Inhibition of Rift Valley Fever Virus Replication and Perturbation of Nucleocapsid-RNA Interactions by Suramin

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Rift Valley fever virus (RVFV) is an emerging infectious pathogen that causes severe disease in humans and livestock and has the potential for global spread. There are currently no proven safe and effective treatment options for RVFV infection. Inhibition of RNA binding to RVFV nucleocapsid protein (N) represents an attractive antiviral therapeutic strategy because several essential steps in the RVFV replication cycle involve N binding to viral RNA. In this study, we demonstrate the therapeutic potential of the drug suramin by showing that it functions well as an inhibitor of RVFV replication at multiple stages in human cell culture. Suramin has been used previously to treat trypanosomiasis in Africa. We characterize the dynamic and cooperative nature of N-RNA binding interactions and the dissociation of high-molecular-mass ribonucleoprotein complexes using suramin, which we previously identified as an N-RNA binding inhibitor in a high-throughput screen. Finally, we elucidate the molecular mechanism used by suramin *in vitro* to disrupt both specific and nonspecific binding events important for ribonucleoprotein formation.

Rift Valley fever virus (RVFV) is a mosquito-transmitted bunyavirus (genus *Phlebovirus*) that causes severe disease in humans and ruminant livestock. In humans, disease symptoms can range from a mild flu-like illness to hemorrhagic fever, encephalitis, neurological disorders, and blindness (1). Pregnant livestock are at risk for miscarriage, and high mortality rates among newborn animals have been reported (2–4). RVFV can be transmitted by a diverse set of mosquito species, and outbreaks have been more severe outside the historically defined areas of endemicity of sub-Saharan Africa. The potential for RVFV to cause devastating epidemics worldwide is evidenced by its classification as a category A high-priority disease agent by the National Institute for Allergy and Infectious Diseases (NIAID) (5, 6). There are currently no proven safe and effective treatment options for RVFV-infected people or livestock. Increasing our understanding of the basic molecular virology of this important pathogen represents an essential step toward identification of new drug targets and the development of more efficacious antiviral therapeutic compounds.

The single-stranded RNA genome of RVFV is composed of three segments (L, M, and S) that appear circular by electron microscopy due to complementarity between the 5'- and 3'-terminal regions (7). The L segment encodes the RNA-dependent RNA polymerase responsible for transcription and replication of the viral genome. The M segment encodes glycoproteins (Gn and Gc) involved in entry into the host cell and nonstructural proteins NSm and 78-kDa protein (8). The S segment utilizes an ambisense strategy to encode the nucleocapsid (N) and NSs proteins, while the genes of the M and L segments are in the negative sense (9). Among all viral gene products, the importance of N is underscored by its involvement during many stages of the RVFV replication cycle. N is an RNA binding protein that plays important roles during transcription, translation, replication, and encapsidation of the viral genome into virions (10–15). During replication, N monomers bind along the entire length of the viral genome and antigenome. This encapsidation event protects the viral RNA from degradation and prevents the formation of double-stranded RNA intermediates that could activate the host innate immune response (16). To discriminate between full-length viral RNA and

the plethora of other abundant species of RNAs present in the host cell, it is thought that N must initially recognize some distinct sequence and/or secondary structural element(s) specific to viral RNA. Subsequently, additional N molecules bind the entire length of the viral RNA. Studies conducted in other bunyaviruses, including Bunyamwera, Sin Nombre, Hantaan, and La Crosse viruses, identified single- or double-stranded structures within the terminal noncoding regions of the RNA genome as important recognition elements for N (17–22). Our laboratory used an *in vitro* selection technique, SELEX (i.e., systematic evolution of ligands by exponential enrichment) to identify and amplify RNAs called “aptamers” that bind to RVFV N with high affinity without any *a priori* assumptions with regard to sequence or structure. In the *in vitro* selection scheme, we observed a recurring GAUU motif that was found to be important for N recognition and binding to many, but not all, of these aptamer RNAs (23). Several lines of evidence suggest that our aptamers bind to N in a physiologically relevant way. First, aptamers can be displaced by RNA constructs that mimic the viral panhandle structure (23), which is widely believed to be a recognition element for N. Second, many of the aptamers selected in the original study have sequences that resemble portions of the RVFV genome or antigenome by BLAST analysis (23). Finally, a survey of aptamer selections against diverse nucleic acid binding proteins demonstrated that aptamers invariably bound to the bona fide nucleic acid binding region of the protein unless deliberate steps were taken to prevent aptamer binding there (24–26).

A high-resolution crystallographic structure of N bound to RNA was recently published (27). Analysis of this structure re-

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vealed an RNA binding groove containing several highly conserved basic amino acid residues located within the C-terminal core domain, but the structure did not lend insight into how N may recognize RNAs in a sequence- or structure-dependent fashion. The N monomer also contained a flexible N-terminal arm that interacts with the neighboring N monomer; this protein-protein interaction likely facilitates cooperative N binding (27, 28). However, the details of how RVFV N and other viral nucleocapsid proteins interact with their cognate RNAs are not well understood. We hypothesize that N binds RNA in a biphasic manner. After an initial specific binding event, subsequent N monomers bind in a nonspecific mode and coat the entire length of the viral genome or antigenome. Furthermore, we propose that the inhibition of RNA binding to N represents an attractive antiviral therapeutic strategy because several essential steps in the RVFV replication cycle involve N binding to viral RNA.

In this study, we show that suramin, a small molecule identified by our laboratory in a high-throughput screen as an N-RNA binding inhibitor, decreases RVFV replication in human cell culture (29). Suramin has been used in Africa to treat trypanosomiasis, although not without some undesirable side effects (30). Using time-of-addition analysis, we determine that suramin exerts its inhibitory effect both by interfering with N-RNA binding and by blocking virus uptake into cells and/or other later steps that have not yet been described. We characterize the cooperative assembly of N monomers onto RNA using mutational analysis, biochemical binding assays, and RNA structure probing techniques. Finally, we elucidate the mechanism utilized by suramin to disrupt both the initial specific binding event, as well as subsequent nonspecific binding events important for ribonucleoprotein (RNP) complex formation.

