

A Novel Endonuclease Inhibitor Exhibits Broad-Spectrum Anti-Influenza Virus Activity *In Vitro*

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Antiviral drugs are important in preventing and controlling influenza, particularly when vaccines are ineffective or unavailable. A single class of antiviral drugs, the neuraminidase inhibitors (NAIs), is recommended for treating influenza. The limited therapeutic options and the potential risk of antiviral resistance are driving the search for additional small-molecule inhibitors that act on influenza virus proteins. The acid polymerase (PA) of influenza viruses is a promising target for new antivirals because of its essential role in initiating virus transcription. Here, we characterized a novel compound, RO-7, identified as a putative PA endonuclease inhibitor. RO-7 was effective when added before the cessation of genome replication, reduced polymerase activity in cell-free systems, and decreased relative amounts of viral mRNA and genomic RNA during influenza virus infection. RO-7 specifically inhibited the ability of the PA endonuclease domain to cleave a nucleic acid substrate. RO-7 also inhibited influenza A viruses (seasonal and 2009 pandemic H1N1 and seasonal H3N2) and B viruses (Yamagata and Victoria lineages), zoonotic viruses (H5N1, H7N9, and H9N2), and NAI-resistant variants in plaque reduction, yield reduction, and cell viability assays in Madin-Darby canine kidney (MDCK) cells with nanomolar to submicromolar 50% effective concentrations (EC₅₀s), low toxicity, and favorable selective indices. RO-7 also inhibited influenza virus replication in primary normal human bronchial epithelial cells. Overall, RO-7 exhibits broad-spectrum activity against influenza A and B viruses in multiple *in vitro* assays, supporting its further characterization and development as a potential antiviral agent for treating influenza.

Influenza A and B viruses are significant human pathogens affecting approximately 5% to 10% of the global adult population annually (1). Vaccination programs are important for preventing and controlling influenza; however, vaccines lose their efficacy when there is an antigenic mismatch with the circulating viruses (2, 3) or when they are administered to high-risk groups, such as the elderly and very young (4). Therefore, antiviral drugs represent a critical, additional line of defense against seasonal influenza viruses and against emerging subtypes for which no vaccine may be available.

Two classes of virus protein-specific antiviral drugs are available for treating influenza, but of these, the drugs in the first class, the adamantanes (amantadine hydrochloride [amantadine] and rimantadine), are largely ineffective because of widespread virus resistance (5). The drugs in the second class, the neuraminidase (NA) inhibitors (NAIs), are currently the only drugs recommended for prophylaxis and treatment of influenza virus infection (6). Until 2007, a low (<0.3%) frequency of circulation of NAI-resistant influenza viruses was reported (7), but during the 2007–2009 seasons, naturally occurring oseltamivir-resistant A(H1N1) viruses with an H274Y NA substitution (N2 numbering is used here and throughout the text) were detected with high prevalence (approximately 90%) worldwide (5, 8). This lineage was later supplanted by the novel swine-origin pandemic A(H1N1)pdm09 influenza virus, which was susceptible to oseltamivir (5, 9). Antiviral surveillance reported a low (0.1% to 3%) frequency of biologically fit oseltamivir-resistant A(H1N1)pdm09 viruses between 2009 and 2015, but the possibility of the establishment and spread of such strains remains a major concern (10, 11). With NAIs being the only therapeutic option for treatment of influenza virus infection, the potential development of NAI-resistant strains poses a grave threat to public health. Thus, there is an urgent need to identify and characterize novel influenza antiviral drugs.

The past decade has witnessed a renewed interest in identifying and characterizing experimental influenza virus inhibitors that target viral proteins or host factors. Several virus protein-targeted compounds, including inhibitors of virus binding, entry, fusion, or genome replication, are undergoing preclinical or clinical evaluation (8). The latter group includes compounds that target viral polymerase functions and represent a promising avenue for antiviral exploration. Such compounds are already used to treat other viral infections, including those by human immunodeficiency virus type 1 (HIV-1) (12, 13) and hepatitis B and C viruses (14, 15). Influenza A viruses have three polymerase proteins: basic polymerase 1 (PB1) is an RNA-dependent RNA polymerase (RdRp); basic polymerase 2 (PB2) binds host-cell capped mRNAs; and the acid polymerase (PA) possesses endonuclease activity that results in removal of the mRNA caps (16). The drugs in the nucleoside analogue class of polymerase inhibitors, including ribavirin and favipiravir (T-705), inhibit influenza virus replication (17). However, ribavirin is not recommended for clinical use because of its high toxicity (17–19), and T-705 is still under clinical evaluation in the United States (20) and has only a narrow range of approved

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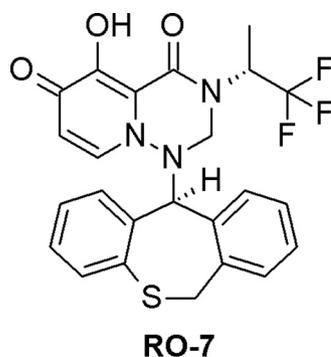


FIG 1 Chemical structure of RO-7.

uses (against NAI-resistant pandemic viruses) in Japan (21). The recent availability of high-quality structural information on the influenza virus PB1, PB2, and PA proteins has led to the development of non-nucleoside analogue inhibitors that directly interact with the polymerases to disrupt protein-protein interactions (PA and PB1; 22–24) or inhibit the function(s) of the proteins. The vast majority of these experimental polymerase inhibitors target the PA protein (reviewed in references 25, 26, and 27).

The PA protein of influenza A and B viruses possesses endonuclease activity that is necessary to cleave host mRNA caps in order to initiate viral transcription (28). This process is essential to completing the replication cycle and is widely recognized as a prime antiviral target. PA endonuclease inhibitors, including the 4-substituted 2,4-dioxobutanoic acid derivatives (29, 30) and the substituted 2,6-diketopiperazine natural product flutimide (31, 32), were characterized 2 decades ago in enzymatic, cell-based, and limited *in vivo* assays (29), where they demonstrated potent (micromolar 50% inhibitory concentrations [IC₅₀s]) and cap-dependent inhibition of influenza A and B viruses. Subsequently, marchantins, catechins (33, 34), hydroxamic acid and N-hydroxyimides (35, 36), 3-hydroxypyridin-2(1H)-one derivatives (37, 38), 3-hydroxyquinolin-2(1H)-one derivatives (39), fullerene derivatives (40), and carboxamide derivatives (41) were identified as PA protein inhibitors. In many of these studies, a library of compound derivatives was screened with a limited number of influenza viruses and/or assays, making it difficult to compare the reported efficacies accurately (42). Here, we evaluated the *in vitro* anti-influenza virus potency of a novel compound, RO-7 (Fig. 1), which is a representative of a new class of highly active influenza inhibitors with a mechanism of action different from that of the inhibitors that are currently approved to treat influenza virus infection (18, 43). We present a characterization of the mechanism of action of RO-7 (i.e., inhibition of the PA endonuclease activity) and demonstrate that it is active against a wide range of influenza A and B viruses, including seasonal subtypes, subtypes with pandemic potential, and oseltamivir-resistant viruses.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells and human embryonic kidney (HEK293T) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in modified Eagle's medium (MEM; Cellgro, Manassas, VA) and Opti-MEM (Fisher, Grand Island, NY), respectively, supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT). Primary normal human bronchial epithelial (NHBE) cells (Lonza, Walkersville, MD) were obtained from 2 donors

