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Computational prediction and biochemical characterization of novel RNA aptamers to Rift Valley fever virus nucleocapsid protein

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Abstract

Rift Valley fever virus (RVFV) is a potent human and livestock pathogen endemic to sub-Saharan Africa and the Arabian Peninsula that has potential to spread to other parts of the world. Although there is no proven effective and safe treatment for RVFV infections, a potential therapeutic target is the virally encoded nucleocapsid protein (N). During the course of infection, N binds to viral RNA, and perturbation of this interaction can inhibit viral replication. To gain insight into how N recognizes viral RNA specifically, we designed an algorithm that uses a distance matrix and multidimensional scaling to compare the predicted secondary structures of known N-binding RNAs, or aptamers, that were isolated and characterized in previous *in vitro* evolution experiment. These aptamers did not exhibit overt sequence or predicted structure similarity, so we employed bioinformatic methods to propose novel aptamers based on analysis and clustering of secondary structures. We screened and scored the predicted secondary structures of novel randomly generated RNA sequences *in silico* and selected several of these putative N-binding RNAs whose secondary structures were similar to those of known N-binding RNAs. We found that overall the *in silico* generated RNA sequences bound well to N *in vitro.* Furthermore, introduction of these RNAs into cells prior to infection with RVFV inhibited viral replication in cell culture. This proof of concept study demonstrates how the predictive power of bioinformatics and the empirical power of biochemistry can be jointly harnessed to discover, synthesize, and test new RNA sequences that bind tightly to RVFV N protein. The approach would be easily generalizable to other applications.

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Keywords

RNA structure prediction; aptamers; Rift Valley fever virus; nucleocapsid protein; viral inhibition

1. Introduction

1.1

Rift Valley fever is a zoonotic disease caused by Rift Valley fever virus (RVFV) found primarily in sub-Saharan Africa. However, the observation that RVFV has recently become endemic on the Arabian Peninsula [1, 2], and its ability to be transmitted by many different mosquito species [3] underscores its potential for spread beyond its historical boundaries. Transmission of RVFV most often occurs via the bite of a mosquito, although agricultural workers who handle infected animals or animal products are also at risk. The RVFV genome is a tripartite, negative-sense RNA encapsidated by a nucleocapsid protein (N). N is important at several points in the virus life cycle (reviewed in [4]) and disruption of the interaction of N with RNA could lead to a possible antiviral therapeutic treatment. An important step toward exploiting N as a target for antiviral chemotherapy involves understanding how N preferentially recognizes RVFV genomic RNA.

In an effort to understand the molecular determinants of how N recognizes its target RNA in the cellular milieu where non-viral RNA vastly outnumbers viral RNA, we previously carried out an *in vitro* evolution (SELEX) scheme that selects and amplifies RNAs that bind strongly to N from a starting random library comprising 10^{14} different RNA sequences synthesized *in vitro* (Ellenbecker et al., 2012). The output of that study was a partially degenerate list of about 100 RNA sequences that were observed empirically to bind with high affinity to N. These tight binding RNAs are called *aptamers*. Understanding the common structural or primary sequence features of these aptamers would be useful for future efforts to understand viral replication and to develop antiviral strategies that interfere with N's ability to bind viral RNAs. Although we observed several short sequence motifs that were repeated in several aptamers, we were not able to propose a set of general sequence or secondary structural rules that could be used to predict RNA binding characteristics.

We reasoned that we could use the data contained within the sequences of known aptamers to develop an algorithm to recognize good N-binding RNAs and, further, to discover new RNA sequences that are potentially good binders. Initial *in silico* analysis of the known aptamer sequences and their computationally predicted secondary structures was carried out using FOLDALIGN [5, 6]. FOLDALIGN uses both energy minimization folding of RNA sequences and sequence similarity to identify RNAs with similar structures (and by extension, similar functions) even when the primary sequences are not highly related. Local and global pairwise folding guides alignment of different RNA by identifying similar secondary structure motifs. This algorithm is particularly useful for the present application, as aptamer RNAs have in common high affinity binding characteristics, but they may have completely unrelated primary sequences. The FOLDALIGN output data demonstrated a conspicuous clustering of the numerical scores, which were based on structural similarity,

on a multidimensional scaling (MDS) plot. Although the plot did not reveal the primary sequence and/or secondary structural features that these sequences have in common, we hypothesized that this method could discriminate potentially good binders from poor binders among a new, randomly generated *in silico* population of RNA sequences depending on whether or not they cluster with the known binders on the MDS plot.

