MARV genomes were analyzed with CFX Manager Software, and data are shown as + or – for GEq (Table 1). To create the GEq standard, RNA from MARV stocks was extracted and the number of MARV genomes was calculated using Avogadro’s number and the molecular weight of the MARV genome.

Virus titration was performed by plaque assay with Vero E6 cells from all serum samples as previously described (16, 17, 22, 24). Briefly, increasing 10-fold dilutions of the samples were adsorbed to Vero E6 monolayers in duplicate wells (200 µl); the limit of detection was 15 PFU/ml.

5′-RACE PCR
5′-RACE PCR was performed as previously described (9), with the exception that RNA was isolated from human hepatocellular carcinoma cell line HepG2 cells infected with MARV-Angola at a multiplicity of infection of 0.1. Cells were treated with 1.0 and 10 nM of NP-718m–LNP 24 hours before infection. The gene-specific primer used for reverse transcription was 5′-TTGCGGCAAAGTGTACCGAGA-3′. PCR primers used were RNA adaptor primer (5′-CGACTGGAGCAGGAGACACTGTA-3′) and MARV NP reverse primer (5′-GCTGAGGACGGCGATGTCT-3′). The sequencing primer used was 5′-CGCGGAGTGTCGACTGTGTTG-3′.

Hematology, serum biochemistry, and blood coagulation
Total white blood cell counts, white blood cell differentials, red blood cell counts, platelet counts, hematocrit values, total hemoglobin concentrations, mean cell volumes, mean corpuscular volumes, and mean corpuscular hemoglobin concentrations were analyzed from blood collected in tubes containing EDTA using a laser-based hematologic analyzer (Beckman Coulter). Serum samples were used for concentrations of albumin, amylase, ALT, AST, ALP, GGT, glucose, cholesterol, total protein, total bilirubin, BUN, CRE, and CRP by using a Piccolo point-of-care analyzer and Biochemistry Panel Plus analyzer discs (Abaxis). Citrated plasma samples were analyzed for coagulation parameters PT, APTT, thrombin time, and fibrinogen on the STart4 instrument using the PTT Automate, STA Neoplastine CI Plus, STA Thrombin, and Fibri-Prest Automate kits, respectively (Diagnostica Stago). Citrated plasma levels of D-dimers were measured by enzyme-linked immunosorbent assay according to the manufacturer’s recommendations (Diagnostica Stago).

Histopathology and immunohistochemistry
Necropsy was performed on all subjects. Tissue samples of all major organs were collected for histopathologic and immunohistochemical examination, immersion-fixed in 10% neutral buffered formalin, and processed for histopathology as previously described (22). For immunohistochemistry, specific anti-MARV immunoreactivity was detected using an anti-MARV VP40 protein rabbit primary antibody (Integrated BioTherapeutics) at a 1:4000 dilution. In brief, tissue sections were processed for immunohistochemistry using the Dako Autostainer. The secondary antibody used was biotinylated goat anti-rabbit immunoglobulin G (IgG) (Vector Laboratories).
at 1:200 followed by Dako LSAB2 streptavidin–horseradish peroxidase (Dako). Slides were developed with Dako DAB chromogen and counterstained with hematoxylin. Nonimmune rabbit IgG was used as a negative control.

Statistical analyses
Statistical analyses were conducted using GraphPad Prism software (version 6.0). Mixed model repeated-measures two-way ANOVA was used to determine whether differences in means were present, followed by post hoc Bonferroni correction for multiple comparisons to identify which comparisons were significant. Fisher’s exact test was used to compare differences in survival between treated and control groups.

SUPPLEMENTARY MATERIALS
www.sciencetranslationalmedicine.org/cgi/content/full/6/250/250ra116/DC1

RESEARCH ARTICLE

2. A. Timen, M. P. Koopmans, A. C. Vossen, G. J. van Doornum, S. Günther, F. van den Berkmortel, L. Dong, D. V. Mourich, S. Crumley, D. K. Nichols, P. L. Iversen, S. Bavari, Advanced antisense LNP to identify which comparisons were significant. Fisher's exact test was used to determine whether differences in means were present, followed by post hoc Bonferroni correction for multiple comparisons to identify which comparisons were significant. Fisher's exact test was used to compare differences in survival between treated and control groups.

REPRESENTATIONS AND NOTES
2. A. Timen, M. P. Koopmans, A. C. Vossen, G. J. van Doornum, S. Günther, F. van den Berkmortel, L. Dong, D. V. Mourich, S. Crumley, D. K. Nichols, P. L. Iversen, S. Bavari, Advanced antisense LNP to identify which comparisons were significant. Fisher's exact test was used to determine whether differences in means were present, followed by post hoc Bonferroni correction for multiple comparisons to identify which comparisons were significant. Fisher's exact test was used to compare differences in survival between treated and control groups.

SUPPORTING MATERIALS
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analyzed the data. K.A.F. performed histologic and immunohistochemical analysis of the data. E.P.T., C.E.M., I.M., and T.W.G. wrote the paper. All authors had access to all of the data and approved the final version of the manuscript. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the UTMB.


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Medicating Marburg

Marburg virus (MARV) and the closely related filovirus Ebola virus cause severe and often fatal hemorrhagic fever; however, there are currently no vaccines or drugs approved for human use to treat these devastating infections. Thi et al. now report that a lipid-encapsulated siRNA can treat MARV in nonhuman primates after symptom onset. They use lipid nanoparticles to deliver siRNA targeting the MARV nucleoprotein to treated animals at various time points after virus exposure. All animals that received the therapy survived MARV infection, including those that were treated 3 days after infection—a stage when animals are viremic and demonstrate the first clinical signs of disease. Therefore, this approach holds promise as a strategy to treat filovirus infection in humans.