Targeting a Novel RNA-Protein Interaction for Therapeutic Intervention of Hantavirus Disease*

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An evolutionarily conserved sequence at the 5′ terminus of hantaviral genomic RNA plays an important role in viral transcription initiation and packaging of the viral genome into viral nucleocapsids. Interaction of viral nucleocapsid protein (N) with this conserved sequence facilitates mRNA translation by a unique N-mediated translation strategy. Whereas this evolutionarily conserved sequence facilitates virus replication with the assistance of N in eukaryotic hosts having multifaceted antiviral defense, we demonstrate its interaction with N presents a novel target for therapeutic intervention of hantavirus disease. Using a high throughput screening approach, we identified three lead inhibitors that bind and induce structural perturbations in N. The inhibitors interrupt N-RNA interaction and abrogate both viral genomic RNA synthesis and N-mediated translation strategy without affecting the canonical translation machinery of the host cell. The inhibitors are well tolerated by cells and inhibit hantavirus replication with the same potency as ribavarin, a commercially available antiviral. We report the identification of a unique chemical scaffold that disrupts a critical RNA-protein interaction in hantaviruses and holds promise for the development of the first anti-hantaviral therapeutic with broad spectrum antiviral activity.

Hantaviruses are segmented negative strand RNA viruses carried by rodents, shrews, moles, and bats. Inhalation of aerosolized excreta from infected rodent hosts causes hantavirus infection in humans (1–4). Hantavirus infections cause hemorrhagic fever with renal syndrome and hantavirus cardiopulmonary syndrome (HCPS)5 with mortality rates of 15 and 50%, respectively (1). Annually, 150,000–200,000 cases of hantavirus infections are reported worldwide, and there is no treatment for hantavirus diseases at present. Although 17 hantavirus species are known to cause HCPS, the disease is predominantly caused by Sin Nombre virus (SNV) and Andes virus in North and South America, respectively. There is no Food and Drug Administration-approved vaccine or anti-hantaviral therapeutic available for hantavirus infection.

The hantavirus genome is composed of three negative sense RNA segments (S, M, and L) that encode viral nucleocapsid protein (N), glycoprotein precursor, and RdRp, respectively (5). The glycoprotein precursor is post-translationally cleaved at a highly conserved WAASA motif, generating two glycoproteins, Gn and Gc (6). The RdRp synthesizes viral mRNA by a unique cap-snatching mechanism. RdRp cleaves the host mRNA 10–14 nucleotides downstream of the 5′-cap and uses the cleaved capped RNA fragment as primer to initiate transcription (7, 8). The capped RNA primers mostly contain a G residue at the 5′ terminus (7, 8). However, the decapping machinery of the host cell actively removes 5′-caps from host cell transcripts destined for degradation. We previously reported that N binds to the host mRNA 5′-caps and protects them from the attack of cellular decapping machinery (8). The rescued capped RNA fragments are stored in cellular P-bodies by N and are efficiently used as primers by the RdRp. The nascent viral transcripts have to compete with the host cell mRNA for translation by the same translation machinery. We have recently found that hantaviruses have evolved a unique N-mediated translation strategy that facilitates mRNA translation without the requirement of eIF4F complex (9, 10). Our published results suggest that the N-mediated translation strategy probably facilitates the translation of viral transcripts with the assistance of viral mRNA 5′-UTR. Thus, N plays crucial roles in both transcription and translation of viral mRNA. Moreover, N encapsidates the viral genome and generates nucleocapsids that are used as templates by the RdRp for both the mRNA synthesis and replication of the viral genome (11, 12). Due to its multifunctional role in hantavirus replication, N is an important target for therapeutic intervention of hantavirus diseases. A unique interaction between N and a highly conserved sequence at the viral genomic RNA (vRNA) 5′-UTR was identified as a novel target for therapeutic intervention of hantavirus disease.

We developed a tractable fluorescence anisotropy-based high throughput screening assay to monitor the interaction between N and the viral UTR. The assay was used to screen a...
chemical library to identify inhibitors that interrupt N-UTR interaction. Here, we report the identification of several compounds that specifically bind to N and block its interaction with the viral UTR, thereby inhibiting viral RNA synthesis and N-mediated translation strategy, although other functions of N may also be perturbed. The launch of a structure-activity relationship (SAR) campaign resulted in the identification of a novel chemical scaffold that has potential for further modification and development of broad-spectrum antivirals. The most potent lead inhibitor harboring the scaffold structure (K31) showed antiviral activity comparable with ribavirin. This lead inhibitor specifically binds to N and induces structural perturbations. K31 failed to impact the replication of HIV and adenovirus, demonstrating its selectivity for hantaviruses.

Results

Development of a Tractable Fluorescence-based High Throughput Screening Assay to Monitor the N-UTR Interaction—Hantaviruses contain a highly conserved triplet repeat sequence UAGUAGUAG at the 5′-UTR of both vRNA and viral mRNA. We previously reported that interaction of N with the hantanucleotide region GUAGUAG of the triplet repeat sequence facilitates the translation of viral mRNA (9, 10, 13, 14, 16). We also reported that interaction of N with this region in vRNA plays a role in transcription initiation in conjunction with viral RdRp (17). However, other investigators have suggested that interaction of N with the 5′-UTR of vRNA is required for selective encapsidation of vRNA into viral nucleocapsids (18, 19). Based on these observations, it is likely that interruption in the N-UTR interaction will inhibit virus replication by the abrogation of viral transcription, encapsidation of vRNA, and translation of viral mRNA by an N-mediated translation strategy. We developed a fluorescence anisotropy-based in vitro assay to monitor the interaction of purified N with the conserved sequence in the viral UTR. Briefly, a 40-nucleotide-long RNA molecule containing the N binding site was synthesized and labeled at the 5′ terminus with 6-carboxyfluorescein (6-FAM). The RNA molecule at a fixed concentration of 3 nM was incubated with increasing input concentrations of N in the reaction buffer, and the fluorescence polarization anisotropy signal (mP) was recorded, as described under “Experimental Procedures.” The fluorescence data were plotted to generate the binding profile for the estimation of binding affinity. As shown in Fig. 1A, N bound to the RNA with a dissociation constant (K_d) of 21 ± 9 nM. The assay was further developed and validated for high throughput screening of chemical libraries to identify molecules that inhibit N-UTR interaction, as discussed in the plate uniformity assay (see “Experimental Procedures”). The optimized assay was finally used in high throughput mode to screen a total of 106,848 chemical compounds from five compound libraries (see “Experimental Procedures” for details). The percentage inhibition of N-UTR interaction was calculated for each tested compound and plotted as scattergram (Fig. 1B). We identified a total of 394 compounds that inhibited N-UTR interaction >3 SD values above the plate median, resulting in a hit rate of 0.39%. To reconfirm the inhibition of N-UTR interaction by the primary screen actives, a dose-response experiment was performed, and K_{50} (the concentration of the compound at which 50% N-UTR interaction was inhibited) was calculated for each compound. Structure-activity-based cluster analysis was performed, and seven major clusters with the best activities across a range of analogs were identified. Based on the ease of synthetic routes, 10 compounds from four clusters were further tested by a fluorescence anisotropy assay and verified by a secondary radioactive filter binding assay. This analysis revealed that two compounds, K31 and K34, from one of the clusters showed moderate inhibition of N-UTR interaction (Fig. 2). Similar observations were made by a fluorescence polarization anisotropy (FPA) assay (Table 1).

