

A Novel Small-Molecule Inhibitor of the Avian Influenza H5N1 Virus Determined through Computational Screening against the Neuraminidase

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Computational molecular docking provides an efficient and innovative approach to examine small molecule and protein interactions. We have utilized this method to identify potential inhibitors of the H5N1 neuraminidase protein. Of the 20 compounds tested, 4-(4-((3-(2-amino-4-hydroxy-6-methyl-5-pyrimidinyl)-propyl)amino)phenyl)-1-chloro-3-buten-2-one (**1**) (NSC89853) demonstrated the ability to inhibit viral replication at a level comparable to the known neuraminidase inhibitor oseltamivir. Compound **1** demonstrated efficacy across a number of cell-lines assays and in both the H1N1 and H5N1 viruses. The predicted binding of **1** to the known H5N1 neuraminidase structure indicates a binding interface largely nonoverlapping with that of oseltamivir or another neuraminidase inhibitor zanamivir. These results indicate that **1** or similar molecules would remain effective in the presence of virus mutations conferring resistance to either oseltamivir or zanamivir and also vice versa.

Introduction

Recent global outbreaks of the highly pathogenic avian influenza H5N1 virus in birds and the increasing cases of bird-to-human transmission pose a pandemic threat to the public.¹ Since the first documented cases of direct transmission of the H5N1 virus from bird to humans in 1997, the severity of the disease and the high mortality rate associated with the avian virus infection have been of great concern to human health. High viral load and intense inflammatory responses in H5N1-infected patients are associated with pathogenesis of the fatal disease.² In the last century, three influenza pandemics, including H1N1 in 1918, H2N2 in 1957, and H3N2 in 1968 cumulatively had caused 50 million deaths. The highly pathogenic H5N1 virus is the most likely candidate to cause the next influenza pandemic.³ Influenza virus can be classified by the antigenic properties of two surface glycoproteins, namely hemagglutinin and neuraminidase. Sixteen subtypes have currently been defined for the hemagglutinin protein (H1–H16) and nine for the neuraminidase protein (N1–N9).⁴ The hemagglutinin antigen binds to the sialic acid receptor on the cell surface, which mediates the virus entry.⁵ The neuraminidase cleaves the specific linkage of the sialic acid receptor, resulting in the release of the newly formed virions from the infected cells. Additionally, the neuraminidase may function to facilitate the early process of influenza virus infection of lung epithelial cells.⁶ Hence, neuraminidase inhibitor has been an attractive target for the development of novel anti-influenza drugs.

The current anti-influenza drugs, oseltamivir and zanamivir, are successful examples of structure-based drug design, and both exert its antiviral effects through the inhibition of neuraminidase activities of the influenza A and B viruses.^{7,8} The influenza A neuraminidases are divided phylogenetically into two distinct subtypes, group-1 and group-2.⁹ Both oseltamivir and zanamivir were developed on the basis of the crystal structures of group-2 neuraminidase proteins (N2 and N9). Because those drugs have shown similar activity against the N1 neuraminidase, it was thought that the active sites of those proteins are similar. However, recently crystal structures of the group-1 neuraminidase (N1, N4, and N8) have been solved by crystallography and the structures reveal a novel, large cavity adjacent to the active site in group-1 but not in group-2 proteins crystallography, suggesting new opportunities for drug design that targets this cavity in addition to the known active site.¹⁰

Results

In Silico Screen and Compounds Selection. We conducted large scale molecular docking-based library screen to identify compounds that could bind to the new open-form binding site of the group-1 neuraminidases. Molecular docking software ICM (version 3.4–2d)^{11,12} was used to screen approximately 230000 compounds from the National Cancer Institute (NCI), USA, database.¹³ The binding pocket of the protein (receptor) is represented by a set of grid maps, and each compound (ligand) of the database was docked to the pockets (described in Materials and Methods section). It is a flexible-ligand/rigid-receptor docking. Receptor flexibility is treated by using multiple receptor conformations determined by crystallography. A score reflecting the “fitting quality” of ligand–receptor complex was calculated. After the database screening, the best scoring compounds were inspected visually and evaluated according to their chemical and drug-like properties as well as three-dimensional conformations of the docked ligand–receptor complex. Then 28 compounds were selected and obtained from

