Supporting Information

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SI Materials and Methods

Virus Binding Assay and HA/Neuraminidase Specificity Assays. ELISA to measure the direct binding activity of influenza A/WSN/33 virus was performed using a modified literature procedure (1). Briefly, microtiter plates (Corning Polystyrene Universal-BIND Microplate) were incubated with 50 μL of varying dilutions of the multivalent inhibitor in PBS at 4 °C overnight and irradiated with 254-nm UV light for 5 min. The solution was then aspirated, and the plates were washed thrice with 2% (wt/vol) BSA (Sigma) and 0.05% (vol/vol) Tween 20 in PBS (PBST), followed by a further 3-h blocking step with 0.3 mL of PBST at room temperature. The plates were then washed thrice each with PBST and 2% (wt/vol) BSA in PBS (PBS-BSA), followed by incubation with a solution containing influenza virus in PBS-BSA at 4 °C overnight. Polyclonal antibodies to the virus diluted in PBS-BSA were subsequently added to the plates and incubated for 5 h at 4 °C. The plates were then washed with PBS-BSA thrice and incubated with HRP-conjugated secondary antibodies in PBS-BSA for 2 h at 4 °C. The plates were washed as above with PBS-BSA before addition of substrate. Colorimetric development of 50 μL of 1-Step Ultra TMB (Thermo Scientific) at room temperature was stopped with 50 μL of 0.2 M H_2SO_4 after incubation for 30 min, and the absorbance was determined at 450 nm.

For the HA/neuraminidase (NA) binding specificity assays, the microtiter plates were covalently conjugated with 50 μL of 10 μg/mL polymeric inhibitor and blocked as above. For the HA specificity assay, His-tagged A/WSN/33 (WSN) (H1N1) HA protein (eEnzyme), primary (mouse anti-His tag IgG; Abcam) and secondary (HRP-conjugated goat anti-mouse IgG; Biolegend) antibodies were mixed in the ratio 4:2:1 and incubated on ice for 20 min. Similarly, His-tagged A/Cal/04/2009 (H1N1) NA (Sino Biological), primary and secondary antibodies were mixed in the ratio 4:2:1, and incubated on ice for 20 min in the NA specificity assay. The mixtures of precomplexed HA or NA were then diluted to varying concentrations with PBS-BSA, and 50 μL was added to each well and incubated for 2 h at room temperature. The wells were washed four times with PBST, and HRP activity was measured as in the whole-virus binding assay above. The experiment was repeated with varying dilutions of the multivalent inhibitor conjugated to the plate, with concentrations of the precomplexed proteins constant at 5 μg/mL. NA inhibition assay was performed and analyzed as described previously (2).

Virus Release Assay. Madin-Darby canine kidney (MDCK) cells were incubated with the WSN virus on ice for 60 min to allow binding, and the cells were washed thrice with PBS to remove unbound virus. The cells were then moved to 37 °C to begin the infection process. After 3 h postinfection (hpi), the infection media was replaced with that containing either poly-L-glutamine zanamivir (PGN-ZA), ZA-linker, or PBS. After 4 h, the supernatant was harvested, and the viral titer quantified by virus plaque assay.

Flow Cytometry. To quantify single-cycle infection by flow cytometry, MDCK cells were infected with WSN virus at a multiplicity of infection (MOI) of 20 for 1 h on ice, followed by washing three times with ice-cold PBS to remove unbound virus. Infection medium was then added and the temperature raised to 37 °C to allow infection to begin. The inhibitors were added at −1, 0, or 1 hpi. To remove them, the cells were washed four times with prewarmed PBS. Mock-infected and WSN-infected/untreated (PBS) samples acted as controls. At 3 hpi, the MDCK cells were trypsinized, washed with PBS twice, and fixed with 2% (wt/vol) paraformaldehyde in PBS. The fixed cells were washed with PBS containing 2% (vol/vol) FBS (PBS-FBS) twice and resuspended in 0.1% (wt/vol) saponin in PBS-FBS (permeabilization buffer). After 10 min at room temperature, the samples were centrifuged and resuspended in 80 μL of the permeabilization buffer containing 1 μg/mL anti-NP (AbD Serotec) and anti-M1 (Abcam) monoclonal antibodies. Following a 1-h incubation in the dark at room temperature, unbound antibodies were removed by two washes with 1 mL of the permeabilization buffer. The cells were then incubated with 50 μL of phycoerythrin-linked anti-mouse IgG antibody (Biolegend) for 30 min at room temperature. Unbound antibodies were again removed by two washes of 1 mL of the permeabilization buffer. Finally, the cell pellets were resuspended in PBS-FBS and analyzed on the Accuri C6 flow cytometer. The analytical gatings between infected and uninfected cells were determined from the PE fluorescence intensity histograms of the mock-infected negative controls. The extent of influenza infection was quantified as the fraction of cells with fluorescence intensity above the analytical gating. All samples were normalized to the mean of three infected, untreated (PBS) controls.

