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Identification of small molecules interfering with H1N1 influenza A viral replication

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Abstract

Successful Influenza A viral replication requires both viral proteins and host cellular factors. Here we utilized a cellular assay to screen for small molecules capable of interfering with any of such necessary viral or cellular components. We employed an established reporter assay assessing influenza viral replication by monitoring the activity of co-expressed luciferase. We screened a diverse chemical compound library, resulting in the identification of compound **7**, inhibiting a novel yet elusive target. Quantitative real-time PCR studies confirmed the dose dependent inhibitory activity of compound **7** in a viral replication assay. Furthermore, we showed that compound **7** was effective in rescuing high dose influenza infection in an in vivo mouse model. As oseltamivir-resistant influenza strains emerge, compound **7** could be further investigated as a possible novel scaffold for the development of anti-influenza agents acting on novel targets.

Keywords

Influenza virus; Drug discovery; Ugi reaction; tetrazole formation

Introduction

Influenza infection poses a significant threat to public health in annual epidemic and potentially devastating pandemic outbreaks. The Center of Disease Control and Prevention (CDC) estimates that influenza infections cause around 30,000 deaths per year in the United States alone, especially in the elderly, the infants, and people with weak or compromised immune system (<http://www.cdc.gov>). The main causal pathogen of influenza infection is the influenza A virus, which carries a genome consisting of eight negative single stranded RNA segments. For our study, we mainly focused on human influenza H1N1, in particular, the A/WSN/33 strain utilized in the cellular reporter assay. There are a total of eleven viral proteins encoded in the A/WSN/33 strain: basic polymerase 2 (PB2), basic polymerase 1 (PB1), PB1-F2, acid polymerase (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), non-structural protein 1 (NS1), and nuclear export protein (NEP or formerly NS2) (Figure 1A). All the above-mentioned viral proteins are potential drug targets against influenza infections. Indeed, the current anti-influenza drugs on the market selectively act on two classes of viral proteins:

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the M2 ion channel and neuraminidase. Amantadine and rimantadine belong to the class that targets the M2 ion channel, which is necessary to release viral RNA into the cytoplasm and proper maturation of viral hemagglutinin.^[1,2] Oseltamivir (Tamiflu[®], Roche) and zanamivir (Relenza[®], GlaxoSmithKline) both inhibit neuraminidase function, which is required to release new virions. Lacking a proof-reading mechanism, the plastic nature of influenza virus allows fast emergence of mutations and, therefore, drug resistance. Unfortunately, the emergence of drug resistance strain often renders drugs of the same class ineffective. Consequently, the quest for both the next generation inhibitors against novel targets^[3] and/or molecules that can overcome the mutations remains pressing.

To explore novel therapeutics against influenza infection, the attention has expanded to include not only viral proteins, but also many host cellular components that are collectively involved in multiple signaling pathways. A central hypothesis is that disruption of any necessary component, either viral or cellular, can lead to abolishment of viral propagation.^[4] Taking both the pathogen and host factors into consideration, we therefore decided to screen a diverse chemical compound library in an unbiased cellular assay. The objective was to identify novel chemicals that may possess structurally diverse scaffolds compared to existing drugs, and/or that may act on a completely different drug target. Recent efforts aimed at the identification of host cellular pathways involving in influenza infection all utilized cell-based assays in combination with siRNA technologies.^[5] A WSN-Ren luciferase assay was established and utilized to identify novel cellular pathways at the early stage of influenza replication with a genome-wide siRNA screen.^[4] The modified reporter virus is derived from the influenza A/WSN/33 strain, and it carries a *Renilla* luciferase gene in the place of hemagglutinin (HA) (Figure 1A). Upon infection with this virus, the production of luciferase in the cell corresponds to the expression level of other viral proteins. Here, using the modified virus and an engineered MDCK cell line (Figure 1B), we sought to identify novel inhibitors of viral replication. By adopting a medium-throughput screening campaign of ~14,000 compounds, we have identified compound **7** as a putative anti-influenza agent. Here we report the identification, synthesis, characterization, and initial SAR studies relative to compound **7**.

Results

An in-house chemical compound library consisting of approximately 14,000 compounds (Asinex Corp.) was screened in the WSN-Ren luciferase assay, which uses high luminescence as a read-out of robust viral replication (Figure 1B).^[4] The amantadine resistant A/WSN/33 strain (Supplementary Figure 1) was chosen in hopes to identify small molecules that can overcome resistance, due the S31N mutation in the M2 ion channel, by acting on a different viral and/or cellular target. We performed the WSN-Ren luciferase screen in a 96-well assay plate format. An engineered HA-expressing MDCK cell line (MDCK-HA) was utilized to allow assessment of multiple cycles of influenza infection and replication. To further increase the throughput of the cellular assay, we pooled the 14,000 compounds into mixtures of 24 compounds per well (total 584 pools). This considerably reduced the number of plates to be tested, making it amenable to a medium throughput screening campaign.

In order to assess the cytotoxicity of the compounds, we tested in parallel each pool of mixtures at 10 μ M against the MDCK-HA cell line using a cytotoxicity ATPlite assay (PerkinElmer), where high luminescence correlates to cell survival. Of the ~600 pools of compounds tested, 354 of them showed at least 80% luminescence in the ATPlite assay, indicating that MDCK-HA cell viability was not significantly affected. Within those 354 pools, we obtained 321 mixtures with significant viral replication inhibition activities at 10 μ M in the WSN-Ren luciferase assay. Eventually 15 pools (out of the 321 wells) still

inhibited the viral replication significantly at a lower concentration (1 μ M). Therefore, the compounds in these 15 wells were de-convoluted and individually tested at 1 μ M. Active compounds were further tested in dose response experiments. Using this screening procedure, we identified compounds **7** and **8** (Table 1) as effective inhibitors of influenza viral replication with EC₅₀ values in the mid nanomolar range (45.6 nM and 86.7 nM) (Figure 1C and Table 1). Interestingly, the structural difference between compound **7** and compound **8** is only by two methyl groups. We therefore focused our further efforts on compound **7**, which is slightly more potent than **8** and possesses a good cytotoxicity profile (Supplementary information figure 2) and reasonable preliminary ADME properties (Supplementary information Table 1).