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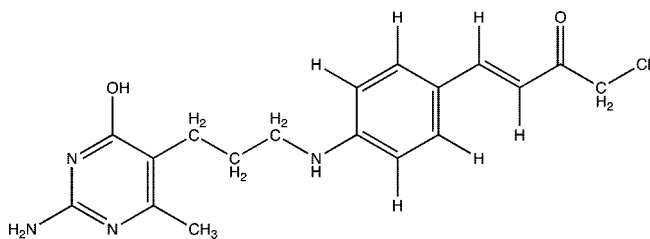


Figure 1. Structure of compound **1**.

the developmental therapeutics program of the National Cancer Institute, USA.

Cytotoxicity Assay. Prior to examining their ability to inhibit influenza virus replication, the compounds were tested for solubility in dimethylsulfoxide (DMSO). Cytotoxicity assays then were performed by incubating the individual compounds with primary human blood macrophages or Madin–Darby canine kidney (MDCK) cells for 48 h. The cell viability was determined by using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Eight of these compounds were not soluble in DMSO, while two were excluded from further testing due to cytotoxicity. The remaining 18 compounds were soluble and did not cause toxicity in primary macrophages or MDCK cells. Cells treated with DMSO were used as solvent controls (Table S1, Supporting Information).

In Vitro Validation. To determine their antiviral activities, the 18 compounds were added individually to MDCK cells infected with H5N1 or H1N1 virus (Table S2, Supporting Information). Subsequently, the culture medium was harvested at 48 h postinfection. The viral titer was first determined by hemagglutination assay. The top scored compound, **1** (NSC89853,¹⁴ Figure 1), demonstrated the ability to inhibit viral replication at a level comparable to that of oseltamivir, while the remaining compounds did not show antiviral activity (data not shown). In addition, the viral titer was quantified by a tissue culture infectious dose (TCID₅₀) assay. Compound **1** showed a significant inhibition on the replication of the H5N1 or H1N1 viruses, and the antiviral effect was demonstrated in a dose-dependent manner in reproducible experiments (Figure 2A,B).

Furthermore, compound **1** inhibition of virions production in H5N1- or H1N1-infected cells was examined by using transmission electron microscopy at 18 h postinfection. Consistent to the results by TCID₅₀, the levels of H5N1 or H1N1 viruses found on the surface of MDCK cells were lower in the cells treated with compound **1** or oseltamivir compared with those of the DMSO-treated cells (Figure 3A–F). Compound **1** has previously been reported to be an inhibitor of dihydrofolate reductase.¹⁵ The in vivo antitumor screening data from NCI indicated that compound **1** is not overtly toxic—mice survived on a toxicity evaluation being injected up to 400 mg/kg/injection.¹⁶

In neuraminidase activity assays, we determined that compound **1** at 100 μM showed inhibitory action on the catalytic activity of group 1 neuraminidase, but the action was less than that of oseltamivir. However, using oseltamivir at a lower concentration of 1 μM, compound **1** displayed an additive effect on the inhibition of neuraminidase activity (Figure 4). These observations suggest that the mechanism of compound **1** action may differ from that of oseltamivir and zanamivir, both of which are sialic acid analogues, and further investigation of the mode of action of this compound is ongoing.