Cellular Toxicity and Inhibition of Viral RNA Synthesis—Both the compounds K31 and K34 were examined for cytotoxicity on four cell lines (HEK293T, HeLa, TMZ-B1, and HUVEC), as described under “Experimental Procedures.” The cytotoxicity of 103772, another compound discussed later, was also similarly examined. As shown in Fig. 3, the CC_{50} (the concentration of the compound at which 50% cell death occurred) for K34 was above 100 μM, suggesting considerable tolerance of this compound by all four cell lines. The cytotoxic curve of K34 could not be completed due to its limited solubility in the medium. The cytotoxicity of K31 was around 90 μM. The compounds were further assayed for antiviral activity. Briefly, HUVECs in 24-well plates were infected with Andes virus at an MOI of ~1.0 along with increasing concentrations of the compound of interest. The compounds were added to cells below the cytotoxicity range to ensure that viral inhibition observed was not due to cytotoxicity. Cells were washed 1 h postinfection and incubated for an additional 24 h with fresh medium con-
Virus replication was monitored by quantitative estimation of viral S-segment RNA by real-time PCR, as described under “Experimental Procedures.” A plot of percentage of viral inhibition versus input concentration of the compound was used for the calculation of VIC50, the concentration of the compound at which 50% virus replication was inhibited. As shown in Figs. 2 and 3, both the compounds K31 and K34 inhibited N-UTR interaction in vitro and also inhibited virus replication in cell culture. However, the impact upon virus replication was much more pronounced for K31, with a VIC50 value of 4.6 μM.

We TABLE 1
Structures and activities of analogs of K31 and K34
The compounds were tested by in vitro FPA and filter binding assays for the inhibition of N-UTR interaction. The compounds were also tested in cell culture for virus inhibition and cytotoxicity, as described under “Experimental Procedures.” The parameters K_{50}, VIC_{50}, and CC_{50} were calculated as described under “Experimental Procedures.” Selective index (SI) = CC_{50}/VIC_{50}. NA, not available due to inactivity of the compound in that assay.

| In vitro assays | FPA | Filter binding inhibition |
|----------------|-----|--------------------------|
| Compound       | K_{50} (μM) | VIC_{50} (μM) | CC_{50} (μM) | SI |
| K31            | 4.0 ± 2 ± 3 | 4.6 ± 0.1 | 86 ± 6 | 18.6 |
| 103772         | 36.9 ± 95.6 | 13.7 ± 0.3 | 36.5 ± 4 | 2.7 |
| 101676         | 63.9 ± 204 | ~30.3 ± 14 | 37.4 ± 11 | 1.2 |
| 107884         | ~30 | 26.3 ± 5.7 | ~50 | >100 | >~2 |
| K34            | 25 ± 92 ± 13 | 34 ± 2.6 | >100 | >2.9 |
| 102014         | 54.7 ± 54.7 ± 9.2 | NA | >100 | NA |
| 104088         | 64.1 ± >100 | NA | >100 | NA |
| 103753         | 111.3 ± >100 | NA | >100 | NA |
| 103980         | ~100 ± 61 ± 4.5 | NA | 47 ± 0.7 | NA |
| 103961         | >500 | NA | >100 | NA |
| 104005         | NA | NA | >100 | NA |
| 100605         | NA | NA | NA | 65 ± 1.7 | NA |
| 103941         | 85.2 ± 112 ± 3 | >40 | 40 ± 4 | NA |
| 102020         | 3.2 ± 6 ± 0.6 | 20 ± 4.6 | 51 ± 1.9 | 2.6 |

FIGURE 2. Filter binding analysis. A filter-binding assay shows the dissociation of N-UTR complex at increasing input concentrations of compounds K31 (A), K34 (B), and 103772 (C). The K_{50} values were calculated as described under “Experimental Procedures.”

Lead Scaffold structure.
next compared the potency of K31 and K34 with ribavarin. The impact of ribavarin on Andes virus replication in HUVECs was quantified by real-time PCR, as described above. We observed that ribavarin inhibited Andes virus replication with a VC50 value of 4.7 ± 0.5 μM, demonstrating that both K31 and ribavarin inhibit Andes virus replication in cell culture with similar potency (data not shown).

Analogs of K31 and K34 Provide Insights into Lead Scaffold Structure—Because K31 and K34 showed activity in both in vitro and in vivo assays, we selected an additional 10 active and 2 inactive analogs of these two compounds from the initial screen for further analysis to obtain insights into SARs (Table 1). Both K31 and K34 and their analogs share a common structural scaffold composed of two benzyl rings A and C attached to a heterocyclic ring B, where different substituents are attached to rings A, B, and C. The analogs were tested for the inhibition of N-UTR interaction in vitro, cytotoxicity on HUVECs, and inhibition of virus replication in cell culture (Table 1). The three analogs 103772, 101676, and 107884 structurally resembling K31 showed inhibition of both N-UTR interaction and virus replication in cell culture, although the potency was relatively weaker compared with the parent K31 compound. Relocation of Br from the -m to the -o position in benzyl A ring (compound 101676) impacted the inhibition of both the N-UTR interaction and virus replication with enhanced cytotoxicity. The dichloro substitutions at the -m and -o positions in the benzyl A ring (compound 103772) slightly impacted the activity with elevated cellular toxicity (Fig. 3). These observations suggest that the location of the halogen atom at the -m position in the benzyl A ring is important for activity. The substitution of the electron-withdrawing group NO2 at the -m position of the benzyl A ring (compound 107884) affected virus replication, although the compound was well tolerated by cells. The remaining analogs structurally resembled K34 and had modifications in the benzyl A ring (Table 1). None of these derivatives except for compounds 102020 and 103941 inhibited virus replication in cell culture. The main difference between K31 and K34 is the location of a COOH group at the second and third position of the benzyl C ring, respectively, suggesting that location of the COOH group at the second position may be more suitable for antiviral activity. Based on selective index, the potency of four lead inhibitors, K31 > K34 > 103772 > 102020, follows a decreasing order and provides key insights into further modifications of the lead scaffold for the development of more potent antivirals. The compounds K31, 103772, and K34 were further evaluated for mode of action.