MATERIALS AND METHODS

Cell culture studies. Human 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Vero cells were maintained in minimum essential medium (MEM) supplemented with 10% FBS, penicillin, and streptomycin. The Rift Valley fever virus (RVFV) vaccine strain MP-12 was kindly provided by Brian Gowen (Utah State University, Logan, UT). During infection, human 293 cells and virus were maintained in DMEM supplemented with 2% FBS, penicillin, and streptomycin. Incubations were carried out at 37°C and 5% CO₂ unless otherwise stated.

Virus yield reduction assays. Growth medium from confluent monolayers of human 293 cells in a 96-well plate format was removed and replaced with DMEM supplemented with 2% FBS. Cells were treated with 1 μ l of various concentrations of suramin (390 nM to 400 μ M final concentration) or dimethyl sulfoxide (DMSO). Immediately following the addition of suramin, cells were infected with RVFV at a multiplicity of infection (MOI) of 0.1 and incubated for 2 h. After incubation medium was removed, cells were washed with phosphate-buffered saline (PBS), and fresh medium and 1 μ l of suramin or DMSO was added. Supernatants of virus-infected and uninfected cells were harvested at 2 and 3 days postinfection (dpi). The amount of virus present in the supernatant was quantitated using plaque assays. In parallel, plates were treated with various concentrations of suramin in the absence of virus for cytotoxicity assays. After 2 and 3 days, the cytotoxicity of suramin was measured using resazurin fluorescence. Resazurin (0.08 mM final concentration) was added to wells containing cells treated with either suramin or DMSO, and the fluorescence was measured at 590 nm using a Biotek Synergy 2 plate reader.

Time-of-addition virus yield reduction assays. To determine if suramin blocks an early or late step in the virus replication cycle, we

modified the protocol described in the previous paragraph. Growth medium from confluent monolayers of human 293 cells grown in the 96-well plate format was removed, and cells were washed with cold DMEM. Next, 100 μ l of cold DMEM supplemented with 2% FBS was added to each well. Either ammonium chloride (an inhibitor of viral entry [12 mM final concentration]) or suramin (50 or 100 μ M final concentration) was added to cells that received drug before infection. Immediately following the addition of ammonium chloride or suramin, cells were infected with RVFV using an MOI of 1.0 and incubated for 1 h at 4°C to allow virus to attach but not enter cells. After the incubation medium was removed, cells were washed with cold PBS to remove unbound virus, and 200 μ l of prewarmed medium was added to each well. Ammonium chloride and suramin were added back to wells that received drug before infection, and the plate was incubated for 1 h to allow the virus to enter the cells. After 1 h, either ammonium chloride or suramin was added to experimental wells that received drug after the virus entered the cells. Supernatants of virus-infected cells were harvested the next morning.

Plaque assays. Supernatants from virus-infected and uninfected cells were serially diluted in MEM and subsequently used to infect confluent monolayers of Vero cells grown in the 6-well plate format. After a 2-h incubation, the cells were overlaid with MEM containing 1% agarose and incubated for 7 days. Cells were fixed with 9.25% formaldehyde, and plaques were revealed using crystal violet stain.

Overexpression and purification of RVFV WT and mutant N proteins. The wild-type (WT) N protein sequence used in this study corresponds to the experimental live attenuated RVFV MP-12 vaccine strain (31). A mutated variant of N lacking the 33 amino acid residues that according to the crystal structure comprise the N-terminal oligomerization domain (Δ 33 mutant) and a mutant that is unable to bind RNA [RNA binding (–) mutant] were also used (28). Three conserved basic residues in the RNA binding cleft of N were changed to aspartic acid (R64D, K67D, and K74D) to abolish the RNA binding activity of N. Sequences for the WT N and mutant constructs were cloned into the N-terminal (His-Asn)₆-tagged pEcoli plasmid (Clontech, Mountain View, CA). Plasmids encoding WT, Δ 33, or RNA binding (–) mutant RVFV N protein were transformed into *Escherichia coli* strain BL21-CodonPlus. Transformed cells were grown at 37°C in LB medium containing 100 μ g/ml ampicillin and 50 μ g/ml chloramphenicol until the optical density at 600 nm (OD₆₀₀) reached 0.6 to 0.8. Protein expression was induced by addition of IPTG (isopropyl- β -D-thiogalactopyranoside) to a final concentration of 0.5 mM, and cultures were grown overnight at room temperature (24°C). Cells were harvested by centrifugation and stored at –80°C.

The thawed cell pellets were lysed by resuspension in lysis buffer (50 mM sodium phosphate [pH 8.0], 50 mM NaCl, 10 mM imidazole, 1 M urea, 1% vol/vol Triton X-100) in the presence of protease inhibitors. Benzonase (Novagen) was added to the lysis buffer at a concentration of 12 U/ml to degrade DNA and free RNA. Insoluble cell debris was removed from the lysate by centrifugation at 10,000 rpm for 30 min at 4°C. The (His-Asn)₆-tagged proteins were batch purified from the cell lysate using Co²⁺-charged Talon resin (Clontech). The resin was washed 4 times with a mixture of 50 mM sodium phosphate (pH 8.0), 500 mM NaCl, 10 mM imidazole, and 1 M urea, and the protein was eluted with a mixture of 25 mM HEPES (pH 8.0), 500 mM NaCl, and 300 mM imidazole. The eluate was dialyzed overnight against the storage buffer (500 mM NaCl, 10% vol/vol glycerol, 50 mM Tris-HCl [pH 8.0], two buffer changes) and concentrated using Amicon centrifugal filters (10,000 molecular weight cut-off [MWCO]). After flash freezing with liquid nitrogen, the aliquots were stored at –80°C. The purity of the protein was checked by SDS-PAGE, and the protein concentration was determined by the absorbance at 280 nm using extinction coefficients obtained from the ExPASy website: 33,000 M^{–1} cm^{–1} for the WT and RNA binding (–) mutant and 24,750 M^{–1} cm^{–1} for the Δ 33 mutant.