Here, we implemented an algorithm that combines energy minimization based RNA folding, a pairwise structural scoring distance matrix, and multidimensional scaling to graphically visualize the clustering of RNA sequences with a common experimentally determined property. We then injected novel randomly generated sequences and selected several that spatially clustered with the known binders. We enzymatically synthesized these novel RNAs whose sequences were generated *in silico* and tested their binding properties compared to those of known N-binding RNAs. Finally, we show that introducing these novel *in silico* aptamers into cells in culture inhibits viral replication, providing proof of concept that this combined bioinformatic and biochemical approach can lead to experimentally and physiologically useful outcomes.

2. Materials and Methods

2.1. In silico generation of putative novel aptamers that are similar to known aptamers

The underlying concept for generating candidate aptamers *in silico* is a simple one. By chance, a small subset of randomly generated sequences of appropriate length should adopt a similar secondary structure to known N-binding RNA aptamers [7]. If a mechanism by which N binds a particular RNA involves specific recognition of a structural feature, those *in silico* aptamers whose structures are similar to the structure(s) of the family of known aptamers potentially represent good binders of N themselves. To screen for new *in silico* derived aptamers, we generated random RNA sequences (within a Python script, randomly drawing from the four nucleotides at each position) 30 nucleotides in length. A pairwise comparison was performed of the structures of these random sequences with those of the known aptamers.

2.1.1. What is “similar”?—FOLDALIGN [5, 6], a tool for aligning RNA structures, was utilized to determine similarity between aptamers. The output of the tool is an alignment score, but our approach, including some of the clustering and visualization techniques, requires a dissimilarity metric. For this reason the alignment score was converted to a distance using Equation 1 where *x* is the alignment score, *d* is distance, and *max* and *min* are the maximum and minimum encountered alignment scores, respectively. In this way, the maximum encountered score becomes a zero distance and the minimum becomes one (largest distance associated with smallest alignment score).

$$d=\frac{min-x}{max-min}+1 \quad \text{(Equation 1)}$$

Most alignments were so dissimilar that FOLDALIGN yielded no alignment score. For these, the distance was set at two times the maximum distance of known aptamers prior to normalization. While it is clear that this distance metric does not represent complete

coverage of the search space, it serves our purposes in that it is bounded by the arbitrary 2X no-alignment scores and it maintains the relative distances between aptamers.

2.1.2. What is "similar enough"?—In order to set an appropriate threshold for an *in silico* generated RNA sequence to be considered a potential novel high affinity RNA aptamer, all pairwise distances between the known aptamers characterized in Ellenbecker et al. (2012) were first calculated and then the aptamer that had the largest average distance from all other aptamers was identified. In fact, the known aptamers formed a cloud of points in a multidimensional vector space whose inter-point distances match those of the FOLDALIGN-determined distances. The known aptamer with the largest average pairwise distance was found to reside on the periphery of that cloud (Figure 1).

Any *in silico* generated candidate aptamer that has a smaller average distance to all known true aptamers would reside inside the "cloud" of true aptamers. Such a randomly generated aptamer proved so rare (the non-constant region of the aptamers is 30 nt in length, therefore there are 4^{30} possible random aptamers) that a threshold of five times this maximum average pairwise distance was set in order to consider the *in silico* generated aptamer a candidate for further study. The five virtual sequences that clustered most closely with the known aptamers using this process were named AS1-AS5. Their sequences are shown in Table 1.

2.2. Synthesis of in silico predicted aptamers

The synthesis of novel RNA molecules that were predicted by the algorithm above to be *aptamer-like* was carried out essentially as previously described [7]. Briefly, we constructed DNA templates of the predicted *in silico* aptamers that contained an *Eco*R1 restriction site at the 5′ end followed by the promoter sequence for T7 RNA polymerase, followed by the predicted aptamer sequence, followed by an *Xba*I restriction site. DNA oligomers to make these constructs were purchased from Integrated DNA Technologies (Coralville, IA). The DNA fragments were cloned into pUC19 and resulting plasmids were amplified in *E. coli*. Plasmids were cut with *Xba*I and RNA run-off transcriptions were performed using MegaShortscript T7 RNA transcription kit according to the manufacturer's instructions (Ambion/Life Technologies).

