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ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · MARCH 2012

Impact Factor: 5.45 · DOI: 10.1021/jm2013503 · Source: PubMed

Journal of Medicinal Chemistry

Article

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Design, Synthesis, and in Vitro Biological Evaluation of 1*H*-1,2,3-Triazole-4-carboxamide Derivatives as New Anti-influenza A Agents Targeting Virus Nucleoprotein

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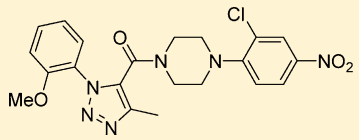
Supporting Information

ABSTRACT: The influenza virus nucleoprotein (NP) is an emerging target for anti-influenza drug development. Nucleozin (**1**) and its closely related derivatives had been identified as NP inhibitors displaying anti-influenza activity. Utilizing **1** as a lead molecule, we successfully designed and synthesized a series of 1*H*-1,2,3-triazole-4-carboxamide derivatives as new anti-influenza A agents. One of the most potent compounds, **3b**, inhibited the replication of various H3N2 and H1N1 influenza A virus strains with IC_{50} values ranging from 0.5 to 4.6 μM. Compound **3b** also strongly inhibited the replication of H5N1 (RG14), amantidine-resistant A/WSN/33 (H1N1), and oseltamivir-resistant A/WSN/1933 (H1N1, 274Y) virus strains with IC_{50} values in sub-μM ranges. Further computational studies and mechanism investigation suggested that **3b** might directly target influenza virus A nucleoprotein to inhibit its nuclear accumulation.



INTRODUCTION

Influenza is a seasonally epidemic acute respiratory disease persistently threatening public health caused by infection of ribonucleic acid (RNA) influenza viruses of *orthomyxoviridae* family. The epidemic and pandemics of influenza have caused serious impact on worldwide morbidity, mortality, and economy.[1] Three distinct types of influenza viruses are classified based on their serological subtypes, among which influenza A viruses are the most virulent human pathogens. Influenza A viruses possesses eight segmented RNA genomes. The continuous genetic mutation and reassortment of influenza A viruses may cause the evasion of primary human immunities and lead to the resistance against current therapies. More importantly, the viral genetic shift may eventually cause the breakthrough of interspecies and interhuman transmission barriers which will be disastrous to public health.[2−5]

Currently, two types of small molecular drugs are available for the treatment of influenza A including M2 ion channel blockers (i.e., amantidine and rimanitidine) and neuraminidase inhibitors (i.e., oseltamivir and zanamivir).[6−8] However, the application of M2 ion channel blockers, particularly for amantidine, has been strictly limited because of the rapid emergence of drug resistance and the occurrence of central nervous side effects.[9] Oseltamivir resistant viruses have also been reported since 2005,[10,11] and almost all the seasonal influenza H1N1 viruses circulating in the United States were resistant to Oseltamivir in the 2008−2009 flu season.[12−17] Most recently, cases of reassortment between swine influenza and 2009 influenza A (H1N1) in human were reported,[18] which highlights the emergent need for developing new anti-influenza agents with new mechanism of action.

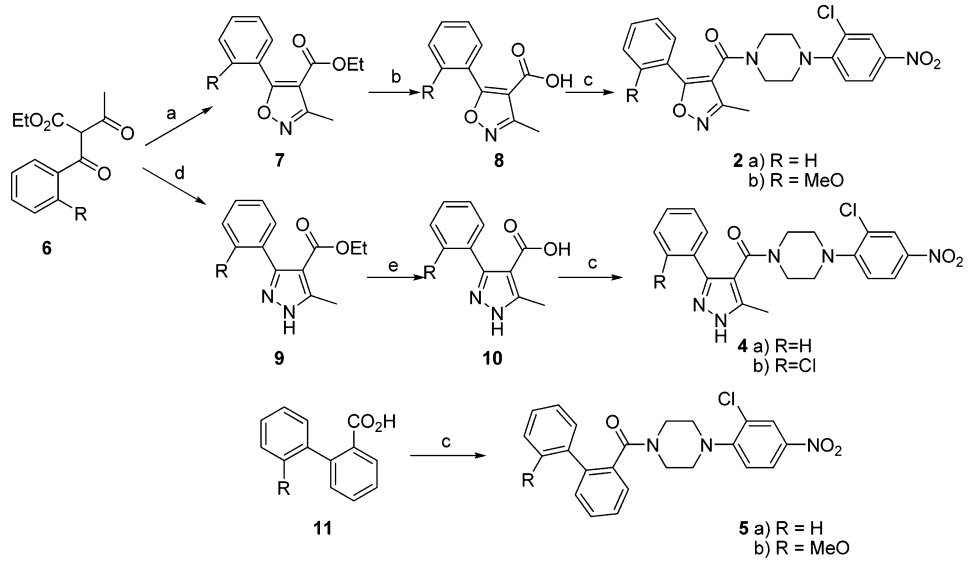
The influenza virus nucleoprotein (NP) is encoded by the fifth genome segment and abundantly expressed during the course of infection. Comparing with the viral surface spike proteins like hemagglutinin and neuraminidase, the influenza virus nucleoprotein is highly conserved.[20−22] During the viral life cycle, nucleoprotein binds with influenza viral RNA segments and polymerase subunit proteins (PB1, PB2, and PA) to form virus ribonucleoprotein (vRNP) complex, which is transported to host cell nucleus to trigger viral RNA transcription, replication, and virion assembly.[19] Owing to its indispensable roles in numerous stages of viral multiplication, influenza virus nucleoprotein has been considered as a novel target for new anti-influenza drug development. Several nucleoprotein inhibitors have been reported to display promising anti-influenza virus activities.[23−28]

Nucleozin (**1**)[24] is the arguably first small molecular influenza virus nucleoprotein inhibitor reported by Yuen and Su independently.[24,25] Studies have demonstrated that **1** and its related derivatives could potently trigger the aggregation of nucleoprotein and inhibit its nuclear accumulation to display

Received: October 8, 2011
Published: February 14, 2012

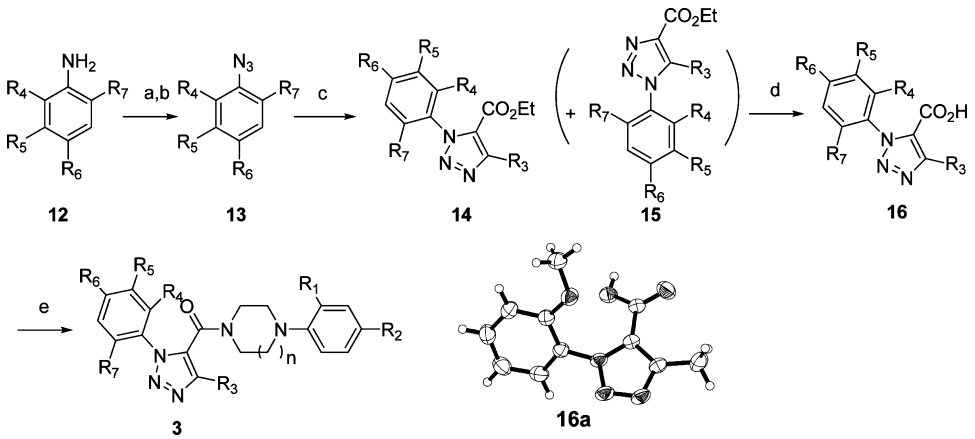
dx.doi.org/10.1021/jm2013503 | *J. Med. Chem.* 2012, 55, 2144−2153

Scheme 1. Synthesis of Compounds 2, 4, and 5[a]



[a]Reagents and conditions: (a) $NH_2OH{\cdot}HCl$, ethanol aq, 60 °C overnight, 76.4%; (b) NaOH, MeOH:THF:H_2O = 1:1:3, overnight, 95.7%; (c) $(COCl)_2$, dichloromethane, 2.0 h, then 1-(2-chloro-4-nitrophenyl)piperazine, Et_3N, dichloromethane, 3.0 h, 60−80%; (d) $N_2H_4{\cdot}H_2O$, ethanol, reflux overnight, 76.4%; (e) BBr_3, DCM, −10 °C, 3.0 h, 56.2%.