Synthesis of aptamer RNA constructs. Two PCR and ligation steps were conducted to build an RNA construct that contains an aptamer RNA

FIG 1 Analysis of the effects of suramin on virus yield and cell viability in human cell culture. (A) Monolayers of cells were treated with either DMSO (no drug) or 100 μ M suramin and subsequently infected (MOI, 0.1) with RVFV. Supernatants of virus-infected cells were harvested at 2 and 3 days postinfection and diluted 1/450 and 1/1,350, respectively. Diluted supernatants were added to monolayers of cells grown in the 6-well plate format, and plaque assays were conducted to determine the number of infectious viral particles produced. (B) Monolayers of cells were treated with various concentrations of suramin (390 nM to 400 μ M) and subsequently infected (MOI, 0.1) with RVFV. Supernatants of virus-infected cells were harvested at 3 days postinfection, and plaque assays were conducted to determine the number of infectious viral particles produced from cells. Error bars represent standard deviations from the average number of infectious viral particles based on the

sequence (previously shown to bind N with high affinity) attached to the 3' end of the human 5S rRNA sequence. First, a sense primer containing an Sall site (5'-CCA TAC GAG TCG ACG GCA TTA CGG CCG GG-3') and an antisense primer containing an XbaI site (5'-GCT CTA GAA GAC GC-3') were used to amplify a 55-nucleotide (nt)-long sequence that corresponds to MBE59 aptamer RNA (23). The PCR fragment was agarose gel purified, digested with Sall and XbaI, and ligated into a pAV plasmid using the Quick ligation kit (New England BioLabs). The pAV plasmid containing the 5S rRNA gene followed by Sall and XbaI restriction sites for insertion of sequences encoding test RNAs was a kind gift from David Engelke (University of Michigan, Ann Arbor) (32). Second, a sense primer containing an EcoRI site with a T7 RNA polymerase promoter (5'-CGG AAT TCT AAT ACG ACT CAC TAT AGG GTC TAC GGC CAT ACC ACC-3') and an antisense primer containing a BamHI site (5'-ACA GGA TCC GCT CTT CCA AAA GCG GAC CGA AG-3') were used to amplify the 200-nt-long segment corresponding to the human 5S rRNA gene plus the MBE59 aptamer RNA sequence. The PCR product was agarose gel purified, digested with EcoRI and BamHI, and ligated into the pUC18 plasmid. The MBE59/5S plasmid DNA construct was verified by DNA sequencing.

RNA was synthesized *in vitro* using the MBE59/5S construct digested with XbaI as a template for T7 RNA polymerase. MEGashortscript (Ambion) was used to synthesize nonradioactive RNA, and MAXIscrip (Ambion) was used to synthesize internally labeled RNA in the presence of radioactive [α - 32 P]UTP (800 Ci/mmol [PerkinElmer]). The transcription reaction mixture was DNase treated and purified by phenol-chloroform extraction and ethanol precipitation. The RNA was further purified by denaturing gel electrophoresis. The band corresponding to the RNA was visualized by UV shadowing, excised, eluted into a mixture of 0.5 M ammonium acetate, 1.0 mM EDTA (pH 8.0), and 0.2% SDS, and ethanol precipitated.

EMSAs. For electrophoretic mobility shift assays (EMSAs), a 35-nt-long fluorescently labeled aptamer-based RNA was used (sequence, 5'-CGG GCU GUU UAC UGA ACU AUG AUA CAA AGA CCC CG-fluorescein-3' [previously described in reference 23]). N protein (50 μ M final concentration) was incubated with 6-carboxyfluorescein (FAM)-RNA (1.0 μ M final concentration) in a binding buffer (10 mM HEPES [pH 7.3], 150 mM NaCl, 20 mM KCl, 5 mM MgCl₂ final concentration) at 10°C for various amounts of time. Samples for competitive binding experiments were incubated at room temperature (~24°C) for various amounts of time before competitor aptamer RNA (25 μ M final concentration) was added, and the reaction mixture was incubated for 1 more hour. After incubation, the reaction mixtures were placed on ice, and glycerol loading dye was added to each tube. Samples were loaded onto prechilled 6% acrylamide-1 $\times$ TB (Tris-borate buffered) gels that were run in the cold room at 120 V for 75 min. Gels were visualized and quantified on a Fuji FLA3000G image analyzer.

Fluorescence polarization measurements. Aliquots of WT and Δ 33 N were serially diluted to various concentrations in a binding buffer (10 mM HEPES [pH 8], 150 mM NaCl, 20 mM KCl, 5 mM MgCl₂, 10% vol/vol glycerol). To determine the equilibrium dissociation constant of N-FAM-RNA complex, various dilutions of N were added to 10 nM FAM-labeled aptamer RNA that had been subjected to denaturation at 90°C for 2 min followed by snap cooling on ice. Reaction mixtures were incubated at room temperature (24°C) for 1 h. Samples for competitive

quantification of at least two dilutions of virus from each treatment. (C) Cytotoxicity assay to measure the effects of suramin on cell viability. Resazurin was added to wells treated with either DMSO (dark gray bars) or 100 μ M suramin (light gray bars), and the fluorescence was measured at 590 nm. The experiment was conducted in duplicate, and error bars represent the standard deviation from the mean. (D) Cytotoxicity assay to measure the effects of suramin on cell viability conducted in duplicate. Resazurin was added to wells treated with various concentrations of suramin (390 nM to 400 μ M), and the fluorescence was measured at 590 nm. Error bars represent $\pm$ standard deviation from the mean.

binding experiments were prepared by incubating either the WT (15 μ M) or Δ 33 mutant (50 μ M) with 10 nM FAM-labeled RNA in binding buffer at room temperature for 1 h. Next, various concentrations of suramin were added (524 pM to 588 μ M final concentration), and the reaction mixture was incubated for 1 h at room temperature. Fluorescence polarization values were measured using a Synergy 2 plate reader (BioTek). Binding profiles were plotted, and apparent K_d (dissociation constant) and 50% effective concentrations (EC_{50} s) were calculated using GraphPad Prism software.