For the flow cytometry-based binding and internalization studies, MDCK cells were trypsinized, resuspended in DMEM, and exposed to WSN virus at MOI of 20 on ice for 1 h. For internalization studies, the cells were then moved to 37 °C for 30 min to allow endocytosis of the bound virions. To differentiate between internalized and surface-bound virions, bacterial sialidase was introduced to remove surface-bound virions. The cells were washed twice with DMEM to remove unbound viruses and inhibitor and treated with *Arthrobacter ureafaciens* (20 mU/100 μL) and *Vibrio cholera* (25 mU/100 μL) neuraminidase for 1 h at 37 °C. For subsequent flow cytometry analysis, the cells were fixed, processed, and analyzed as described above.

Virus plaque assays were performed using a modified literature procedure (2–4). Briefly, for the early-stage inhibition samples (−1 to 1 h), equal volumes of viruses and inhibitors of various concentrations were preincubated for 1 h before cell inoculation. Thereafter the inoculum was aspirated, and the cells were washed four times with prewarmed PBS to remove any residual inhibitor or viruses. The cells were then overlaid with agar solution with no inhibitor. In the case of the late-stage inhibition samples (1–72 h), there was no pretreatment, and the initial 1-h infection was also done in the absence of inhibitors. After infection, the cells were overlaid with agar solution containing the appropriate concentrations of inhibitor. For the combination samples (−1 to 72 h), the inhibitor was present throughout the assay, from pretreatment through the agar overlay.

Transmission Electron Microscopy. The WSN virus was sonicated, and remaining viral aggregates were removed using a 0.2-μm-pore filter. The virus was then incubated at room temperature for 1 h with either PBS or PGN-ZA at a concentration exceeding 10 × IC_{50} (1.8 μM of ZA). The surface of a carbon/formvar film supported on a Cu grid was treated with a drop of the influenza virus solution for 1 min. The surface was washed by successively dipping the grid in three drops of water and stained with either 0.75% (wt/vol) uranyl acetate or 1% (wt/vol) phosphotungstic acid. A drop of the stain was placed on the surface of the grid for 45 s and then removed by absorption onto a piece of filter paper. The samples were allowed to dry overnight and analyzed using a Tecnai G^2 Spirit Biotwin transmission electron microscopy (TEM) instrument.

Fluorescence Microscopy. The labeling process was modified from a previously published protocol (5). The WSN virus was labeled with Alexa Fluor 647 carboxylic acid succinimidyl ester dye (Invitrogen) in a carbonate buffer (pH 9.3) at room temperature for 1 h with gentle shaking. Unbound dye was removed by a buffer exchange with 50 mM Hepes buffer (pH 7.4, 145 mM NaCl) using Nap5 gel-filtration columns (GE Healthcare). Viral aggregates were removed by filtration immediately before experiments using a 0.2-μm filter. MDCK cells were exposed to the dye-labeled WSN at an MOI of 20 on ice for 1 h to allow binding. Unbound virus and inhibitors were removed by three washes of cold PBS. Medium containing Lysotracker (Invitrogen) and either PBS or PGN-ZA were added to the samples before they were immediately moved to a 37 °C water bath to begin infection. Samples were washed, fixed at 0, 5, 15, 30, and 60 min postinfection with 2% (wt/vol) paraformaldehyde, and the cell boundaries labeled with GFP-tagged E-cadherin. The samples were then cured overnight with DAPI Prolong Gold (Invitrogen). Images were taken on Applied Precision DeltaVision Ultimate Focus Microscope with a 60× objective. The images taken were deconvolved to visualize individual virus peaks. For quantitative analysis, the number of viruses per cell was quantified using ImageJ and normalized to the PBS controls. Amantadine, an inhibitor of the M2 ion channel, was used as a positive control. The assay was done the same way except that Alexa Fluor 488-labeled A/Turkey/MN/80 (TKY) virus and 125 μM amantadine were used. The TKY virus had to be used because WSN is resistant to amantadine.

Inactivation of Virus by Acidic Treatment. The low-pH inactivation of influenza virus was performed as previously described (6), and the virus was titrated with the plaque assay.

Statistical analysis was performed using two-tailed *t* tests (7).