Mode of Action—To examine the effect on virus replication in previous experiments (Table 1), the compound of interest and
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FIGURE 4. Effect of lead compounds K31, K34, and 103772 upon hantavirus replication. HUVECs seeded in 24-well plates were infected with Andes virus (MOI of 1.0) and treated with either K31 (10 μM), 103772 (10 μM), or K34 (10 μM). The compounds were delivered by either co-incubation or postincubation or the co-/postincubation method, as described under “Methods.” Virus replication was monitored 24 h postinfection by the microscopic visualization of N protein using immunofluorescence staining.

viral inoculum were simultaneously added to HUVECs in culture and incubated for 1 h. Cells were washed and further incubated with fresh medium containing the compound of interest. The compounds tested using this approach (co-/postincubation) would perturb either virus entry or postentry steps of virus replication or both. To dissect the mode of action, the three lead compounds were further examined for virus inhibition using three different approaches. 1) A fixed concentration of the compound and Andes virus inoculum were simultaneously added to cells and incubated for 1 h. Cells were washed and further incubated with fresh medium lacking the inhibitor (co-incubation). The effect exerted on virus replication in this approach would be mostly due to the interference in the entry step of virus replication. 2) The virus inoculum was first incubated with cells in the absence of inhibitor. Cells were washed to remove the unattached virus and were further incubated with fresh medium containing the inhibitor. The effect observed on virus replication using this approach would be mostly due to interference in postentry steps of virus replication. 3) The inhibitors were tested using a co-/postincubation approach as described above. Virus replication in infected cells was monitored 24 h postinfection by the visualization of N protein using immunofluorescence staining (Fig. 4). It is evident that all three compounds had no impact upon virus replication when delivered by the co-incubation approach (Fig. 4), suggesting that these inhibitors may not act at the entry step of virus replication. They probably exert their antiviral effects by perturbing postentry steps of the virus replication cycle. All three compounds inhibited virus replication by either postincubation or co-/postincubation treatments, although the effect was quite noticeable for K31. The trend in virus inhibition (K31 > 103772 > K34) is evident from both microscopy (Fig. 4) and real-time PCR (Table 1) approaches. Among all three compounds, K31 strongly inhibited both N-UTR interaction and virus replication.

Interaction between N and Lead Inhibitors—All three lead inhibitors (K31, K34, and 103772) inhibit N-UTR interaction (Table 1). It is likely that these inhibitors either compete with the RNA for the same binding site on N or bind to a different site and induce a conformational change in N. The resulting conformationally altered N may be deficient in binding to RNA. Alternatively, the inhibitors may bind to RNA and block its interaction with N protein. To test these hypotheses, we used a biolayer interferometry to examine the binding of lead inhibitors with N and RNA, as described under “Experimental Procedures.” The compound 100605, which inhibited neither N-UTR interaction nor virus replication in cell culture, was used as a negative control. It is evident in Fig. 5 (A–D) that, unlike the negative control, all three inhibitors bound to N protein. The kinetic data were fit to a 1:1 binding model for the calculation of on-rate and off-rate constants that were used to estimate the binding affinity (Fig. 5E). All four inhibitors bound to N protein with relatively similar on-rates and mostly differed in the off-rate, which was noticeably slower for the compound K31. Analysis of the kinetic data revealed that binding affinities of all three inhibitors with the N protein were in the order of K31 > 103772 > K34, which was consistent with their respective inhibitory potential of virus replication (VIC50) (Table 1 and Fig. 3). Similar analysis carried out by biolayer interferometry revealed that none of the compounds bound to RNA (Fig. 5G).

We next employed 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid, dipotassium salt (bis-ANS) as a probe to monitor the conformational alterations induced in N protein due to binding with these inhibitors. The fluorescence signal of bis-ANS increases upon binding to hydrophobic pockets of proteins and has been widely used to probe conformational changes induced in proteins due to binding with diverse ligands (16, 21–23). The increase in bis-ANS fluorescence signal observed in Fig. 5F is due to the binding of bis-ANS at the hydrophobic pockets of N protein. It is evident that the fluorescence signal of bis-ANS increases with high magnitude after binding to free N protein as compared with N-inhibitor complex. The decreased fluorescence signal suggests that binding of all three inhibitors reduces the number of available hydrophobic binding pockets for bis-ANS. It is likely that either inhibitor binding induces a conformational change that reduces the hydrophobic pockets in N, or the inhibitor binding sites are themselves hydrophobic in nature. It is equally possible that bis-ANS and the compounds share a common binding site on N. Under such circumstances, bis-ANS similar to the compounds should inhibit N-UTR interaction. The impact of bis-ANS upon the dissociation of N-UTR complex was monitored by a filter binding assay. It is evident from Fig. 5H that bis-ANS, unlike the compounds, failed to inhibit N-UTR interaction, suggesting that bis-ANS and the compounds do not compete for the same binding site on N protein.

Uninfected DMSO
Co-Incubation Post-Incubation Co/Post-Incubation
K-31
103772 K-34
Lead Inhibitors Selectively Inhibit N-mediated Translation Mechanism—The N-UTR interaction is reported to facilitate mRNA translation (10). Our published data support a model that monomeric and trimeric N molecules individually associated with the RPS19 of the 40S ribosomal subunit and viral mRNA 5'-UTR undergo N-N interaction to facilitate the efficient loading of N-associated ribosomes onto the viral mRNA 5'-UTR without the requirement of eIF4F cap binding complex (14) (Fig. 6C). In contrast, the translation initiation by the canonical translation machinery of the host cell requires the recruitment of eIF4F complex at the mRNA 5'-cap (Fig. 6B). To examine the specificity of lead inhibitors for N, we asked