(healthy males aged 2 and 4 years) and grown in culture in an air-liquid interface (ALI) system on Transwell inserts (Corning, Tewksbury, MA). The apical surfaces of the cells were exposed to humidified 95% air and 5% CO₂ for 6 weeks before use. The basal surfaces were maintained in bronchial epithelial basal medium (BEBM; Lonza) supplemented with SingleQuots growth factors (Lonza).

Viruses. Influenza A and B viruses were obtained from the St. Jude Children's Research Hospital influenza virus repository and propagated in MDCK cells for 48 h at 33 to 37°C in serum-free MEM containing L-tosylamido 2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington, Lakewood, NJ) (1 µg/ml).

Laboratory facilities. Experiments using highly pathogenic influenza A(H5N1) or minimally pathogenic A(H7N9) and A(H9N2) viruses were conducted in a biosafety level 3 enhanced containment laboratory in accordance with USDA 9 CFR 121 and USDA 7 CFR 331.

Antiviral compounds. RO-7 and oseltamivir carboxylate (oseltamivir) were synthesized at Hoffmann-La Roche Ltd. (Basel, Switzerland) in collaboration with WuXi AppTec (Wuhan, China). RO-7 was prepared as a 10 mM stock in dimethyl sulfoxide (DMSO) and was soluble when diluted in various reaction mixtures and cell culture media. Final DMSO concentrations in antiviral assays ranged from 0.05% to 0.2% at the highest RO-7 concentrations tested; these concentrations of DMSO were replicated in mock-infected and vehicle control wells and did not induce a loss of cell viability or cytopathic effect (CPE). Oseltamivir was prepared as a 5 mM stock in distilled water. Ribavirin (Sigma-Aldrich, St. Louis, MO), epigallocatechin gallate (EGCG; Sigma-Aldrich), and amantadine hydrochloride (amantadine; Sigma-Aldrich) were prepared as 10 mM stocks in distilled water. All compound stocks were stored at –20°C until use. In tissue culture assays, RO-7 and ribavirin were added after virus adsorption, while amantadine was present 1 h before, during, and after virus adsorption.

Cytotoxicity and cell viability assays. MDCK cells (1.5 × 10⁴) were plated in 96-well plates and treated with RO-7 (1 pM to 1 mM) in serum-free or 5% FCS-containing MEM. At 48 h postinoculation (hpi), the cell viability was measured with a CellTiter-Glo luminescent assay (Promega, Madison, WI). To determine the viability of virus-inoculated cells in the presence of RO-7, MDCK cells were plated as described above and inoculated with influenza virus (multiplicity of infection [MOI] of 0.01), and the cell viability was determined at 48 hpi. The 50% cytotoxic concentrations (CC₅₀s) were determined by using the log (inhibitor) versus response logistic nonlinear regression equation in GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA). For cell viability assays, the metabolic activity of the negative-control wells (with no drug) was set at 100%, and the percentage of reduction was calculated for each RO-7 concentration; the 50% effective concentrations (EC₅₀s) were calculated in the same manner as the CC₅₀ values. For each assay, the mean of 3 or 4 independent determinations for each RO-7 concentration was used for the calculations.

Plaque number reduction assays. MDCK cells (1 × 10⁶) were plated in 6-well plates and inoculated with A/California/04/2009 (H1N1)pdm09 virus (MOI of 0.01), washed 3 times with phosphate-buffered saline (PBS), and overlaid with MEM containing 0.45% immunodiffusion-grade agarose (MP Biomedical, Solon, OH), 1% bovine serum albumin (BSA), 1 µg/ml TPCK-trypsin, and RO-7 (0.01 to 500 nM). At 48 hpi, the overlays were removed and the cell monolayers were stained with 1% crystal violet–10% formaldehyde. The mean number of plaques in each well was calculated, and the EC₅₀s were determined by using the log (inhibitor) versus response logistic nonlinear regression equation in GraphPad Prism 6.0 software. In each case, the selective index (SI) was determined by calculating the ratio of the CC₅₀ to the EC₅₀.

Virus yield reduction assay. MDCK cells (2.5 × 10⁵) were plated in 24-well plates, inoculated with influenza viruses (MOI of 0.01), and grown in culture in MEM containing 1% BSA, 1 µg/ml TPCK-trypsin, and RO-7 (0.01 to 100 nM). At 48 hpi, the supernatants were collected and the 50% tissue culture infectious doses (TCID₅₀s) were determined by the

TABLE 1 RO-7 reduction of influenza A and B virus infectious yield and CPE in MDCK cells

Influenza virus genus/subtype	Strain	Oseltamivir susceptibility ^a	Virus yield reduction assay (degree of inhibition, log ₁₀ TCID ₅₀ /ml) ^b		CPE reduction assay (EC ₅₀ ± SD, nM) ^c
			5 nM	50 nM	
A(H1N1)	A/Mississippi/03/2001	R (H274Y)	2.9	7.0	2.3 ± 0.1
	A/Memphis/13/2006	S	1.5	8.8	5.0 ± 0.2
	A/Brisbane/59/2007	S	2.1	8.2	21.6 ± 1.8
	A/Hawaii/28/2007	R (H274Y)	<	8.1	1.1 ± 0.6
	A/Georgia/20/2006	R (H274Y)	0.8	7.9	11.7 ± 4.9
Avg			1.8 ± 0.9	8.0 ± 0.7	8.3 ± 8.5
A(H1N1)pdm09	A/Perth/265/2009	S	0.8	6.0	3.9 ± 1.4
	A/Perth/261/2009	R (H274Y)	2.6	6.9	1.2 ± 0.5
	A/California/7/2009	S	1.5	8.2	1.3 ± 0.2
	A/Denmark/524/2009	S	3.2	6.5	3.2 ± 3.2
	A/Denmark/528/2009	R	0.8	5.1	ND
	A/New York/3467/2009	R (H274Y)	1.7	8.5	9.7 ± 2.3
	A/New York/1692/2009	R (H274Y)	0.9	8.3	12.9 ± 2.3
	A/Memphis/43/2013	S	<	7.3	19.4 ± 1.4
A/Tennessee/F5034/2014	S	1.3	6.9	4.1 ± 2.4	
Avg			1.6 ± 0.9	7.1 ± 1.1	7.0 ± 6.5
A(H3N2)	A/Fukui/20/2004	S	2.2	5.7	7.5 ± 2.7
	A/Fukui/20/2004	R (E119V)	5.0	7.1	12.6 ± 4.5
	A/Perth/16/2009	S	1.6	6.3	ND
	A/Victoria/361/2011	S	2.3	6.8	ND
	A/Tennessee/F4039/2013	S	2.8	5.4	3.8 ± 2.9
	A/Memphis/2/2015	S	2.3	7.4	ND
A(H3N2)v	A/Indiana/08/2011	S	2.2	8.2	9.9 ± 8.0
Avg			2.6 ± 1.1	6.7 ± 1.0	8.5 ± 4.0
B	B/Lee/1940	S	<	6.2	20.1 ± 4.3
	B/Victoria/02/87	S	<	5.7	14.2 ± 6.0
	B/Perth/211/2001	S	<	6.7	10.5 ± 5.4
	B/Perth/211/2001	R (D198E)	<	6.5	13.2 ± 2.9
	B/Brisbane/60/2008	S	0.9	5.8	13.3 ± 2.1
	B/Wisconsin/01/10	S	<	7.8	5.2 ± 3.0
B avg			<	6.4 ± 0.8	12.8 ± 4.9
A avg			2.0 ± 1.0	7.2 ± 1.1	7.7 ± 6.3

