Supplementary Information

Novel small-molecule binds to the influenza A virus RNA promoter and inhibits viral replication

Supplementary Materials and Methods

RNA preparation

RNA was synthesized by *in vitro* transcription using T7 polymerase from DNA templates which included the T7 polymerase promoter (Integrated DNA Technologies, Inc). The transcribed RNA was purified by standard denaturing PAGE and dialyzed against NMR buffer (10 mM potassium phosphate pH 6.1, 50 mM NaCl, and 0.1 mM EDTA). The uniformly [^{13}C,^{15}N]-labeled RNA and partially deuterated RNA (H6/H8, H1', H2', but D3', D4', D5'/D5", and D5) were prepared by using [^{13}C,^{15}N]-labeled ribonucleotide triphosphates (rNTPs, Cambridge Isotope Laboratories, Inc.) and partially deuterated rNTPs. All RNA samples for NMR were analyzed at a final concentration of 1-1.5 mM. The partial alignment of RNA-ligand complex for RDC data was performed by addition of the filamentous bacteriophage Pf1 (ASLA biotech) to [^{13}C,^{15}N]-labeled RNA at room temperature.

Small molecule screening

RNA samples for small molecule screening were prepared and dialyzed against 10mM potassium phosphate buffer at pH 6.1 including 50mM NaCl and 0.1mM EDTA. RNA samples at concentrations of 50μM were used for screening a fragment library from the SBMRI. The scaffold library is composed of 3677 compounds that were selected based on their anticipated use as building blocks or scaffolds components of further optimized molecules. The scaffold library was acquired from three different commercial sources and the chemical structures of the library are deposited into PubChem (AID_1637, http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1637&loc=ea_ras#a Protocol). In line with the general NIH Molecular Libraries Screening Centers Network (MLSCN) library, the library also included a collection of 602 Natural Products (MicroSource, http://www.msdiscovery.com/natprod.html) that could be screened by NMR. Ligand binding was monitored by comparing the imino region of 1D ^{1}H NMR spectra of a 40 μM Influenza A promoter RNA solution (100 mM sodium phosphate buffer at pH 6.25 containing 90%/10% $H_2O/^2H_2O$; T = 287 K) in the presence and absence of compounds tested at a final concentration of 100 μM. The fragments are dissolved at 100 mM in DMSO-d6, used as stock solutions. Small volumes of these stocks (or a diluted 10 mM stock depending on the final concentration) are dissolved in the assay buffer. Compounds were initially tested in mixtures of 20, then individual compounds for those mixtures that caused significant perturbations in the spectrum were subsequently tested. Initially, seven small molecules that bind to the RNA were identified. The hit rate is relatively low, probably due to the nature of the library, which was not designed to target RNAs. The binding affinity (K_d) of the strongest binding molecule, shortened as DPQ, was determined by 1D NMR experiments by a titration with 200μM RNA sample. Changes in peak intensity were analyzed by VnmrJ and SigmaPlot programs and the K_d was determined by single site saturation mode.

NMR spectroscopy

Conventional NMR experiments used for spectral assignments of both the free and DPQ-bound RNAs were performed on Avance 600 MHz and 500 MHz spectrometers (Bruker) equipped with TXI-HCN cryogenic probes. Exchangeable 2D NOESY spectra were recorded in 90% H_2O/10% D_2O including 10 mM potassium phosphate buffer (pH 6.1), 50 mM NaCl, and 0.1 mM EDTA. The non-exchangeable 2D NOESY, 2D TOCSY, and 2D ^{1}H,^{13}C-HSQC spectra were recorded in 100% D_2O buffer at 298 K. Both exchangeable and non-exchangeable 2D NOESY spectra were collected at various mixing times (150–400 ms) to facilitate spectral assignments by comparison of cross-peak intensities. Intermolecular NOEs for the complex were identified by comparison of 2D NOESYs recorded from the ligand, free RNA, and RNA-ligand complex; they were straightforward to identify because the DPQ resonances are observed upfield of the RNA peaks. The NMR data were processed and analyzed using NMRPipe [1] and Sparky [2]. In order to measure residual dipolar couplings (RDCs), the in-phase-antiphase (IPAP) HSQC spectra of [^{13}C, ^{15}N]-labeled RNAs were collected in the unaligned sample and the partially aligned sample using Pf1 phage. The RNA-ligand complex sample was partially aligned in the presence of 25 mg/mL of phage.