FIGURE 5. The lead inhibitors bind to N and induce conformational changes after binding. The C-terminally His-tagged Andes virus N was immobilized on a Ni-NTA probe. Compounds K31 (A), 103772 (B), K34 (C), and 100605 (D) were passed over the immobilized N surface at the indicated concentrations. The association and dissociation kinetics were monitored, as described under “Experimental Procedures.” E, the data points were fit to a 1:1 binding model for the calculation of association (k_a) and dissociation rate constants (k_d). The binding affinity (K_D = k_d/k_a) was calculated from the kinetic data. F, fluorescence titration of hydrophobic fluorophore (bis-ANS) with N or N-inhibitor complexes. The fluorophore was excited at 399 nm, and emission was recorded at 485 nm. Shown are the titration curves of bis-ANS binding with free N (blue circle), N-K31 complex (green square), N-K34 complex (yellow triangle), or N-103772 complex (red triangle). See “Experimental Procedures” for details. G, the lead inhibitors K31, K34, and 103772 do not bind to RNA. The viral UTR was synthesized by T7 transcription and biotenylated during synthesis as described under “Experimental Procedures.” The biotenylated RNA was immobilized on a streptavidin biosensor. Compounds K31 (black), 103772 (red), and K34 (green) were passed over the immobilized RNA surface at a concentration of 350 μM each. The association and dissociation kinetics was monitored, as described under “Experimental Procedures.” H, bis-ANS does not dissociate N-RNA complex. Preformed N-RNA complex was incubated with increasing concentrations of bis-ANS at room temperature for 30 min. The dissociation of the complex was monitored by a filter binding assay, as described under “Experimental Procedures.”

Table 3. Kinetic parameters for the interaction of Andes virus N protein with the compounds K31, K34 and 103772 using Biolayer interferometry.

| Compound | k_a (M) | k_d (s^-1) | K_D (μM) |
|----------|---------|------------|-----------|
| K31      | (1.7 ± 0.2) x 10^7 | (9.6 ± 0.2) x 10^3 | 5 ± 2     |
| K34      | (9.1 ± 0.4) x 10^7 | (1.9 ± 0.2) x 10^3 | 70 ± 10   |
| 103772   | (9.8 ± 0.2) x 10^7 | (2.1 ± 0.1) x 10^3 | 13 ± 7    |
whether they selectively target N-mediated translation mechanism inside the cell. The viral S-segment mRNA 5'-UTR was fused with the mCherry reporter upstream of the AUG codon (Fig. 6A). The resulting reporter transcript was expressed either alone or along with N in HeLa cells. Without N protein expression, the reporter mRNA is translated by the canonical translation machinery of the cell. However, in N-expressing cells, the mRNA is preferentially translated by N-mediated translation strategy. HeLa cells expressing mCherry reporter were challenged with lead inhibitors, K31, 103772, and K34. An examination by fluorescence microscopy revealed that none of the lead inhibitors affected the canonical translation machinery of the host cell (Fig. 6D, iii–v; see quantified mCherry expression in xi). In contrast, a treatment with 4E1RCat, a well known chemical inhibitor for cap-dependent canonical translation that interferes with the recruitment of eIF4F cap binding complex at the mRNA 5'-cap, dramatically inhibited the translation of mCherry reporter mRNA (Fig. 6D, vi and xi). It is evident from Fig. 6D (vii and xi) that 4E1RCat partially inhibited the translation of mCherry reporter mRNA in HeLa cells expressing N, demonstrating the translation of reporter mRNA by both canonical and N-mediated translation strategies. The resistance imparted by the N protein expression against 4E1RCat-induced translation shutdown confirms the previously reported independence of N-mediated translation strategy on the eIF4F complex. To inhibit the translation of reporter mRNA in N-expressing cells, both canonical and N-mediated translation strategies need to be inhibited. The requirement for

FIGURE 6. The lead inhibitors selectively inhibit N-mediated translation strategy. A, schematic representation of mCherry reporter mRNA having Andes virus S-segment mRNA 5’-UTR upstream of the AUG codon (shown in black). An additional 12 nucleotides representing cap-snatched sequence (green) were fused upstream of the viral UTR sequence. The mRNA was expressed from a polymerase II promoter. B, simple schematic representation of canonical cap-dependent translation initiation. Some initiation factors are not shown for simplicity. The chemical inhibitor 4E1RCat inhibits the formation of eIF4F complex, composed of eIF4E, eIF4G, and eIF4A. C, schematic representation of N-mediated translation strategy. Monomeric and trimeric N molecules (pink) bind to the mRNA 5’-cap and conserved heptanucleotide sequence GUAGUAG of the viral mRNA 5’-UTR. An additional N-molecule binds to the RPS19 of the 40S ribosomal subunit. N-associated ribosomes are selectively loaded onto the viral mRNA 5’-UTR without the assistance of the eIF4F complex. D, HeLa cells seeded in a 12-well plate were transfected with either pTriEx1.1 empty vector (i–vi) or pTriEx-SNVN, expressing SNV N (vii–x). Twelve hours post-transfection, cells were again transfected with pTriEx1.1 empty vector (i) or pTriEx-mCherry plasmid (ii–x), expressing mCherry reporter. Six hours after the second transfection, medium was replaced with fresh medium containing DMSO along with either K31 (10 μM), K34 (15 μM), 103772 (10 μM), 4E1RCat, or 4E1RCat along with K31 (10 μM), K34 (15 μM), or 103772 (10 μM), as indicated, at the bottom of each panel. The mCherry expression in i–x was quantified and plotted in xi. To ensure that changes in mCherry expression in i–x were not due to alterations in mRNA levels, the experiment was repeated, and mCherry mRNA levels were quantified by real-time PCR as shown in xii. E, HeLa cells either stably expressing N or deficient in N expression were seeded in 12-well plates. Cells were transfected with pTriEx-mCherry plasmid. Six hours post-transfection, medium was replaced with fresh medium containing DMSO along with 4E1RCat or K31 or both K31 and 4E1RCat, as shown at the bottom of each panel. Cells were washed with 1× PBS and visualized under a fluorescence microscope 12 h after treatment with chemical inhibitors. F, the mCherry signal in E was quantified and plotted. Error bars, S.E.
the combined treatment of 4E1Rcat along with either K31 or 103772 or K34 to inhibit mCherry reporter in N-expressing cells (Fig. 6D, viii–x and xi) clearly demonstrates that lead inhibitors selectively target the N-mediated translation strategy. To ensure that changes in mCherry expression observed by microscopy (Fig. 6D, i–x) are not due to alterations in mRNA levels, the experiment was repeated, and mCherry mRNA levels were quantified by real-time PCR. It is evident from Fig. 6D (xii) that mCherry mRNA levels were not affected by the treatment with 4E1Rcat and test compounds of interest, further confirming the selectivity of lead inhibitors toward the N-mediated translation strategy.