^a S, susceptible to oseltamivir with absence of known markers of NAI resistance; R, resistant to oseltamivir with indicated NA substitution, N2 numbering.

^b Data represent reductions of virus yield (log₁₀ TCID₅₀ per milliliter) from infected MDCK cells (MOI of 0.01, *n* = 3 wells/drug concentration/virus) at 48 hpi as determined by titration in MDCK cells. Average values are presented with standard deviations. <, titers were below the assay limit of detection (0.75 log₁₀ TCID₅₀/ml).

^c Data represent RO-7 reductions of virus-induced (MOI of 0.01) CPE in MDCK cells (*n* = 3 wells/drug concentration/virus) as determined by CellTiter-Glo luminescent cell viability assay at 48 hpi. ND, not done.

method of Reed and Muench (44) and used to determine EC₅₀s by using the log (inhibitor) versus response logistic nonlinear regression equation in GraphPad Prism 6.0 software. For screening multiple viruses (Tables 1, 2, and 3), data from the virus yield reduction assay were expressed as degrees of inhibition of virus replication (log₁₀ TCID₅₀ per milliliter reduction) by RO-7 at either 5 and 50 nM (MDCK cells) or 3 and 30 nM (NHBE cells) (45). SI values were determined as in the plaque reduction assays (described above).

Antiviral activity in NHBE cells. The apical surfaces of NHBE cells were washed with PBS and equilibrated at 37°C for 30 min with infection medium (BEBM supplemented with 0.5% BSA). Cells were inoculated with influenza viruses (MOI of 0.01) for 1 h and then washed with 2 acidic

washes (PBS, pH 2.2) and 3 neutral washes (PBS, pH 7.4) to remove unbound virus. RO-7 (3 and 30 nM) was then added to the basal medium. At 48 hpi, 300 μl of BEBM was added to the apical surfaces for 30 min and then harvested for virus titration in MDCK cells. The degree of inhibition of virus replication (log₁₀ TCID₅₀ per milliliter reduction) was determined in the manner described above. For each assay in the different donor cells, the means of the results determined for 2 independent cell inserts for each RO-7 concentration were used for the EC₅₀ calculations.

Time-of-addition assay. MDCK cells (2.5 × 10⁵) were plated in 24-well plates overnight and then washed with PBS. RO-7 (50 or 500 nM) or ribavirin (100 μM) was added at the following different times before or

TABLE 2 RO-7 reduction of emerging influenza A virus infectious yield and CPE in MDCK cells

Influenza A virus subtype	Strain	HA clade ^a	Oseltamivir susceptibility ^b	Virus yield reduction assay (degree of inhibition, log ₁₀ TCID ₅₀ /ml) ^c		CPE reduction assay (EC ₅₀ ± SD, nM) ^d
				5 nM	50 nM	
A(H5N1)	A/Hong Kong/483/1997	0	S	2.8	8.4	14.9 ± 1.5
	A/Vietnam/1203/2004	1	S	1.5	6.9	15.7 ± 0.4
	A/Shenzen/1/2011	2.3.2.1	S	1.8	7.8	4.9 ± 2.7
	A/Hong Kong/5923/2012	2.3.2.1	S	1.9	7.5	19.0 ± 14.4
	A/Egypt/MOH-NRC-7305/2014	2.2.2	S	1.1	6.4	14.9 ± 4.5
Avg				1.8 ± 0.6	7.4 ± 0.8	13.9 ± 5.3
A(H7N9)	A/Anhui/1/2013	N/A	S	1.0	5.5	17.4 ± 4.7
	A/Shanghai/1/2013	N/A	R (R292K)	1.6	8.1	13.3 ± 1.8
Avg				1.3 ± 0.4	6.8 ± 1.8	15.4 ± 2.9
A(H9N2)	A/Hong Kong/1073/1999	G1	S	2.2	8.2	12.2 ± 2.3
Overall avg				1.7 ± 0.6	7.3 ± 1.0	14.0 ± 4.3

^a Hemagglutinin (HA) clade designations refer to the respective subtypes. N/A, not applicable.

^b S, susceptible to oseltamivir with absence of known markers of NAI resistance; R, resistant to oseltamivir with indicated NA substitution, N2 numbering.

^c Data represent reductions of virus yield (log₁₀ TCID₅₀/ml) from infected MDCK cells (MOI of 0.01, *n* = 3 wells/drug concentration/virus) at 48 hpi as determined by titration in MDCK cells. Average values are presented with standard deviations.

^d Data represent reductions of virus-induced (MOI of 0.01) cytopathic effect in MDCK cells (*n* = 3 wells/drug concentration/virus) as determined by CellTiter-Glo luminescent cell viability assay at 48 hpi.

after inoculation with A/California/04/2009 (H1N1)pdm09 virus (MOI of 2.0): 1 h before inoculation (−1); during virus adsorption (0); or 1, 2, 4, or 6 h after inoculation (+1, +2, +4, or +6 h). At 10 hpi, the supernatants were harvested and the virus titers were assessed by TCID₅₀ assay in MDCK cells. The mean titers were calculated from triplicate measures for each RO-7 concentration and time point of addition.