Scheme 2. Synthesis of Compounds 3 and the X-Ray Structure of Intermediate 16a[a]



[a]Reagents and conditions: (a) H_2O/ethyl acetate, then HCl,$NaNO_2$; (b) NaN_3, 0 °C, 2.0 h,59.0%; (c) toluene, reflux, overnight, 80.9% (**14** + **15**); (d) LiOH, MeOH:THF:H_2O = 1:1:3, overnight, 96%; (e) $(COCl)_2$, dichloromethane, DMF cat., 2.0 h, then 1-(2-chloro-4-nitrophenyl)piperazine, Et_3N, dichloromethane, 3.0 h, 58.0%.

potent anti-influenza virus activities. Further mutational studies suggested that these inhibitors might directly bind to nucleoprotein and "induce formation of higher-order nucleoprotein oligomers" that are unable to migrate into the nucleus.[24−27] However, it remains elusive that how the inhibitors induced the formation of nucleoprotein oligomers.[27] None of the current inhibitors has been approved for clinical investigation. It is highly desirable to identify new inhibitors for to further validate nucleoprotein as novel molecular target for anti-influenza A development. Herein, we report the structural design, synthesis, and in vitro biological evaluation of 1*H*-1,2,3-triazole-4-carboxamide derivatives as new anti-influenza A agents using **1** as the lead compound.[27]

CHEMISTRY

Compounds **2** and **4** were prepared by using ethyl 2-benzoyl-3-oxobutanoate as the same starting material (Scheme 1). The condensation/cyclization of ethyl 2-benzoyl-3-oxobutanoate (**6**) with hydroxylamine hydrochloride or hydrazine hydrate yielded ethyl 3-methyl-5-phenylisoxazole-4-carboxylate (**7**) or ethyl 5-methyl-3-phenyl-1*H*-pyrazole- 4-carboxylate (**9**), respectively. Compound **7** or **9** was hydrolyzed and reacted with 1-(2-chloro-4-nitrophenyl)piperazine to produce the designed (4-(2-chloro-4-nitrophenyl) piperazin-1-yl)(3-methyl-5-phenylisoxazol-4-yl)methanone (**2**) or (4-(2-chloro-4-nitrophenyl)-piperazin-1-yl) (5-methyl-3-phenyl-1*H*-pyrazol-4-yl) methanone (**4**). (4-(2-Chloro-4-nitrophenyl) piperazin-1-yl)(2′-biphenyl)methanone (**5**) was readily prepared by a direct condensation of 2-phenyl benzoic acid (**11**) with 1-(2-chloro-4-nitrophenyl)piperazine.

Compounds **3** were synthesized by using a traditional "3 + 2" cycloaddition as the key step.[29−31] Briefly, the anilines **12** were diazotized and then reacted with NaN_3 to produce the azidobenzenes **13**. Compounds **13** were directly reacted with

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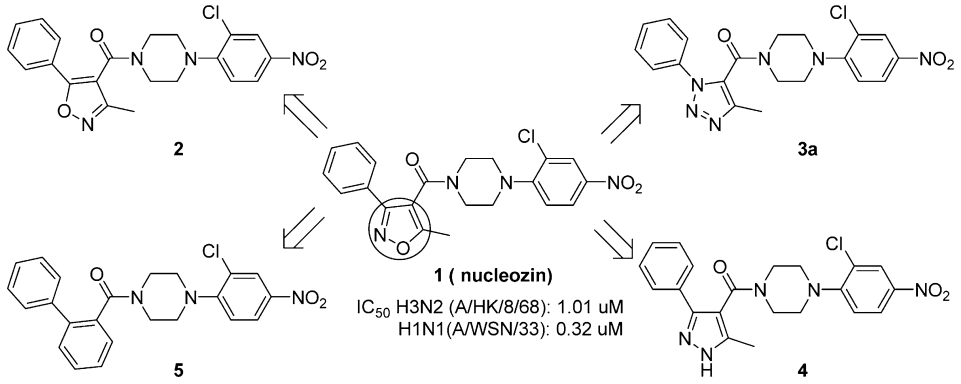
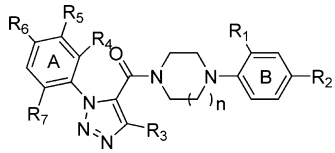


Figure 1. Design of new anti-influenza A agents using a scaffold-hopping strategy.

Table 1. Inhibitory Activities of the Compounds against Influenza A Replication



compd	n	R_1	R_2	R_3	R_4	R_5	R_6	R_7	CC_{50}[c] (μM)	IC_{50} (μM) H3N2[a]	IC_{50} (μM) H1N1[b]
1									15.9	1.08	0.32
2a										6.00	4.83
2b										2.62	0.71
4a										8.64	3.38
4b										2.87	0.72
5a										7.61	5.24
5b										3.68	1.57
3a	1	Cl	NO_2	Me	H	H	H	H	>100	5.16	3.13
3b	1	Cl	NO_2	Me	OMe	H	H	H	>100	1.97	0.68
3c	1	Cl	NO_2	Me	H	OMe	H	H	>100	13.77	5.35
3d	1	Cl	NO_2	Me	H	H	OMe	H	>100	14.72	5.61
3e	1	Cl	NO_2	Me	Cl	H	H	H	>100	5.65	1.20
3f	1	Cl	NO_2	Me	H	Cl	H	H	>100	9.38	4.89
3g	1	Cl	NO_2	Me	H	H	Cl	H	>100	>50	4.93
3h	1	Cl	NO_2	Me	OH	H	H	H	>100	7.84	1.58
3i	1	Cl	NO_2	Me	Me	H	H	H	>100	4.92	2.40
3j	1	Cl	NO_2	Me	OEt	H	H	H	>100	8.97	3.22
3k	1	Cl	NO_2	Me	OiPr	H	H	H	>100	16.16	8.42
3l	1	Cl	NO_2	Me	COMe	H	H	H	>100	14.80	13.72
3m	1	Cl	NO_2	Me	OMe	H	H	OMe	>100	2.42	1.34
3n	1	Cl	NO_2	H	OMe	H	H	H	>100	>50	8.36
3o	1	Cl	NO_2	Et	OMe	H	H	H	>100	>50	>50
3p	1	Cl	H	Me	OMe	H	H	H	>100	>50	48.12
3q	1	H	NO_2	Me	OMe	H	H	H	>100	>50	16.98
3r	1	H	H	Me	OMe	H	H	H	>100	>50	>50
3s	1	OMe	NO_2	Me	OMe	H	H	H	>100	>50	>50
3t	2	Cl	NO_2	Me	OMe	H	H	H	>100	>50	35.45
AMD[d]									>100	2.22	>50

[a]Influenza A/HK/8/68 (H3N2) stains. [b]Influenza A/WSN/33 (H1N1) stains. [c]Antiproliferation against MDCK cells. [d]AMD: amantidine. The data are means of results from at least three independent experiments.

different alkynoates to obtain the key intermediates ethyl 3-phenyl-3*H*-1,2,3-triazole-4-carboxylates (**14**) and the byproducts ethyl 1-phenyl-1*H*-1,2,3-triazole-4-carboxylates (**15**). Compounds **14** and **15** could be separated by silica gel column chromatography, and the structures of **14** were further determined by X-ray crystallographic analysis of a representative compound **16a** (Scheme 2).[32] With the key intermediates **14** in hands, compounds **3** were readily obtained by hydrolysis and coupling with substituted piperazines or 1, 4-diazepane with good yields.

RESULTS AND DISCUSSION

Taking compound **1** as the lead compound, we initially designed isoxazol-4-carboxamide (**2a**), 1*H*-1,2,3-triazole-4-

dx.doi.org/10.1021/jm2013503 | J. Med. Chem. 2012, 55, 2144−2153

carboxamide (**3a**), 1*H*-pyrazol-4-carboxamide (**4a**), and 2-biphenyl carboxamide (**5a**) as new anti-influenza A agents by using a scaffold-hopping strategy (Figure 1). The anti-influenza A virus activities of the compounds were preliminarily evaluated using Madin−Darby canine kidney (MDCK) cell-based CPE (cytopathic effect protection) assays. Under the screening conditions, **1** inhibited the replication of H3N2 (A/HK/8/68) and H1N1 (A/WSN/33) strains, with IC_{50} values of 1.01 and 0.32 μM, respectively, which were comparable to the reported data.[24,25] The results also suggested that all the four designed compounds potently inhibited the replication of H3N2 (A/HK/8/68) and H1N1 (A/WSN/33) strains, among which 1*H*-1,2,3-triazole- 4-carboxamide (**3a**) displayed the greatest potency and inhibited the replication of H3N2(A/HK/8/68) and H1N1 (A/WSN/33) strains, with IC_{50} values of 5.16 and 3.13 μM, respectively (Table 1). Although compound **3a** is about 5-fold less potent than the original lead compound **1**, it represented a new anti-influenza A agent with a different chemical scaffold. Therefore, further structural optimization was conducted to improve its anti-influenza activity. The results were summarized in Table 1.