In Fig. 6D (vii–x), the cotransfection was carried out using mCherry reporter plasmid at a concentration 20-fold higher than N expression plasmid. Under such transfection conditions, most cells probably received the mCherry reporter plasmid, but a limited number of cells probably received both the mCherry and N expression plasmids. Thus, in Fig. 6D (vii), the treatment with 4E1Rcat inhibited mCherry translation in cells lacking N but probably failed to do so with same efficiency in cells expressing N. To further confirm the specificity of lead inhibitors for the N-mediated translation strategy, we generated a stable cell line constitutively expressing N. The cell line was transfected with the reporter plasmid at a concentration 10-fold lower than the concentration used in Fig. 6D to enable the preferential translation of reporter mRNA by the N-mediated translation strategy. It is evident from Fig. 6 (E and F) that a treatment with 4E1Rcat almost completely inhibited mCherry expression in cells lacking N. However, it failed to do so in cells constitutively expressing N, clearly demonstrating the preferential translation of reporter mRNA by the N-mediated translation strategy. Again, the requirement for the combined treatment of both 4E1Rcat and K31 to inhibit mCherry expression in an N-expressing stable cell line further confirms the selectively of K31 for the N-mediated translation strategy. Taken together, the observations form Fig. 6 support the selectivity of lead inhibitors for N and demonstrate that interruption in N-UTR interaction abrogates the N-mediated translation mechanism.

Specificity of K31—To determine whether K31 has broad-spectrum antiviral activity, we examined its impact upon the replication of HIV, adenovirus, and vesicular stomatitis virus (VSV) as described under “Experimental Procedures.” Briefly, HIV, VSV, and adenovirus were allowed to propagate in cell culture along with increasing concentrations of K31. Virus replication was monitored by either microscopy or quantification of viral genomic RNA by real-time PCR analysis. It is evident from Fig. 7 (A and B) that K31 did not affect the replication of HIV and adenovirus even at 50 μM concentration. However, K31 had a moderate impact upon the replication of VSV (Fig. 7C). These observations suggest that K31 may also target other negative strand RNA viruses.

Discussion

In this study, we attempted to identify a small molecule inhibitor for hantavirus nucleocapsid protein. As mentioned in the Introduction, hantavirus nucleocapsid protein plays diverse roles in the establishment of virus infection in the host cell and is thus an important target for therapeutic intervention. Due to their multifunctional nature, nucleocapsid proteins in other viruses, such as HIV-1 (24, 25) and Rift Valley fever virus (26), have been targeted for therapeutic intervention. The small molecule compound Azodicarbonamide that targets HIV-1 nucleocapsid protein was introduced in Phase I/II clinical trials (27, 28). The proposed roles of N-UTR interaction in transcription initiation in conjunction with hantaviral RdRp, encapsidation and packaging of hantavirus genome and selective translation of viral mRNA by an N-mediated translation strategy, lead to the basis for N-UTR interaction as a novel target for therapeutic intervention of hantavirus disease. The specific binding of N to the heptanucleotide region (GUAGUAG) of the viral 5'-UTR was used for the development of a high throughput screening assay to identify molecules that interrupt N-UTR interaction.

The assay was used to screen chemical libraries that lead to the identification of several compound clusters inhibiting N-UTR interaction in vitro. Based on the selective index and competence to inhibit both N-UTR interaction and virus replication in cell culture, we identified two potent compounds, K31 and K34, from one of the clusters that supported the launch of a SAR campaign, which resulted in the identification of a lead scaffold structure (Table 1) having potential for further modification and generation of high potency derivatives. The three lead inhibitors, K31, K34, and 103772, harboring the lead scaffold structure, were further tested for specificity. An examination by biolayer interferometry revealed that all three compounds bind to N protein and not to the RNA. The analysis by
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bis-ANS binding revealed a possible conformational change induced in N due to the binding with all three lead inhibitors. Their relative binding affinities with N were in agreement with their relative potencies to inhibit vRNA synthesis by the RdRp in infected cells. The exact mechanism by which lead inhibitors inhibit vRNA synthesis is not clear from the present study. However, the requirement of N in RdRp function of negative strand RNA viruses has been established in numerous reverse genetic systems.

The specific binding and inhibition of N-UTR interaction was evident from the selective inhibition of the N-mediated translation mechanism in cells (Fig. 6). The mCherry reporter mRNA harboring the viral S-segment mRNA 5′-UTR was co-expressed along with Andes virus N protein in cells. The test mRNA is translated in host cells by both the canonical and N-mediated translation strategies. The shutdown of canonical cap-dependent translation by 4E1RCat completely inhibited the translation of reporter transcript in cells lacking the N protein. However, the N-mediated translation strategy resisted the 4E1RCat-mediated translation inhibition of the reporter mRNA in N-expressing cells, further confirming the previously reported independence of the N-mediated translation strategy on eIF4F complex (9). Inhibition of reporter mRNA translation in N-expressing cells required the interruption of the N-mediated translation strategy by the lead inhibitors K31, K34, and 1037772. This observation clearly demonstrated that reporter mRNA was preferentially translated by the N-mediated translation strategy, which was selectively inhibited by the lead inhibitors K31, K34, and 1037772. Nonetheless, the off target effects of lead inhibitors cannot be ruled out.

K31 is the most active lead inhibitor with the highest selective index, inhibiting hantavirus replication with same efficiency as ribavarin. The comparison of K31 with ribavarin might be slightly complicated because ribavarin needs to be activated by cellular enzymes to yield the antiviral nucleotide that must then be recognized by the viral RdRp. Whereas ribavarin is an inhibitor for RdRp, the K31 targets the N. However, the impact of K31 upon RdRp function is evident from Fig. 4, most likely due to the requirement of N for the RdRp function. Although ribavarin has been reported to reduce disease severity in a Syrian hamster model exposed to Andes virus, it has been shown to cause hemolytic anemia in patients that may worsen the hantavirus-induced cardiac disease and lead to fatal myocardial infarction. Due to significant manifestations of heart and lungs during hantavirus infection, the efficacy of ribavarin is surely questionable for hemorrhagic fever with renal syndrome and HCPs. The efficacy of K31 was tested in cell culture for HIV and adenovirus, Andes virus, and SNV. K31 inhibited SNV replication with similar efficiency as Andes virus. This was expected because hantavirus N is highly conserved, and K31 inhibited N-UTR interaction in vitro for both Andes virus and SNV with similar potency (not shown). K31 did not show any impact upon the replication of adenovirus and HIV but showed moderate inhibition of VSV, suggesting that K31 might have broad spectrum antiviral activity toward other negative strand RNA viruses. These observations hold promise for further modification of the lead scaffold structure to generate high potency derivatives with improved target binding affinity and efficacy. A co-crystal structure of N-K31 complex will provide critical insights for selective modification of the substituent functional groups of K31 to aid the synthesis of potential high efficacy derivatives.

