ACS Medicinal Chemistry Letters

Letter

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Identification and Synthesis of Quinolizidines with Anti-Influenza A Virus Activity

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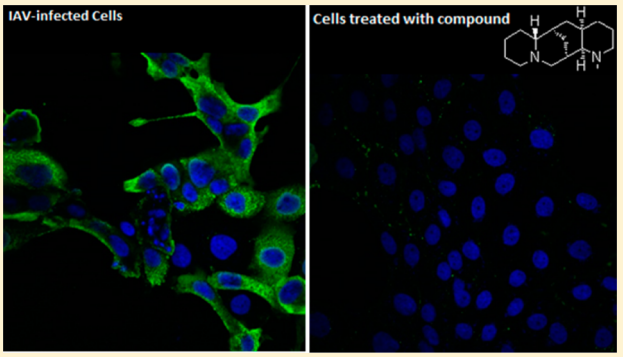
S *Supporting Information*

ABSTRACT: Influenza A virus infection causes a contagious respiratory illness that poses a threat to human health. However, there are limited anti-influenza A therapeutics available, which is further compounded by the emergence of drug resistant viruses. In this study, Sophora quinolizidine alkaloids were identified as a new class of anti-influenza A virus agents. Among the tested Sophora alkaloids, dihydroaloperine exhibited the most potent activity with an EC_{50} of 11.2 μM. The potency of the quinolizidine alkaloids was improved by approximately 5-fold with chemical modifications on the aloperine molecule. These compounds were effective against an H1N1 influenza A virus that was resistant to the two antiflu drugs oseltamivir and amantadine. The identification of the quinolizidine alkaloids as effective and novel anti-influenza A agents may aid in the development of new therapeutics.



KEYWORDS: *Influenza, influenza inhibitor, nucleoprotein*

Influenza A virus (IAV) infection causes a contagious pandemic respiratory illness that afflicts millions of people annually. IAV infection could have a lethal consequence, as evidenced by the 1918 pandemic that caused 30 to 50 million deaths worldwide.[1] The 2009 pandemic swine flu (H1N1) resulted in the death of an estimated 284,000 people.[2] IAV evolved a promiscuous entry process that uses sialic acid-containing molecules as receptors to enter cells.[3] This allows the virus to infect cells across various animal species. Avian influenza viruses, such as H5N1, have circulated in recent years. Although transmission of avian flu viruses to humans is inefficient, they are highly pathogenic and may pose a threat if the viruses acquire the ability for efficient human-to-human transmission.

The influenza A virus genome contains eight negative-stranded RNA segments, which encode for 11 viral proteins: hemagglutinins (HA1; HA2), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), nonstructural protein 1 (NS-1), polymerase acidic protein (PA), polymerase basic proteins (PB1; PB2; PB1-F2), and neuraminidase (NA).[4] Two NA inhibitors, oseltamivir and zanamivir, are the FDA-approved influenza antiviral drugs recommended by the US CDC for use against recently circulating influenza viruses.[5] However, emerging drug resistance and limited effectiveness associated with the NA inhibitors were reported.[6−8] There are other older drugs, amantadine and rimantadine targeting the M2 ion channel, approved for treatment and prevention of influenza A. However, many strains of influenza, such as the 2009 H1N1 influenza virus, are resistant to these two drugs.[9] Thus, novel anti-influenza virus agents are needed to circumvent the limitations of current drugs.