Similar to the previous observation on the derivatives of compound **1**,[25,27] a substituted group in phenyl ring A might have great impact on the anti-influenza activity of the 1*H*-1,2,3-triazole-4-carboxamide compounds. For instance, when a methoxyl group was introduced at R_4 position of compound **3a**, the anti-influenza activity was obviously improved and the resulting compound **3b** displayed IC_{50} values of 1.96 and 0.68 μM against the replication of H3N2 (A/HK/8/68) and H1N1 (A/WSN/33) strains, respectively, which was about 3 times more potent than the original compound **3a**. However, when a methoxyl group was introduced in R_5 or R_6 position (**3b** and **3c**), the potency was decreased. The R_4-Cl− substituted compound **3e** was also more potent than the corresponding R_5- or R_6-substituted compound **3f** or **3g**, which suggested that R_4 might be a feasible position for further optimization. Therefore, a variety of other moieties such as hydroxyl (**3h**), methyl (**3i**), ethoxyl (**3j**), or acetyl (**3k**) groups were introduced for further investigation. However, it was disappointing that none of these substitutions further improved the potency. The 2′,6′-dimethoxyl compound **3m** (R_4, R_7-disubstituted compound) also displayed good potency against the replication of H3N2 (A/HK/8/68) and H1N1 (A/WSN/33) strains. The impact of R_3 was also investigated, and the results indicated that removal of the methyl group (**3n**) or replacing it with a slightly bigger ethyl group (**3o**) caused dramatic decrease of the potency. Investigation on the substituted group in phenyl ring B revealed that both the nitro- (R_2) and chloro- (R_1) were necessary for the anti-influenza activity. The potencies were significantly lost by removal of the nitro or/and chloro groups (**3p**−**3s**). It was also demonstrated that replacement of the piperazine linker with 1,4-diazenpane (**3t**) led to about 50-time decrease of the potency (comparing with **3b**).

Utilizing the knowledge of the structure−activity relationship on compounds **3**, methoxyl or chloro substituted derivatives of compounds **2a**, **4a**, and **5a** were also design and synthesized. The resulting compounds **2b**, **4b**, and **5b** displayed improved anti-influenza activities against H3N2 (A/HK/8/68) and H1N1 (A/WSN/33) strains, with IC_{50} values comparable to that of **3b**.

Antiproliferative activities of the compounds against MDCK cells were also evaluated to monitor the potential cytotoxic effects. Under the assay conditions, **1** displayed moderate toxicity against the MDCK cells with IC_{50} value of 16 μM. However, none of the 1*H*-1,2,3-triazole-4-carboxamide compounds showed obvious cellular growth inhibition against MDCK cells under 100 μM (Table 1), indicating that the compounds beard great selectivity indexes (SI = CC_{50}/IC_{50}).

Although **3b** was about 2 times less potent than compound **1**, it displayed equal potency with amantidine against the replication of influenza A/HK/8/68 (H3N2) stains with great selectivity index. Furthermore, **3b** also potently inhibited the replication of A/WSN/33 (H1N1) stains which were resistant to the clinical M2 blocker amantidine (Table 1), suggesting that this compound might be used a new lead compound for novel anti-influenza drug discovery. Therefore, further biological evaluations were performed to validate its anti-influenza activity.

Plague counting based viral yield reduction assay is one of the most direct and reliable methods for evaluating the inhibitory effects on viral replication. Therefore, the anti-influenza function of **3b** was further validated by using a plaque reduction assay. As shown in Figure 2, compound **3b** dose-

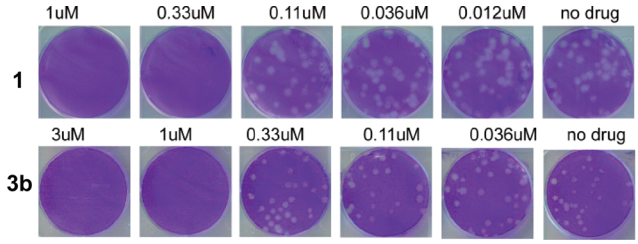


Figure 2. Compound **3b** potently inhibited the plaque formation of influenza virus infected MDCK cells. Monolayer MDCK cells were infected with A/WSN/33 (H1N1) at 0.01 MOI (multiplicity of infection) and overlaid with 1% agar containing various concentrations of nucleozin (**1**) (upper panel) and **3b** (bottom panel). At 72 h postinfection, plaques were visualized by stained with crystal violet. The results shown are representative of at least three independent experiments.

dependently inhibited the plaque formation of MDCK cells by A/WSN/33 (H1N1) influenza virus infection. The plaque formation was completely inhibited by **3b** under a concentration of 1.0 μM. Highly consistent with the results from our CPE assay, compound **1** displayed better plague suppressive effect than **3b** and induced a complete abrogation of viral plagues at 0.3 μM. The viral yield reduction activities of **1** and **3b** were further validated by using microplate counting of infectious particles (McIP) assay,[25] and the deduced IC_{50} values were 0.10 and 0.23 μM, respectively.

The anti-influenza activities of **3b** were also evaluated against a panel of other influenza A virus strains, and the results were summarized in Table 2. It was shown that **3b** also potently inhibited several other H1N1 and H3N2 influenza A virus strains with varied IC_{50} values in low μM range, except for the H3N2 (Br/10/09) strains (IC_{50} > 50 μM). It was also noteworthy that **3b** potently inhibited the replication of H5N1 (RG14) strains, tamiflu resistant WSN (274Y) A/WSN/1933 (H1N1) strains, and A/Guangdong/1996 Chicken H9N2 influenza virus strains with IC_{50} values of 0.69, 1.11, and 4.62 μM, respectively.

Compound **1** had been reported as a nucleoprotein inhibitor that triggered the aggregation and inhibited the nuclear accumulation of influenza virus nucleoproteins.[24,25] Given the structural similarity of the 1*H*-1,2,3-triazole-4-carboxamide

dx.doi.org/10.1021/jm2013503 | J. Med. Chem. 2012, 55, 2144−2153

Table 2. Antiinfluenza Activities of Compound 3b against a Panel of Influenza A Viruses

strains	IC_{50} values against influenza A virus (μM)[a]	
	1	3b
A/Guangzhou/2009 (H1N1)[b]	0.54	0.52
A/Brisbane/10/2007 (H1N1)[b]	1.59	1.86
A/Weiss/43 (H1N1)[b]	0.20	0.66
A/Mal/302/54 (H1N1)[b]	0.52	0.90
A/New Jersey/8/76 (H1N1, NP 289H)[b]	>100	>100
A/Brisbane/10/2007 (H3N2)[b]	12.4	>50
A/Udorn/1972 (H3N2)[b]	0.34	1.17
A/RG14 (H5N1)[b]	0.15	0.69
A/Guangdong/96 (H9N2)[b]	3.87	4.62
A/WSN/33 (neuraminidase, 274Y)[c]	0.66	1.11
rWSN (NP, 52Y)[c]	0.49	1.40
rWSN (NP, 52H)[c]	>50	>50

[a]A/RG14 (H5N1): the NIBRG14 reasorant strains that harbors the hemagglutinin and neuraminidase genes from A/VietNam/1194/2004 and other influenza genes from PR8 viruses. H9N2 (GD/96), A/Chicken/Guangdong/1996 H9N2 strains; rWSN(52Y), rWSN virus with wild-type NP (52Y); rWSN (52H), rWSN virus with mutated NP (52H); WSN (274Y), A/WSN/1933 (H1N1) with tamiflu resistant neuraminidase (274Y). [b]Data obtained by CPE prevention assay. [c]Data obtained by measurements of NP antigen reduction assay. The data are means of results from two or three independent experiments.

derivatives to that of **1**, we hypothesized that the new compounds might also target influenza A nucleoproteins. Therefore, the effect of **3b** on nucleoproteins was inspected under fluorescence microscopy (Figure 3). Not surprisingly, **3b** potently inhibited the nuclear accumulation of influenza virus A