Gel filtration chromatography. Purification of recombinant WT N from bacteria results in a heterogeneous population of monomers and multimers in a complex with *E. coli* RNA. For some experiments, isolation of the lowest-molecular-weight species (monomeric population) was required, and gel filtration chromatography was used to fractionate N. A Superose 12 10/300 GL column (GE Healthcare Life Sciences) was equilibrated in fast protein liquid chromatography (FPLC) running buffer (10 mM HEPES [pH 8.0], 500 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$) using an ÄKTA FPLC system (Amersham Biosciences). After elution from the cobalt resin, N was concentrated to approximately 10 mg/ml, and 200 μ l was loaded onto the column. Protein was eluted at a flow rate of 0.2 ml/min and monitored by absorbance at 280 nm. The following standard proteins were used to calibrate the column: carbonic anhydrase (29 kDa), alcohol dehydrogenase (ADH) monomer (44 kDa), albumin (66 kDa), and ADH dimer (74 kDa) and tetramer (150 kDa). Blue dextran (2,000 kDa) was used to determine the void volume.

The Superose 12 column was also used analytically to compare the multimeric state of N in the presence and absence of suramin. First, the column was equilibrated in FPLC running buffer. Purified WT or RNA binding (–) mutant N was adjusted to a concentration of 5 mg/ml in 10 mM HEPES (pH 8.0)–500 mM NaCl–20 mM KCl–5 mM $MgCl_2$ and incubated at room temperature for 1 h in either the presence or absence of various concentrations of suramin. N was loaded onto the column (600 μ g; 120 μ l) and eluted at a flow rate of 0.2 ml/min, and absorbance at 280 nm was measured. Fractions (0.5 ml) were collected and analyzed for RNA content by phenol-chloroform extraction, ethanol precipitation, and denaturing gel electrophoresis. Gels were stained with ethidium bromide and scanned using a Fuji FLA-3000G phosphorimager, and RNA content was quantified by measuring band intensities using Image Gauge software.

Time-dependent RNA structure probing. First, MBE59/5S was subjected to denaturation at 90°C for 2 min followed by snap cooling on ice. Next, WT monomeric N (30 μ M) and RNA (0.5 μ M) were incubated in a binding buffer (10 mM HEPES [pH 8], 150 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$) for 5 min or 5 h at 10°C to allow ribonucleoprotein (RNP) complexes to form. The MBE59/5S alone and RNP complexes were probed using either RNase T₁ or RNase A. T₁ RNase reaction mixtures were each incubated with 0.18 U of enzyme per tube for 4 min at 10°C, and RNase A reaction mixtures were each incubated with 6.25 ng of enzyme per tube for 2 min at 10°C. Reactions were stopped by phenol-chloroform extraction followed by ethanol precipitation. Samples were resuspended in Nanopure water and stored at –20°C.

Analysis of probing experiments. The probed RNA samples were reverse transcribed using three different γ -³²P-labeled DNA oligonucleotides located 10 to 40 nucleotides upstream of the region of interest on the RNA and avian myeloblastosis virus reverse transcriptase (AMV-RT) (Promega). The sequences of the primers used are 5'-TCT AGA AGA CGC CCC-3', 5'-ACG CCT ACA GCA CCC-3', and 5'-AGG CCC GAC CCT GC-3'. After 30 min of primer extension at 42°C, NaOH was added at a 0.1 N final concentration to each sample, and the mixture was incubated at 65°C for 10 min. Samples were ethanol precipitated, dried, resuspended in formamide loading dye, and subjected to denaturing gel electrophoresis. Gels were exposed using a Fuji FLA-3000G phosphorimager, and quantification of band intensity (which corresponds to the reactivity of RNA bases) was performed using Image Gauge software. Band intensities were adjusted to eliminate small variations in lane loading by divid-

FIG 2 Time-of-addition assays to determine the stage(s) of the virus replication cycle inhibited by suramin. (A) Plaque assay showing the effects of drug concentration and time of addition on the ability of suramin to inhibit viral replication. Cells were either pretreated (before entry) with suramin or ammonium chloride or treated 1 h postinfection (after entry) with suramin or ammonium chloride. Cells were infected with RVFV using an MOI of 1.0, and supernatants of virus-infected cells were harvested for plaque assays at 15 h postinfection. (B) Quantification of the plaque assay in Fig. 2A. Cells either were treated with DMSO ("untreated" [dark gray bar]), pretreated with suramin or ammonium chloride (light gray bars), or treated 1 h postinfection with suramin or ammonium chloride (medium gray bars). Error bars represent the standard deviation from the average number of infectious viral particles based on the quantification of at least two dilutions of virus from each treatment. (C) Cytotoxicity assay to measure the effects of suramin and ammonium chloride on cell viability conducted in duplicate. Resazurin was added to wells either treated with DMSO ("untreated" [dark gray bar]), pretreated with suramin or ammonium chloride (light gray bars), or treated 1 h postinfection with suramin or ammonium chloride (medium gray bars), and the fluorescence was measured at 590 nm. Error bars represent standard deviations from the mean.

ing all bases affected by N by an internal control value. In other words, the intensity of each experimental band was divided by the intensity of a control band present in each lane. For control bands, we used naturally occurring reverse transcriptase (RT) pause/stop sites referred to as K-

FIG 3 Characterization of N-RNA binding interactions. (A) EMSA analysis of fluorescently (3'-FAM) labeled RNA binding affinity for N after incubation for various amounts of time. The bands that migrate faster on the gel are unbound RNA species, and the slower-migrating bands represent N-RNA complexes. (B) Competitive EMSA to test the ability of an unlabeled competitor RNA to displace FAM-RNA that was preincubated with N for various amounts of time.

bands. These bands were identified by their appearance in the MBE59/5S control lanes (K-lanes), where samples were not treated with RNases. Since the intensities of the K-bands are consistent for each sample, these bands were used to normalize experimental bands and compensate for any differences in band intensity that could be attributed to experimental error. After normalization of all experimental bands, the intensity of bands in samples incubated with N for 5 min or 5 h were compared to the band intensities of the control with RNA alone.