Influenza mini-replicon assay. The NP, PA, PB1, and PB2 genes from A/California/04/2009 (H1N1)pdm09 influenza virus were cloned into the pHW2000 plasmid, propagated in One Shot Top10 chemically competent *Escherichia coli* (Invitrogen), and purified (Qiagen, Valencia, CA). The

polymerase activity in the presence of RO-7 (4 to 500 nM) or ribavirin (100 μM) was measured as described previously (46). Briefly, HEK293T cells were treated with compounds for 3 h at 37°C and then transfected with 0.1 μg of virus-gene plasmids, pHW72-luciferase (under the control of the influenza M gene noncoding region), and pCMV-β-galactosidase (under the control of a constitutively active cell promoter) by using TransIT-LT1 transfection reagent (Mirus, Madison, WI). The inhibitory compound concentrations were maintained during the transfection process, and after 24 h, the cells were disrupted with lysis buffer (Promega). Luciferase activity (detected with the Promega luciferase assay system) and

TABLE 3 RO-7 reduction of influenza A virus infectious yield in primary NHBE cells

Influenza virus genera/subtype	Strain	Oseltamivir susceptibility ^a	Virus yield reduction assay (degree of inhibition, log ₁₀ TCID ₅₀ /ml) ^b			
			Donor 1		Donor 2	
			3 nM	30 nM	3 nM	30 nM
A(H1N1)	A/Brisbane/59/2007	S	2.4	7.1	2.3	4.4
	A/Hawaii/28/2007	R (H274Y)	2.6	7.3	<	7.8
A(H1N1)pdm09	A/Perth/265/2009	S	<	3.1	1.3	1.8
	A/Perth/261/2009	R (H274Y)	1.6	2	1.5	3.6
A(H3N2)	A/Fukui/20/2004	S	2.4	5.6	1.5	3
	A/Fukui/20/2004	R (E119V)	1.4	3.1	ND	ND
A(H5N1)	A/Vietnam/1203/04	S	4.6	4.6	1	5.4
Influenza B	B/Perth/211/2001	S	<	3.6	1	2.4
	B/Perth/211/2001	R (D198E)	<	6.5	<	6.5
Avg			2.5 ± 1.1	4.8 ± 1.9	1.4 ± 0.5	4.4 ± 2.1

^a S, susceptible to oseltamivir with absence of known markers of NAI resistance. R, resistant to oseltamivir with indicated NA substitution, N2 numbering.

^b Data represent reductions of virus yield (log₁₀ TCID₅₀/ml) from infected NHBE cells (MOI of 0.01, *n* = 2 wells/drug concentration/virus) at 48 hpi as determined by titration of virus yield in MDCK cells. Average values are presented with standard deviations. <, titers were below the assay limit of detection (0.75 log₁₀ TCID₅₀/ml); ND, not done.

β -galactosidase activity (detected with *o*-nitrophenyl β -D-galactopyranoside [ONPG] substrate [Sigma-Aldrich]) were measured on a Synergy 2 plate reader (BioTek, Winooski, VT). The luciferase values were normalized to β -galactosidase activity. The data are presented as values representing the mean polymerase activity of triplicate measures for RO-7 and are representative of the results of 3 independent experiments. The EC₅₀ was determined as described above.

vmRNA and vRNA analysis. MDCK cells (2.2×10^5) were plated in 24-well plates, inoculated with influenza viruses (MOI of 5.0) as described above, and grown in culture in MEM containing 1% BSA and 1 μ g/ml TPCK-trypsin. RO-7 (50 to 5,000 nM) or ribavirin (100 μ M) was added to each well. At 7 hpi, the cells were lysed and the total RNA was isolated (RNeasy minikit; Qiagen). The levels of strand-specific virus mRNA (vmRNA) and virus genomic RNA (vRNA) for the nucleoprotein (NP) gene were quantified as described previously (47). Briefly, cDNAs complementary to each RNA species were synthesized from 200 ng total RNA by using gene-specific primers that were 5' tagged with 18 to 20 nucleotides of a non-influenza virus sequence in hot start, saturated trehalose (48) reverse transcription reactions. Quantitative PCR (qPCR) was performed with Power SYBR green PCR master mix (Invitrogen) using cDNA templates, the non-influenza virus "tag" primer, and an NP gene-specific primer. The numbers of RNA copies were determined from the cycle threshold values (ΔC_T) as fold changes in values relative to those determined for the controls (virus infected, no drug treatment) as follows: $[2^{(\text{treated } \Delta C_T - \text{control } \Delta C_T)}]$ (49).

Endonuclease inhibition assay. Endonuclease activity assays were performed as described previously (33, 34, 42, 50). Briefly, the 209-residue N-terminal domain of the influenza A virus PA protein (PA_N) was cloned and His tag purified (42). Recombinant PA_N was kindly provided by Stephen White (St. Jude Children's Research Hospital). PA_N (2 μ g) was incubated with the single-stranded M13mp18 DNA plasmid (TaKaRa, Shiga, Japan) (0.3 μ g) in the presence of RO-7 (0 to 10 μ M) or EGCG (100 μ M) (as a positive control) (33) in digestion buffer (20 mM Tris [pH 7.3], 100 mM NaCl, 10 mM β -mercaptoethanol, 2 mM MnCl₂ [pH 8.3]) for 1.5 h at 37°C. The total reaction volume was 19 μ l, and the final concentration of PA_N in the mixture was approximately 4.4 μ M. The reactions were quenched with 20 mM EDTA and resolved on 1.0% agarose gels. PA-mediated endonuclease activity was indicated by the digestion and laddering of the M13mp18 band (approximately 2 kb). The data presented are representative of the results of at least 3 independent experiments.

NAI susceptibility. Oseltamivir susceptibility was determined by a modified fluorometric assay using the fluorogenic substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (Munana; Sigma-Aldrich). Influenza viruses were standardized to equivalent NA activity and incubated with oseltamivir (5×10^{-7} to 2 μ M). The fluorescence of the released 4-methylumbelliferone was measured in a Synergy 2 microplate reader (Biotek) at excitation and emission wavelengths of 360 nm and 460 nm, respectively. The IC₅₀s were determined as described above and compared to the values reported for a panel of reference influenza A and B viruses provided by the Antiviral Group of the International Society for Influenza and Other Respiratory Virus Diseases (ISIRV) (51).

Statistical analysis. The data presented are representative of or consist of combined data from at least 3 independent experiments, as indicated above. The results represent the means \pm standard deviations of duplicate (for NHBE cell data) or at least triplicate (for all other data) determinations. The inhibitory activity 50% endpoints in each assay were determined by nonlinear regression curve fitting using the log (inhibitor) versus response logistic equation using GraphPad Prism 6.0 software.