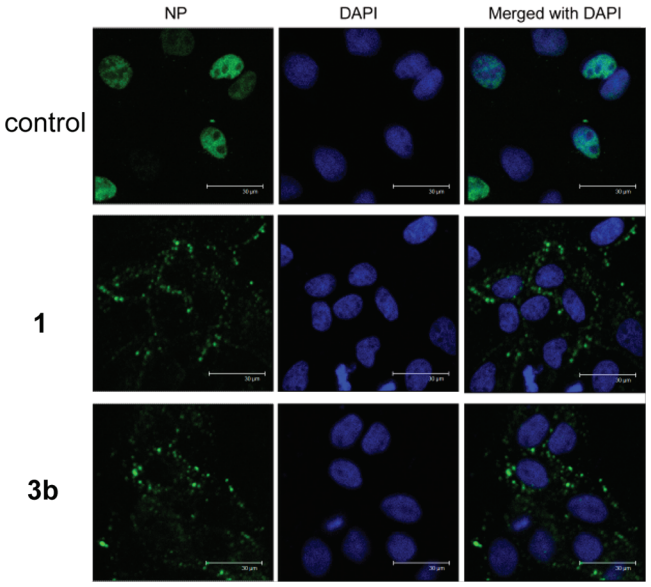


Figure 3. Compound **3b** blocked nuclear accumulation of Influenza A nucleoproteins. MDCK cells were infected with A/WSN/33 (H1N1) viruses at multiplicity of infection (MOI) of 10 in the absence or presence of 10 μM **1** (middle row) or **3b** (bottom row). At 4.0 h post infection, cells were fixed and stained with DAPI for nucleus (blue), and FITC-tagged anti-influenza A NP antibodies specific for viral nucleoprotein (green), respectively. Images were visualized by confocal microscopy. The illustration is representative of experiment in triplicate.

nucleoprotein and caused the nucleoproteins to be trapped in the cytoplasm and scattered randomly in H1N1 (A/WSN/33) virus infected MDCK cells, indicating nucleoproteins might be the molecular target of the 1*H*-1,2,3-triazole-4-carboxamide analogues.

Most recently, the X-ray crystal structure of nucleoprotein complex with a compound **1** analogue was disclosed. It was demonstrated that each inhibitor bridged two molecules of NP (i.e., NP_A and NP_B) protein to form a hexameric complex.[27] The interactions of the inhibitor with the Y289 region in NP_A and the Y52 region in NP_B were critical for binding of the inhibitor to interface of NP_A and NP_B (Figure 4A,B). The importance of the Y289 and Y52 regions in nucleoprotein had also been observed by previous mutational studies.[24,25]

To understand the potential interaction of **3b** with influenza nucleoproteins, a computational study was performed. The results suggested that **3b** bound to nucleoproteins with a similar mode to that of the nucleozin analogues (Figure 4). The carbonyl group of **3b** formed an essential hydrogen bond with the OH group of the S376 residue in NP_B. The 4-nitro-2-chloro-phenyl moiety might form strong $\pi-\pi$ stacking interaction with the Y289 residue of NP_A, while the 2-methoxyl phenyl group and the piperazine moiety could achieve important hydrophobic effect with the Y52 side chain of NP_B. To further validate the proposed interaction, the anti-influenza activity of **3b** were determined against A/New Jersey/8/76 strains bearing Y289H mutation and recombinant WSN viruses harboring either wild-type NP (52Y) or mutated NP (52H) using **1** as a reference compound. Similar to compound **1**, **3b** is a potent anti-influenza agent to the WSN virus with wild-type NP (52Y). However, both Y52H mutated isogenic WSN virus and Y289H mutated A/New Jersey/8/76 strains were resistant to **3b** (IC_{50} > 50 μM), highlighting the critical interaction of **3b** with these two residues. These results were highly consistent to our computational modeling results and further supported that virus nucleoprotein might be the molecular target of the 1*H*-1,2,3-triazole-4-carboxamide anti-fluenza agents.

CONCLUSION

In summary, using scaffold-hopping and bioisosteric replacement strategies, we successfully designed and synthesized a series of 1*H*-1, 2, 3-triazole-4-carboxamide derivatives as new anti-influenza A agents based on the chemical structure of compound **1**. One of the most potent compounds **3b** potently inhibited the replication of various H3N2 and H1N1 influenza A virus strain with IC_{50} values ranging from 0.5 to 4.6 μM. Furthermore, **3b** also potently inhibited the replication of H5N1 (RG14), amantidin-resistant A/WSN/33 (H1N1), and tamiflu-resistant WSN (274Y) A/WSN/1933 (H1N1) with IC_{50} values of 0.69, 0.68, and 1.11 μM, respectively. Further computational study and mechanism investigation suggested that **3b** might directly target influenza virus A nucleoprotein to inhibit its nuclear accumulation. Our study provided a new series of lead compounds for anti-influenza drug development targeting influenza virus nucleoproteins.

EXPERIMENTAL SECTION

Reagents and solvents were obtained from commercial suppliers and used without further purification. Flash chromatography was performed using silica gel (300−400 mesh). All reactions were monitored by TLC, and silica gel plates with fluorescence F_{254} were used and visualized with UV light. ^{1}H or ^{13}C NMR spectra were

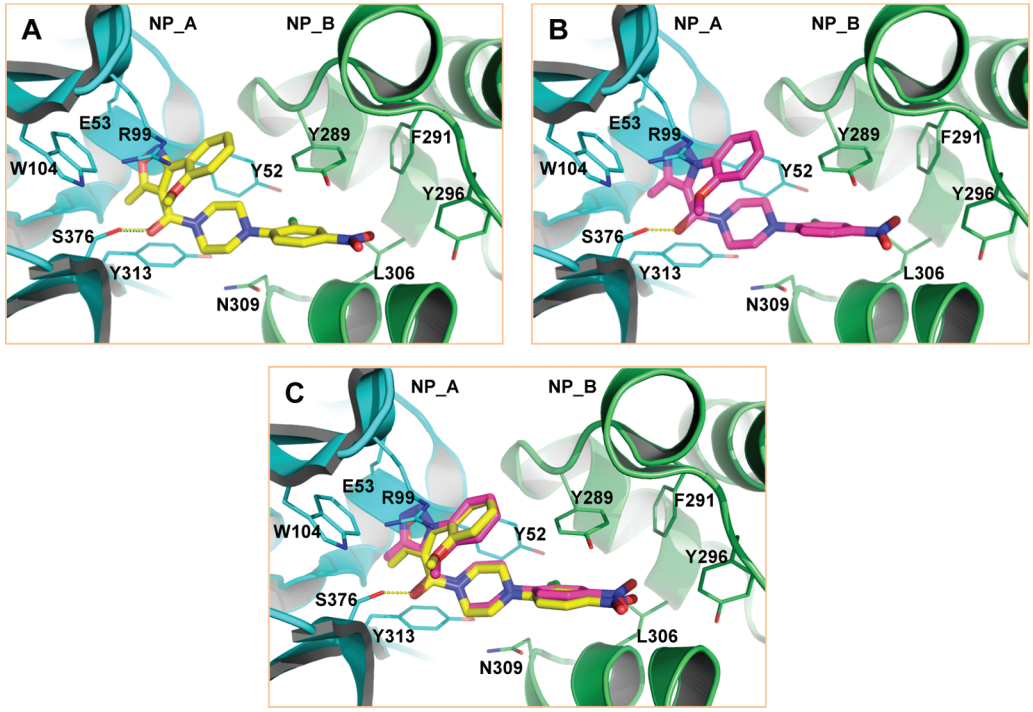


Figure 4. Compound **3b** binds to nucleoprotein with similar mode as that of the nucleozin analogue. (A) nucleozin analogue−NP derivative complex structure; (B) **3b**−NP complex structure predicted by molecular docking. (c) Overlay of **3b** and the nucleozin analogue in the nucleoprotein dimer interface pocket.