A high throughput screen targeting the nucleocapsid protein–RNA interaction in Rift valley fever virus, another member of the Bunyaviridae family, identified lead inhibitors suramin sodium salt (C51H34N6Na6O23S6), topotecan (C23H23N3O5), and quinacrine dihydrochloride (C23H28Cl2N3O2H2Cl), which significantly inhibited the replication of Rift valley fever virus (26). These inhibitors are structurally different and unrelated to K31 lead inhibitor. Similarly, azodicarbonamide (C2H4N4O2) that targets HIV-1 nucleocapsid protein (27, 28) has a structure completely different from that of K31. To our knowledge, K31 is a unique molecule that has potential for further modification to generate broad-spectrum antivirals.

Experimental Procedures

Expression and Purification of Hantavirus N—Expression of SNV N was carried out from the pSNVN vector, as described previously (16). Briefly, BL21 (DE3) cells transformed with pSNVN vector were induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside upon entering into exponential growth phase (A600 = 0.4). Cells were allowed to grow for another 4 h at 37 °C and were harvested by centrifugation at 3000 rpm for 30 min at room temperature. Cells were resuspended in lysis buffer (20 mM HEPES, pH 8.0, 300 mM NaCl, 2 mM CHAPS, 8 mM urea, 10 mM imidazole, and protease inhibitors (HaltTM protease inhibitor mixture, Thermo Scientific). Cells were lysed using a French press and homogenized by mild sonication. Cleared lysates were applied to Ni-NTA beads that were evenly packed and equilibrated with lysis buffer. The beads were washed three times with lysis buffer containing increasing concentrations of 25, 50, and 100 mM imidazole. The bound protein was finally eluted with lysis buffer containing 250 mM imidazole. Purified protein was refolded by step dialysis in 20 mM HEPES, pH 8.0, 200 mM NaCl, 5% glycerol, and 1 mM DTT with a gradual decrease in the concentration of urea. Vero E6 cells infected with Andes virus strain Chile-9717869 were lysed, and total RNA extracted from cell lysates was reverse transcribed using random primers. Andes virus N gene was PCR-amplified from the resulting cDNA using a forward F primer (5′-GGAAACCCCATGGATGAGCCACCTCCAAGAATTGC) and reverse R primer (5′-GGAAACCTCGAGCATTAAAGTGCTCTTGTT). The PCR product was gel-purified and cloned in pTrEx 1.1 backbone (Novagen) between NcoI and HindIII restriction sites. The expression and purification of Andes virus N was carried out similarly to SNV N, as described above.

FPA Assay Development—A 40-nucleotide-long UTR fragment harboring the N binding site at the 5′ terminus (5′-6-FAM/GUAUGAUAUGCUUCUUGAAAGCAAUCA/GAAGUUACCUU/ACGUU) was commercially synthesized and labeled with 6-FAM at the 5′-end. The FPA assay was developed using a 384-well plate to study the binding of purified N with the 5′-FAM-labeled RNA. The binding reactions containing labeled RNA (3 nM) and N protein (100 nM) in 40 μl of assay buffer (40 mM HEPES, pH 7.4, 80 mM NaCl, 20 mM KCl, and 1 mM DTT and RNAsin) were incubated for 90 min at 25 °C. The
fluorescence polarization anisotropy value for each sample in the 384-well plate was quantified using a PerkinElmer Life Sciences Envision plate reader. The plate reader was calibrated for G values that generated a value of 27 mP for 1 nM fluorescein. The mP values were calculated using the equation, 

$$mP = 1000 \times \frac{(S - P \times G)}{(S + P \times G)}$$

where S represents fluorescence signal parallel to the excitation plane (S-channel), P is fluorescence signal perpendicular to the excitation plane (P-channel), and G is the instrument G-factor. The assay was optimized for the time of incubation and DMSO sensitivity.

**Plate Uniformity Assay**—Plate uniformity assays were performed to study the signal variability between plates and between days the assays were set up. Two 384-well plates were set up independently twice for 3 days. In both of the plates, 128 wells out of 384 wells each contained binding reactions that would generate maximum signal, minimum signal, and signal in the mid-range. Fluorescence polarization measurements were recorded after the incubation of RNA and N for 90 min. The mean of maximum, minimum, and mid-range signals; S.D. value; and coefficient of variation were calculated for each plate. Both the signal windows and Z’ factor were computed for each plate over a period of 3 days.

**Compound Libraries**—The following five compound collections were used in the optimized FPA assay to identify the compounds that inhibit the interaction between N and RNA: 1) MicroSource Spectrum (2,320 compounds containing Food and Drug Administration-approved drugs, bioactive, natural products); 2) Prestwick Chemical Library (1200 compounds, Prestwick Chemical, Washington, D. C.); 3) University of Kansas Center of Excellence in Chemical Methodologies and Library Development (KU-CMLD; 3360 compounds with novel diverse scaffolds); 4) ChemBridge Library (43,736 drug-like diverse chemical structures); and 5) ChemDiv Library (56,232 compounds, diversity set from ChemDiv Corp.).

**High Throughput Screening**—The optimized FPA assay was used to screen a collection of 106,848 compounds. The screening was performed in 384-well format. The first two columns of the assay plates did not contain any compounds and were used for in–plate controls (no protein controls, no RNA controls, protein plus RNA in the presence of 0.4% DMSO). The compounds from the library plates were transferred to the assay plates acoustically by ECHO 550, followed by the addition of reaction mixtures containing 6-FAM-labeled 40-nucleotide-long RNA (3 nM) and N (120 nM). The reaction contents were mixed for 2 min at room temperature. The library was screened at a final concentration of 10 μM containing DMSO at a final concentration of 0.4%. The controls in each assay plate were used to determine whether a plate passed or failed in the assay and were also used to determine plate statistics.