Quinolizidine alkaloids are known principal constituents in *Sophora* species (*Fabaceae*).[10,11] Crude extractions from some *Sophora* species containing quinolizidine alkaloids have been used in traditional Chinese medicinal preparations for centuries.[12] Modern pharmacological studies have shown that natural products isolated from Sophora plants may protect against viral infections. For example, some flavonoids isolated from *Sophora flavescens* were found to inhibit the neuraminidase of IAV[13] or suppress the IAV-induced pro-inflammatory chemokine production in lung epithelial cells.[14] Several quinolizidine alkaloids including oxymatrine and matrine are known to have antihepatitis B virus (HBV) activity.[15] Thus, to investigate if the quinolizidine alkaloids can inhibit the IAVs,

Received: June 3, 2014
Accepted: June 30, 2014
Published: June 30, 2014

   dx.doi.org/10.1021/ml500236n | *ACS Med. Chem. Lett.* 2014, 5, 942−946

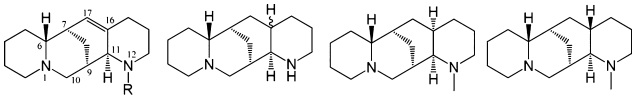
several Sophora alkaloids were tested for their antiviral activity against IAV. The results of this study indicated that aloperine (**1**), a lupine alkaloid found in *Sophora* species, exhibited the best anti-IAV activity.[16] Aloperine was subsequently used as a scaffold to derive compounds with improved activity. Compounds with improved activity were also used in mechanism of action studies, and the results suggest that these compounds may inhibit influenza virus infection by targeting the nucleoprotein of IAV.

Quinolizidine Alkaloids and Their Antiviral Activities. Pure alkaloids from the *Sophora* species, including aloperine, matrine, and oxymatrine, were tested for their activity against a mouse-adapted influenza virus A/Puerto Rico/8/34 (H1N1) (PR8). These structurally related alkaloids are called quinolizidine alkaloids because of their common bicyclic quinolizidine core. Ring extensions from the quinolizidine core form subtypes of quinolizidine alkaloids including tricyclic compounds such as cytisine (**5**) and tetracyclic compounds such as (+)- and (−)-sparteine (**3** and **4**), aloperine (**1**), matrine (**8**), and oxymatrine (**7**) (Table 1). Among the tested quinolizidine alkaloids, aloperine protected MDCK cells from being killed by IAV PR8 at an EC_{50} of 14.5 μM. Hydrogenation of aloperine at C16/C17 double bond resulted in a racemic mixture of dihydroaloperine (**2**), which was slightly more potent than aloperine with an EC_{50} of 11.2 μM. Sparteines also showed activity against this IAV strain but were approximately 2-fold less potent than aloperine. Matrine, oxymatrine, cytisine, and methyl cytisine did not inhibit IAV PR8 infection of MDCK cells. The two antiflu drugs oseltamivir and amantadine were ineffective against the PR virus, with EC_{50} greater than 80 μM (Table 1). The compounds listed in Table 1 were not toxic to MDCK cells at 80 μM. These results suggest that some quinolizidine alkaloids, such as aloperine and sparteines, can inhibit IAV infection.

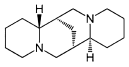
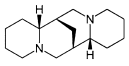
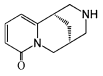
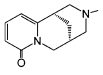
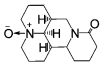
Improved Anti-IAV Potency of Aloperine Derivatives. Among the active compounds (**1**−**4**), sparteines (**3** and **4**) are more compact and lack convenient functional groups for further modifications. In contrast, **1** and **2** possess a secondary amine that is easy to modify.[17] To further increase the anti-IAV activity, a series of N12 modified aloperine derivatives (**9**−**18**) were synthesized by using a reductive amination method with suitable aldehydes and sodium triacetoxyborohydride (Scheme 1 and also described in the Supporting Information). Compounds **9**−**14** are aloperine derivatives with varied N12 alkyl groups.

The small substitution on N12 increased the potency slightly as shown by **9** (*N*-methyl) and **10** (*N*-ethyl) when compared to aloperine (NH). Larger *N*-substitution also resulted in derivatives with anti-IAV activity as shown by **11**−**16**. Among them, **16** exhibited the most potent activity against IAV PR8 with an EC_{50} of 2.4 μM. Hydrogenation of **9** yielded **17** and **18** as a mixture of stereoisomers at C16, which were separated by HPLC. The *cis* and *trans* configurations (C11/C16) of the decahydroquinonoline subunit in **17** and **18** were determined based on NMR data included in the Experimental Section (Supporting Information). Compound **17** was one of the more potent compounds and had preferred physicochemical properties among the synthesized derivatives (Mass = 248; Log *P* = 2.05). Compound **17** inhibited the oseltamivir resistant IAV PR8 strain with an EC_{50} of 6.2 μM, which was greater than 2-fold more potent than **1** with an unmodified N12 amine and unsaturated C16−C17 bond. Compound **17** was also slightly more potent than its C16−C17 unsaturated analog **9**.