recorded on a Bruker AV-400 spectrometer at 400 MHz or Bruker AV-500 spectrometer at 125 MHz, respectively. Coupling constants (J) are expressed in hertz (Hz). Chemical shifts (δ) of NMR are reported in parts per million (ppm) units relative to the internal control (TMS). The low or high resolution of ESIMS was recorded on an Agilent 1200 HPLC-MSD mass spectrometer or Applied Biosystems Q-STAR Elite ESI-LC-MS/MS mass spectrometer, respectively. The purity of compounds was determined by reverse-phase HPLC analysis confirming to be over 95% (>95%). HPLC instrument: DIONEX SUMMIT HPLC. Column: Diamonsil C18, 5.0 mm, 4.6 mm × 250 mm (Dikma Technologies). PDA-100 photodiode array detector. Detector: ASI-100 autoinjector. Pump p-680A. Elution, MeOH in water; flow rate, 1.0 mL/min.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(3-methyl-5-phenylisoxazol-4-yl)methanone 2a. To a stirred solution of 3-methyl-5-phenylisoxazole-4-carboxylic acid **8** (207.9 mg, 1.02 mmol, Supporting Information) in dry dichloromethane (DCM, 10 mL) was added oxalyl chloride (0.26 mL, 3.07 mmol) and two drops of DMF. The reaction mixture was stirred at room temperature for 2 h, and the solvent was removed under vacuum. The resulting residue was diluted with dry DCM (10 mL) and added 164.2 mg (0.68 mmol) 1-(2-chloro-4-nitrophenyl)piperazine in 2.0 mL dry DCM and followed by 0.28 mL (2.04 mmol) diethylethanamine in the ice bath. The reaction mixture was stirred at room temperature for 3 h, and the organic solvent was removed under vacuum. The resulting residue diluted with water (30 mL) and extracted with ethyl acetate (2 × 50 mL). The combined organic layer was washed with dilute sodium hydroxide solution and then with water, dried over anhydrous sodium sulfate, and concentrated under vacuum to get the crude product which was purified by column chromatography over silica gel to afford pure compound 320.4 mg (yield 73.5%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, J = 2.8 Hz, 1 H), 8.07 (dd, J = 2.8, 8.8 Hz, 1 H), 7.74−7.71 (m, 2 H), 7.49−7.48 (m, 3 H), 6.93 (d, J = 8.8 Hz, 1 H), 4.02−4.00 (m, 2 H), 3.42 (br, 2 H), 3.23−3.20 (m, 2 H), 2.83 (br, 2 H), 2.37 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.8, 162.5, 159.1, 153.8, 142.9, 131.0, 129.2, 128.0, 126.7(2C), 126.6, 123.4, 119.6, 110.2, 50.7, 50.4, 46.7, 41.8, 10.4. HRMS (ESI) calcd for $C_{21}H_{20}ClN_4O_4$ $[M + H]^+$ 427.1168; found 427.1167; HPLC analysis: 80:20 methanol−water, 9.04 min, 97.5%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(5-(2-methoxyphenyl)-3-methylisoxazol-4-yl)methanone 2b. Yield 76.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.21 (d, J = 2.4 Hz, 1 H), 8.06 (dd, J = 2.4, 8.8 Hz, 1 H), 7.68 (dd, J = 1.6, 7.6 Hz, 1 H), 7.47 (dt, J = 1.6, 7.8 Hz, 1 H), 7.08 (t, J = 7.6 Hz, 1 H), 6.99 (d, J = 8.4 Hz, 1 H), 6.90 (d, J = 8.8 Hz, 1 H), 3.91 (br, 2 H), 3.84 (s, 3 H), 3.31 (br, 2 H), 3.15 (br, 2 H), 2.73 (br, 2 H), 2.39 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.5, 163.1, 159.4, 156.4, 153.9, 142.7, 132.5, 129.6, 127.9, 126.5, 123.3, 121.1, 119.5, 116.2, 111.9, 111.3, 55.3, 50.4, 46.6, 41.5, 10.5. HRMS (ESI) calcd for 457.1273 $[M + H]^+$; found 457.1276. HPLC analysis: 80:20 methanol−water, 8.84 min, 95.3% in purity.

Synthesis of 1*H*-1,2,3-Triazole-4-carboxamide Derivatives 3. Method A: The procedure was similar to that of **2**.

Method B: To a stirred solution of 1-phenyl-1*H*-1,2,3-triazole-5-carboxylic acids **16** (1 mmol, Supporting Information) and aryl poperazine (1.1 mmol) in dry DCM (10 mL) was added 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) (1.5 mmol), 1-hydroxybenzotriazole anhydrous (HOBt) (1.2 mmol), and triethylamine (3 mmol). The resulting mixture was stirred at room temperature for 3 h, and the organic solvent was removed from the reaction mixture under vacuum. The resulting residue was diluted with water and extracted with ethyl acetate (2 × 50 mL).The combined organic layer was washed with dilute sodium hydroxide solution and then with water, dried over anhydrous sodium sulfate, and concentrated under vacuum to get the crude product which was purified by column chromatography over silica gel to afford pure compound (yield 58−96%).

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(4-methyl-1-phenyl-1*H*-1,2,3-triazol-5-yl)methanone 3a. Method A, yield 58.1%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23−8.21 (m, 1 H), 8.09−8.06 (m, 1 H), 7.60−7.50 (m, 5 H), 6.87(d, J = 8.8 Hz, 1 H), 3.89 (br, 2 H), 3.24 (br, 6 H), 2.45 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.0, 153.6, 143.0 (2C), 136.4, 129.8, 129.7, 128.2 (2C), 126.6, 123.4, 123.3, 119.6, 50.4, 50.2, 46.4, 42.0, 10.5. HRMS (ESI) calcd for $C_{20}H_{20}ClN_6O_3$ $[M + H]^+$ 427.1280; found 427.1278. HPLC analysis: 75:25 methanol−water, 8.01 min, 95.3%.