RESULTS

RO-7 inhibits transcription and genome replication steps in the influenza virus cycle. Compound RO-7 (Fig. 1), a polycyclic carbamoyl pyridone derivative with good physicochemical properties (kinetic solubility, 130 μ g/ml; Log D, 2.6; pK_a, 7.8), represents a novel class of influenza replication inhibitors with a mechanism

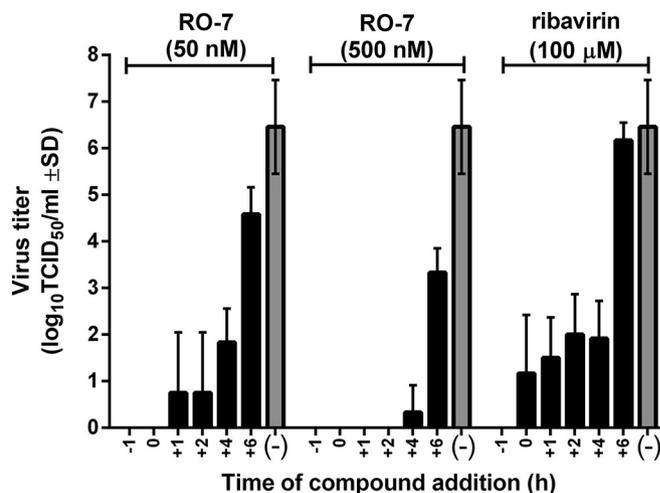


FIG 2 Effect of time of RO-7 addition on inhibition of A/California/04/2009 (H1N1)pdm09 influenza virus replication in MDCK cells. MDCK cells were inoculated with A/California/04/2009 (H1N1)pdm09 virus (MOI of 2) in medium containing RO-7 (50 or 500 nM) or ribavirin (100 μ M) that was added 1 h before inoculation (-1 h), during virus adsorption/inoculation (0), or 1, 2, 4, or 6 h after inoculation (+1, +2, +4, +6 h). Untreated controls [0 μ M drug; (-)] are indicated by gray bars. At 10 hpi, the supernatants were collected and the virus titers were determined in MDCK cells. Values are means \pm standard deviations (SD) and representative of the results of at least 3 independent experiments.

of action different from that of currently marketed compounds (18). To assess the stage of the influenza viral replication cycle that is affected by RO-7, a time-of-addition experiment was performed with A/California/04/2009 (H1N1)pdm09 virus. As a positive control, we used ribavirin (100 μ M), a purine analog that inhibits the replication of influenza viruses by multiple mechanisms, including the inhibition of RdRp (45, 52). Adding RO-7 before virus inoculation, during virus adsorption, or at 2 to 4 hpi resulted in a dose-dependent inhibition of virus replication in MDCK cells (Fig. 2). These early events encompass virus binding and entry, as well as the transcription of viral mRNA (vmRNA) and replication of viral genomic RNA (vRNA), which are dependent on the viral polymerases. RO-7 was less effective when added after 4 hpi, resulting in inhibition levels of only 29% at 50 nM and 49% at 500 nM compared to the levels seen with untreated controls (Fig. 2), and is thus less effective at late stages of replication (when vRNA replication predominates) and/or genome packaging (53). A similar trend was observed with ribavirin, which had no inhibitory activity when added later than 4 hpi (Fig. 2).

RO-7 also inhibited activity of the influenza polymerase complex in a virus-free and cell-free mini-genome system (46). HEK293T cells were transfected with A/California/04/2009 (H1N1)pdm09 (NP, PA, PB1, and PB2) plasmids and an influenza M gene-driven luciferase reporter plasmid. RO-7 exhibited a dose-dependent inhibition of polymerase activity compared to control results (Fig. 3A), with a mean IC₅₀ of 12.0 nM (Fig. 3B). Further, RO-7 treatment specifically inhibited the products of the influenza virus polymerase complex, i.e., synthesis of vmRNA and vRNA. Virus-inoculated cells treated with RO-7 or the control drug ribavirin (100 μ M) yielded fewer copies of both RNA species, resulting in copy number levels that were up to 3.6-fold or 2.5-fold lower than those seen with controls (0 μ M drug), respectively

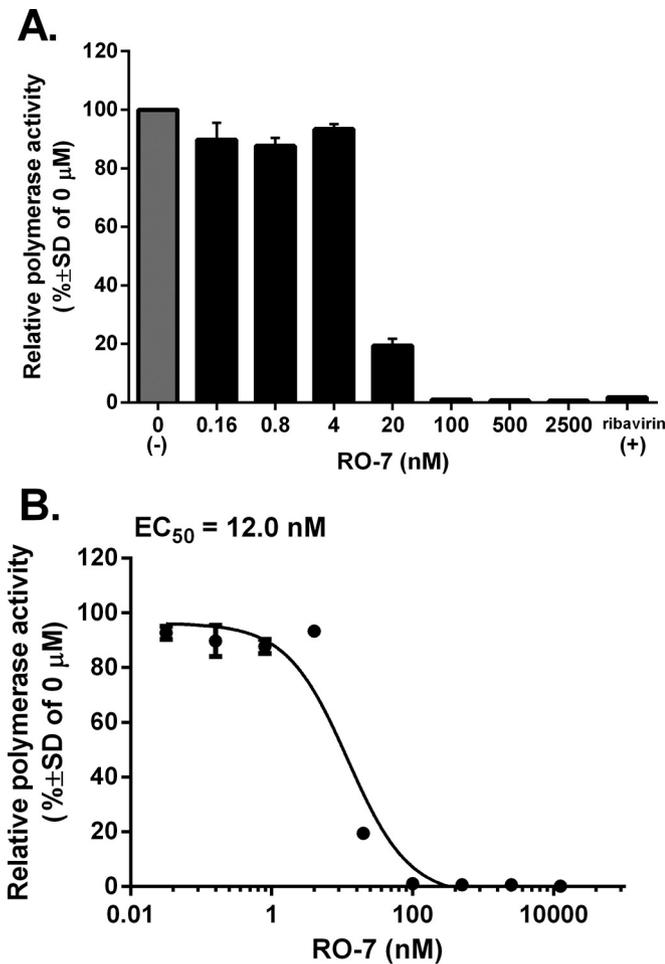


FIG 3 RO-7 inhibition of influenza A/California/04/2009 (H1N1)pdm09 influenza virus polymerase activity in the mini-genome assay. HEK293T cells were transfected with plasmids expressing viral proteins (NP, PA, PB1, PB2) along with luciferase and β -galactosidase reporters and were treated with RO-7 (0.16 nM to 10 μ M) or ribavirin (100 μ M) 3 h before and 24 h after transfection. The polymerase activity was measured relative to that in the untreated control (0 μ M drug, gray bar) as luciferase activity normalized to β -galactosidase activity (A) and was used to construct a dose-response curve and determine IC_{50} values (B). Values are means \pm SD of triplicate determinations and representative of the results of 3 independent assays.

(Fig. 4). Thus, we showed that RO-7 is an influenza virus replication inhibitor that is active during those stages in the viral replication cycle encompassing transcription and genome replication.