Table 1. Anti-IAV Activities of Quinolizidine Alkaloids and Derivatives[a]



1, 9 -16 2 17 18

Compd[b]	Structure or R	EC_{50}	CC_{50}	LogP[c]
1	H	14.5±4.2	>80	1.27
2		11.2±3.3	>80	1.67
3		19.7±4.9	>80	
4		21.3±5.6	>80	
5		>80	>80	
6		>80	>80	
7		>80	>80	
8		>80	>80	
9	Me	9.6±2.3	>80	1.65
10	Et	7.7±2.4	>80	1.98
11	*i*-Bu	34.5±7.2	>80	2.87
12		15.1±4.5	>80	2.37
13		18.8±3.9	>80	2.79
14		29.3±6.8	>80	3.62
15		5.5±1.8	>80	2.74
16		2.4±0.83	>80	3.63
17		6.2±2.1	>80	2.05
18		8.1±2.2	>80	2.05
Nucleozin		0.71±0.155	>80	
Oseltamivir		>80	>80	
Amantadine		>80	>80	

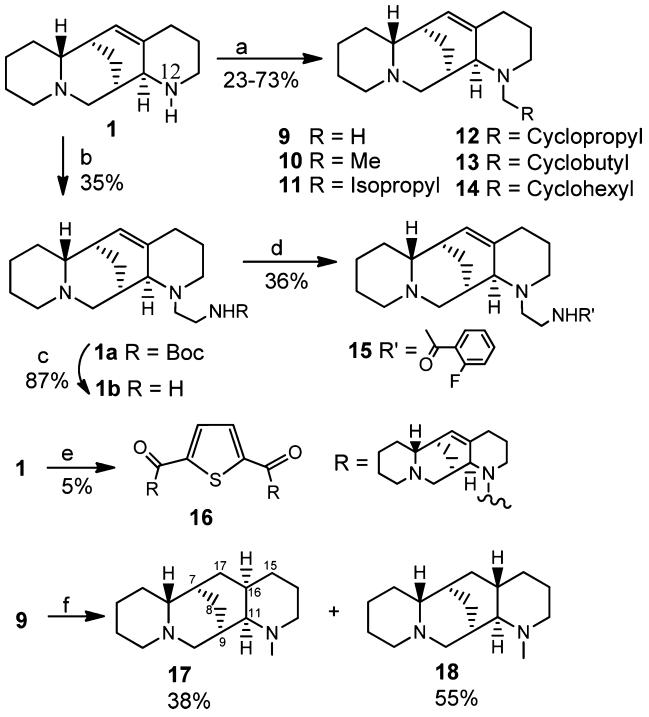
[a]A mouse-adapted influenza virus strain A/Puerto Rico/8/1934, PR8, was used in the assays; EC_{50} (μM) is the concentration required to protect the cytocidal effect of the influenza virus PR8 by 50%, which was presented as mean ± SD from three independent tests; CC_{50} (μM) is the concentration that reduced the viability of MDCK cells by 50%. [b]Compounds **1**−**9** are known compounds. [c]Log *P* values were estimated by using ChemBioDraw Ultra v. 12.0.2 (CambridgeSoft).

Compound **18**, the *trans* isomer of **17**, exhibited comparable anti-PR8 activity to that of **17** (Table 1).