RESULTS

Suramin inhibits RVFV replication in cell culture. Plaque assays were conducted to measure the ability of suramin to inhibit the production of viable virus over the course of several days. Human 293 cells were infected at a low multiplicity of infection (MOI of 0.1) in the presence or absence of suramin, and the number of infectious viral particles produced was quantitated using a plaque assay. The cytotoxicity of suramin was measured using resazurin fluorescence. The metabolic activity of living cells converts resazurin into a fluorescently active compound that can be detected at 590 nm using a microplate reader. Suramin (100 μ M) decreased the number of PFU per ml by approximately 1 log at 2 and 3 days postinfection (dpi) and was not cytotoxic to human 293 cells (Fig. 1A and C). The ability of various concentrations of suramin (390 nM to 400 μ M) to inhibit viral replication was also tested using plaque assays. The viral EC_{50} of suramin was 58.5 μ M, and a 50% cytotoxic concentration (CC_{50}) of 200 μ M was determined using resazurin fluorescence (Fig. 1B and D), yielding a therapeutic index of approximately 3.4.

Suramin blocks both early and late steps in the RVFV replication cycle. Time of addition experiments were performed to determine if suramin blocks an early and/or late step during virus replication. First, human 293 cells were treated with ammonium chloride (12 mM), a known potent blocker of viral entry, either before or at various time points after infection to determine the amount of time required for RVFV MP-12 to enter cells. We found that at 1 h postinfection, ammonium chloride was no longer able to block infection, suggesting that entry is complete within 1 h (data not shown). Next, cells were treated with ammonium chloride or suramin either before or after the virus (MOI, 1.0) entered cells. Supernatants of infected cells were harvested the next morning (15 h postinfection), and the number of infectious viral particles produced was quantitated using a plaque assay. The

results showed that 50 μ M suramin added before infection effectively blocked viral replication, but when added 1 h postinfection, the inhibitory effect was significantly diminished (Fig. 2A and B). However, if a higher concentration of suramin was used (100 μ M), the drug was able to effectively inhibit replication when added either before or after virus entry into cells (Fig. 2A and B). The concentrations of ammonium chloride and suramin used in these experiments had no effect on cell viability (Fig. 2C). These data suggest that suramin is able to prevent RVFV replication at multiple stages during virus replication. Suramin inhibits an early step during viral replication, as well as one or more later steps that have yet to be determined.

Competitor RNA is unable to displace prebound RNA in mature Rift Valley fever virus ribonucleoprotein complexes. An electrophoretic mobility shift assay (EMSA) was conducted to test the ability of a fluorescently (3'-FAM) labeled RNA to bind Rift Valley fever virus (RVFV) nucleocapsid (N) protein and visualize the ribonucleoprotein (RNP) complexes of various sizes that form during an N-RNA binding event. A constant amount of FAM-RNA and N was incubated at 10°C for various amounts of time (1 min to 5 h). The binding reaction mixture was incubated at 10°C to decrease the speed of RNP formation because previous experiments conducted in our laboratory indicated that N binds RNA rapidly at room temperature (data not shown). After incubation, the samples were subjected to nondenaturing gel electrophoresis at 4°C. The bands that migrate faster on the gel correspond to free RNA, and the higher-migrating bands represent higher-molecular-mass RNA-protein complexes. The results showed that when the binding reaction mixture was incubated for 1 min to 2 h, two distinct N-RNA complexes were formed. However, after a 5-h incubation period, the bands that corresponded to the lower-molecular-mass N-RNA complex and free RNA species were significantly reduced. These data suggest that over time the RNP complex becomes larger (Fig. 3A), as additional molecules of N bind along the length of the RNA molecule and eventually incorporate almost all of the FAM-RNA into the highest-molecular-mass N-RNA complexes.

Since N bound RNA efficiently, we next chose to study the stability of N-RNA complexes. A competitive binding experiment was performed to test the ability of an unlabeled RNA to bind N and displace a fluorescently (FAM) labeled RNA. A constant

FIG 4 RNase T₁ and RNase A structure probing analysis of an N-RNA binding interaction. Lanes designated A, C, G, and U are sequencing lanes. K lanes are primer extensions of MBE59/5S untreated with RNases. Lanes 5' (i.e., 5 min) and 5 h indicate the length of time that N and RNA were incubated together before probing. Arrows indicate reactive nucleotides, and asterisks indicate bands used to normalize band intensities for quantification purposes, as described in Materials and Methods. (A to C) Primer extension analysis of MBE59/5S probed with RNase T₁ in duplicate. (D to F) Primer extension analysis of MBE59/5S probed with RNase A in duplicate.

amount of FAM-RNA was incubated at room temperature with N for various amounts of time (5 to 60 min), and then competitor aptamer RNA was added. The reaction mixtures were incubated at room temperature for an additional hour and subjected to non-denaturing gel electrophoresis. The results showed that competitor RNA was able to bind N and displace some FAM-RNA when added 5 or 10 min into the incubation period. However, when FAM-RNA and N were incubated for 30 to 60 min prior to addition of competitor, significantly less FAM-RNA was displaced (Fig. 3B). These data suggest that after an initial N-RNA binding event, the RNP complex undergoes a structurally uncharacterized maturation to a more robust and impenetrable complex. It is also possible that competitor RNA is unable to displace prebound RNA after prolonged incubation because the dissociation rate (k_{off}) of the mature complex is very slow. To further study the formation of high-molecular-mass N-RNA complexes, we used

RNA structure probing to visualize changes in the reactivity of RNA bases that occur over time as a result of N binding.

A specific RNA binding event triggers cooperative binding between N monomers. Previously, we used an *in vitro* selection technique (SELEX) to generate high-affinity 55-nt-long RNA ligands, or aptamers, to RVFV N. Mutational analysis of aptamer RNAs identified a GAUU motif found in many of the aptamers as an important recognition sequence for N (23). To create a longer (~200-nt) RNA molecule with additional nonspecific N-binding sites and additional primer binding sites for structure probing studies, we added the human 5S rRNA sequence to the 5' end of an aptamer RNA with a duplicated GAUU motif (MBE59/5S). The 5S rRNA molecule was chosen because the structure is well characterized, robust, and folds readily and stably into its native structure. Therefore, addition of new sequence to its terminus does not alter the structure of the attached aptamer RNA.