RO-7 inhibits endonuclease activity of influenza A virus PA protein. The endonuclease activity of the influenza virus PA polymerase protein is critical for cleaving mRNA caps to initiate vRNA transcription (28). We tested the ability of RO-7 to inhibit the enzymatic activity of the N-terminal endonuclease domain (PA_N). PA_N endonuclease activity with respect to a single-stranded DNA substrate was inhibited in the presence of the positive-control EGCG compound (100 μ M) (Fig. 5) (33). PA_N endonuclease activity was also variably inhibited by RO-7 at a concentration of approximately 10 nM (Fig. 5), similarly to cell-based assays, demonstrating its endonuclease inhibitory activity.

Cytotoxicity and antiviral activity of RO-7 in MDCK cells. It is critical that novel therapeutic compounds have no adverse effect

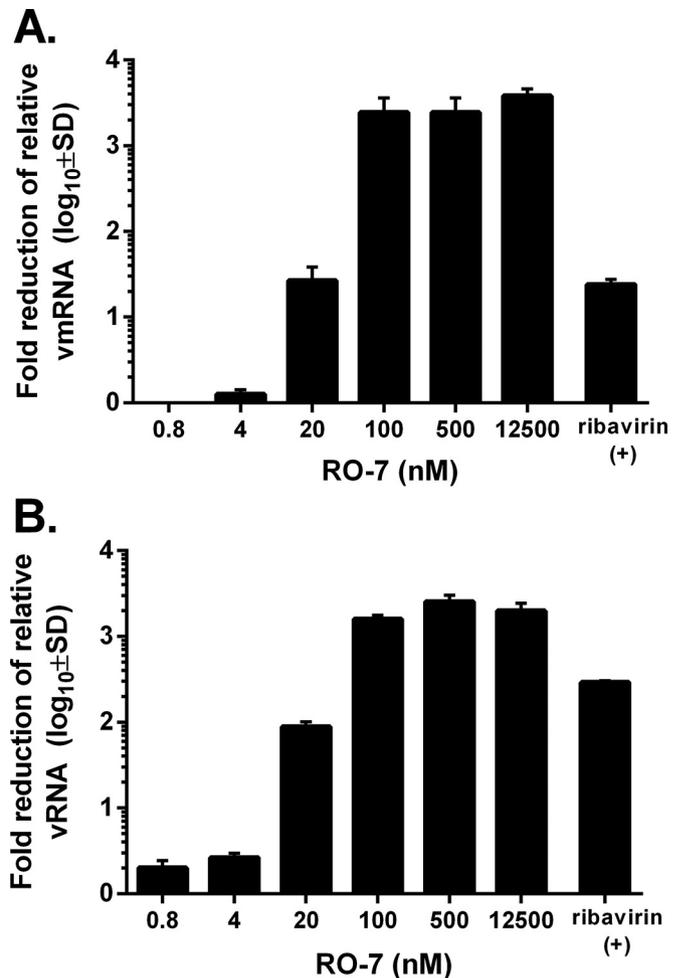


FIG 4 Inhibition of viral mRNA (vmRNA) and viral genomic RNA (vRNA) synthesis by RO-7. MDCK cells were inoculated with A/California/04/2009 (H1N1)pdm09 virus (MOI of 5.0) in the presence of RO-7 (0.8 nM to 12.5 μ M) for 6 to 7 hpi, and the total RNA was isolated from lysates. The vmRNA (A) and vRNA (B) levels were quantified by quantitative PCR using a SYBR green platform and are presented as the fold reduction (\log_{10}) of levels of relative RNA species compared to control levels (0 μ M drug). Values are means \pm SD of triplicate determinations and representative of the results of 3 independent assays.

on host-cell processes and cytopathology. Therefore, we determined the cytotoxicity of RO-7 for MDCK cells, the cell line used for the subsequent examination of antiviral activity. In these cells, the CC_{50} was 11.9 μ M in serum-free medium, whereas adding 5% FCS increased the CC_{50} nearly 7-fold (to 86.2 μ M) (Fig. 6A). In all of the experiments whose descriptions follow, we used RO-7 at concentrations of 10 μ M or less in FCS-free medium.

To evaluate the activity of RO-7 against influenza viruses *in vitro*, we initially performed 3 assays in MDCK cells, namely, a plaque number reduction assay, a virus yield reduction (TCID₅₀) assay, and a cell viability assay. Using the A/California/04/2009 (H1N1)pdm09 virus, we observed mean EC_{50} s of 11.3 nM and 16.0 nM in the plaque number and virus yield reduction assays, respectively (Fig. 6B and C). In a liquid culture of the virus yield reduction assay (where the virus spread was not artificially constrained) and in the plaque number reduction assay, RO-7 exhibited similar levels of inhibitory activity. Additionally, in the cell

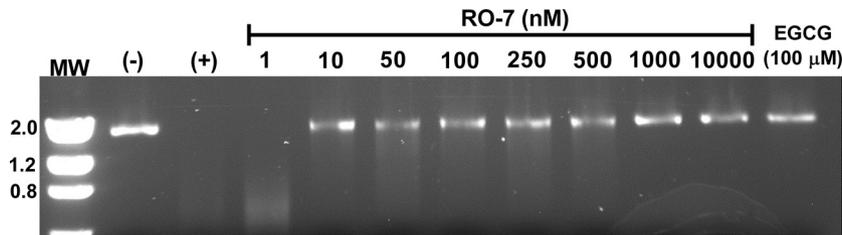


FIG 5 Inhibition of influenza A virus PA_N endonuclease activity by RO-7. The single-stranded M13mp18 DNA plasmid was incubated with (+) or without (-) PA_N in the presence of RO-7 (1 nM to 10 μM) or EGCG (100 μM) (pH 8.3). The samples were resolved on a 1.0% agarose gel with molecular-weight markers (MW, left). The agarose gel image is representative of the results of at least 3 independent experiments.

viability assay, RO-7 protected MDCK cells from death, with a mean EC₅₀ of 3.2 nM (Fig. 6D). The CC₅₀ and nanomolar EC₅₀s of RO-7 resulted in favorable selective indices (SI = CC₅₀/EC₅₀) as determined in the plaque number reduction (SI = 1,053.1), virus yield reduction (SI = 743.8), and cell viability (SI = 3718.8) assays. Thus, RO-7 exhibits activity against influenza A virus in cell-based assays, with minimal cytotoxicity.