Aloperine Derivatives Are Effective against the Oseltamivir Resistant Virus PR8 (H1N1) and Sensitive Virus VR1679 (H3N2). The predominant current subtypes of influenza A viruses found in humans are influenza A (H1N1) and influenza A (H3N2) viruses.[18] The PR8 used in this study is very resistant to oseltamivir, as shown in Table 1. The aloperine derivative **17** was chosen to test its effects on PR8 and VR1679 due to the simplicity of its structure and synthesis.

dx.doi.org/10.1021/ml500236n | ACS Med. Chem. Lett. 2014, 5, 942−946

Scheme 1. Synthesis of Compounds 9−18[a]



[a]Reagents and conditions: (a) $NaBH(AcO)_3$, R-CHO, DCE, RT; (b) *N*-Boc-2-bromo-ethylamine, K_2CO_3, MeCN, 110 °C; (c) 55% TFA/DCM, RT; (d) 2-fluorobenzoic acid, EDC-HCl; DIEA, THF, RT; (e) 2,2-thiophendicarboxylic acid, EDC-HCl; DIEA, THF, RT; (f) H_2/Pd−C, MeOH, RT.

In contrast to PR8, VR1679 was very sensitive to oseltamivir (Figure 1). Oseltamivir inhibited VR1679 with an EC_{50} of 0.33

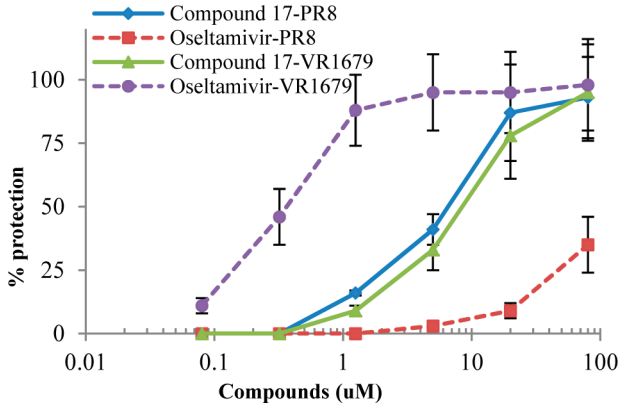


Figure 1. Compound **17** inhibited the oseltamivir resistant IAV PR8. MDCK cells were infected with PR8 or VR1679 at MOI = 1 in the presence of **17** or oseltamivir at various concentrations for 2 days. Protection of MDCK cells from being killed by the virus (% protection) was calculated with a formula detailed in the Supporting Information. Each data point in the figure represents mean ± standard deviation of three independent experiments.

μM, which was at least 200-fold more potent than that against the H1N1 virus PR8. On the other hand, **17** was approximately equally potent against the two IAVs. These results suggest that aloperine derivatives are likely to have a different mechanism of action from that of oseltamivir.

Compound 17 and Oseltamivir Did Not Share the Same Mechanism of Action. Oseltamivir is an NA inhibitor that blocks the release of the influenza virus from infected cells.[19] Therefore, oseltamivir does not prevent IAV entry or production of viral proteins inside the cells.[20] To further investigate the mechanistic differences in IAV inhibition between aloperine derivatives and oseltamivir, VR1679 was used to infect MDCK cells in the presence of the compounds for 6 h. The viral NP was detected using a confocal microscope after immunostaining. NP was stained green with FTIC-anti-NP mAbs, while the cell nuclei were stained blue with diamidino-2-phenylindole (DAPI).

As expected, oseltamivir at 10 μM did not inhibit NP production (Figure 2). In fact, VR1679 infected MDCK cells