Discussion

In silico, compound **1** was predicted to bind to the open-form conformation of N1 neuraminidase (Figure 5). The

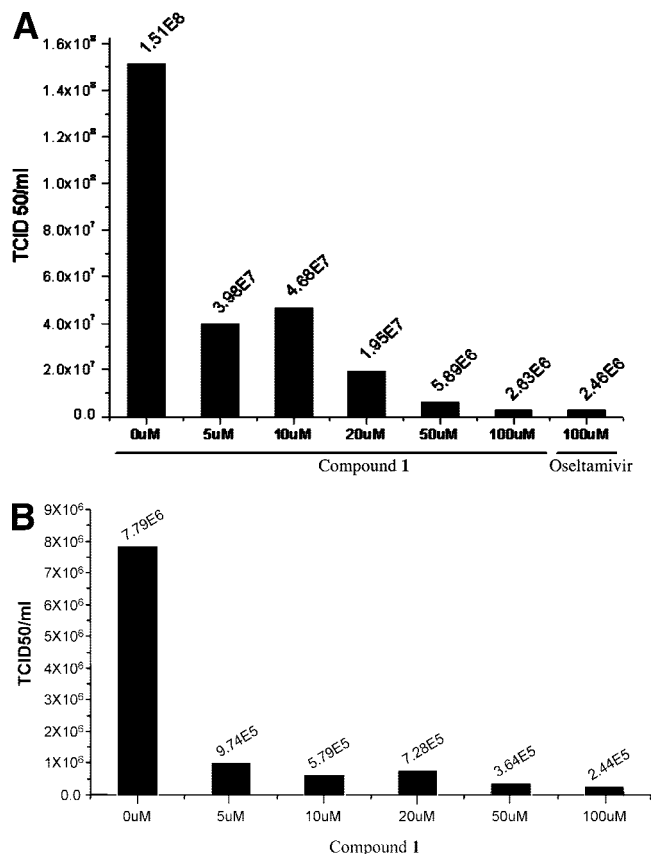


Figure 2. Dosage effects of compound **1** on influenza viral titers. (A) Results on H5N1 virus. MDCK cells were infected with H5N1 virus at a moi of 2. Cells were then incubated with compound **1** at various concentrations as indicated, or with oseltamivir at 100 μM as the standard antiviral agent. Culture supernatants were collected at 48 h postinfection. Viral titers (TCID₅₀) were measured by titration in MDCK cells. Data presented are representative results from three independent experiments. (B) Results on H1N1 virus. MDCK cells were infected with H1N1 virus at a moi of 2. Cells were then incubated with compound **1** at various concentrations as indicated. Culture supernatants were collected at 48 h postinfection. Viral titers (TCID₅₀) were measured by titration in MDCK cells. Data presented are representative results from three independent experiments.

pyrimidine moiety deeply binds to the novel cavity and establishes two hydrogen bonds with the backbone atom of Val116, Thr439, and the side chain of Arg156; the latter is a conserved residue across the neuraminidases and was described by the authors of the new structure paper as a prospective inhibitor binding partner.¹⁰ The other end of the compound takes two distinct conformations depending on the crystal structure used for docking. There are two “open-form” crystal structures available for N1 neuraminidase: an apo structure (protein data bank (PDB)²⁵ code 2hty) and a complex with oseltamivir (2hu0). The conformations of the protein in the two structures are almost identical (the root-mean-square deviation (rmsd) < 0.2). When docked to 2hty, in the best scored conformation, the compound shows minimal overlap over the position of oseltamivir and establishes the same hydrogen bonds (with Arg292 and Arg371) as oseltamivir does (Figure 5, binding mode 1). In contrast, when docked to 2hu0, the best scored conformation does not overlap with oseltamivir at all (Figure 5, binding mode 2). We have docked compound **1** to both the complex (oseltamivir being retained) and the N1 only (oselta-

^a Abbreviations: MD, molecular dynamics; NA, neuraminidase; PDB, protein data bank; rmsd, root-mean-square deviation.