2.3. Filter binding assays to assess aptamer affinity to N

Purified recombinant N protein was serially diluted to varying concentrations in a binding buffer (10mM HEPES pH 7.3, 150mM NaCl, 20mM KCl, and 5mM $MgCl_2$ final concentration). Nine μL of various dilutions of N were added to each tube along with 1 μL of 5′ end-labeled RNA that had been denatured at 90°C for two minutes followed by snap cooling on ice. In each experiment 25,000 cpm/μL of reference RNA (wild type) was added. After incubation at 30°C for one hour, reactions were diluted to 100 μL with ice-cold binding buffer and filtered through pre-soaked nitrocellulose filters (Millipore HAWP). The filters were washed twice with 500 μL ice-cold binding buffer, dried, and the radioactivity retained on filters was measured by scintillation counting.

2.4. Cell culture studies

Human 293 cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. Vero cells were maintained in minimum essential medium (MEM) alpha medium supplemented with 10% FBS, penicillin and streptomycin. The Rift Valley fever virus (RVFV) vaccine strain, MP-12, was provided by Brian Gowen (Utah State University, Logan, UT). A Rift Valley fever reporter virus containing the gene for *Renilla* luciferase was provided by Richard Elliott (University of St. Andrews, St. Andrews, United Kingdom). The *Renilla* luciferase open reading frame was inserted in place of the NSs gene encoded by the S segment of Rift Valley fever virus (RVFV) and is known to not be required for viral infection. 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% CO_2.

2.5. Cell culture and transfection

Confluent monolayers of human 293 cells grown in 24-well plate were transiently cotransfected with 15 pmol RNA/well and 0.5 μg plasmid DNA/well using the Lipofectamine 2000 kit (Life Technologies). The next morning transfected cells were harvested, counted and reseeded in 96-well plate format.

2.6. Luciferase assays

Confluent monolayers of transfected human 293 cells grown in 96-well plate format were infected with RVF-luc virus using a MOI of three. Cells were harvested at eight hours post infection by removing the growth media, adding 20 μL of lysis buffer and rocking the cells for 15 minutes at room temperature. The cell lysate was diluted (5–20x) in reagent buffer and 20 μL was transferred to a white 96-well plate. A Biotek Synergy 2 plate reader was used to autoinject each sample well with 100 μL of *Renilla* luciferase assay reagent (Promega) and measure the luminescence.

2.7. Plaque Assays

Confluent monolayers of transfected human 293 cells grown in 96-well plate format were infected with RVF MP-12 virus using a MOI of 0.1, centrifuged at 2500 RPM for 100 minutes and incubated for 1 hour. After incubation the media was removed and replaced with DMEM supplemented with 2% FBS, penicillin and streptomycin. Supernatants of virus infected cells were harvested at 3 days post infection. The supernatants were serially diluted in MEM and subsequently used to infect confluent Vero cells grown in 6-well plate format. After a 2 hour incubation the cells were overlaid with MEM containing 1% agarose and incubated for 7 days. Cells were fixed with 9.25% formaldehyde and plaques revealed using crystal violet stain.

3. Results

3.1. In silico prediction of novel N-binding RNAs

We analyzed known aptamer sequences [7] and predicted secondary structures using an energy minimization and alignment algorithm called FOLDALIGN [5, 6]. After numerical

scoring of the secondary structure pairwise comparisons, the data output was in the form of a distance matrix. The distance matrix was then imported into R to utilize multidimensional scaling (MDS) to visualize the relationship between the sequences (Figure 1). Almost all of the aptamer sequences were observed to cluster in the MDS plots. Subsequently, additional randomly generated RNA sequences were 'injected' into the aptamer pool of sequences to screen for new sequences that cluster closely to the known aptamers. Weeks of computational time generated several new sequences whose positions in the MDS plots were within a threshold distance to the cluster centroid. It was hypothesized that these sequences would be potentially good binders of N.