To validate the inhibitory activity of compound **7** in an orthogonal assay, quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure mRNA expression of the viral NP as an indicator of viral replication. First, we measured viral NP mRNA expression level under the same conditions of the WSN-Ren luciferase assay. We found that treating MDCK-HA cells with 10 μ M and 0.1 μ M of compound **7** led to a reduction of the NP mRNA expression compared to DMSO treated controls (Figure 2A). Furthermore, cells treated with a higher dose of compound **7** (10 μ M) maintained minimal viral replication even after 26 hour incubation. To evaluate whether compound **7** was also active in a wild type influenza viral infection system, we conducted the same viral replication inhibition assay in the human lung cancer epithelial cell line (A549) with the wild type WSN virus. In wild type system, treatments with 10 μ M, 1 μ M, and 0.1 μ M of compound **7** also reduced NP mRNA expression levels in a dose dependent matter (Figure 2B), thus confirming that compound **7** possesses anti-influenza activity.

Given its interesting anti-viral properties, we subsequently designed, synthesized and purified around 450 mg of compound **7** as described in Scheme 1. In summary, compound **1** was acetylated in the presence of acetic anhydride. Compound **3** was prepared according to the published procedures^[6] through Vilsmeier-Haak cyclization conditions, in a simple and regioselective fashion. Compound **4** was synthesized from compound **3** in the presence of aqueous acetic acid at refluxing temperature. Compound **6** was prepared via compound **5** under reductive amination conditions. Compound **7** was prepared in one pot 4-components under the Ugi reaction conditions (Scheme 1).^[7] Similarly, using the Ugi reaction, we also synthesized compound **23** using the appropriate reagents as shown in Scheme 2. Compound **24**, was prepared from commercially available 3-(aminomethyl)-6,7-dimethylquinolin-2(1*H*)-one using standard amide bond formation conditions as shown in Scheme 3. Compound **25**, was synthesized from compound **4** using (tetrahydrofuran-2-yl)methanamine instead of 2-aminoethanol as a starting material as shown in Scheme 4.

The core of compound **7** is made up of three main portions: a quinolinone ring, an aliphatic chain containing an alcohol group and a tetrazole ring. There is also an alkyl group present between the aliphatic alcohol and the tetrazole ring. However, we focused our attentions mostly on modification of the first three described pharmacophores. To assess the importance of these structural groups, we carried out preliminary structure activity relationship (SAR) studies on a collection of commercially available (compounds **8** to **22**) or in-house synthesized analogs (compounds **6**, **7**, **23**, **24** and **25**) (Table 1 and Schemes 1–4). All compounds were tested in the WSN-Ren luciferase assay and an ATPlite cytotoxicity assay in parallel. We identified seven additional compounds that demonstrated dose-dependent inhibitory activity on viral replication and determined their EC₅₀ values (compounds **9**, **13**, **16**, **17**, **18**, **20**, and **25**) (Table 1). The majority of the active compounds demonstrated inhibitory activity comparable to the original hits with EC₅₀ values in the nanomolar range. However, two compounds (**17** and **25**) demonstrated significantly reduced activity with EC₅₀ values in low micromolar range (Table 1).

These studies suggest that the quinolinone ring is important for the anti-influenza activity of this series because replacement with a benzene ring (compound **22**) resulted in loss of activity. Benzodioxine modification of the quinolinone ring (compound **19**) caused inactivity as well. Moreover, the position of substituents also affects potency. We found that methylation on carbon 5 appeared to have a significantly negative impact, as illustrated by compound **17** (EC₅₀ value around 3.8 μ M). On the other hand, methylation on carbon 6 and/or 7 seemed to be well tolerated, as illustrated by compounds **8**, **9**, **13** and **20**, all being active at the nanomolar range. Even with a methoxy substitution on carbon 6, compound **16** is still quite potent. However, retaining only the quinolinone ring but not the aliphatic alcohol and tetrazole moieties is not sufficient (compound **24**) (Table 1).

We then investigated the relevance of the aliphatic alcohol revealing in SAR studies that it was also required for the anti-influenza activity. Complete elimination of the alcohol side chain resulted in inactivation of compound **23** (Table 1). Replacement with bulky aromatic side chain (compounds **10**, **11**, **12**, **14**, **15** and **21**) also resulted in compounds with abolished inhibitory activity. Almost all active compounds possessed an aliphatic alcohol with the exception of compound **25**, which contains a tetrahydrofuran in place of the alcohol and demonstrated a much higher EC₅₀ value at $\sim$ 3 μ M.

Finally, SAR studies suggested that the portion containing the tetrazole together with the alkyl side chain is important because compound **6** did not elicit any anti-influenza activity in our assay. Among the active compounds, replacement of alkyl side chain with an ethyl group (compound **8** and **13**) is tolerated. The substitution on the tetrazole seemed well tolerated since analogues containing a tetrazole substituted with a cyclopentane, a cyclohexane, a t-butyl, or a 1,1 dimethyl propyl substituent (compounds **9**, **13**, **16**, **17**, **18**, **20**, and **25**) still retained activity in the cell-based WSN-Ren luciferase assay (Table 1).