**Data Analysis**—The actives from the primary screen were cherry-picked from mother plates and retested for the inhibition of N-RNA interaction using the FPA assay in an eight-concentration dose-response mode (2-fold dilution starting at 30 mM). The IC50 (50% inhibition) data from concentration response curves was used for preliminary scaffold analysis and clustering using the Tripos Selector program via the Jarvis Patrick routine, using default parameters. From each preliminary cluster, the largest conserved substructure present in at least half of the cluster members was identified.

**Cheminformatics Cluster Analysis**—A cluster analysis was performed in which 772 compounds were grouped into common scaffold structures using SYBYL X 2.0 software. Clusters containing multiple compounds with a range of inhibitory activities versus hantavirus nucleocapsid protein were prioritized. A PubChem promiscuity analysis was performed for the cherry-picked clusters to triage any scaffolds that may possess off-target liabilities. The remaining scaffolds were analyzed via a pan-assay interference compound filter (29) to identify substructural features known to be frequent hitters in biochemical high throughput screens. These scaffolds were not triaged but were noted as possessing these potential liabilities. The scaffolds were then rank-ordered by ligand efficiency, a metric calculating the amount of biological activity contributed per heavy atom (30). Taking into account these analyses and potential synthetic routes for future structure-activity relationship optimization, clusters 1, 2, 3, and 5 were selected for follow-up testing to confirm inhibitory activity.

**Filter Binding Secondary Assays to Confirm the Positive Hits**—The RNA molecule harboring the hantaneucleotide sequence of viral mRNA 5’-UTR was synthesized by an *in vitro* T7 transcription reaction. The RNA was body-labeled with [α-32P]GTP during synthesis. The RNA was purified using TRIzol reagent (Ambion) followed by column purification using the miRNAeasy kit (Invitrogen). A fixed concentration of the RNA (20,000 cpm) was incubated with the fixed concentration of N (500 nM) for 30 min at room temperature in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% DMSO to allow the formation of N-RNA complex. The resulting complex was incubated with increasing concentrations of the test compound of interest at room temperature for an additional 30 min. DMSO was incorporated into the binding buffer to ensure the solubility of the test compound. The mixture was filtered through a nitrocellulose filter, and the amount of RNA retained on the filter was quantified by the scintillation counter, as reported previously (9, 10). The amount of RNA retained on the filter at each input concentration of the test compound was normalized relative to the control lacking the test compound. The percentage of intact N-RNA complex at each input concentration of the test compound was plotted versus the log (test compound) concentration, and the data points were fit to a dose-response equation using nonlinear least-squares analysis (GraphPad Prism software). The IC50 value represented the concentration of the compound at which 50% of N-RNA complex was dissociated. Each experiment was repeated twice for the calculation of S.E., represented as error bars (31). The impact of bis-ANS upon N-RNA complex was studied similarly, except the test compounds were replaced by bis-ANS.

**Cytotoxicity**—The viability of HUVEC, HEK293T, TMZ-bI, and HeLa cells was determined using CellTiter-Glo® luminescent assay reagent according to the manufacturer’s instructions (Promega). Briefly, 10,000 cells were seeded in each well of a 96-well plate and incubated for 48 h in 100 μl of medium containing increasing concentrations of the compound of interest. Control wells containing medium with increasing concentrations of the compound without cells were also prepared. The
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plate was equilibrated at room temperature for 30 min, followed by the addition of 100 μl of the Cell titer-Glo reagent. The Cell titer-Glo reagent was prepared following the manufacturer’s instructions. The plate was incubated for 2 min on an orbital shaker at room temperature to induce cell lysis. The plate was incubated for an additional 10 min at room temperature to stabilize the luminescent signal. The luminescence was recorded on a plate reader. The luminescence signal for each sample was subtracted from the corresponding negative control. Because the small molecule inhibitors were dissolved in 1% DMSO, the cell viability in each well was normalized relative to viability observed at 1% DMSO without the compound of interest.

Andes Virus Propagation—All hantavirus experiments were carried out in a bio-safety level-3 laboratory following appropriate laboratory guidelines. Andes virus (strain Chile-9717869) was propagated in Vero E6 cells by infecting confluent monolayers of cells with Andes virus at an MOI of 0.01. Virus-infected cells were cultured for 13 days in viral growth medium (DMEM/high glucose from Thermo Scientific, catalog no. SH30243.01, containing 2.5% fetal bovine serum). The viral growth medium containing budded virus particles was harvested, cleared by low speed centrifugation, and stored in 1-ml aliquots at −80 °C in DMEM containing 10% FBS. Andes virus was quantified as described previously (32).

Andes Virus Replication Assay—Impact of the compound of interest upon Andes virus replication was assayed in HUVECs. Briefly, HUVECs seeded in 24-well plates were infected with Andes virus at an MOI of ~1.0 for 1 h with brief shaking every 15 min. The virus inoculum was removed and replaced with fresh HUVEC growth medium (Lonza EBM, CC-3121). To examine the effect of test compounds upon the virus replication, the compound was delivered to virus-infected cells in a dose-dependent manner. Because the test compounds were dissolved and stocked in 100% DMSO, the final concentration of DMSO in HUVEC cell growth medium was 0.1% after the addition of test compound of interest. Thus, the growth medium of virus-infected HUVECs lacking the test compound was also supplemented with 0.1% DMSO as a control. Cells were harvested 24 h postinfection, and total RNA was extracted by TRI reagent (Ambion), following the manufacturer’s protocol. Virus replication was monitored by quantitative estimation of viral S-segment RNA by real-time PCR using the relative quantification method, as reported previously (33). The primers used for the quantification of Andes virus S-segment RNA were 5′-CAGCTCGTGACTGCTCGGC (forward) and 5′-GTAGACACAGCTGCCGACTC (reverse). The primers used for the quantification of U6RNA as internal control were 5′-TCGTCGCTCTGAGCTCTCATAC (forward) and 5′-CAATCG-ATTGCGGTGTCATCTCTTG (reverse). All experiments were repeated twice for the calculation of S.E., represented as error bars (31). Similarly, the quantification of SNV S-segment RNA was carried out as reported previously (33) to examine the effect of test compounds upon SNV replication.

Adenovirus Replication Assay—The effect of the compound of interest upon the replication of adenovirus was monitored in HEK293T cells, maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), as described above. Briefly, HEK293T cells were infected with adenovirus (MOI of ~1.0) expressing GFP reporter. The reporter adenovirus a gift from Dr. Jianming Qiu (University of Kansas Medical Center, Kansas City, KS). Cells were incubated for 1 h with the viral inoculums containing the compound of interest. One hour postinfection, cells were replenished with fresh medium containing the compound of interest at a concentration of either 0.5, 5, 20, or 50 μM. Cells were examined for adenovirus-mediated GFP expression 48 h postinfection using fluorescence microscopy.