dx.doi.org/10.1021/jm2013503 | *J. Med. Chem.* 2012, 55, 2144−2153

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(2-methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3b. Method A, yield 82.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 2.0 Hz, 1 H), 8.10 (dd, J = 2.4, 8.8 Hz, 1 H), 7.53 (d, J = 7.6 Hz, 1 H) 7.48 (t, J = 8.0 Hz, 1 H), 7.12 (t, J = 7.6 Hz, 1 H), 7.06 (d, J = 8.4 Hz, 1 H), 6.97 (d, J = 9.2 Hz, 1 H), 3.81 (br, 5 H), 3.47 (br, 2 H), 3.13 (br, 2 H), 2.93 (br, 2 H), 2.46 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.2, 153.8, 152.6, 142.9, 141.9, 131.3, 129.8, 128.1, 127.6, 126.6, 125.6, 123.4, 121.4, 119.6, 112.2, 56.1, 50.7, 50.3, 46.8, 42.0, 11.0. HRMS (ESI) calcd for $C_{21}H_{22}ClN_6O_4$ $[M + H]^+$ 457.1386; found 457.1384. HPLC analysis: 75:25 methanol−water, 7.60 min, 97.1%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(3-methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3c. Method A, yield 66.8%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26−8.20 (m, 1 H), 8.11−8.04 (m, 1 H), 7.40 (t, J = 8.0 Hz, 1 H), 7.15 (s, 1 H), 7.11 (d, J = 4.0 Hz, 1 H), 7.00 (d, J = 8.4 Hz, 1 H), 6.89 (d, J = 8.8 Hz, 1 H), 3.91 (br, 2 H), 3.84 (s, 3 H), 3.24 (br, 4 H), 2.43 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.5, 160.0, 153.6, 143.0, 142.9, 137.3, 130.5, 128.1 (2C), 126.6, 123.4, 119.6, 115.5, 115.0, 108.9, 55.7, 50.4, 50.2, 46.4, 42.0, 10.5. HRMS (ESI) calcd for $C_{21}H_{22}ClN_6O_4$ $[M + H]^+$ 457.1386; found 457.1379. HPLC analysis: 80:20 methanol−water, 6.89 min, 97.8%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(4-methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3d. Method A, yield 88.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J = 2.8 Hz, 1 H), 8.08 (dd, J = 2.8, 8.8 Hz, 1 H), 7.49 (d, J = 8.8 Hz, 2 H), 7.02 (d, J = 8.8 Hz, 2 H), 6.91 (d, J = 8.8 Hz, 1 H), 3.90 (br, 2 H), 3.85 (s, 3 H), 3.26 (br, 2 H), 3.11(br, 2 H), 2.75 (br, 2 H), 2.44 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.4, 160.0, 153.6, 142.9, 142.6, 129.4, 128.2, 128.1, 126.5, 124.7, 123.3, 119.6, 114.7, 55.6, 50.5, 50.2, 46.4, 41.9, 10.5. HRMS (ESI) calcd for $C_{21}H_{22}ClN_6O_4$ $[M + H]^+$ 457.1386; found 457.1386. HPLC analysis: 75:25 methanol−water, 9.07 min, 97.2%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(2-chlorophenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3e. Method A, yield 72.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.26 (d, J = 2.4 Hz, 1 H), 8.11 (dd, J = 2.4, 8.8 Hz, 1 H), 7.58−7.48 (m, 4 H), 7.01 (d, J = 9.2 Hz, 1 H), 3.85 (br, 2 H), 3.66 (br, 2 H), 3.14 (br, 4 H), 2.48 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.3, 153.7, 143.0, 141.4, 134.2, 131.5, 130.3, 130.1, 129.9, 129.3, 128.1, 128.0, 126.6, 123.4, 119.7, 50.9, 50.3, 47.0, 42.0, 11.2. HRMS (ESI) calcd for $C_{20}H_{19}Cl_2N_6O_3$ $[M + H]^+$ 461.0890; found 461.0878. HPLC analysis: 80:20 methanol−water, 6.75 min, 96.0%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(3-chlorophenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3f. Method A, yield 82.0%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J = 2.8 Hz, 1 H), 8.10 (dd, J = 2.4, 8.8 Hz, 1H), 7.63 (s, 1 H), 7.52−7.46 (m, 3 H), 6.94 (d, J = 9.2 Hz, 1 H), 3.94 (br, 2 H), 3.33 (br, 2 H), 3.16 (br, 2 H), 2.83 (br, 2 H), 2.45 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.7, 153.5, 143.0, 137.2, 135.5, 130.8, 129.7, 128.2, 128.1, 126.6, 123.4, 121.2, 119.7, 50.6, 50.2, 46.5, 42.1, 10.5. HRMS (ESI) calcd for $C_{20}H_{19}Cl_2N_6O_3$ $[M + H]^+$ 461.0890; found 461.0874. HPLC analysis: 80:20 methanol−water, 8.42 min, 97.5%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(4-chlorophenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3g. Method A, yield 63.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (s, 1 H), 8.10 (d, J = 8.8 Hz, 1 H), 7.56−7.50 (m, 4 H), 6.94 (d, J = 9.2 Hz, 1 H), 3.92 (br, 2 H), 3.30 (br, 2 H), 3.14 (br, 2 H), 2.83 (br, 2H), 2.44 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.8, 153.5, 143.1, 142.9, 135.7, 134.8, 130.0, 128.2(2C), 126.6, 124.5, 123.4, 119.7, 50.7, 50.3, 46.5, 42.0, 10.6. HRMS (ESI) calcd for $C_{20}H_{19}Cl_2N_6O_3$ $[M + H]^+$ 461.0890; found 461.0878. HPLC analysis: 80:20 methanol−water, 8.25 min, 97.3%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(2-hydroxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3h. Method A, yield 32.5%. ^{1}HNMR (400 MHz, DMSO-d_6) δ 10.51 (br, 1 H), 8.27 (d, J = 2.8 Hz, 1 H), 8.20 (dd, J = 2.8, 8.8 Hz, 1 H), 7.43 (dd, J = 2.4, 8.0 Hz, 1 H), 7.36−7.32 (m, 1 H), 7.29 (d, J = 8.8 Hz, 1 H), 7.02 (d, J = 8.0 Hz, 1 H), 6.99 (t, J = 8.0 Hz, 1 H), 3.70 (br, 2 H), 3.55 (br, 2 H), 3.20 (br, 4 H), 2.35 (s, 3 H). ^{13}CNMR (125 MHz, DMSO-d_6) δ 159.3, 154.1, 150.4, 141.9, 140.1, 130.6, 130.0 126.7, 126.4, 126.0, 124.1, 123.8, 120.7, 119.3, 116.5, 50.0, 49.7, 46.6, 41.4, 10.5. HRMS (ESI) calcd for $C_{20}H_{20}ClN_6O_4$ $[M + H]^+$ 443.1229; found 443.1224. HPLC analysis: 70:30 methanol−water, 9.53 min, 96.0%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(4-methyl-1-*o*-tolyl-1*H*-1,2,3-triazol-5-yl)methanone 3i. Method A, yield 92.8%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J = 2.8 Hz, 1 H), 8.09 (dd, J = 2.8, 8.8 Hz, 1 H), 7.43−7.37 (m, 2 H), 7.34−7.30 (m, 1 H), 7.26 (d, J = 8.4 Hz, 1 H), 6.93 (d, J = 8.8 Hz, 1 H), 3.82 (br, 2 H), 3.44 (br, 2 H), 3.06 (br, 2 H), 2.86 (br, 2 H), 2.47 (s, 3 H), 2.22 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.6, 153.6, 143.0, 141.9, 135.3, 135.0, 131.6, 130.4, 129.6, 128.2, 126.7, 126.6, 126.4, 123.4, 119.7, 50.8, 50.3, 46.6, 42.0, 17.8, 10.8. HRMS (ESI) calcd for $C_{21}H_{22}ClN_6O_3$ $[M + H]^+$ 441.1437; found 441.1428. HPLC analysis: 75:25 methanol−water, 9.24 min, 95.1%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(2-ethoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3j. Method B, yield 95.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 2.4 Hz, 1 H), 8.10 (dd, J = 2.4, 8.8 Hz, 1 H), 7.50−7.44 (m, 2 H), 7..11 (t, J = 7.6 Hz, 1 H), 7.06 (d, J = 8.0 Hz, 1 H), 6.92 (d, J = 9.2 Hz, 1 H), 4.08 (q, J = 7.2 Hz, 2 H), 3.79 (br, 2 H), 3.37 (br, 2 H), 3.08 (br, 2 H), 2.79 (br, 2 H), 2.48 (s, 3 H), 1.30 (t, J = 7.2 Hz, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$), δ 160.1, 153.8, 152.6, 142.9, 142.4, 131.4, 129.9, 128.1, 127.9, 126.6, 125.9, 123.4, 121.1, 119.6, 113.4, 65.0, 50.6, 50.3, 46.7, 42.0, 14.5, 10.9. HRMS (ESI) calcd for $C_{22}H_{24}ClN_6O_4$ $[M + H]^+$ 471.1542; found 471.1534. HPLC analysis: 75:25 methanol−water, 9.23 min, 96.7%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(2-isopropoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3k. Method B, yield 93.4%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 2.8 Hz, 1 H), 8.09 (dd, J = 2.4, 8.8 Hz, 1 H), 7.48−7.43 (m, 2 H), 7.11−7.06 (m, 2 H), 6.90 (d, J = 9.2 Hz, 1 H), 4.57−4.48 (m, 1 H), 3.79 (br, 2 H), 3.35 (br, 2 H), 3.08 (br, 2 H), 2.77 (br, 2 H), 2.48 (s, 3 H), 1.24 (s, 3 H), 1.22 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$), δ 159.9, 153.8, 152.1, 142.9, 142.5, 131.3, 130.0, 128.1(2C), 126.9, 126.6, 123.4, 121.1, 119.5, 115.3, 72.5, 50.7, 50.3, 46.7, 42.0, 21.9, 10.9. HRMS (ESI) calcd for $C_{23}H_{26}ClN_6O_4$ $[M + H]^+$ 485.1699; found 485.1695. HPLC analysis: 80:20 methanol−water, 7.70 min, 97.1%.

1-(2-(5-(4-(2-Chloro-4-nitrophenyl)piperazine-1-carbonyl)-4-methyl-1*H*-1,2,3-triazol-1-yl)phenyl)ethanone 3l. Method A, yield 64.1%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (d, J = 2.8 Hz, 1 H), 8.07 (dd, J = 2.4, 8.8 Hz, 1 H), 7.76 (dd, J = 1.2, 6.8 Hz, 1 H), 7.65−7.61 (m, 2 H), 7.55 (dd, J = 1.6, 7.6 Hz, 1 H), 6.90 (d, J = 4.8 Hz, 1 H), 3.85 (br, 2 H), 3.66 (br, 2 H), 3.06 (br, 2 H), 2.88 (br, 2 H), 2.53 (s, 3 H), 2.45 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.6, 159.6, 153.8, 142.8, 141.6, 136.7, 133.6, 131.8, 130.4, 130.1, 128.4, 128.1, 126.6, 123.3, 119.5, 51.0, 50.3, 46.7, 42.0, 29.4, 10.8. HRMS (ESI) calcd for $C_{22}H_{22}ClN_6O_4$ $[M + H]^+$ 469.1386; found 469.1383. HPLC analysis: 75:25 methanol−water, 7.57 min, 98.4%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(2,6-dimethoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3m. Method A, yield 85.7%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (d, J = 2.4 Hz, 1 H), 8.10 (dd, J = 2.4, 8.8 Hz, 1 H), 7.41 (t, J = 8.8 Hz, 1 H), 6.96 (d, J = 8.8 Hz, 1 H), 6.68 (d, J = 8.4 Hz, 2 H), 3.78 (s, 10 H), 3.51 (br, 2 H), 3.08 (br, 2 H), 2.96 (br, 2 H), 2.47 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.9, 155.9, 153.9, 142.9, 141.3, 131.8, 130.4, 128.1, 126.6, 123.4, 119.6, 114.5, 104.6, 56.4, 50.9, 50.5, 46.7, 41.9, 11.1. HRMS (ESI) calcd for $C_{22}H_{24}ClN_6O_5$ $[M + H]^+$ 487.1491; found 487.1492. HPLC analysis: 75:25 methanol−water, 7.13 min, 97.2%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(1-(2-methoxyphenyl)-1*H*-1,2,3-triazol-5-yl)methanone 3n. Method B, yield 86.5%. ^{1}H NMR (400 MHz, $CDCl_3$), δ 8.27 (d, J = 2.4 Hz, 1 H), 8.12 (dd, J = 2.4, 8.8 Hz, 1 H), 7.88 (s, 1 H), 7.59 (d, J = 7.6 Hz, 1 H), 7.49 (t, J = 7.6 Hz, 1 H), 7.15 (t, J = 7.6 Hz, 1 H), 7.06 (d, J = 8.4 Hz, 1 H), 7.01 (d, J = 8.8 Hz, 1 H), 3.85 (br, 2 H), 3.81 (s, 3 H), 3.69 (br, 2 H), 3.18 (br, 2 H), 3.06 (br, 2 H). ^{13}C NMR (125 MHz, $CDCl_3$), δ 159.1, 153.8, 152.3, 143.0, 132.9, 132.3, 131.3, 128.1, 127.4, 126.7, 125.5, 123.5, 121.4, 119.7, 112.1, 56.0, 50.7, 50.3, 47.1, 42.0. HRMS (ESI) calcd for $C_{20}H_{20}ClN_6O_4$ $[M + H]^+$ 443.1229; found 443.1232. HPLC analysis: 70:30 methanol−water, 9.82 min, 95.9%.