In our initial attempts to identify possible targets for compound **7**, we subsequently investigated its potential inhibitory properties against a panel of enzymes. Since the quinolinone moiety has been reported to be included in kinase inhibitors^[8], we selected a panel of representative kinases. Compound **7** was tested against 13 kinases which are involved in one or multiple cellular signaling pathways required to establish a successful viral replication.^[4] However, when tested at concentrations up to 5 μ M, compound **7** did not significantly inhibit any of the selected enzymes (Supplementary information Table 2). We then focused on the existing two viral protein targets, namely neuraminidase and the M2 ion channel. A fluorescent neuraminidase assay was set up using recombinant neuraminidase and 2 -4-methylumbelliferyl- -*N*-acetylneuraminic acid (MUNANA) as a substrate. The assay conditions were validated using the known neuraminidase inhibitor zanamivir, which gave an EC₅₀ value of 3.4 nM comparable to previously reported data (Supplementary information Figure 3A).^[9] However, tested in serial dilutions from 100 μ M to 6.4 nM, compound **7** remained ineffective against neuraminidase (Supplementary information Figure 3B). To assess if compound **7** physically interacted with the M2 ion channel, we utilized a 1D proton NMR approach. It was reported that once the M2 ion channel was reconstituted in micelles, the conformational changes caused by the binding of amantadine or rimantadine could be detected by monitoring the chemical shift perturbation (from 10.4 ppm to 10.9 ppm) of the epsilon hydrogen (H ϵ) of tryptophan 41.^[10] Indeed, the 1D ¹H NMR spectra of a synthetic M2 peptide reconstituted in micelles were clearly affected by the binding of amantadine, with a new peak rising at 10.9 ppm with serial addition (from 1mM to 10mM), representing a positive control for the binding assay. However, there was no peak formation at 10.9 ppm when even the highest soluble concentration of compound **7** (4 mM) was added to the same system (Supplementary Information Figure 4). Hence, despite these efforts, the viral or cellular target(s) for compound **7** remains elusive at this moment.

Nonetheless, given the encouraging cellular activity of this compound series, we investigated whether compound **7** would also possess *in vivo* efficacy prior to pursuit further target identification studies. For this purpose, a mouse influenza infection model was set up by infecting mice using mouse adapted A/PR/8 viral strain (H1N1) and assessing survival as outcome.^[11] Female C57BL/6 mice were challenged with a high intranasal dose of influenza virus. Each group of mice was then treated i.p. every 12 hours for 5 days with control medium (5% DMSO, 43% PEG400 in PBS) or compound **7** (100 mg/kg/day, 5% DMSO, 43% PEG400 in PBS). As a control, oseltamivir phosphate (1 mg/kg/day, 5% DMSO, 43% PEG400 in PBS) was used. Oseltamivir phosphate, a potent anti-neuraminidase agent, is a prodrug with its active form being oseltamivir acid. It was advised to use 1 mg/kg/day oseltamivir acid as a control;^[11] hence, we calculated the equal molar concentration for oseltamivir phosphate and prepared 1 mg/kg/day for our *in vivo* experiment. The EC₅₀ value of oseltamivir phosphate was determined in the WSN-Ren assay to be around 0.5 nM, which is roughly 100 fold more potent than our best compounds (Table 1). Therefore we decided to use a concentration 100 fold higher than oseltamivir phosphate for compound **7**, which we referred as 100 mg/kg/day treatment in the *in vivo* model.

The mice were monitored for body weight at every treatment and other significant clinical signs of distress and illness were also recorded. The majority of the mice started to lose weight around day 3 and 4 after high dose influenza challenge. Almost all mice lost significant amount of body weight by day 11, when some mice succumbed to the disease. By the end of the experiment (day 22), only 30% of control medium treated mice survived the infection. The rest of the control medium-treated mice reached the maximum allowable weight loss and exerted significant clinical signs of illness such as emaciation, hunch-back posture and ruffled fur coat appearance.

The SAR studies were limited due to the available starting materials and commercially availability of analogues. In our preliminary studies, we demonstrated that the quinolinone and the aliphatic alcohol were necessary for the anti-influenza activities comparing compound **7** against compounds **22** and **23** (Table 1). The portion containing the tetrazole ring and an alkyl side chain is also required for the anti-influenza activity while the substituents on the tetrazole rings were flexible to a certain degree.

When preparing the stock solution for the *in vivo* studies, we noticed formation of white precipitation of compound **7** in the formulated solution (5% DMSO, 43% PEG400 in PBS). We suspected that compound **7** did not dissolve completely and we determined the highest soluble concentration of compound **7** to be between 10 to 15 μ M in PBS (with 2% DMSO) using 1D proton NMR solubility experiment (data not shown). However, we believe that 43% of PEG400 increased the solubility significantly. Unfortunately the effect of PEG400 could not be assessed using the same NMR method. Hence we will take into account when improving the solubility of compound **7** in future SAR studies.

Nonetheless, using these doses, the survival rates were 50% and 80% for mice treated with compound **7** and oseltamivir phosphate respectively (Figure 3). The surviving mice receiving the treatment of 100mg/kg/day of compound **7** maintained smooth fur coat appearance, demonstrated agile movements, and eventually recovered from the infection by regaining the lost body weight, similar to the treatment with Oseltamivir (albeit the latter was given at a lower concentration).

Discussion and Conclusions

Our unbiased cellular screen resulted in the identification of a novel series of active agents with interesting anti-viral activity *in vivo*. Mice challenged with high dose infection of

mouse adapted influenza virus usually succumbed to death around day 6; nevertheless, i.p. administration with isotonic fluid such as PBS could delay the endpoint. Since all i.p. treatments used PBS based formulation, we expected to see maximal weight loss at around day 8 for control medium treated mice and/or later (day 9 to 11) for compound treated animals. Nevertheless, regardless of treatment groups, all mice should lose close to 30% of their body weight and then either succumb to the infection or recover. Indeed most of the control animals succumbed to the disease around day 11; while the compound treated groups had less and/or delayed death and the surviving mice eventually recovered. Treatment with oseltamivir phosphate and compound **7** rescued more than 50% of the mice in each group. Compound **7** treated mice had prolonged median time of survival from 11 to 21 days. Taken together, these data support that compound **7** and its analogues could be potentially optimized into viable candidates for the development of novel anti-influenza agents.