Structure calculations

Structures of the influenza A virus promoter-DPQ complex were calculated with Xplor-NIH [3] using the experimental restraints summarized in Table 1. Distance restraints were classified as strong (1.8-2.9 Å), medium (2.5-4.5 Å), weak (3.5-5.0 Å), and very weak (4.0-7.0 Å) based on cross-peak intensities. Dihedral angle restraints were experimentally established using standard methods. [4] Structures were originally calculated without RDCs; then these restraints were applied with a harmonic potential well to refine the structure. The powder pattern-like distribution of RDCs was used to calculate starting values for the orientation parameters that were then optimized with PALES.[5,6] The amplitude of alignment tensor (D_a) and rhombicity (R) values are -9.37 Hz and 0.43, respectively. The experimental RDC values were well fit to the back-calculated RDC values with correlation factor (R^2) of 0.959. The final 20 converged structures of the complex were selected from the population of structures with no NOE restraint violations and with the lowest total energy. Analysis of RNA helical parameters such as bending angle and groove width in the final 20 structures was performed with CURVES 5.2 and CURVES+. [7]

Cytopathic effect (CPE) inhibition assay

Standard antiviral compounds were commercially available. Oseltamivir carboxylate (OSV-C; the active form of oseltamivir phosphate) was purchased from US Biological (Swampscott, MA), and ribavirin (RBV) and amantadine (AMT) from Sigma-Aldrich (St. Louis, MO). MDCK cells confluently grown in 96-well plates were infected with approximately 50 plaque-forming units (PFU) of influenza viruses suspended in 100 µl of serum-free MEM for 1 h. Unabsorbed virus was removed and serial 3-fold dilutions of each compound in MEM containing 2 µg/ml TPCK-trypsin were added to the cell cultures. After 3 days of incubation, cell viability was measured using the fluoresceine diacetate (FDA) method. Briefly, culture supernatants were removed and cells were incubated with 100 µl of a 30 µg/ml FDA solution (Sigma-Aldrich) for 20 min at 37°C. Fluorescence intensity was read at 485/538 nm (excitation/emission). The 50% cytotoxic concentration (CC_{50}; the concentration of compound required to reduce

cell viability by 50%) and the EC_{50} (the concentration of compound required to reduce the level of virus-induced CPE by 50%) values were analyzed using Softmax Pro 5.3 software (Molecular Devices, Sunnyvale, CA).

Cells and viruses

Mardin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA) were grown in minimum essential medium (MEM; Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco-Invitrogen). Influenza viruses A/Taiwan/1/86 (H1N1) (TW) and B/Panama/45/90 (PNM) were obtained from the Korea National Institute of Health, South Korea, and A/Hong Kong/8/68 (H3N2) (HK) was from ATCC. They were propagated by infection of MDCK cells in the presence of 2 μg/ml TPCK-trypsin (Sigma-Aldrich) at 33°C (for TW and PNM) or 35°C (for HK). Viral stocks were stored at -70°C and titrated by plaque assay before use.

Plaque reduction assay

Confluent cultures of MDCK cells in 48-well plates were infected with influenza A virus strains, A/Taiwan/1/86 (TW) and A/Hong Kong/8/68 (HK), for 1hr at 33°C (TW) or 35°C (HK). Cells were washed with PBS and overlaid with serum-free overlay medium [MEM containing 0.5% carboxymethyl cellulose (Sigma-Aldrich) and 2μg/ml TPCK-trypsin] in the presence or absence of increasing amounts of the test compounds. After 3 days, cells were stained with 1% crystal violet solution to visualize viral plaques.

WSN-Ren luciferase assay

We used an engineered MDCK cell line expressing influenza hemagglutinin (MDCK-HA) and a modified WSN virus, which will be referred to as WSN-Ren luciferase virus.[8] The hemagglutinin coding region is replaced by the *Renilla* luciferase sequence in the WSN-Ren luciferase virus, which expresses luciferase in the same way as other viral proteins. Thus, the amount of luciferase expression is indicative of viral replication levels. In a 96-well plate, 25,000 MDCK-HA cells were seeded in each well with addition of DPQ, other tested compounds or DMSO. Then each well was infected with WSN-Ren luciferase virus at MOI ~0.69 and incubated at 37ºC for 24 hour. EnduRen™ live cell substrate (Promega, catalog number: E6482) was added at least 4 hours before measuring luminescence. The luminescence was measured in a Perkin Elmer 2030 multilabel reader and the half maximal inhibitory concentrations (IC_{50} values) were calculated using Prism.