HIV Replication Assay—The effect of test compound upon HIV-1 replication was monitored on TZM-bl cells. TZM-bl indicator cells enable quantitative analysis of HIV replication using β-galactosidase reporter (20). Briefly, TZM-bl cells were seeded in 12-well plates and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), as described above. Cells were infected with HIV-1 at an MOI of ~2.0. Both HIV-1 and TZM-bl cell line were obtained from Dr. Edward Stephens (University of Kansas Medical Center). The viral inoculums containing the test compound of interest at increasing concentrations were incubated with cells for 3 h with periodic shaking. Three hours postinfection, cells were replenished with fresh medium containing the test compound of interest. Cells were washed twice with PBS 48 h postinfection and incubated for 5 min at room temperature with PBS containing 0.25% glutaraldehyde and 0.8% formaldehyde. Cells were washed three times with PBS and further incubated with staining solution (400 μg/ml X-gal, 4 mM MgCl2, 4 mM K3Fe(CN)6, 4 mM K4Fe(CN)6-3H2O) for 2 h at 37 °C. Cells were washed twice with PBS and visualized under a microscope. The blue-stained cells indicated HIV infection.

VSV Replication Assay—The effect of K31 upon the replication of VSV was monitored in HeLa cells, maintained in DMEM (Invitrogen). Briefly, HeLa cells grown in 6-well plates were infected with VSV (MOI ~1.0). The VSV was a gift from Dr. Edward Stephens (University of Kansas Medical Center). The viral inoculums containing K31 at a concentration of either 10, 20, or 50 μM were incubated with HeLa cells for 3 h with periodic shaking. Cells were replenished with fresh medium containing K31 at the same concentrations. Cells were lysed 48 h postinfection, and total RNA was purified. VSV replication was examined by the quantification of viral genomic RNA using real-time PCR analysis, as reported previously (15).

Immunofluorescence Staining—Immunofluorescence staining was carried out to detect Andes virus N in infected cells. Briefly, HUVECs were infected with Andes virus at an MOI of ~1.0. The virus was allowed to replicate in the presence or absence of the test compound of interest or DMSO as a negative control. Infected cells were fixed 24 h postinfection for 10 min using an acetone/methanol mixture at a ratio of 1:1. Cells were washed three times with PBS, permeabilized with 0.5% Triton X-100 in PBS, and blocked with 5% BSA for 2 h. Cells were incubated with rat anti-N antibody (1:1000) in 5% BSA for 2 h at room temperature. Cells were washed three times with PBS and incubated for 1 h with FITC-conjugated goat anti-rat IgG (catalog no. 112-096-071, Jackson ImmunoResearch Laboratories). Cells were again washed and visualized under a fluorescence microscope.
Bis-ANS Binding—Fluorescence studies of the hydrophobic fluorophore bis-ANS (Sigma, catalog no. 65664-81-5) were carried out in a Shimadzu spectrofluorometer (RF-5301PC). The fluorophore was dissolved in DMSO to a final concentration of 7.4 mM. The fluorophore was excited at 399 nm, and the emission spectrum was recorded from 420 to 600 nm. To a fixed concentration of N (800 nM) in reaction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% DMSO), small aliquots of bis-ANS were added from a higher concentration stock, and the fluorescence intensity at 485 nm was recorded at each input concentration of bis-ANS. Similarly, N was first incubated with the test compound of interest at room temperature for 45 min, followed by the addition of small aliquots of bis-ANS from higher concentration stock, and the fluorescence value at 485 nm was recorded. To determine the change in fluorescence signal of bis-ANS due to binding with N, the fluorescence signal of free bis-ANS in reaction buffer without N was subtracted.

Bi-layer Interferometry—Bi-layer interferometry was carried out in a FortéBio BLItz® instrument to monitor the interaction of N with the test compound of interest. His-tagged N was immobilized on a Ni-NTA probe (FortéBio, 18-5102), followed by the incubation of the probe in the solution of the compound of interest. All reactions were carried out in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% DMSO at room temperature. All reaction cycles were composed of 30 s of initial baseline, 600 s of N immobilization onto the Ni-NTA probe, 30 s of baseline, and 300 s of association of the compound of interest with the immobilized N at the required concentration, followed by 450 s of dissociation phase. Each reaction cycle was carried out at three different concentrations of the compound of interest. The association and dissociation phases of each reaction cycle were analyzed by fitting the data into a 1:1 binding model, using BLItz® software. Each reaction was repeated twice for the calculation of S.E.

To examine the binding of the test compounds with the viral UTR, the UTR sequence was synthesized in vitro by T7 RNA polymerase. The RNA was biotinylated during synthesis by the addition of biotinylated CTP (catalog no. 04739205001, Roche polymerase. The RNA was biotenylated during synthesis by the UTR, the UTR sequence was synthesized

Each reaction was repeated twice for the calculation of S.E.

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N immobilization onto the Ni-NTA probe, 30 s of baseline, and
300 s of association of the compound of interest with the immo-
obilized N at the required concentration, followed by 450 s of dis-
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and dissociation phases of each reaction cycle were analyzed by
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Each reaction was repeated twice for the calculation of S.E.

To examine the binding of the test compounds with the viral UTR, the UTR sequence was synthesized in vitro by T7 RNA polymerase. The RNA was biotinylated during synthesis by the addition of biotinylated CTP (catalog no. 04739205001, Roche Applied Science) to the reaction mixture. The biotinylated RNA was purified using the miRNA Easy kit (Invitrogen). The RNA was immobilized on a high precision streptavidin biosensor (FortéBio), followed by the incubation of the sensor in the solution of the compound of interest at a concentration of either 350 or 700 μM. The binding reactions for each compound were carried out in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5% DMSO at room temperature, following the reaction cycle described above.

Author Contributions—N. N. S. prepared Figs. 2, 3 (J–O), 5 (A–F), and 7 (A and B). N. N. S. also prepared Table 1 and S. G. prepared Figs. 6 and 7. A. R. prepared Fig. 1. The FPA data in Table 1 were provided by A. R. S. J. prepared Figs. 4, 5 (G and H), and 6 (D (xi–xii) and F). N. N. S. wrote the first draft of the manuscript. M. A. M. supervised the research, prepared Fig. 3 (A–I), and wrote the manuscript. All authors read and approved the manuscript for publication.

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