While the cellular target(s) for the compound series remained elusive, exploratory SAR studies may provide initial insights into the possible nature of the interactions that confer activity to the compounds. Excluding viral neuraminidase and M2 ion channel as the putative targets of compound **7** (Supplementary Figures 3 and 4), the potential targets of compound **7** could possibly be other viral proteins or any component(s) of host cellular pathways involved in different stages of influenza replication.^[5]

In the future, attempts to cultivate resistant viral strains could help identify or exclude other viral proteins as direct targets of compound **7**. However, a more systematic approach is required to design experiments to elucidate host cellular targets, which might have been reported in literature or have yet to be discovered as players involved in influenza replication.

In summary, we report here the identification of a new class of anti-influenza agents acting on either viral or cellular targets. Compound **7** consists of three major pharmacophores - a quinolinone, an aliphatic alcohol, and a tetrazole ring - all are required for this compound series to be active. Compound **7** currently is not particularly soluble in PBS; yet it is partially solubilized in a formulation contained 43% PEG400 and possessed *in vivo* efficacy in partially rescuing mice from high dose influenza infection. Therefore we believe that compound **7** and its active analogs are potentially interesting candidates for further investigations as anti-influenza agents and as pharmacological tools to eventually identify novel potential targets for novel therapeutics.

Significance

The identification of compound **7** provides a new direction in development of anti-influenza agents. The currently circulating strains in the North American continent are all resistant to amantadine treatment and the resistance against neuraminidase inhibitors is rising rapidly. Therefore, the development of a new class of pharmacological tools is essential. However even though all the viral and cellular components could potentially be drug targets, not many of them are validated as druggable due to the structural or functional nature of the proteins. Here we bypassed target identification and validation and delivered compound **7** to be active in cellular and *in vivo* models of influenza infection.

Experimental procedures

Chemical compound library screening

Detailed design and generation of the WSN-Ren luciferase virus and MDCK-HA cell line was described in the previous publication⁴. In summary, this modified virus produces *Renilla* luciferase along with all other viral proteins but hemagglutinin. As viral replication

occurs in this system, the amount of luciferase production correlates with viral replication. EnduRen™ live cell substrate (Promega, catalog number E6482) was used to measure and quantify the presence of the *Renilla* luciferase following the manufacturer's protocol. To manage 14,000 compounds in one screen, we convoluted 20 mother plates into 1 daughter plate making each well consist of 24 different chemicals at equal concentration (1 mM). DMSO and amantadine were used as negative and positive controls at indicated or equal concentration as the compounds. In a 96-well white flat bottom plate, each well was added in the order of 25,000 MDCK-HA cells, compound mixtures and WSN-Ren virus in post infection media in DMEM (1% FBS, 1% P/S, 0.3% albumin and 20 mM HEPES). DMEM was purchased from Cellgro (Catalog number 10-013-CU) with 4.5 g/L glucose, L-glutamine and sodium pyruvate. EnduRen™ live cell substrate was added 4 hour after infection in post infection media to reach final concentration of 8 μ M. The luminescence was measured using Perkin Elmer 2030 Victor™ X5. The compound mixture was first screened at 10 μ M in WSN-Ren luciferase assay and an ATPlite assay in parallel at 24 h and 48 h. We then picked the 354 wells that showed proliferation profiles greater than 80% and screened them at a final concentration of 1 μ M. From the second screen, 15 wells showed less than 40% viral replication in the WSN-Ren luciferase assay and more than 50% survival in the ATPlite assay at 24 h or 48 h. We then deconvoluted the 15 wells of compounds. The resulting 360 individual compounds were screened again at a final concentration of 1 μ M and 10 compounds showed less than 40% luminescence in WSN-Ren luciferase assay. All 10 chemicals were then characterized in the WSN-Ren assay for dose dependent inhibitory activities and EC₅₀ values.

Quantification of mRNA level of nucleoprotein through qRT-PCR

Influenza replication inhibition assay was performed and the cell lysates were collected for measuring viral mRNA level in quantitative real time PCR (qRT-PCR). Two cell lines were used for infection by two strains of influenza A virus. One system was MDCK-HA cell and the WSN-Ren luciferase virus, which were components of the WSN-Ren luciferase assay.

In a transparent 96well flat bottom plate, MDCK-HA cells were seeded at 20,000 cells per well overnight in post infection media. Compound **7** was diluted to 1 mM and 10 μ M in DMSO and added to the well to make final concentrations of 10 μ M and 0.1 μ M in 1% DMSO. WSN-Ren luciferase virus was used to infection the cells at MOI: 1.15. QIAGEN RNeasy 96 Kit (Catalog number: 74181) was used to harvest cell lysates and to prepare total RNA at 3, 7, 11, 14 and 26h post infection. Before cell lysates were harvested, visual inspection on cell viability was performed and the wells were washed with PBS to remove dead cells. QIAGEN QuantiTect Rev Transcription kit (catalog number: 205311) was used to convert total RNA into cDNA. In quantitative real-time polymerase chain reaction (qRT-PCR), the mRNA of viral NP was measured in each lysate as indication of viral replication (forward primer sequence: 5'-TGGCACTCCAATTTGAATGAT-3' and reverse primer sequence: 5'-TCCATTCTGTGCGAACAAG-3') and the level of mRNA of canine β -actin was also measured in each lysate, accounting for total amount of cells (forward primer sequence: 5'-GGGGCATCCTGACCCTGAAGTACC-3' and reverse primer sequence: 5'-GAGGATCTTCATGAGGTAGTCGGTC-3').^[12,13] For each lysate, the mRNA level of NP was standardized against the level of β -actin to allow comparison between different wells. The fluorescence was measured with 7900H1 Fast Real-time PCR machine from Applied Biosystems.