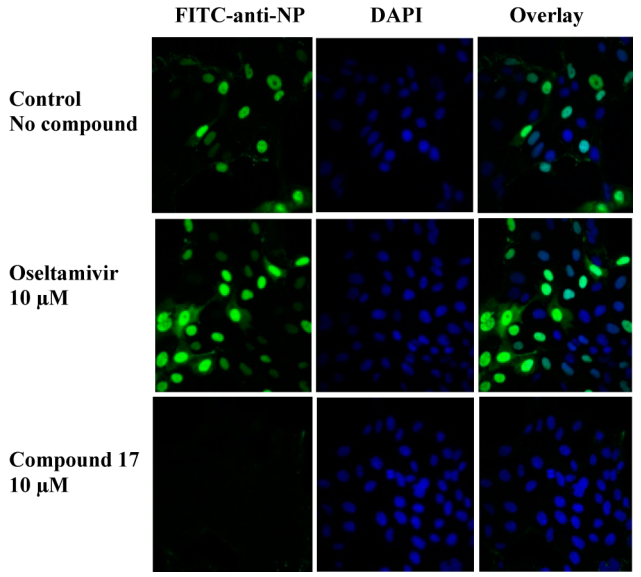


Figure 2. Compound **17** inhibited the accumulation of NP in MDCK cells. MDCK cells were infected with VR1679 at MOI = 1 in the presence of 10 μM oseltamivir or compound **17** for 6 h. The cells were stained with FITC conjugated anti-NP antibodies and DAPI (nuclear staining). The three images in the top panels were used as controls with viral infection in the absence of compounds. The confocal images were acquired using a Nikon TE2000-U laser-scanning confocal microscope.

appeared to accumulate more NP in the presence of oseltamivir than without the drug. It is likely that inhibition of IAV release from infected MDCK cells resulted in a backlog of NP within the cells. NP had been shown to induce apoptosis in cells.[21] Thus, oseltamivir might not be effective in protecting the cells after viral entry. In contrast, compound **17** at 10 μM completely inhibited the production of VR1679 NP (Figure 2). These results further support the notion that aloperine derivatives inhibit IAV with a different mechanism of action from that of oseltamivir.

Compound 17 Inhibited IAV at Multiple Stages of the Viral Life Cycle. Lack of NP expression in the presence of **17** suggests that the compound inhibits a relatively early step of the IAV life cycle. To further dissect the drug sensitive phase of the IAV life cycle, a time of addition assay was performed. The life cycle of IAV begins with entering into the host cells, which involves viral HA and M2 proteins.[19,20] After entry, the viral ribonucleoprotein particles (vRNPs) translocate into the nucleus. The vRNP includes viral RNAs, NP, and polymerases.

dx.doi.org/10.1021/ml500236n | ACS Med. Chem. Lett. 2014, 5, 942−946

Following transcription and replication of the viral genome, new vRNPs are exported out of the nucleus. The viral life cycle is completed after the viruses are assembled and budded at the plasma membrane of host cells. Oseltamivir targets this very last step of viral replication cycle by inhibiting NA, which is required for IAV release from infected cells.

The results of the time of addition assays indicated that both oseltamivir and **17** were fully active when added before or at 2 h post VR1679 virus infection (Figure 3). Both compounds