FIG 5 Summary of the nucleotide reactivities superimposed on an Mfold predicted secondary structure model of MBE59/5S. MBE59/5S is an RNA construct containing an aptamer RNA sequence with a GAUU motif attached to the 3' end of the human 5S rRNA sequence. The GAUU motif located within the 55-nt-long aptamer RNA sequence is underlined. Circles represent RNase A probing of cytidine and uridine residues, and stars represent RNase T₁ probing of guanosine residues. Open symbols represent enhanced reactivity, and closed symbols represent a decrease in reactivity. The number of symbols next to a residue indicates the relative level of reactivity of the residue after incubation with N for either 5 min (nonshaded symbols) or 5 h (shaded and boxed symbols) compared to the RNA-alone control. Band intensities were quantitated using ImageGauge software, and the number of symbols used is based on the percentage of change in band intensity compared to the intensity of the RNA-alone control: 0 to 25%, 1 symbol; 26 to 50%, 2 symbols; 50 to 75%, 3 symbols; and 76 to 100%, 4 symbols.

The MBE59/5S RNA was incubated with N in binding buffer for 5 min or 5 h at 10°C to allow RNP complexes to form. The complexes were then probed using either RNase T₁ (which cleaves after single-stranded guanosine residues) or RNase A (which cleaves after single-stranded uridine and cytidine residues). The probed RNA samples were reverse transcribed using a γ -³²P-labeled DNA primer and AMV reverse transcriptase so that the relative reactivity of the individual bases to the RNase enzymes in the presence and absence of N could be analyzed. By comparing cDNA band patterns produced by primer extension of probed RNA alone and probed RNA complexed with N, nucleotides exhibiting either reduced or enhanced reactivities in the N-RNA complex were identified. (A cleavage by the RNase results in a shortened cDNA produced by the RT and is identified as a strong stop on the gel.) This information was then used to deduce the RNA sequences specifically contacted by N, as well as secondary structural rearrangements that occurred when N bound RNA.

When N was incubated with MBE59/5S and then probed with RNase T₁, there was a strong and very rapid protection of the two G residues in each of the duplicated GAUU motifs (G156 and G152), suggesting that this sequence is immediately recognized and bound by N (Fig. 4C and 5). However, G residues located away from the GAUU motif (G75, G48, and G47) exhibited little to no protection at the 5-min time point but after an extended incubation showed moderate (G75) and significant (G48 and G47) decreases in reactivity (Fig. 4A and B and 5). These data are consistent with the idea that an initial specific binding event triggers subsequent binding of N monomers and/or that the highest-affinity binding sites are occupied first followed by binding to lower-affinity sites.

A pattern similar to the one described above was observed when RNP complexes were probed with RNase A. For example, a U residue present in the GAUU motif (U159) was strongly pro-

tected at both the 5-min and 5-h time points, whereas several C and U residues located closer to the 5' end of the RNA construct and farther away from the GAUU motif exhibit weak protections at the 5-min time point but became more protected over time (Fig. 4D to F and 5). Interestingly, residues U130 and U76 located approximately in the middle of the RNA construct initially exhibited enhanced reactivity and then became protected after 5 h (Fig. 4E and F and 5).

Suramin inhibits RNA binding to wild-type and mutant N protein. To determine whether suramin inhibits RNA binding *per se* or N-N interactions essential for RNA binding, a fluorescence polarization experiment was performed to compare the ability of the wild type (WT) and a mutated version of RVFV N that lacks the amino-terminal 33 amino acids (Δ 33) important for N oligomerization to bind RNA. A fluorescently labeled (3'-FAM) RNA previously shown to bind wild-type RVFV N was incubated in binding buffer with various concentrations of WT and Δ 33 N at 30°C for 1 h, and fluorescence polarization (FP) was measured. Titration of WT N with FAM-RNA gave an apparent K_d of 1.2 μ M and a Hill coefficient of 2.2. Titration of the Δ 33 mutant version of N gave an apparent K_d of 9.0 μ M and a Hill coefficient of 1.4 (Fig. 6A). The 7.5-fold decrease in the apparent K_d of WT N compared to the Δ 33 mutant shows that removal of the N-terminal arm decreases the ability of N to bind RNA. The Hill coefficients for both WT and mutant N proteins were greater than 1, indicating that N binds RNA in a positively cooperative manner. The N-terminal arm may play an important role in the ability of N to oligomerize because the WT N protein exhibited a steeper binding curve and larger Hill coefficient than the Δ 33 mutant.

We next performed competitive binding experiments by incubating a constant amount of N (15 μ M WT, 50 μ M Δ 33 N) with 10 nM RNA at room temperature for 1 h. The binding experiments discussed above (Fig. 6A) showed that N concentrations of

FIG 6 Analysis of the effects of suramin on WT and $\Delta 33$ N-RNA binding interactions using fluorescence polarization. (A) Binding profile for the association of WT (filled squares) and $\Delta 33$ mutant (open circles) N with fluorescently (FAM) labeled RNA. (B) A competition assay in which a fixed concentration of the WT (15 μ M [solid squares]) and $\Delta 33$ mutant (50 μ M [open circles]) N was incubated with FAM-RNA. Various concentrations of suramin were added, and the FP signal was plotted versus suramin concentration. The results are the average of two independent experiments, and the error bars represent $\pm$ standard deviation from the mean.

15 μ M (WT) and 50 μ M ($\Delta 33$ N) were required to achieve plateau binding of the FAM-RNA and were therefore the constant concentrations of N chosen for the competitive binding assays. Increasing concentrations of suramin (524 pM to 588 μ M) were added, and the reaction mixture was incubated for 1 more hour at room temperature. FP measurements were taken, and the results showed suramin competed with aptamer RNA for N binding. The 50% inhibitory concentration (IC_{50}) value of suramin for WT N was approximately 3-fold higher than that for the $\Delta 33$ mutant protein (22.3 μ M for the WT versus 7.1 μ M for the $\Delta 33$ mutant) (Fig. 6B). These data show that the absence of the N-terminal arm increases the ability of suramin to disrupt an N-RNA binding interaction and suggest that the N-terminal arm may play an important role in stabilizing the RNP complex.