The inhibitory activity of compound **7** was further investigated in human lung epithelial cell line A549 using wild type WSN influenza virus. For each well, 25,000 cells were seeded in a transparent 96-well plate overnight. We treated cells with 0.1 μ M, 1 μ M and 10 μ M of compound **7** immediately before and during infection of wild type WSN virus at MOI of

0.032. Visual inspection of cell viability was performed, cells were washed and cell lysates were harvested at 3.5, 7, 11, 23, 27 and 31 h post infection. Total RNA extraction and reverse transcription were performed as mentioned above and the level of NP and human TATA box binding protein (forward primer sequence: 5'-CCACTCACAGACTCTCACAAC-3' and reverse primer sequence: 5'-CTGCGGTACAATCCCAGAACT-3') were measured in qRT-PCR with iTaq SYBR green supermix with Rox reagent (Bio-Rad, catalog number: 1725850). The mRNA level of viral NP was standardized against human TATA box binding protein, which corresponded to total cell amount in each lysate. The fluorescence was measured in a ViiATM 7 real time PCR system from Life Technologies.

Structure activity relationship studies

A small set of commercially available or synthesized derivatives of quinolin-2 (1H)-one was assembled. Each of the 18 derivatives (Table 1) was tested in the WSN-Ren luciferase assay to assess the inhibitory activities over a range of concentrations (ranging from 10 μ M to 0.001 nM). For those that demonstrated dose dependent inhibitory activities, the EC₅₀ values were further determined in the WSN-Ren assay using GraphPad Prism Version 5.01 for Windows, GraphPad Software, San Diego California USA.

Synthetic chemistry

General—Unless otherwise indicated, all anhydrous solvents were commercially obtained and stored in Sure-seal bottles under nitrogen. All other reagents and solvents were purchased as the highest grade available and used without further purification. Thin-layer chromatography (TLC) analysis of reaction mixtures was performed using Merck silica gel 60 F254 TLC plates, and visualized using ultraviolet light. NMR spectra were recorded on Varian 300 or 500 MHz instruments. Chemical shifts (δ) are reported in parts per million (ppm) referenced to ¹H (Me₄Si at 0.00). Coupling constants (*J*) are reported in Hz throughout. Mass spectral data were acquired on Shimadzu LCMS-2010EV for low resolution, and on an Agilent ESI-TOF for either high or low resolution. Purity of all compounds was obtained in a HPLC Breeze from Waters Co. using an Atlantis T3 3 μ m 4.6 $\times$ 150 mm reverse phase column. The eluant was a linear gradient with a flow rate of 1 mL/min from 95% A and 5% B to 5% A and 95% B in 15 min followed by 5 min at 100% B (Solvent A: H₂O with 0.1% TFA; Solvent B: acetonitrile with 0.1% TFA). The compounds were detected at λ = 254 nm or 220 nm. Purity of key compounds was established by elemental analysis as performed on a Perkin Elmer series II-2400. Combustion analysis was performed by NuMega Resonance Labs, San Diego, CA, USA.

Synthesis of *N*-(3,4-dimethylphenyl)acetamide (2)

A mixture of 3,4-dimethylaniline (1.21 g, 10 mmol) and Et₃N in dichloromethane (15 mL), Ac₂O (1.4 mL, 11.1 mmol) was added dropwise. The mixture was stirred at room temperature for 1 h, concentrated at reduced pressure. The residue was purified by column chromatography (silica gel, DCM) to give compound **2** as white-grey solid (1.05 g, 64%). Melting point: 96–97 °C; ¹H NMR (400 MHz, DMSO-*d*₆) δ 2.24 (s, 3 H), 2.46 (s, 3 H), 2.48 (s, 3 H), 7.21 (s, 1 H), 7.71 (d, *J* = 8.5 Hz, 1 H), 7.92 (d, *J* = 8.5 Hz, 1 H), 11.20 (s, 1 H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 19.2, 20.2, 24.6, 117.8, 121.8, 130.2, 132.9, 135.8, 137.2, 168.9; HRMS calcd for C₁₀H₁₄NO (M+H) 164.1075, found 164.1077.

Synthesis of 2-chloro-6,7-dimethylquinoline-3-carbaldehyde (3)

Compound **2** (1 g, 6.10 mmol) was added portion wise to a mixture of POCl₃ (6.53 g, 42.7 mmol) and DMF (1.1 g, 15.20 mmol). Then reaction mixture was heated at 90 °C for 16 hours, cooled down to room temperature and it was poured into ice-water (50 mL) and stirred for 40 min. Formed precipitate was collected by filtration, washed with cold water

and purified by column chromatography (silica gel, DCM/MeOH) to afford compound **3** as white solid (450 mg, 34%). Melting point: 156–157 °C; ¹H (400 MHz, DMSO-*d*₆) 2.24 (s, 3 H), 2.38 (s, 3 H), 7.08 (s, 1 H), 7.58 (s, 1 H), 8.48 (s, 1 H), 10.25 (s, 1 H, CHO); HRMS calcd for C₁₂H₁₁ClNO (M+H) 220.0529, found 220.0522.

Synthesis of 6,7-dimethyl-2-oxo-1,2-dihydroquinoline-3-carbaldehyde (**4**)

A suspension of 2-chloro-6,7-dimethylquinoline-3-carbaldehyde **3** (440 mg, 2 mmol) in acetic acid (12 mL) was added H₂O (0.72 mL) and the mixture was refluxed for 5 h (TLC control), formed precipitate collected by filtration, washed with acetic acid and dried on the air to give compound **4** as yellow powder (350 mg, 87%). ¹H NMR (400 MHz, DMSO-*d*₆) 2.23 (s, 3 H), 2.30 (s, 3 H), 7.11 (s, 1 H), 7.62 (s, 1 H), 8.35 (s, 1 H), 10.19 (s, 1H, CHO), 12.07 (s, 1 H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆) 18.9, 19.9, 116.1, 116.9, 125.1, 130.8, 132.1, 140.3, 142.3, 144.8, 162.1, 190.2; MS *m/z* 202 (M+H⁺); HRMS calcd for C₁₂H₁₂NO₂ (M+H) 202.0868, found 202.0862; Anal. calcd for C₁₂H₁₁NO₂: C 71.63, H 5.51, N 6.96, found: C 71.48, H 5.65, N 6.89; purity 95% (HPLC).