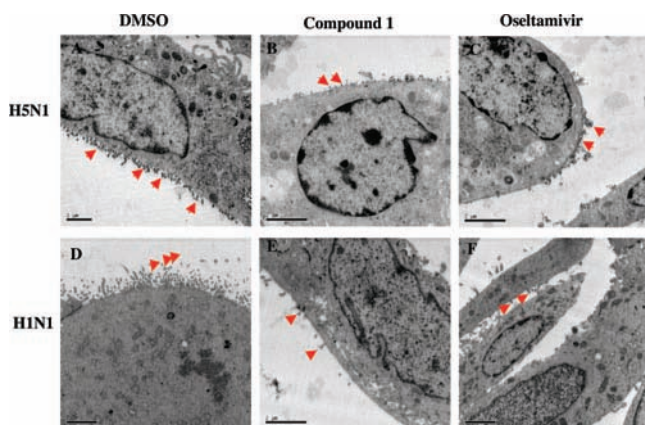


Figure 3. Transmission electron microscopy of influenza virus infected cells. MDCK cells were infected with H5N1 (A–C) or H1N1 (D–F) virus at a moi of 2. Cells were then treated with compound **1** (100 μ M), oseltamivir (100 μ M), or DMSO. At 18 h postinfection, the cells were collected for ultrastructural examination by transmission electron microscopy. Arrows indicate the virions. Magnification: A, 14000 \times ; B–F, 8900 \times .

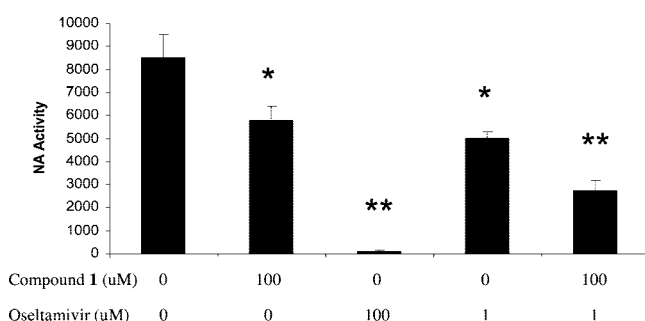


Figure 4. Inhibitory effect of compound **1** on the group 1 neuraminidase. H1N1 virus was incubated with compound **1**, oseltamivir, or both inhibitors at indicated concentrations and assayed by the influenza neuraminidase inhibitor resistance detection kit. Values represent the mean \pm SD of three samples and statistically analyzed by two tailed, paired *t*-test. *: $p < 0.05$; **: $p < 0.01$.

mir being removed before molecular docking). The results show that the best scored conformations are binding mode 2, regardless of the presence or absence of oseltamivir. The molecular docking results imply that compound **1** may be able to bind to the protein simultaneously with oseltamivir or zanamivir because it possesses an alternative binding mode that is distinct from the known drugs.

The neuraminidase is a flexible protein. Additional to the crystal data that captured the open and closed conformations of the 150 loop, computational investigation using molecular dynamics (MD) suggests that this loop is able to open even wider than the open conformation in the crystal structures.¹⁷ This research also suggests that another loop (430 loop, which forms part of the pocket where our predicted compound bound in the binding mode 2) is also flexible. Further research, which combined MD and computational solvent mapping, identified “hot spots” within those flexible loops which are putative drug-binding regions.¹⁸ Directly modeling the protein flexibility in molecular docking remains challenging due to the conformational space is too large to be sampled. A practical approach is using multiple receptor conformations determined either experimentally and/or computationally.¹² We take advantage of multiple available crystal structures of the group-1 neuraminidases to set up the receptor conformations for ligand screening. In binding modes shown in Figure 5, compound **1** is predicted

to establish hydrogen bonds with several residues that are predicted “hot spots”: they are V116, R156 of 150 loop, and T439 of 430 loop.

We also performed additional neuraminidase (NA) activity assays to elucidate whether this compound is competitive with oseltamivir. Two types of treatment, sequential treatment (treatment with oseltamivir first and then compound **1** after 20 min) and cotreatment (treatment with compound **1** and oseltamivir simultaneously) were conducted with the same experimental conditions. The hypothesis being that if both compounds competed with each other, a difference in NA activity between the two experiments would be observed. However, no significant difference was observed. These experiments were repeated three times (data not shown). These results suggest that the two compounds are not mutually competitive.