3.2. Binding characteristics of in silico aptamers to RVFV N protein

The N-binding curves of several aptamers are shown in Figure 2. We incubated each of the radiolabeled RNAs with increasing amounts of N and determined the amount of RNA bound by N using a nitrocellulose filter binding assay. Each of the *in silico* aptamers exhibited measurable binding to N. While none of the *in silico* aptamers bound as well as the MBE87 sequence, a true aptamer that survived 16 rounds of selection in the original SELEX experiment [7], the *in silico* aptamers displayed significant binding, especially aptamers AS-1, AS-2, and AS-4. Binding of *in silico* aptamers AS-3 and AS-5 in the nitrocellulose filter binding assay was weaker, but was significantly above background.

3.3. In silico selected RNAs inhibit viral replication and production of viral protein in human cell culture

To determine if RNAs selected to bind with high affinity to RVFV N protein using a computer algorithm could inhibit viral replication and/or production of viral protein during an infection, we cotransfected cells with a green fluorescent protein (GFP) reporter plasmid and either non-binding RNAs (JSR0), RNAs that have been selected by a computer algorithm to bind N with high affinity (AS-5, AS1 and AS-4) or an aptamer RNA that has undergone 16 rounds of an *in vitro* selection and binds N with high affinity in biochemical assays (MBE87). The GFP reporter plasmid was used to verify that equal and high levels of transfection efficiency were achieved. After transfection in 24-well plate format cells were harvested, counted and reseeded in two 96-well plates. To test the ability of selected RNAs to inhibit viral replication over the course of several days, a 96 well plate of transfected cells was infected with the MP-12 strain of RVFV using a low MOI (0.1) and the number of infectious viral particles produced at three days post infection was measured using a plaque assay. The results show that RNAs that were selected to bind N both *in vitro* and *in silico* were able to inhibit virus replication during a long term (three day) infection. Transfection of AS-1 caused the largest (35 fold) decrease in virus replication when compared to cells transfected with a known non-N-binding RNA (JSR0). An approximately 15 fold decrease in virus replication was observed in cells transfected with AS-4 and MBE87 whereas a more modest (5 fold) decrease was observed in cells transfected with AS-5 (Figure 3A and B). The transfections and plaque assays were repeated 2–4 times each, with similar results.

In parallel, to determine if aptamer RNAs could inhibit production of luciferase protein that is encoded in the viral genome, a second 96-well plate of aptamer-transfected cells was infected with a RVF reporter virus that contains the gene for *Renilla* luciferase (RVF-luc).

Cells were harvested and lysed at 8 hours post infection and the amount of protein produced by RVF-luc virus was quantified by adding a substrate to each sample that, when oxidized by *Renilla* luciferase, produces light proportional to the luciferase present. The results show that transfection of non-binding RNA into cells does not decrease the production of viral protein when compared to cells that were transfected with only plasmid DNA. Transfection of *in silico* selected aptamer AS-5 caused a slight (1.4 fold) decrease in viral protein production. However, transfection of AS-1, AS-4 and MBE87 RNA caused 3.4, 2.9 and 2.8 fold decrease in virally encoded luciferase yield, respectively (Fig 3C).

To verify that selected RNAs specifically inhibit production of viral (and not cellular) protein, cells were cotransfected with a plasmid containing the gene for *Renilla* luciferase (phRL-CMV; Promega) and either GFP, non-binding or selected RNA. After transfection in 24-well plate format, cells were harvested, reseeded in 96-well plate format and infected with the MP-12 strain of RVFV using a MOI of 3. At 8 hours post infection, the amount of protein translated by the host cell was quantified by adding substrate to lysed cells and measuring luminescence (as described above). The results demonstrated that the presence of non-binding RNA has no effect on host cell translation. Transfection of *in silico* and *in vitro* selected aptamers causes an approximately 1.5-fold decrease in host cell protein synthesis (data not shown). The decrease in translation of viral protein is significantly greater than the observed decrease in host cell translation for AS-1, AS-4 and MBE87 RNA, suggesting that these RNAs are specifically targeted to and inhibiting RVFV. However, a significant difference in between viral and host cellular translation was not observed for AS-5 RNA.