Synthesis of 3-((2-hydroxyethylamino)methyl)-6,7-dimethylquinolin-2(1H)-one (**6**)

To a suspension of 6,7-dimethyl-2-oxo-1,2-dihydroquinoline-3-carbaldehyde **4** (2.01 g, 10 mmol) in AcOH (20 mL), 2-aminoethanol (1.20 g, 20 mmol) was added and stirred at 80 °C for 3 h. Reaction mixture was concentrated at reduced pressure. The residue was dissolved in EtOH (20 mL) and NaBH₄ (760 mg, 20 mmol) was added portionwise, then the mixture was stirred at room temperature for 2 h. Reaction mixture was purified by prep HPLC (C18, acetonitrile/water) giving compound **6** (1.60 g, 67%) as white powder. ¹H NMR (400 MHz; DMSO-*d*₆) 2.07 (br s, OH), 2.23 (s, 3H); 2.26 (s, 3H); 2.58 (t, *J* = 5.2 Hz, 2H); 3.48 (t, *J* = 5.3 Hz, 2H); 3.54 (s, 2 H), 4.46 (br s, 1H, NH); 7.03 (s, 1H); 7.36 (s, 1H); 7.70 (s, 1H); 11.60 (br s, 1 H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆) 18.8, 19.8, 48.3, 51.2, 60.5, 115.1, 117.4, 127.3, 130.1, 131.1, 135.1, 136.2, 138.6, 161.9; MS *m/z* 247 (M+H)⁺; HRMS calcd for C₁₄H₁₉N₂O₂ (M+H) 247.1447, found 247.1439; Anal. calcd for C₁₄H₁₈N₂O₂: C 68.27, H 7.37, N 11.37, found: 68.19, H 7.45, N 11.31; purity 97% (HPLC).

Synthesis of 3-(((2-hydroxyethyl)(2-methyl-1-(1-tert-pentyl-1H-tetrazole-5-yl)propyl)amino)methyl)-6,7-dimethylquinolin-2(1H)-one (**7**)

A mixture of 3-((2-hydroxyethylamino)methyl)-6,7-dimethylquinolin-2(1H)-one **6** (750 mg, 3.0 mmol), isobutyraldehyde (235 mg, 3.05 mmol) in dry MeOH (7 mL) was stirred at 50 °C for 30 min. Then azidotrimethylsilane (400 mg, 3.50 mmol) and *t*-amylisocyanide (345 mg, 3.50 mmol) were added and reaction mixture was stirred at 50 °C for 3 h. The precipitate was collected by filtration, washed with MeOH, ether and dried to give title compound as a white powder (645mg, 48%). melting point: 205–206 °C (decomp.); ¹H NMR (400 MHz; DMSO-*d*₆) 0.52–0.58 (m, 6H); 1.14 (d, *J* = 6 Hz, 3H); 1.53 (s, 3H); 1.57 (s, 3H); 1.87–1.94 (m, 2H); 2.14 (s, 3H); 2.17 (s, 3H); 2.40 (s, 3H); 2.65–2.69 (m, 1H); 2.94–2.98 (m, 1H); 3.01–3.09 (m, 1H); 3.72 (s, 2H); 4.18 (d, *J* = 9 Hz, 1H); 4.38 (t, *J* = 5.4 Hz, 1H); 6.96 (s, 1H); 7.17 (s, 1H); 7.50 (s, 1H); 11.55 (s, 1H); ¹³C NMR (100 MHz, DMSO-*d*₆) 8.2, 18.8, 19.8, 20.2, 20.5, 27.2, 28.1, 31.5, 35.2, 50.3, 55.4, 60.3, 62.8, 64.9, 115.1, 117.3, 127.2, 130.1, 130.8, 134.6, 135.9, 138.6, 155.1, 161.7; MS *m/z* 463 (M+ Na)⁺, 441 (M+H)⁺, 405, 337, 253, 191, 169, 146, 107, 87, 85; HRMS calcd for C₂₄H₃₇N₆O₂ (M+H) 441.2972, found 441.2973; Anal. calcd for C₂₄H₃₆N₆O₂: C 65.43, H 8.24, N 19.07, found: C 65.38, H 8.31, N 19.01; HPLC-UV: t_R 11.66 min, purity 99% (254 nm) (Supplementary Information Figure 5).

Following above mentioned procedure and the appropriate starting materials and reagents used; compounds **8** to **22** were prepared and purchased from Asinex (Russia).

Synthesis of 6,7-dimethyl-3-(((2-methyl-1-(1-(*tert*-pentyl)-1*H*-tetrazole-5-yl)propyl)amino)methyl)quinolin-2(1*H*)-one (23)

A mixture of 3-(aminomethyl)-6,7-dimethylquinolin-2(1*H*)-one (616 mg, 3.05 mmol), isobutyraldehyde (235 mg, 3.05 mmol) in dry MeOH (7 mL) was stirred at room temperature for 3 h. Then azidotrimethylsilane (400 mg, 3.50 mmol) and *t*-amylisocyanide (345 mg, 3.50 mmol) were added and reaction mixture was stirred at room temperature for 40 h. The precipitate was collected by filtration, washed with MeOH, diethyl ether and dried to title compound as a white powder (603 mg, 50%). ¹H NMR (400 MHz, DMSO-*d*₆) 0.56 (t, *J* = 6.8 Hz, 3 H), 0.87 (d, *J* = 6.8 Hz, 3 H), 1.03 (d, *J* = 6.8 Hz, 3 H), 1.59 (s, 3 H), 1.61 (s, 3 H), 1.85–1.92 (m, 2 H), 2.20 (s, 3 H), 2.22 (s, 3 H), 3.12 (d, *J* = 5.3 Hz, 2 H), 4.06–4.11 (m, 1 H), 7.01 (s, 1 H), 7.31 (s, 1 H), 7.67 (s, 1 H), 11.55 (br s, 1 H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆) 8.1, 17.7, 18.8, 19.8, 20.1, 27.1, 27.4, 31.8, 34.3, 44.3, 57.8, 64.2, 115.1, 117.3, 127.3, 130.1, 130.6, 134.5, 136.1, 138.7, 156.9, 161.6; HRMS calcd for C₂₂H₃₃N₆O (M+H) 397.2716, found 397.2710; Anal. calcd for C₂₂H₃₂N₆O: C 66.64, H 8.13, N 21.19, found: C 66.56, H 8.19, N 21.11; Purity 99% (HPLC).