Suramin inhibits a specific N-RNA binding interaction. Our previous results have shown that N specifically recognizes and binds to GAUU motifs on selected aptamer RNAs. RNA structure probing experiments were conducted (as described above) in the presence of various concentrations of suramin (0.8 to 800 μ M) to determine if suramin is able to inhibit this specific binding interaction. Consistent with the results obtained previously, G156 and G152 in the GAUU motif were reactive in the absence of N and became strongly protected when N bound MBE59/5S. When suramin was added to the binding reaction mixture, the protective effect of N was abolished and G156 and G152 regained their susceptibility to cleavage by RNase T₁ (Fig. 7). These data suggest that

FIG 7 RNase T₁ probing analysis of the effects of suramin on an N-RNA binding interaction. RNase T₁ structure probing followed by primer extension was used to visualize the effects of increasing concentrations of suramin (0.8 to 800 μ M) on the ability of N to bind the GAUU motif of MBE59/5S. Lanes A, C, and G are sequencing lanes, the K lane is a primer extension of untreated MBE59/5S, and arrows indicate reactive guanosine nucleotides.

suramin disrupts the ability of N to specifically recognize and bind its cognate RNA. Next we sought to determine if suramin was able to disrupt nonspecific N-RNA interactions that are important for the formation of high-molecular-mass RNP complexes.

Suramin disrupts high-molecular-mass N-RNA complexes. Purification of recombinant WT N protein (31 kDa) from bacteria results in a heterogeneous population of N dimers and multimers in a complex with *E. coli* RNA. Gel filtration chromatography yielded two protein/RNA fractions with apparent molecular masses of $\sim$ 153 and 90 kDa based on elution volumes from a Superose 12 (S-12) column that was calibrated with protein standards (Fig. 8A). The calibrated S-12 column was used to compare the multimeric states of wild-type N in the presence and absence of suramin. Our results showed that when N was incubated with increasing concentrations of suramin, higher-molecular-mass ribonucleoprotein complexes were disrupted, resulting in the formation of lower-molecular-mass complexes (Fig. 8A). The peak at 10.7 ml in the absence of suramin corresponded to a molecular mass of approximately 90 kDa. (N copurifies with RNA from the cells from which it is expressed; the 90-kDa peak likely represents two molecules of N bound to a single molecule of RNA.) When 10 mM suramin was added, this peak decreased in size significantly and a peak at 14.8 ml appeared, corresponding to a molecular mass of $\sim$ 37 kDa (Fig. 8A). To compare the amounts of RNA in higher-molecular-mass N-RNA complexes versus lower-molecular-mass complexes formed by incubation with 10 mM suramin, fractions of N were collected and phenol-chloroform extracted, and the amount of RNA in each fraction was determined by denaturing gel electrophoresis followed by densitometry. Next, the protein concentration was plotted against the elution volume of the gel filtration column, and the relative amounts of copurifying RNA were observed at each point. Large amounts of RNA were present in the fractions that corresponded to the 90-kDa peak; however, the 37-kDa peak that formed during incubation with 10 mM suramin was devoid of RNA (Fig. 8B and C). These data show that suramin is able to displace the RNA from the higher-molecular-mass N-RNA complexes, resulting in the formation of a lower-molecular-mass species that could be an N monomer bound to suramin.

To determine whether the observed shift to a lower-molecular-

FIG 8 Characterization of the effects of suramin on the multimeric state of N using gel filtration chromatography. (A) Gel filtration profile of wild-type (WT) N under the following conditions: no suramin, solid line; 1 mM suramin, dashed line; 10 mM suramin, dotted line. (B) Analysis of the RNA content of fractionated WT N in the absence of suramin. The RNA level (dashed line; measured by gel band intensities in arbitrary units) and protein concentration (solid line; reported in milliabsorbance units [mAU] at 280 nm)

mass species is caused by suramin blocking the RNA binding site of RVFV N protein or inhibiting N-N interactions required for multimerization, we tested the ability of suramin to disrupt a dimer structure formed by a mutated N that is unable to bind RNA. The predominant peak for the RNA binding (–) mutant corresponded to a molecular mass of 54 kDa and likely represents an N dimer without RNA (Fig. 8D). The gel filtration profiles of the mutated N were identical in both the presence and absence of suramin, suggesting that suramin was unable to disrupt the protein-protein interactions important for the formation and stabilization of the N dimer structure (Fig. 8D). Together, these data suggest that suramin disrupts formation of high-molecular-mass N-RNA complexes by obstructing the RNA binding cleft.

DISCUSSION

In the present study, we demonstrate that suramin, a drug used successfully in the past to treat human patients with trypanosomiasis and onchocerciasis (33, 34), functions well as an inhibitor of RVFV N-RNA interactions *in vitro* and inhibits viral replication in cell culture. Furthermore, characterization of the mechanism of suramin inhibition allows us to dissect N-RNA binding interactions and their relations to the roles of N in RVFV RNA encapsidation. We demonstrate that suramin inhibits the initial specific N-RNA binding event and is also capable of dissociating high-molecular-mass N-RNA complexes. These results add significantly to a growing body of evidence that viral nucleocapsid protein is an attractive antiviral therapeutic target.