4. Discussion and Conclusions

Here we demonstrate that secondary structure data from a collection of aptamers derived from a SELEX protocol can be used to guide *in silico* prediction of novel N-binding RNAs. We used numerical scoring of secondary structure similarity among aptamers, representation of these scores on multidimensional scaling plots, and subsequent calculations of distances between the cluster of known aptamers and potential new aptamers derived from randomly generated sequences *in silico*. Potential *in silico* aptamers whose structure scores placed them within a threshold distance from the cluster of known aptamers were then selected for synthesis in the laboratory and their N-binding properties were assessed through biochemical and virological methods. We found that the *in silico* derived aptamers were indeed recognized by N protein *in vitro*, as measured by a nitrocellulose filter binding assay, and they inhibited viral function when they were introduced into live cells prior to infection with RVFV, suggesting that they competitively bound to N in infected cells and interfered with viral replication.

These results are significant for several reasons. First, they demonstrate that it is possible to predict new RNA sequences that have desired functional characteristics based on secondary structure and alignment scores and relative distances of these scores to those of known aptamers. Using this approach, detailed sequence analysis of individual aptamers is not necessary. Rather the matrix distance of the scores of new proposed aptamers from the scores of all of the known aptamers is sufficient to select which sequences will be good binders. Our original SELEX dataset was not amenable to direct sequence alignment

because of very limited primary sequence similarity [7], the present method of plotting secondary structure scores using multidimensional scaling represents a powerful tool for the analysis of SELEX results. In the present case, we can conclude that RNA secondary structure is a powerful determinant for recognition by N, since the *in silico* aptamers were selected by their structural scores. It is notable that all five of the potential aptamers that were identified in the *in silico* screen bound to N with higher affinity than random or un-selected RNAs. These *in silico* aptamers were rare; it took several weeks of pairwise distance calculations of approximately 2.5 million random RNAs to find these five sequences that clustered with the true aptamers. It is also intriguing that the aptamers bound to N with different affinities. We suspect that the presence of the GAUU motif, which was also seen to be overrepresented in the original aptamer dataset, in aptamers AS1 and AS4 contributed to the higher binding affinity along with subtle differences in structure among the aptamers.

Second, these results show that N is an attractive potential target for antiviral therapeutic interventions. When aptamer RNAs were transfected into cells and subsequently infected with either RVFV or with a mutant RVFV that expresses the reporter protein luciferase, the robustness of the infection and the production of virally-encoded protein were dramatically reduced. Transfection of an RNA that does not bind to N did not have the same effect. These data suggest that the aptamer RNAs competitively bind to N in infected cells, and thereby interfere with N's normal functions in viral replication. Nucleocapsid protein has been regarded as a potential antiviral target in many viruses [9–12], these results emphasize that perturbing N function is an effective way to inhibit viral replication.

Finally, this work represents a proof of concept for a synergistic bioinformatics/biochemical partnership that allows direct testing of *in silico* predictions in the laboratory. In turn, results from laboratory experiments could be used to inform and refine algorithms used to make the next generation of predictions. While many bioinformatics studies consist wholly of analysis and extraction of patterns from large datasets, this system enables iterative and direct testing of the hypotheses derived from bioinformatic analysis. This partnership forms the basis for a model system for future bioinformatics/laboratory endeavors in the future.

Although the present approach to screening randomly generated RNA sequences for similarity to known aptamers gave intriguing and apparently biologically relevant results, this undirected screening was computationally expensive and time consuming. We are currently exploring the use of Evolutionary Computation techniques to iteratively evolve initially random RNA sequences to become similar in secondary structure to the known aptamers. To iteratively select 'desirable' secondary structure characteristics *in silico* we propose using a genetic algorithm to fix the desirable secondary structure elements regardless of sequence, while allowing the rest of the nucleotides in the RNA molecule vary in a subsequent generation of *in silico* aptamers. This evolutionarily directed approach should yield exact matches in secondary structure (as predicted using UNAFold[13]) much faster than screening randomly generated RNA sequences for structures that are, by chance, identical to a target structure. This approach will also allow for the forced inclusion of specified motifs, making it possible to test more directly whether structure or sequence of

the RNA, or both, are primary drivers of the affinity of nucleocapsid protein for its cognate RNA.