The experimental data has demonstrated two interesting aspects of the compound. First, it significantly inhibits the replication of the H5N1 and H1N1 viruses, but it is not a potent neuraminidase inhibitor compared with oseltamivir. Second, it demonstrated an additive effect with oseltamivir based on neuraminidase activity assays, suggesting that this compound and oseltamivir bind to the N1 protein simultaneously. The biological activity data generally supports the computational modeling results, although compound **1** is a weak NA inhibitor. The precise binding mode of N1 and compound **1** will be determined by using alternative NA inhibitory assay, and ligand–receptor structure analysis will be the subject of further studies.

To date, at least four drug-resistant mutations have been reported for oseltamivir: R292K, N294S, E119V, and H274Y.¹⁹ Among them, H274Y is the only one reported for group-1 neuraminidase (from H1N1), but it is of very high frequency. The influenza virus found in seven of 43 oseltamivir-treated children developed this mutation,²⁰ and the virus mutation has been associated with death in two out of eight H5N1 infected patients.²¹ To form a pocket for the side chain of oseltamivir, residue E276 must rotate and bond to R224, but the mutation H274Y prevents E276 from rotation. On the basis of the predicted binding modes, this mutation would not appear to affect the compound binding to the N1 protein.

The recently solved structures of group-1 neuraminidases complexed with inhibitors have explained why those inhibitors whose development was guided by the crystal structures of the group-2 neuraminidases remain effective against the group-1 proteins: an induced fitting of the proteins occurs when binding to the inhibitors. This induced fitting closed the open conformations and resulted in almost the same inhibitor binding sites as the group-2 enzymes possess, allowing the group-1 neuraminidases to bind to the inhibitors as strongly as the group-2 proteins do. However, if mutations arise, within or outside of the active site, in the N1 protein preventing this induced fitting, the current drugs will be less effective or totally ineffective to the viruses containing group-1 neuraminidases such as H5N1. In this scenario, compound **1** could be a lead compound for novel inhibitor development.

Conclusions

Neuraminidase inhibitors, including oseltamivir and zanamivir, have been shown to be highly effective against influenza virus infection in humans.²² Until recently, the emergence of oseltamivir-resistant viruses due to neuraminidase mutations,²³ together with the report on the distinct structure of group-1 and group-2 neuraminidases, have led us to search for new drugs that are effective against neuraminidases of the influenza virus.