Synthesis of *N*-((6,7-dimethyl-2-oxo-1,2-dihydroquinolin-3-yl)methyl)benzamide (24)

A mixture of 3-(aminomethyl)-6,7-dimethylquinolin-2(1*H*)-one (56 mg, 0.281 mmol), benzoic acid (34 mg, 0.281 mmol), EDC (64 mg, 0.337 mmol), HOBt (45 mg, 0.337 mmol), DIEA (0.24 mL, 1.40 mmol) in DMF (2 mL) was stirred at 70 °C for 4 h. The reaction mixture was cooled down to room temperature and water was added, precipitated formed, filtered, washed with sodium bicarbonate solution and brine respectively. The residue was further purified over silica gel column chromatography (5–10% MeOH in dichloromethane) to afford a pure product (60 mg, 70%). ¹H NMR (400 MHz, DMSO-*d*₆) 2.21 (s, 3 H), 2.26 (s, 3 H), 4.32 (d, *J* = 5.2 Hz, 2 H), 7.07 (s, 1 H), 7.40 (s, 1 H), 7.46–7.55 (m, 3 H), 7.57 (s, 1 H), 7.90–7.94 (m, 2 H), 8.88 (t, *J* = 5.2 Hz, 1 H, NH), 11.73 (br s, 1 H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆) 18.8, 19.85, 39.1, 115.1, 127.3, 127.8, 128.3, 129.1, 130.5, 131.2, 134.1, 136.2, 161.5, 166.5; HRMS calcd for C₁₉H₁₉N₂O₂ (M+H) 307.1447, found 307.1449; Anal. calcd for C₁₉H₁₈N₂O₂: C 74.49, H 5.92, N 9.14, found: 74.39, H 6.02, N 9.10; Purity 98% (HPLC).

6,7-dimethyl-3-(((2-methyl-1-(1-(*tert*-pentyl)-1*H*-tetrazol-5-yl)propyl)((tetrahydrofuran-2-yl)methyl)amino)methyl)quinolin-2(1*H*)-one (25)

Compound **25** was synthesized from compound **4** using (tetrahydrofuran-2-yl)methanamine (0.5 mmol scale) instead of 2-aminoethanol as a starting material as shown in Scheme 4 as white powder (96 mg, 40%). All starting materials and intermediate compound are available from Asinex (Russia). Spectral and analytical data for compound **25** as follows: ¹H NMR (400 MHz; DMSO-*d*₆) 0.57–0.65 (m, 6H), 1.22–1.38 (m, 5 H), 1.58 (s, 3 H), 1.67 (s, 3 H), 1.84–2.04 (m, 4 H), 2.21 (s, 3 H), 2.25 (s, 3 H), 2.61–2.66 (m, 1 H), 2.90–3.01 (m, 1 H), 3.47–3.96 (m, 6 H), 4.20–4.25 (m, 1 H), 7.03 (s, 1 H), 7.29 (s, 1 H), 7.70 (s, 1 H), 11.63 (br s, 1 H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆) 8.2, 18.7, 19.8, 24.7, 27.2, 28.1, 29.8, 31.5, 62.4, 65.1, 65.2, 66.8, 67.1, 78.9, 115.1, 127.1, 130.1, 130.8, 134.6, 136.1, 138.8, 139.2, 156.2, 162.7; HRMS calcd for C₂₇H₄₁N₆O₂ (M+H) 481.3291, found 481.3289; Anal. calcd for C₂₇H₄₀N₆O₂: C 67.47, H 8.39, N 17.48, found 481.3289; Anal. calcd for C₂₇H₄₀N₆O₂: C 67.47, H 8.39, N 17.48, found: C 67.41, H 8.48, N 17.44; Purity 98% (HPLC).

In vivo studies

Female C57BL/6 mice (c57BL/6J), aged between 6 to 8 weeks, were purchased from Jackson Laboratory USA, and held under specific-pathogen-free conditions in the vivarium at the Sanford Burnham Medical Research Institute. Influenza virus A PR/8/34 (PR8, H1N1) was grown in the allantoic fluid of 10-day-old embryonated eggs (McIntyre Poultry, San

Diego) and store at -80°C until use. Before infection, mice were anesthetized by intraperitoneal injection of 100 μL ketamine/xylazine (14.3 mg/mL Ketaset (Fort Dodge, IA)/2.86 mg/mL Anased (Lloyd Laboratories, IA)). Mice were infected with a high dose ($1.25 \times 10^4 \text{ EID}_{50}$) [14] of influenza virus in 50 μL by intranasal route.