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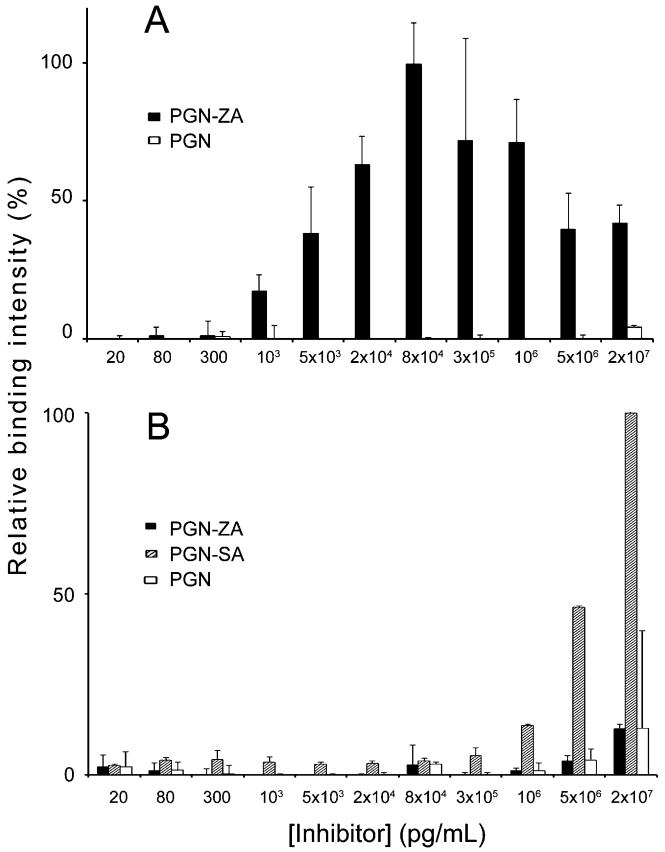

Fig. S1. PGN-ZA binds to NA. Varying dilutions of PGN-ZA, PGN-sialic acid (SA), and PGN were first covalently attached to 96-well plates via UV cross-linking. The relative binding of NA (*A*) and HA (*B*) to the inhibitors was determined using ELISAs. As with Fig. 1, bare PGN was used as a control for nonspecific binding, and PGN-SA was included as a positive control for HA binding. Error bars represent the SEM from two independent experiments.

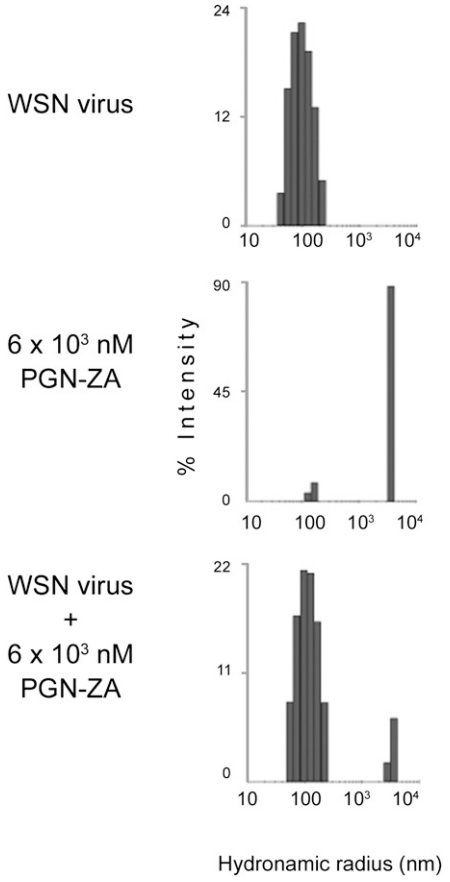

Fig. S2. PGN-ZA does not cause viral aggregation. The hydrodynamic radius of WSN viruses was determined by dynamic light scattering in the presence (*Bottom*) or absence (*Top*) of PGN-ZA (30-fold IC_{50}). PGN-ZA alone was included as a negative control (*Middle*). Results shown are representative of two independent experiments.

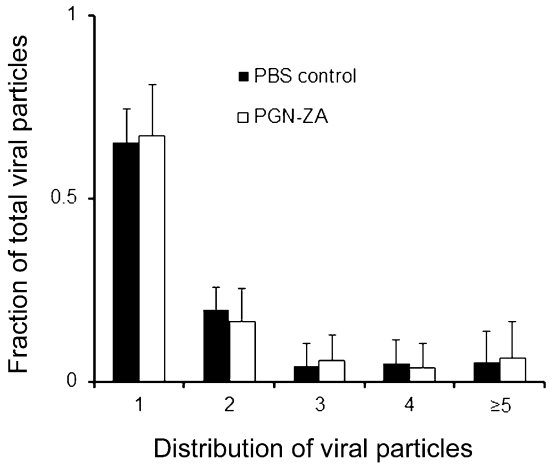

Fig. S3. PGN-ZA does not cause viral aggregation. WSN viruses were stained with phosphotungstic acid and visualized by TEM in the presence or absence of PGN-ZA. Representative TEM images were taken at 4,800× magnifications. The number of viral particles (1, 2, 3, etc.) in every viral cluster was enumerated and normalized to the total number of viral particles in each image. The number of viruses counted for each group is as follows: 1: n = 1,430; 2: n = 401; 3: n = 116; 4: n = 104; ≥5: n = 152.