Suramin was identified in a high-throughput screening assay for compounds that inhibit a specific RVFV N-RNA binding interaction (29). Suramin is a symmetric, polyanionic compound that contains two naphthalene-1,3,5-trisulfonic acid head moieties, and it seemed likely that suramin could bind to N similarly to the mode of binding of a polyanionic RNA molecule. Indeed, we observed that although competitor RNA was unable to disrupt mature N-RNA complexes, suramin was able to penetrate and disrupt N-RNA complexes. Our data suggest that suramin binds directly to the RNA binding cleft to disrupt interactions with RNA. First, N purified by gel filtration chromatography after incubation with suramin had a diminished RNA content. Second, suramin was unable to disrupt a dimer structure formed by mutant N that is unable to bind RNA; therefore, suramin does not act by dissociating N-N protein interactions. A recent crystal structure of N from a related phlebovirus (severe fever with thrombocytopenia syndrome virus [SFTSV]) with a bound suramin showed that the head of the suramin molecule penetrated deeper into the RNA binding slot than an RNA molecule (35). The ability of suramin to penetrate further into the RNA binding groove could explain why we found that suramin is able to eject prebound RNA, albeit at higher concentrations. The propensity of suramin to bind to the nucleic acid binding domain of N is corroborated by

are plotted against the elution volume of the gel filtration column. (C) Analysis of the RNA content of fractionated WT N after incubation with 10 mM suramin. To compare the amounts of RNA in high-molecular-mass N-RNA complexes versus lower-molecular-mass N-RNA complexes formed by incubation with 10 mM suramin, the RNA level (dashed line) and protein concentration (solid line) are plotted against the elution volume of the gel filtration column (graph labeling as in panel C). (D) Gel filtration profile of RNA binding (–) mutant N under the following conditions: no suramin, solid line; 1 mM suramin, dashed line; 10 mM suramin, dotted line.

its ability to inhibit the polymerase active sites of norovirus RNA-dependent RNA polymerase (36) and retroviral reverse transcriptase (37).

We propose that RNA encapsidation by N is triggered by an initial specific binding event and previously reported that N specifically recognizes and binds appropriately positioned GAUU sequences on aptamer RNA molecules (23). Here, time-resolved RNA structure probing experiments allowed us to observe sequential N binding to RNA and showed that N first bound and protected G and U residues located within a GAUU motif from cleavage by RNases. At the same time point, bases adjacent to the initial binding site exhibited a slight increase in reactivity. These enhancements could be a result of the binding of the first N monomer, causing a secondary structural rearrangement that prepares the adjacent binding site for another molecule of N to bind. Sequential binding of N monomers onto our RNA construct was also observed. RNA bases at the N-RNA recognition site near the 3' end of the RNA molecule were protected first, and bases toward the 5' end were protected later. As the complex matured over time, global protection of all reactive RNA bases was observed. These results support a model of RNP formation that involves a specific RNA binding event initiating subsequent binding of N monomers, which then switch to a nonspecific mode of RNA binding that allows N to coat the viral genome.

In support of a bimodal (specific then nonspecific) RNA binding model, our experiments showed that N exhibited positive cooperativity when binding to an RNA molecule, resulting in the formation of high-molecular-mass N-RNA that over time became increasingly resistant to displacement by competitor RNA. A high-resolution structure of N multimers bound nonspecifically to RNA shows that the RNA bases are sequestered in a deep binding groove that is contiguous along adjacent N monomers (27); this arrangement could explain our observation that competitor RNA was unable to displace prebound RNA. However, removal of 33 residues from the amino-terminal domain of N ($\Delta 33$ mutant) decreased the cooperativity of N binding as well as the affinity of N for RNA by approximately 7.5-fold compared to WT N. These results demonstrate that the N terminus plays an important role in N oligomerization, as suggested by the high-resolution structure models, in which the flexible N-terminal arm wraps around the solvent side of an adjacent subunit (27, 28).

During replication, encapsidation of viral RNA by N has been proposed to occur in the 5' to 3' direction (20, 21). We observed sequential binding in the opposite (3' to 5') direction, suggesting either that N is able to oligomerize in both directions or that binding occurred first at a high-affinity site and subsequently at sites further upstream. That N exhibits disparate binding behaviors on different RNAs has been demonstrated in La Crosse virus-infected cells (38). During the later stages of a La Crosse viral infection (24 to 48 h postinfection) as the concentration of N in the host cell increases, viral mRNAs become encapsidated (38). Viral mRNAs are considered secondary targets because at low concentrations of N, only genomes and antigenomes are complexed with N. At higher concentrations, viral but not cellular mRNAs are associated with N protein (38). This suggests that the viral mRNA has a lower-affinity N recognition motif or motifs, but at a critical concentration of N, the mRNA can be bound first specifically and then cooperatively by N. It is thought that encapsidation of viral mRNA is a mechanism to regulate viral protein synthesis because encapsidated mRNAs cannot be translated by the ribosome.

Suramin was not only effective at disrupting RVFV N-RNA binding interactions *in vitro*, it also inhibited viral replication in human cell culture at a clinically relevant concentration (EC_{50} of 58.5 μ M). When suramin was used to treat human trypanosomiasis or HIV, blood plasma concentrations of suramin were at or exceeded 100 μ M (30, 39), which is well above the concentration of suramin that diminishes RVFV replication in cell culture. The relatively low therapeutic index (3.4-fold) in our experiment and the fact that suramin is known to have some undesired side effects in patients suggest that medicinal chemistry efforts to reduce cytotoxicity would make for a more desirable drug.

Using time-of-addition experiments in cell culture, we showed that in addition to its ability to inhibit N-RNA interactions, suramin also interfered with an early step(s) in the viral replication cycle. Polyanionic compounds like suramin are known to interfere with virus adsorption, and in a related phlebovirus, sandfly fever Sicilian virus (SFSV), suramin was shown to inhibit an early step, possibly including virus entry (40). It is also possible that suramin in the media could penetrate the viral particle and begin to disrupt the RNP complex and/or the RNA-dependent RNA polymerase before it enters the cell. A drug that exerts its effects at different points during the viral replication cycle could be effective at concentrations that are lower than expected due to synergistic or additive effects at multiple points in the cycle.

This study demonstrates that suramin is a useful tool to study RVFV N-RNA binding interactions, and it could form the basis for antiviral therapeutic strategies that specifically target this essential interaction. We show here that suramin inhibits RVFV replication at multiple steps in cell culture, and its ability to inhibit replication of several other viruses in previous studies suggests that further development could result in a class of broad-spectrum antiviral drugs.

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