References

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Table S1. Structural statistics for the NMR structure of the influenza A virus promoter–DPQ complex

NOE-derived distance restraints	480
RNA (intramolecular)	412
DPQ-RNA (intermolecular)	26
Hydrogen-bonding restraints	42
Dihedral restraints	242
Dipolar coupling restraints	19
Rmsd (all heavy atoms) [Å]	2.09±0.7
Average rmsd values from experimental restraints	
Distance [Å]	0.055
Angles [°]	0.33

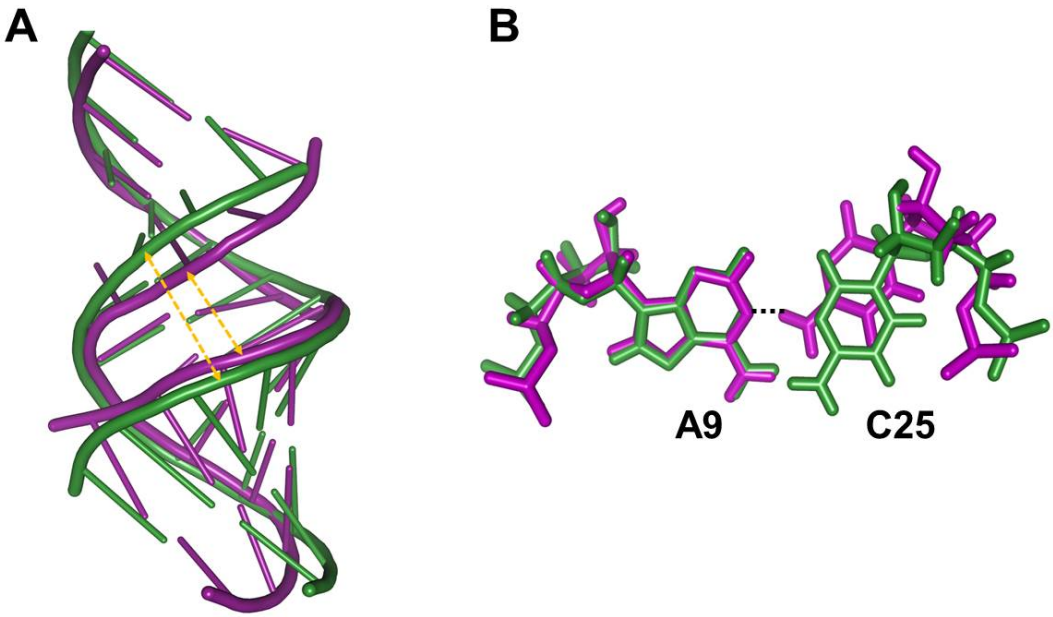

Fig. S1 Comparison of free (purple) and DPQ-bound (green) RNA structures. (**A**) Superposition of backbone structures of free and bound RNA. Dotted lines indicate major groove widths. (**B**) A9-C25 base pair in the free RNA and in the complex. The C25 residue in the bound RNA is displaced compared to the free RNA.

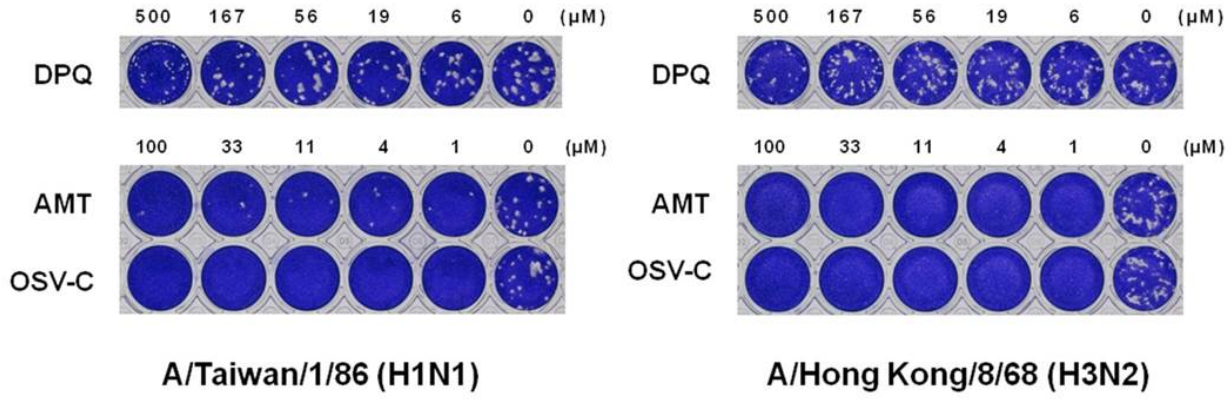

Fig. S2 Plaque reduction assay. MDCK cells were infected with the influenza virus and the effect of DPQ, amantadine (AMT), and oseltamivir carboxylate (OSV-C) on viral plaque formation of A/Taiwan/1/86 (H1N1) and A/Hong Kong/8/68 (H3N2) strains was measured.