Acknowledgments

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Highlights

- A family of RNA aptamers selected for their ability to bind to a viral protein, but whose secondary structures were uncharacterized, was folded and numerically scored *in silico*.
- The predicted structural characteristics of randomly generated RNA sequences were pairwise compared to those of the bona fide aptamers.
- The novel *in silico* aptamers that clustered structurally using multidimensional scaling with the bona fide aptamers were synthesized in the laboratory for further investigation.
- The *in silico* aptamers displayed good binding characteristics to Rift Valley fever virus nucleocapsid protein *in vitro*.
- Several of the *in silico* aptamers exhibited antiviral activity when transfected into cells prior to infection with RVFV.

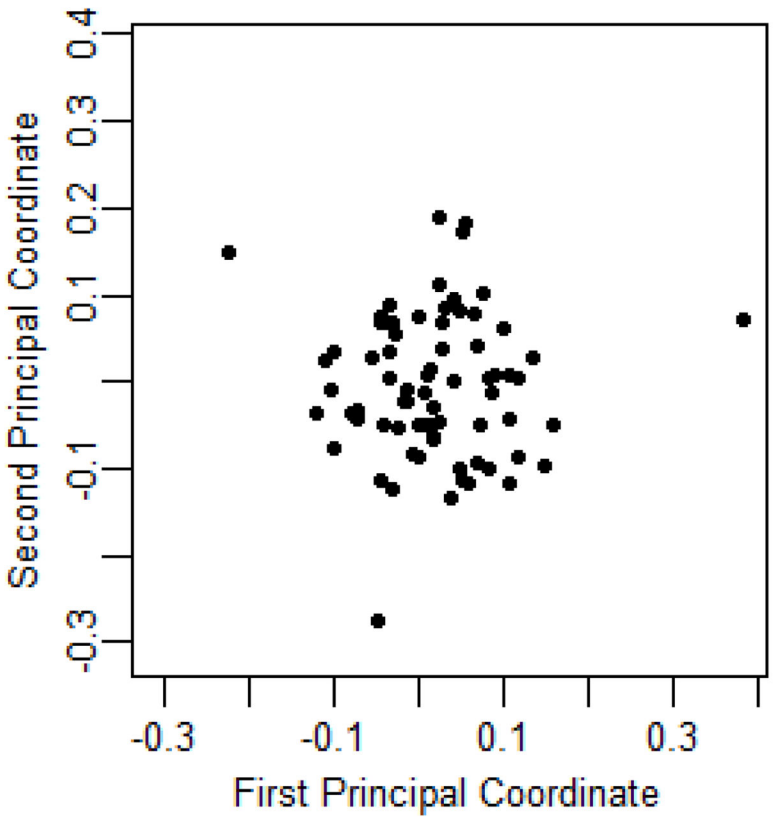

Figure 1.
Multidimensional Scaling [8] representation of the inter-aptamer distances.

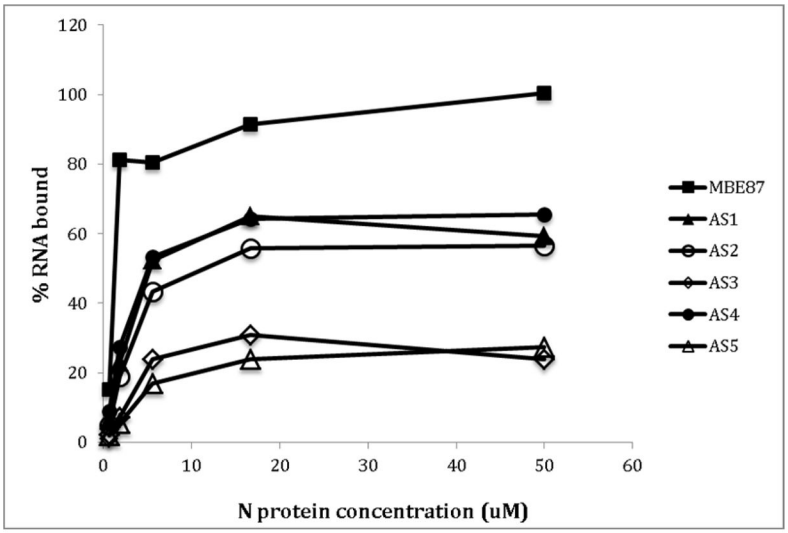

Figure 2.