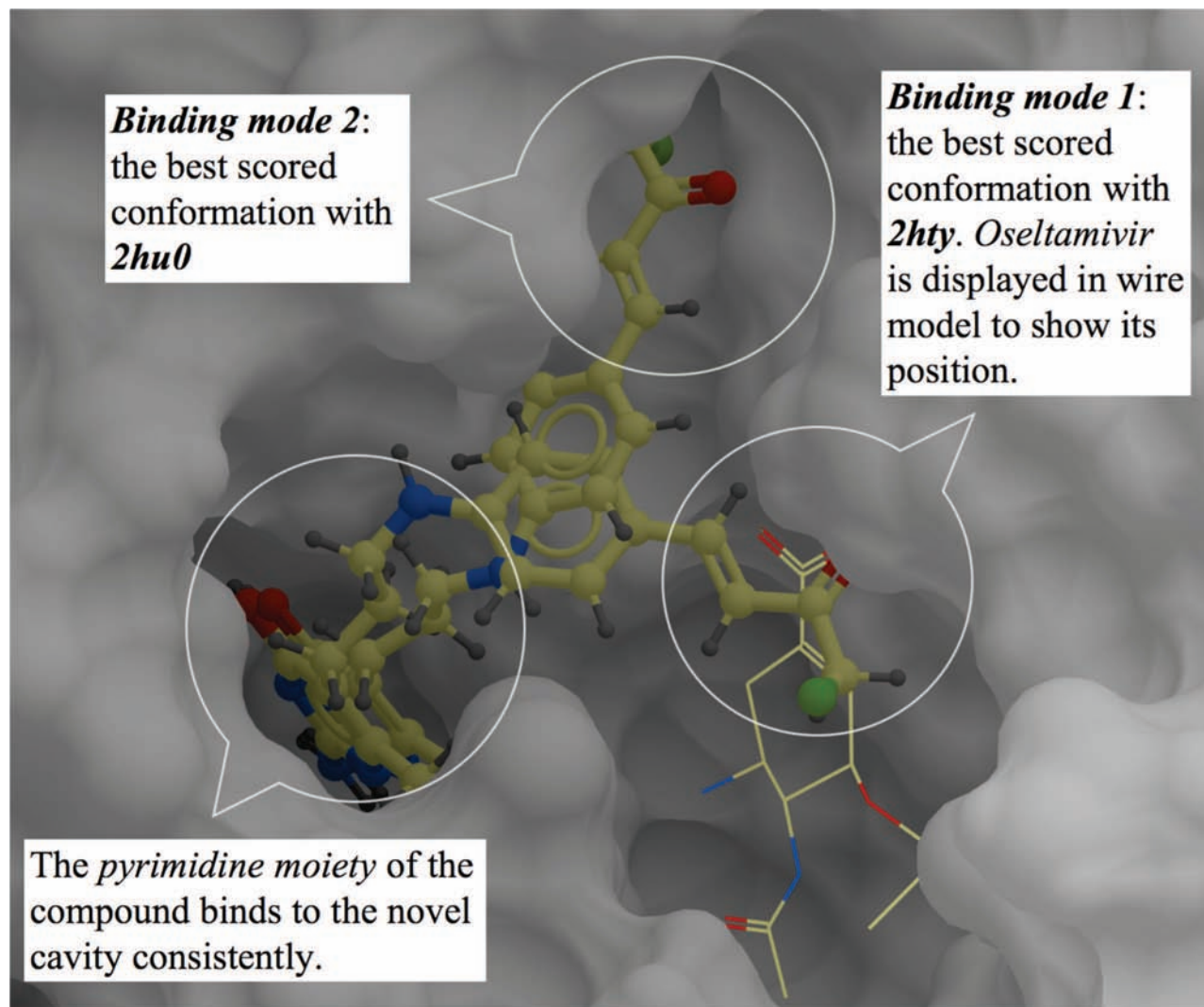


Figure 5. Predicted three-dimensional conformations of compound **1** bound to the open-form N1 neuraminidase. Oseltamivir is displayed as a thin wire to show its relative position to the docked compound **1**. The pyrimidine moiety of compound **1** is deeply fitted in the new cavity, while the other end of the compound takes different conformations.

Our findings suggest that chemical inhibition of viral replication through novel binding to a region of the N1 neuraminidase is possible. This is of significant pharmaceutical interest, as the compound may overcome the drug resistance introduced by H274Y for the N1 protein.

Materials and Methods

Small-Molecule Database and Protein Structures. The NCI database was downloaded from ZINC²⁴ and the crystal structure data of neuraminidase from PDB,²⁵ respectively. We used all the four available, open-form crystal structures for N1, N4, and N8 subtypes of neuraminidase (PDB accessions 2hty, 2hu0, 2htv, and 2ht5), as well as a closed-form, N1-oseltamivir complex (2hu4). The complex 2hu4 was chosen for two reasons: first, to calibrate the docking process by redocking this complex. Second, we were also hoping to discover novel potent inhibitors that bind to the closed form in a similar manner to that of oseltamivir.

Computational Screening and Molecular Docking. The docking and screening software ICM was licensed from Molsoft LLC. A linux-based computer cluster of 200 CPUs was used. The binding pocket was identified by the pocketFinder program,²⁶ which covers both the novel cavity and the known drug/substrate-binding site (for open form structures). Each binding pocket (receptor) is represented by a set of maps for van der Waals (carbon-based and hydrogen-based), electrostatic, hydrogen bonding, and hydrophobic

interactions. Each compound (ligand) of the database was docked to the pockets one by one, and a score (ICM score) reflecting the quality of the docked complex was assigned to each compound.²⁷ The compound is fully flexible, and both the intramolecular ligand energy and the ligand–receptor interaction energy are optimized during the docking. The conformational sampling is based on the biased probability Monte Carlo procedure in the internal coordinate space.²⁸ Because of the nature of Monte Carlo based sampling, we repeated the database screening process five times for each receptor. The results were combined, and the best score for each compound was retained.