dx.doi.org/10.1021/jm2013503 | *J. Med. Chem.* 2012, 55, 2144−2153

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(4-ethyl-1-(2-methoxyphenyl)-1*H*-1,2,3-triazol-5-yl)methanone 3o. Method B, yield 96.2%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.25 (d, J = 2.4 Hz, 1 H), 8.17 (dd, J = 2.8, 8.8 Hz, 1 H), 7.55 (t, J = 8.0 Hz, 1 H), 7.49 (d, J = 7.2 Hz, 1 H), 7.27 (d, J = 8.0 Hz, 1 H), 7.14 (t, J = 7.6 Hz, 1 H), 3.78 (s, 3 H), 3.66 (br, 2 H), 3.48 (br, 2 H), 3.17 (br, 4 H), 2.75 (q, J = 7.6 Hz, 2 H), 1.28 (t, J = 7.6 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 159.5, 154.4, 153.0, 146.5, 142.4, 131.9, 130.0, 127.9, 126.9, 126.4, 125.4, 124.2, 121.3, 121.2, 113.2, 56.5, 50.6, 50.3, 46.8, 41.9, 18.6, 13.6. HRMS (ESI) calcd for $C_{22}H_{24}ClN_6O_4$ [M + H]$^+$ 471.1542; found 471.1545. HPLC analysis: 75:25 methanol−water, 9.17 min, 97.8%.

(4-(2-Chlorophenyl)piperazin-1-yl)(1-(2-methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3p. Method B, yield 91.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, J = 8.0 Hz, 1 H), 7.46 (t, J = 8.0 Hz, 1H), 7.36 (d, J = 8.0 Hz, 1 H), 7.22 (t, J = 7.6 Hz, 1 H), 7.11 (t, J = 7.6 Hz, 1H), 7.05 (d, J = 8.8 Hz, 1 H), 7.01 (t, J = 7.2 Hz, 1 H), 6.92 (d, J = 8.0 Hz, 1 H), 3.81 (br, 5 H), 3.45 (br, 2 H), 2.98 (br, 2 H), 2.80 (br, 2 H), 2.47 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.1, 152.7, 148.2, 141.7, 131.2, 130.7, 130.1, 128.9, 127.7, 127.6, 125.6, 124.5, 121.2, 120.3, 112.1, 56.0, 51.3, 50.8, 47.2, 42.3, 11.0. HRMS (ESI) calcd for $C_{21}H_{23}ClN_5O_2$ [M + H]$^+$ 412.1535; found 412.1537. HPLC analysis: 80:20 methanol−water, 6.21 min, 99.6%.

(1-(2-Methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)(4-(4-nitrophenyl)piperazin-1-yl)methanone 3q. Method B, yield 94.0%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.08 (d, J = 9.6 Hz, 2 H), 7.54−7.48 (m, 2 H), 7.24 (d, J = 8.4 Hz, 1 H), 7.13 (t, J = 7.6 Hz, 1 H), 7.02 (d, J = 9.6 Hz, 2 H), 3.76 (s, 3 H), 3.63 (br, 2 H), 3.51 (br, 2 H), 3.50 (br, 2 H), 3.37 (br, 2 H), 2.30 (s, 3 H). ^{13}CNMR (125 MHz, DMSO-d_6) δ 159.4, 154.7, 152.8, 141.2, 137.8, 131.7, 130.5, 127.7, 126.1, 125.5, 121.3, 113.3, 113.1, 56.5, 46.8, 46.1, 45.9, 41.5, 11.0. HRMS (ESI) calcd for $C_{21}H_{23}N_6O_4$ [M + H]$^+$ 423.1776; found 423.1773. HPLC analysis: 70:30 methanol−water, 5.58 min, 96.4%.

(1-(2-Methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)(4-phenylpiperazin-1-yl)methanone 3r. Method B, yield 86.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (d, J = 7.2 Hz, 1 H), 7.45 (t, J = 7.6 Hz, 1 H), 7.28 (t, J = 7.2 Hz, 2 H), 7.11 (t, J = 7.6 Hz, 1 H), 7.02 (d, J = 8.0 Hz, 1 H), 6.94−6.87 (m, 3 H), 3.79 (br, 5 H), 3.44 (br, 2 H), 3.13 (br, 2 H), 2.94 (br, 2 H), 2.46 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.0, 152.6, 150.6, 141.7, 131.2, 130.0, 129.3, 127.6, 125.6, 121.2, 120.9, 116.8, 112.1, 56.0, 49.7, 49.4, 46.8, 42.0, 11.0. HRMS (ESI) calcd for $C_{21}H_{24}N_5O_2$ [M + H]$^+$ 378.1925; found 378.1921. HPLC analysis: 70:30 methanol−water, 6.88 min, 98.3%.

(4-(2-Methoxy-4-nitrophenyl)piperazin-1-yl)(1-(2-methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3s. Method B, yield 93.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84 (d, J = 7.2 Hz, 1 H), 7.71(s, 1 H), 7.52 (d, J = 7.6 Hz, 1 H), 7.46 (t, J = 8.0 Hz, 1 H), 7.11 (t, J = 7.6 Hz, 1 H), 7.04 (d, J = 8.0 Hz, 1 H), 6.80 (d, J = 8.8 Hz, 1 H), 3.95 (s, 3 H), 3.80 (br, 5 H), 3.46 (br, 2 H), 3.15 (br, 2 H), 2.97 (br, 2 H), 2.46 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 160.1, 152.6, 151.3, 146.2, 142.8, 141.8, 131.2, 129.9, 127.6, 125.6, 121.3, 117.6, 116.9, 112.1, 106.6, 56.0(2C), 50.0, 49.6, 46.8, 42.0, 11.0. HRMS (ESI) calcd for $C_{22}H_{25}N_6O_5$ [M + H]$^+$ 453.1881; found 453.1881. HPLC analysis: 74:26 methanol−water, 5.69 min, 96.6%.