Nitrocellulose filter binding data of *in silico* aptamers AS1-AS5 and *in vitro* selected aptamer MBE87. RNAs were synthesized and radiolabeled as described in Materials and Methods. A fixed amount of radiolabeled RNA (50,000 cpm) was incubated with increasing amounts of RVFV N protein. Free RNAs are not retained on the filter, but RNAs bound to the N protein are retained on the filter. Radioactivity retained on the filter was quantified by liquid scintillation counting. Sequences of novel aptamers that came out of the *in silico* selection and the MBE87 control RNA used in the binding experiments are shown in Table 1.

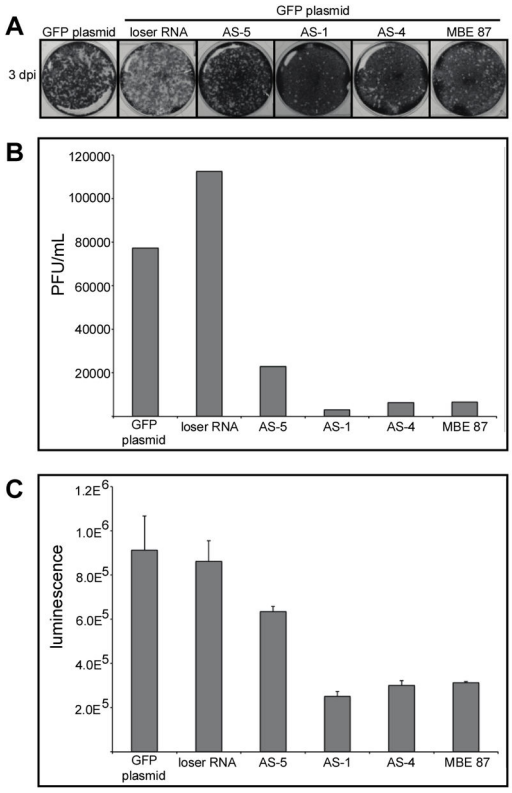

Figure 3. Analysis of the ability of *in silico* and *in vitro* selected RNA to inhibit RVFV replication and protein production in cell culture

A) A representative plaque assay to determine the number of infectious viral particles produced from cells transfected with either plasmid DNA alone (GFP plasmid), non-binding RNA JSR0 (loser RNA), RNA that has been selected to bind N using a computer algorithm (AS-5, AS-1 and AS-4) or true aptamer RNA that was selected to bind N with high affinity (MBE87). B) Quantitation of the representative plaque assay from (A). Similar results were obtained on replicate experiments. C) Luciferase assay showing the effects of selected RNAs on the production of viral protein. Cells were transfected with either plasmid DNA alone (GFP plasmid), non-binding RNA JSR0 (loser RNA), RNA computationally predicted to bind N (AS-5, AS-1 and AS-4) or RNA that has been selected to bind N with high affinity using an *in vitro* binding assay (MBE87). RNA-transfected cells were infected with RVF-luc at a MOI of 3. At 8 hours post infection cells were harvested, lysed and protein quantitated by measuring luminescence using a microplate reader.

Table 1

Sequences of RNAs used in this study

AS1-5 represent sequences that were generated *in silico* as potential N-binding aptamers. MBE87 is a known aptamer, and JSR0 is an RNA sequence that is not an aptamer and does not bind appreciably to N.

Name	Sequence
AS1	5′GGUCGACAGGCUAGGCCGUCUAAAGCUGAUUGCUCCCGGGGAAUAAUUAUUGACAGCU3′
AS2	5′GGAGUAUCUGUCGGCAUCACGCUUGGAUGUGCAAGGCUCUUUAUCUCUAUUAUGCAAG3′
AS3	5′GGUACGCUCGCCUCGCCCAAGAAUCUCACGGUCUAGGGUGCCUAUGGAUGAGCUAGGC3′
AS4	5′GGGGCGCGUUCUAAACAACGAUUGCCGACAAGACGAUUUCUGGGUCAAACUUUAUUCG3′
AS5	5′GGUAGCCAUAUUAGCGCAUAACCAUCACAACCGUGGGCUCAUUGGUGGCCACUGCCAU3′
MBE87	5′ GGCAUUACGGCCGGGAGCCAGUAUUACUGAUUGAUGAUUCUGUGGGCGUCUUCUAG 3′
JSR0	5′ GGGGCAUUACGGCCGGGGCCUUUACACCUGGCUCGGACCACCUAAGCGUCUUCUAG 3′