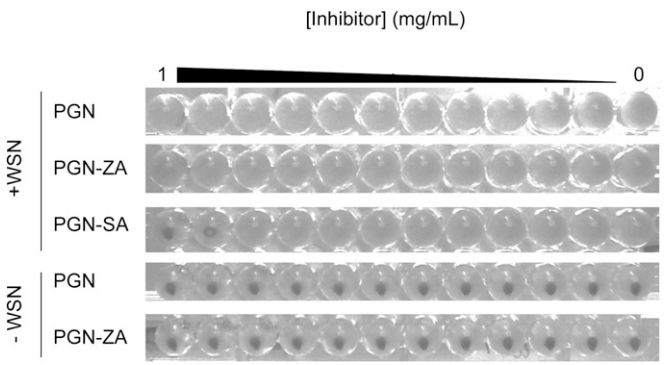

Fig. S4. PGN-ZA does not inhibit hemagglutination. Hemagglutination inhibition assay was used as an additional method to evaluate the effect of the multivalent inhibitors on virus binding to target cells. Four hemagglutinating units of the WSN virus was first preincubated with serial dilutions of inhibitor in a U-bottom 96-well plate for 1 h at room temperature. The inhibitor concentrations in the first 11 wells ranged from 1 mg/mL to 2×10^{-7} mg/mL, using fourfold serial dilutions. The last well is the inhibitor-free PBS control and 50 μL of 1% red blood cells was added to each well. The plate was then placed on ice for 1 h before observing red blood cell agglutination. PGN was included as a control for nonspecific binding, and PGN-SA was included as a positive control for hemagglutination inhibition. Results shown are representative of two independent experiments.

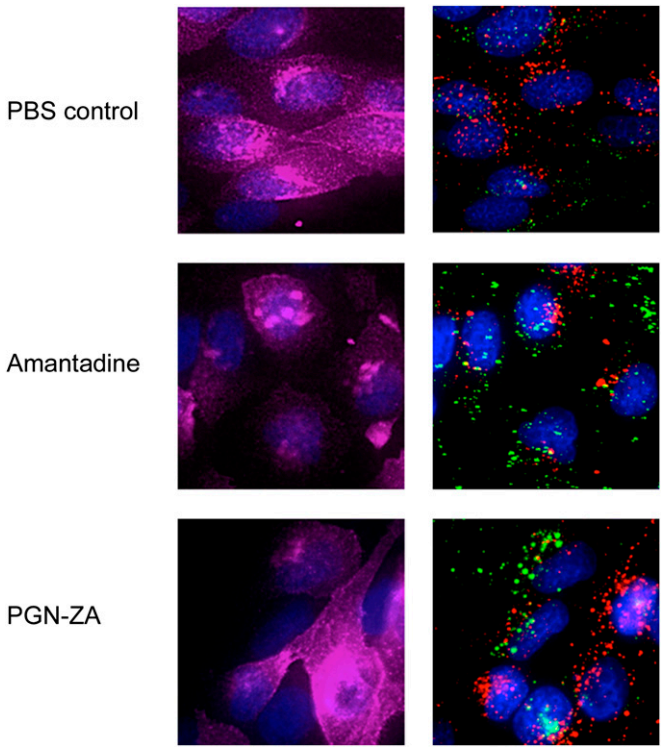

Fig. S5. Amantadine control for virus-endosome fusion assay. Amantadine, an inhibitor of the influenza M2 ion channel, interferes with viral fusion and was used as a positive control for the fusion assay by microscopy. Purified TKY virus was labeled with Alexa Fluor 488 (green), and mixed with either PBS, 125 μM amantadine, or 18 μM PGN-ZA for 1 h at room temperature. The mixtures were then added to MDCK cells at 4 °C for 1 h, washed twice with PBS, and immediately moved to 37 °C in medium containing Lysotracker (red), and either PBS, amantadine, or PGN-ZA to initiate infection. The cells were then washed, fixed at t = 15 min postinfection, and labeled with AF 647-tagged E-cadherin (magenta) to visualize the cell boundary. The samples were then cured overnight, stained with DAPI (blue) to visualize the cell nuclei, and imaged by fluorescent microscopy at 60× magnification. For each sample, the left panel shows E-cadherin (cell boundary) and DAPI (nuclei) staining and the right panel shows viruses, Lysotracker (acidic compartments) and DAPI staining. Representative images are shown.