RO-7 activity against NAI-resistant and -susceptible seasonal influenza A and B viruses. To test the antiviral efficacy of RO-7 against a wide range of influenza viruses, we assessed its inhibition of a panel of seasonal influenza A viruses (H1N1 and H3N2 subtypes [*n* = 21]) and influenza B viruses (Yamagata and Victoria lineages [*n* = 6]). Overall, RO-7 inhibited the replication

of influenza A viruses in MDCK cells by 2.0 log₁₀ TCID₅₀/ml at 5 nM and by 7.2 log₁₀ TCID₅₀/ml at 50 nM, with an EC₅₀ of 7.7 nM in the cell viability assays (Table 1). For influenza B viruses, RO-7 inhibited the replication by 6.4 log₁₀ TCID₅₀/ml at 50 nM, with a mean EC₅₀ of 12.8 nM in the cell viability assays (Table 1). The mean viability assay EC₅₀s were similar to those observed with A/California/04/2009 (H1N1)pdm09 virus (Fig. 6C and D), as were the mean SIs for influenza A viruses (SI = 1,541.5) and B viruses (SI = 933.3). Importantly, influenza A viruses carrying the common NAI resistance-associated H274Y and E119V NA substitutions, as well as influenza B virus with a D198E NA substitution, were susceptible to RO-7, demonstrating the potential of that compound for controlling NAI-resistant viruses. In summary,

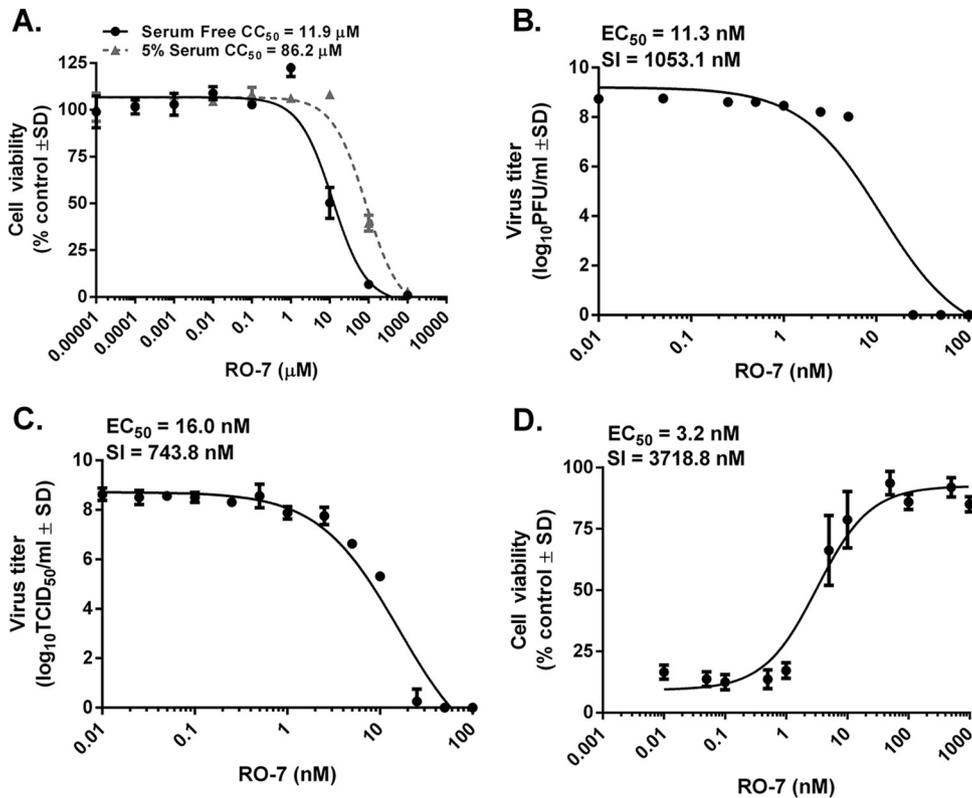


FIG 6 Cytotoxicity and antiviral activity of RO-7 against A/California/04/2009 (H1N1)pdm09 influenza virus in MDCK cells. (A) MDCK cells were treated with RO-7 (1 pM to 1 mM) with or without FCS. Cell viability was determined at 48 hpi, and the data are presented as a percentage of the control (0 μM drug) viability. CC₅₀ values are representative of triplicate dose-response curves ± SD. (B to D) MDCK cells were inoculated with A/California/04/2009 (H1N1)pdm09 (MOI of 0.01), and the inhibitory activity of RO-7 was determined in a plaque number reduction assay (B), a virus yield (TCID₅₀) reduction assay (C), or a cell viability assay (D) at 48 hpi. The EC₅₀s were calculated from triplicate dose response curves, and the SIs were calculated as the ratio of the CC₅₀ to the EC₅₀. Values are means ± SD and representative of the results of at least 3 independent experiments.

RO-7 has potent and selective inhibitory activity against seasonal influenza A and B viruses, including NAI-resistant variants.

RO-7 activity against avian-origin A(H5N1), A(H7N9), and A(H9N2) influenza viruses. Influenza A viruses are zoonotic pathogens, residing most prominently in avian reservoirs. Occasionally, avian-origin influenza A viruses are transmitted to humans, causing severe disease with high mortality. This has occurred with highly pathogenic (HPAI) A(H5N1) (54), with minimally pathogenic A(H9N2) (55), and, most recently, with the A(H7N9) influenza viruses (56). Therefore, we extended the panel of tested viruses and examined the activity of RO-7 against avian-origin human influenza A viruses ($n = 8$). At a 5 nM concentration, RO-7 reduced the yield of H5N1, H7N9, and H9N2 viruses by 1.3 to 1.8 log₁₀ TCID₅₀/ml. At a 50 nM concentration, those yields were reduced by 6.8 to 7.4 log₁₀ TCID₅₀/ml (Table 2). The mean EC₅₀s in the cell viability assays were 13.9 nM for influenza A(H5N1) virus, 15.4 nM for influenza A(H7N9) virus, and 12.2 nM for influenza A(H9N2) virus (Table 2), consistent with the EC₅₀s observed with human seasonal influenza viruses. Additionally, RO-7 was effective against NAI-resistant A/Shanghai/1/2013 (H7N9) virus carrying an R292K NA substitution (57). Thus, the *in vitro* efficacy of RO-7 is not confined to seasonal human viruses; RO-7 is also efficacious against virulent zoonotic subtypes.

RO-7 inhibition of influenza A and B virus replication in differentiated primary NHBE cells. To determine whether RO-7 can inhibit influenza A and B virus replication in primary human cells, we tested its antiviral efficacy in differentiated NHBE cells that retain the physiologic and structural functions of the primary site of influenza virus replication, the human airway (58). RO-7 inhibited replication of a panel of influenza A and B viruses in NHBE cells. At a 3 nM concentration, RO-7 reduced the yield of influenza A viruses by up to 2.5 log₁₀ TCID₅₀/ml and inhibited replication of a single influenza B virus in NHBE cells from one donor. At a 30 nM concentration, RO-7 inhibited replication of the influenza A and B viruses tested by 4.4 to 4.8 log₁₀ TCID₅₀/ml in NHBE cells from both donors (Table 3), suggesting that it is effective in physiologically relevant airway cells infected with different influenza viruses.