(4-(2-Chloro-4-nitrophenyl)-1,4-diazepan-1-yl)(1-(2-methoxyphenyl)-4-methyl-1*H*-1,2,3-triazol-5-yl)methanone 3t. Method A, yield 74.5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (d, J = 2.4 Hz, 1 H), 8.03−7.99 (m, 1 H), 7.47−7.41 (m, 2 H), 7.08−6.94 (m, 3 H), 3.86−3.84 (m, 1 H), 3.76 (br, 4 H), 3.51−3.49 (m, 1 H), 3.45−3.42 (m, 1 H), 3.36 (br, 1 H), 3.29 (br, 1 H), 3.19 (br, 2 H), 2.43 (s, 3 H), 2.05 (br, 1 H), 1.94 (br, 1 H), 1.82 (br, 1 H). HRMS (ESI) calcd for $C_{22}H_{24}ClN_6O_4$ [M + H]$^+$ 471.1542; found 471.1545. HPLC analysis: 80:20 methanol−water, 5.61 min, 99.8%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(5-methyl-3-phenyl-1*H*-pyrazol-4-yl)methanone 4a. Followed the procedure for **3**, method B, yield 60.2%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.49 (br, 1 H), 8.21 (d, J = 2.4 Hz, 1 H), 8.05 (dd, J = 2.4, 8.8 Hz, 1 H), 7.56 (d, J = 6.8 Hz, 2 H), 7.39−7.35 (m, 3 H), 6.82 (d, J = 9.2 Hz, 1 H), 3.92 (br, 2 H), 3.29 (br, 2 H), 3.11 (br, 2 H), 2.52 (br, 2 H), 2.29 (s, 3 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.7, 154.1, 142.7, 128.9, 128.8, 127.9, 127.4, 126.6, 123.4, 119.5, 112.1, 50.2, 46.9, 41.8, 10.7. HRMS (ESI) calcd for $C_{21}H_{21}ClN_5O_3$ [M + H]$^+$ 426.1328; found 426.1327. HPLC analysis: 75:25 methanol−water, 10.73 min, 98.1%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(3-(2-chlorophenyl)-5-methyl-1*H*-pyrazol-4-yl)methanone 4b. Method B, yield 45.6%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.10 (s, 1 H), 8.22 (d, J = 2.0 Hz, 1 H), 8.15 (d, J = 8.8 Hz, 1 H), 7.60−7.42 (m, 4 H), 7.14 (d, J = 8.8 Hz, 1 H), 3.48 (br, 4 H), 2.78 (br, 4 H), 2.32 (s, 3 H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 164.5, 154.1, 147.3, 141.8, 139.3, 132.8, 131.9, 129.5, 127.1, 126.3, 125.9, 123.8, 120.4, 113.0, 49.9(4C), 9.9. HRMS (ESI) calcd for 460.0938 [M + H]$^+$; found 460.0941. HPLC analysis: 80:20 methanol−water, 8.09 min, 95.7% in purity.

Biphenyl-2-yl(4-(2-chloro-4-nitrophenyl)piperazin-1-yl)-methanone 5a. Followed procedure for **2**, yield 80.6%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.19 (d, J = 2.8 Hz, 1 H), 8.04 (dd, J = 2.4, 8.8 Hz, 1 H), 7.53−7.34 (m, 9 H), 6.76 (d, J = 9.2 Hz, 1 H), 4.05−4.01 (m, 1 H), 3.58−3.52 (m, 1 H), 3.18−3.12 (m, 2 H), 3.00−2.96 (m, 1 H), 2.79−2.76 (m, 1 H), 2.67−2.61 (m, 1 H), 1.82−1.76 (m, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.0, 154.2, 142.6, 139.8, 138.3, 134.8, 129.7, 129.3, 128.9, 128.7, 128.0, 127.9, 127.8, 126.5, 123.3, 119.4, 49.9, 46.3, 41.3. HRMS (ESI) calcd for $C_{23}H_{21}ClN_3O_3$ [M + H]$^+$ 422.1266; found 422.1266. HPLC analysis: 80:20 methanol−water, 13.98 min, 98.6%.

(4-(2-Chloro-4-nitrophenyl)piperazin-1-yl)(2′-methoxybiphenyl-2-yl)methanone 5b. Yield 90.9%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (d, J = 2.4 Hz, 1 H), 8.06 (dd, J = 2.2, 8.8 Hz, 1 H), 7.48−7.31 (m, 7 H), 7.04−6.96 (m, 2 H), 6.82 (d, J = 8.8 Hz, 1 H), 3.87−3.81 (m, 1 H), 3.78 (s, 3 H), 3.62−3.60 (m, 1 H), 3.28−3.23 (m, 1 H), 3.13−3.11 (m, 2 H), 2.87−2.84 (m, 1 H), 2.69−2.65 (m, 1 H), 2.10−2.04 (m, 1 H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.1, 156.4, 154.2, 142.5, 135.4, 135.2, 131.5, 131.1, 129.4, 128.9, 128.5, 127.8(2C), 127.7, 126.5, 123.3, 120.7, 119.4, 110.8, 55.4, 50.3, 50.2, 46.4, 41.3. HRMS (ESI) calcd for 452.1372 [M + H]$^+$; found 452.1378. HPLC analysis: 80:20 methanol−water, 14.26 min, 99.2% in purity.

MDCK Cell-Based Anti-influenza Assay (CPE). Cell-based anti-influenza virus inhibitor screening was based on the principle of cytopathic effect (CPE) protection assay as described in Supporting Information.[33]

Cytotoxicity Assay. Procedure is similar to the CPE assay, but no virus was added.

NP Antigen Reduction Assay. Subconfluent MDCK cells were infected with 500 PFU of WSN virus in 96-well black clear bottom plates and treated with varied testing compounds. After a 24 h incubation, the cells were fixed and treated with 0.5% triton X-100 followed by NP antigen staining using anti-NP antibody (Millipore MAB8251) and FITC conjugated second antibody for fluorescence measurement (ex480, em535).

Plaque Reduction Assay. The plaque reduction assay was performed in 6-well tissue culture plates. The MDCK cells were seeded at 6 × 105 cells/well in DMEM (Invitrogen) with 1 mg/mL TPCK and 0.3% BSA and incubated overnight at 37 °C in a humidified 5% CO_2 incubator. The confluent monolayers of MDCK cells were then washed twice with 2 mL of Hanks and inoculated with 0.01MOI A/WSN/33 (H1N1) virus. After 1 h of virus adsorption at 37C, the viral inoculums were removed and the cells were washed twice with PBS. The cell monolayers were then overlaid with MEM medium containing 1% agar and serial compounds dilutions in the presence of 2 mg/mL trypsin and 0.3% BSA. Three days after infection, the monolayers were fixed and stained with 0.1% crystal violet solution. The number of virus-induced plaques was counted.

McIP Assay. The plaque reduction was also determined in a high throughput manner using the microplate counting of infectious particles (McIP) assay.[25] Briefly, the viral yields in the harvested conditional media after anti-influenza treatments were measured as the number of infection centers determined by NP staining after overnight infection of MDCK cells in microwells.

Immunofluorescence Microscopy. MDCK cells were seed to 35 mm culture plate or 6-well plates with coverslips and grow to 70−80% confluence, and then were infected with A/WSN/33 (H1N1) viruses

dx.doi.org/10.1021/jm2013503 | J. Med. Chem. 2012, 55, 2144−2153

at MOI of 10 in the presence or absence of 10 μM compounds. At 2.0, 4.0, and 6.0 h postinfection, the inoculums were aspirated and the cells were fixed by 4% paraformaldehyde at 4 C for 1 h. After fixation, the cells were washed and incubated with blocking buffer for 1 h and then incubated with FITC-tagged NP primary antibody (Abcam, MA, USA) in antibody dilution buffer (dilution 1: 20) for 2.0 h. The coverslips were then washed and counterstained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) for nucleus localization and mounted on slides using antifade mounting medium. The immunofluorescence images were photographed using a Leica laser scanning confocal microscope.

Computational Study. The structure of influenza virus nucleoprotein oligomers was retrieved from the Protein Data Bank (PDB code: 3ROS). The protein was processed using protein preparation wizard, which assigns bond orders and adds hydrogens and missing atoms. Compound **3b** was prepared in LigPrep (LigPrep, version 2.5, Schrödinger, LLC, New York, NY, 2011) module using OPLS-2005 force field. Molecular docking was performed in Glide module (Glide, version 5.7, Schrödinger, LLC, New York, NY, 2011). Compound **3b** was docked into the pocket at the dimer interface with extra precision scoring function.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures for key chemical intermediates, detail procedure for CPE assays, ^{1}HNMR, ^{13}C NMR, and HPLC spectrum for compound **2a**, **2b**, **3a**−**3t**, **4a**, **4b**, **5a**, and **5b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank National Basic Research Program of China (grant 2010CB529706, 2009CB940904), the 100-Talent program of Chinese Academy of Sciences (CAS), and key innovative drug discovery project of Guangzhou city (grant 2009Z1-E911, 2010J-E551) for their financial support. We also thank the scientists from Roche Pharma Research and Early Development (Shanghai) for the assistance to determine the anti-influenza activities of compound **1** and **3b** against A/New Jersey/8/76, A/Weiss/43, and A/Mal/302/54 strains.

■ ABBREVIATIONS USED

NP, nucleoprotein; RNA, ribonucleic acid; vRNP, virus ribonucleoprotein complex; CPE, cytopathic effect protection assays; MDCK, Madin-Darby canine kidney cells; AMD, amantidine; McIP, microplate counting of infectious particles assay; MOI, multiplicity of infection

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