The infected mice were treated with control medium, oseltamivir phosphate (1 mg/kg/day) or compound **7** (100 mg/kg/day) every 12 hours for 5 days by i.p. injection. Mouse weights were recorded before infection, treatment and once a day after the treatment. Mice were terminated at the indicated time point (day 29) or when clinical signs reached predefined endpoints (body weight loss of 30% and severe clinical signs indicating terminal illness) by CO_2 inhalation followed with cervical dislocation. Other clinical signs were also observed and recorded. All experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at the Sanford Burnham Medical Research Institute and were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Fluorescent neuraminidase assay

The fluorescent neuraminidase assay follows the protocol published by McKimm-Breschkin, Trivedi, Hampson, Hay, Klimov, Tashiro, Hayden, and Zambon.^[9] The assay buffer is 32.5 mM MES with 4 mM CaCl_2 , pH 6.5, which is used to prepare all components in this assay. In a black 96-well optiplate, 10 μL of diluted compound or control were added with 10 μL of 250 μM recombinant neuraminidase N1 (A/California/04/09) (Sino Biological catalog number 11058-V07B) and incubated at 37°C for 30 min. Then 30 μL of 100 μM 2 -4-methylumbellifery- -*N*-acetylneuraminic acid (Sigma catalog number 69587) was added to the well and incubated at 37°C for 1 h. 150 μL Stop buffer of 0.14 M NaOH and 83% ethanol was prepared fresh and added to the well right before reading the fluorescence.

1D proton NMR M2 binding assay

1 mM of M2 WT peptides were reconstituted in 100 mM DPC-d38 in buffer 100 mM Tris, pH 7.9 according to established protocol in Schnell and Chou.¹⁰ 1D proton spectra were acquired on 600MHz Bruker Avance III spectrometer equipped with TCI probe and z-shielded coils. All NMR data were processed and analyzed using TOPSPIN2.1 (Bruker Biospin Corp., Billerica, MA, USA). Ligand binding was detected at 300K by comparing the tryptophan peak at 10.4 ppm in the presence or absence of compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Diagrams of the wild type A/WSN/33 virus and the modified WSN-Ren luciferase virus, diagram of WSN-Ren assay and dose response curves of compounds 7 and 8

a) Representative diagrams show the genomic makeup and surface viral proteins of a wild type WSN influenza virus and of a WSN-Ren luciferase virus. The modification is engineered by replacement of viral hemagglutinin (HA) coding sequence with that of *Renilla* luciferase. b) A diagram demonstrates WSN-Ren luciferase assay and c) Dose dependent inhibitory activity of compound 7 and compound 8 in the WSN-Ren luciferase assay with EC_{50} values of 45.6 nM and 86.7 nM respectively.

Figure 2. Inhibition of viral replication by compound 7

a) Influenza NP of WSN strain (WSN-NP) mRNA expression level in MDCK-HA cells after WSN-Ren luciferase virus infection. Cells were treated with indicated concentrations of compound **7** or DMSO during infection. Cell lysates were collected at 3, 7, 11, 14 and 26 hour for total mRNA preparation. The WSN-NP mRNA level was then measured in quantitative real time PCR (qRT-PCR) and standardized against the mRNA level of β -actin.

b) The WSN-NP mRNA expression level in A549 cells after wild type influenza A/WSN/33 virus infection. A549 cells were treated with indicated concentrations of compound **7** or DMSO during infection. Cell lysates were collected at 3.5, 7, 11, 23, 27 and 31 hour for total mRNA preparation. The WSN-NP mRNA level was then measured in qRT-PCR and standardized against the mRNA level of human TATA box binding protein.

Figure 3. Mouse survival curve

Mice were infected with high dose mouse-adapted PR/8 influenza virus at day 0. Mice were treated every 12 hour via i.p. route for 5 days then monitored for survival. Survival curves demonstrated that 50% of compound **7** (100 mg/kg/day) treated mice, 80% of mice treated with oseltamivir phosphate (1 mg/kg/day) and 30% of the mice treated with PBS survived.

Scheme 1. Synthesis of 3-(((2-hydroxyethyl)(2-methyl-1-(1-(tert-pentyl)-1H-tetrazole-5-yl)propyl)amino)methyl)-6,7-dimethylquinolin-2(1H)-one (Compound 7)

Reagents and conditions: (a) Ac_2O , Et_3N , CH_2Cl_2 , R. T., 1 h; (b) POCl_3 , DMF, 90 °C, 16 h; (c) AcOH , H_2O , reflux, 5 h; (d) 2-aminoethanol, AcOH , 80 °C, 3 h; (e) NaBH_4 , EtOH , R. T., 2 h; (f) TMSN_3 , isobutyraldehyde, *t*-amylisocyanide, 50 °C, 2 h.

Scheme 2. Synthesis of compound 23

Reagents and conditions: (a) TMSN_3 , isobutyraldehyde, *t*-amylisocyanide, R. T.

Scheme 3. Synthesis of compound 24

Reagents and conditions: (a) benzoic acid, EDC, HOBT, DIEA, DMF, 70 °C, 4 h.

Scheme 4. Synthesis of (Compound 25)

Reagents and conditions: (a) (tetrahydrofuran-2-yl)methanamine, AcOH, 80 °C, 3 h then NaBH₄, EtOH, R.T., 2 h; (b) TMSN₃, isobutyraldehyde, *t*-amylisocyanide, 50 °C, 2 h.

Table 1
Inhibition results for quinolin-2 (1*H*)-one derivatives against influenza replication

Cpd ID	Structure	EC ₅₀ [a]	Cpd ID	Structure	EC ₅₀ [a]
6		> 100 μM	7		45.6 nM
8		86.7 nM	9		139 nM
10		> 100 μM	11		> 100 μM

Cpd ID	Structure	EC ₅₀ [a]	Cpd ID	Structure	EC ₅₀ [a]
12		>100 μM	13		147 nM
14		>100 μM	15		>100 μM

Cpd ID	Structure	EC ₅₀ [a]	Cpd ID	Structure	EC ₅₀ [a]
16		169 nM	17		3850 nM
18		42 nM	19		>100 μM
20		41.5 nM	21		>100 μM

Cpd ID	Structure	EC ₅₀ ^[a]	Cpd ID	Structure	EC ₅₀ ^[a]
22		>100 μM	23		>100 μM
24		>100 μM	25		2946 nM

^[a] Values are means of at least duplicate or more experiments and SD are within ± 22%.