DISCUSSION

The list of antiviral drugs recommended for the prevention and treatment of influenza infection is limited. NAIs remain the only influenza virus-specific therapeutic agents recommended for use in the United States (6, 18). Therefore, the potential circulation of NAI-resistant virus poses a serious threat, and this justifies the pursuit of novel inhibitors with different viral targets, including the polymerase proteins. Here, we have characterized a novel inhibitor and demonstrated that inhibition of the endonuclease domain of the PA protein confers broad-spectrum activity against influenza A and B viruses of multiple lineages, subtypes, origins, and NAI-susceptibility phenotypes.

In this report, we have characterized the activity of this compound in a variety of assays and platforms: the plaque number reduction, virus yield (TCID₅₀) reduction, and cell viability assays (5, 59). Our results across 36 influenza A and B viruses demonstrate the breadth of anti-influenza activity of RO-7. In all cases, the viruses were highly susceptible to RO-7 and had favorable SIs. Furthermore, the EC₅₀s and SIs were similar for seasonal viruses and for emerging zoonotic A(H5N1), A(H9N2), and A(H7N9)

viruses, suggesting that this inhibitor is potentially useful in a pandemic scenario. A final advantage of our study was the inclusion of primary NHBE cells. In these physiologically relevant cells, RO-7 inhibited all the viruses tested at concentrations in the nanomolar to submicromolar range, and its efficacy should, therefore, be tested in future *in vivo* studies.

An *in vitro* comparison of RO-7 to existing clinical anti-influenza compounds such as the NAIs is difficult because cell culture-based assays are not accurate methods for determining NAI susceptibility (51, 60). However, RO-7 may possess an *in vivo* benefit over NAIs because it acts before the last step in the virus life cycle, preventing a full round of replication and potentially decreasing the magnitude of triggered inflammatory responses. Additional benefits of RO-7 are that it acts upon a protein target different from that seen with the NAIs and is potent against NAI-resistant virus infections and could be used in combination therapy to limit the selection of resistant virus variants (61).

We observed that RO-7 had EC₅₀s *in vitro* that were lower than those of other inhibitors that act upon internal processes and target viral structural proteins. In the CPE reduction assay, RO-7 EC₅₀s were 10.8 nM, which is consistent with previous data (Fig. 1; see also Table S1 in the supplemental material), compared to 0.3 μM for amantadine (amantadine-susceptible viruses) (62, 63) and 31.6 μM for ribavirin (see Table S1). Results of tissue culture assays and conditions can differ greatly between laboratories, and those differences complicate a comparison of RO-7 to other experimental polymerase inhibitors. Nevertheless, RO-7 in our studies was active in the nanomolar range, giving results similar to reported values for cap-binding inhibitors VX-787 in a CPE reduction assay (3.2 nM versus 1.6 nM) (64) and BPR3P0128 in a plaque reduction assay (11.3 nM versus ≥29 nM) (65). RO-7 has low toxicity in MDCK cells that is within the range of that of the endonuclease inhibitors flutimide (CC₅₀, ≥10 μM; 34) and 4-substituted 2,4-dioxobutanoic acid L-742,001 (CC₅₀, >100 μM; 29). The tissue culture EC₅₀s observed with RO-7 were severalfold lower than those observed in published studies performed with flutimide (EC₅₀, 5.9 μM; 32), L-742,001 and its derivatives (0.8 to 11 μM; 29, 30, 41, 66), tetramic acid compounds (EC₅₀, 21 to 100 μM; 36), and marchantins and fullerenes (EC₅₀, 43 to 100 μM; 40). RO-7 inhibited PA_N activity at concentrations as low as 10 nM in non-cell-based plasmid cleavage enzymatic assays conducted at pH 8.3. We note that this function was pH dependent, and assays performed at neutral pH yielded IC₅₀s in the 0.5 to 2 μM range (data not shown).

A critical concern with any antimicrobial agent is the development of drug resistance. Resistance-associated substitutions in the M2 protein of influenza A viruses are quickly generated during amantadine therapy, and the frequency of amantadine-resistant variants among circulating seasonal A(H1N1) and A(H3N2) subtypes is >90% (10, 67). Lessened resistance selection rates are among the advantages of NAIs, but influenza A viruses such as the clade 2B lineage of A/Brisbane/59/2007-like (H1N1) have been shown to escape oseltamivir pressure without a loss of viral fitness (68–70). Oseltamivir-resistant A(H1N1)pdm09 viruses carrying the H274Y NA substitution could be efficiently transmitted via contact and respiratory droplet routes in ferret and guinea pig animal models (71–73). Substitutions that confer resistance to polymerase inhibitors would be expected to significantly decrease virus fitness (74, 75). Serial passage in cells with various nucleoside analogues, such as ribavirin, T-705, 5-azacytidine, and 5-fluorou-

racil, often fails to yield specific substitutions in the polymerase proteins because of the lethal mutagenesis mechanism of these compounds (76–78). However, this mechanism is different from that employed by RO-7; it is unknown to what degree the viral PA protein can escape from RO-7 drug pressure and still retain suitable endonuclease activity. The PA protein is highly conserved among all influenza A viruses (79), with critical enzymatic domains retaining 99% homology (42); it is less likely to tolerate resistant mutations (25, 80, 81). PA inhibitor-resistant mutants have been generated by cell culture passage for L-742,001 (30, 66), a 2-substituted-4,5-dihydropyrimidine derivative (42). This L-742,001-resistant mutant strain (PA T20A) was significantly more impaired in replication capacity (3-fold EC₅₀ change) than the zanamivir-resistant mutant (NA Q199G) that arose through passages in MDCK cells within the same study (~30,000-fold EC₅₀ change), suggesting a profile of lower resistance for this endonuclease inhibitor than for the NAI (66). Additionally, results of a recent study by Song and colleagues suggest that other substitutions beyond PA T20A are unlikely to arise through serial passage in MDCK cells. Amino acid substitutions in the endonuclease domain of the PA protein, such as I79L, F105S, and E119D, were observed only after application of PCR mutagenesis, virus rescue, and sequential passage of rescued viruses in the presence of LL-742,001 (82). These mutations induced a 2- to 29.4-fold change in IC₅₀ or EC₅₀s compared to wild-type virus results. An examination of influenza A virus resistance potential in the face of RO-7 is under way.

Our data from analyses of NHBE cells demonstrate that RO-7 retains its inhibitory activity in physiologically relevant respiratory cells and could, therefore, also be active in animal models. Similar conclusions have been drawn from testing existing NAIs and experimental antiviral compounds in human lung tissue explants (83). Future exploration of the *in vivo* potential of RO-7, including an analysis of dosage, therapeutic window, and route(s) of inoculation, is therefore warranted.

In summary, the novel compound RO-7 is a promising antiviral agent that broadly inhibits influenza virus replication by targeting the viral PA protein endonuclease. Further investigation is warranted of this and other similar inhibitors as potential therapeutic agents against influenza.

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