HPLC Analysis and Chromatographic Conditions. Analysis of the compound was performed by an Agilent 1200 series system (Palo Alto, CA) interfaced to a G1315c photodiode array detector equipped with a Waters Atlantis HILIC silica column (5 μ m, 4.6 mm i.d. \times 150 mm). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA, Sigma-Aldrich, spectrophotometric grade) in Milli-Q H₂O [A] and 0.1% TFA in acetonitrile (Tedia, HPLC grade) [B] using a gradient program of 100–80% B in 0–5 min, 80–50% B in 5–7 min, 50–100% B in 7–10 min at a flow rate of 1 mL/min. The detection wavelength was set at 210, 254, and 280 nm for acquiring chromatograms, and the column temperature was at 22 °C. After analysis, the compound was shown to have a purity of more than 95% (Figure S3, Supporting Information).

Viral Infection. Avian influenza H5N1 virus, A/Vietnam/1203/04, and human influenza H1N1 virus, A/HK/54/98, were prepared as described in our previous reports.^{29,30} MDCK cells were infected with viruses at a multiplicity of infection (moi) of 2 for 30 min. The virus containing supernatants were removed and cells were washed once with PBS. Serum-free medium supplemented with *N*-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)-trypsin and compounds were used to replenish the cell culture. The supernatants and virus-infected cells were harvested for TCID₅₀ assays and electron microscopy examination, respectively.

Tissue Culture Infective Dose (TCID₅₀) Determination. Prior to TCID assays, MDCK cells were seeded at 2×10^4 cells per well on the 96-well plates. Culture supernatants were harvested from virus-infected cells at 48 h postinfection. Serial 2-fold dilutions of the supernatant samples were prepared, and the diluted samples were incubated with MDCK cells for 1 h for virus adsorption. The virus inoculum was then removed. Cells were washed once and replenished with minimum essential medium (MEM), supplemented with 1 μ g/mL TPCK-treated trypsin. After four days of incubation, cells were fixed with 10% formaldehyde for 30 min and stained with 0.5% crystal violet to determine the virus-induced cytopathic effects. TCID₅₀ titers were calculated using the Reed–Muench formula.³¹

Transmission Electron Microscopy. At 18 h postinfection, the culture supernatant was removed and cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 M sodium cacodylate-HCl buffer, pH 7.4) for 15 min on ice. Cells were then scraped from the culture dish, and the pellet was harvested by centrifugation at 5000 rpm for 3 min. Cells were fixed with 2.5% glutaraldehyde in cacodylate buffer at 4 °C overnight and then resuspended in cacodylate buffer with 0.1 M sucrose. After washings with cacodylate buffer, cells were fixed in 1% osmium tetroxide in cacodylate buffer for 30 min at room temperature. Cells were washed three times and then dehydrated with graduated ethanol. After two washings with propylene oxide, cell pellets were embedded in epoxy resin. Ultrathin sections with a thickness of 100 nm were cut. Ultrastructural features of cells were examined under a transmission electron microscope (Philips EM208S).

Neuraminidase Activity Assay. Neuraminidase inhibitory activity was determined by the NA-Star influenza neuraminidase inhibitor resistance detection kit (Applied Biosystems). Briefly, 25 μ L of compound **1** or oseltamivir at two times the desired concentration was added in triplicate to a 96-well microtiter plate. H1N1 virus (TCID₅₀ = 2×10^7 /mL) was diluted 5-fold with the assay buffer. To the plate, 25 μ L of the diluted virus was mixed with the compounds and incubated at 37 °C for 20 min. The substrate was diluted at 1:1000 in assay buffer immediately before use. Then 10 μ L of the diluted substrate were added to each well. The reaction mixtures were incubated at room temperature for 30 min and then activated by adding 60 μ L of accelerator. The chemiluminescent signal was quantified immediately by EnVision (Perkin-Elmer). Results are statistically analyzed by two-tailed, paired *t*-test.

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Supporting Information Available: List of biologically tested compounds and purity data of the active compound. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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