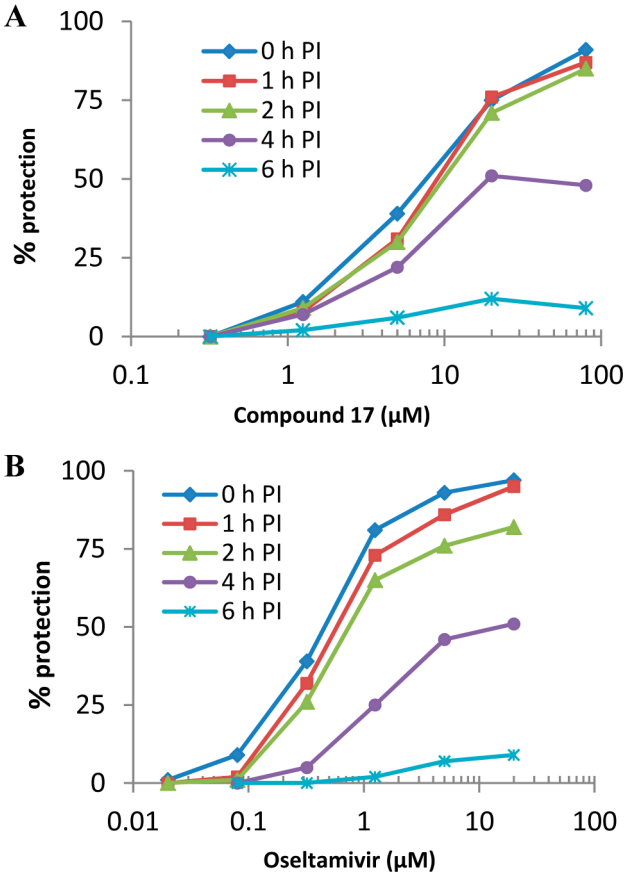


Figure 3. Compound **17** could inhibit IAV at a late stage of the viral life cycle. MDCK cells were infected with VR1679 (MOI = 0.1) in the presence of **17** (**A**) or oseltamivir (**B**) at various time points postinfection (PI). Protection of MDCK cells from being killed by the virus (% protection) was calculated with a formula detailed in the Supporting Information. Each data point in the figure represents the average of two independent experiments.

partially lost activity when they were added 4 h after viral infection. The two compounds were ineffective when they were added 6 h postinfection. These results suggest that compound **17** and oseltamivir can both inhibit IAV at a late stage of the viral life cycle. This is unexpected because the results of confocal imaging of NP suggest that compound **17** inhibits IAV at a relatively early stage of the viral life cycle (Figure 2). One possible explanation of these results is that compound **17** targets a viral protein that is involved in multiple steps of the viral life cycle.

Recombinant NP Abrogated the Anti-IAV Activity of Compound 17. Since targeting multiple steps of the viral life cycle were implicated, we speculated that IAV NP could be the target of **17**. IAV NP is a viral protein involved in multiple stages of the IAV life cycle including intracellular trafficking of the viral genome, viral RNA replication, and virus assembly.[4] Several IAV inhibitors, such as nucleozin and naproxen, were reported to target NP for their antiviral activity.[22,23] Nucleozin protected MDCK cells from being killed by IAV PR8 at an EC_{50} of 0.71 μM (Table 1). To test whether NP was a target of aloperine derivatives, the antiviral activity of **17** was determined in the presence of a recombinant NP (rec-NP). Association of the compounds with the rec-NP in culture medium is expected to decrease the potency of the anti-IAV compounds. Oseltamivir were used as a control in this experiment. As expected, the rec-NP did not affect the anti-IAV activity of oseltamivir, suggesting that the drug did not bind or interact with the rec-NP (Figure 4). In contrast, the rec-NP significantly abrogated the anti-VR1679 activity of **17**. These results support the notion that NP might be the target of aloperine derivatives.

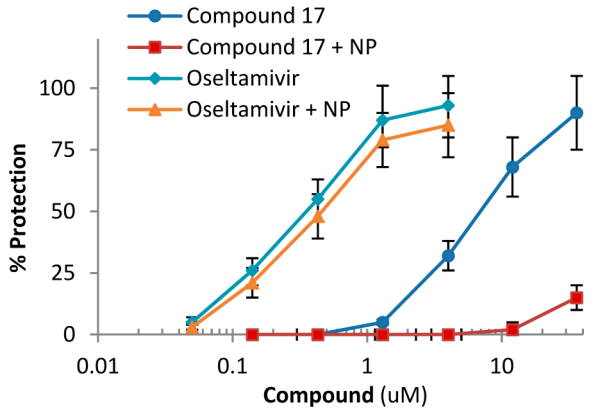


Figure 4. Rec-NP abrogated the anti-IAV activity of **17**. Inhibition of VR1679 infection of MDCK cells (MOI = 1) by compound **17** or oseltamivir was determined in the presence (compound + NP) and absence of a rec-NP at 500 μg/mL (A/Puerto Rico/8/1934, Novus Biologicals). Percent protection of MDCK cells from being killed by the virus was determined 2 days after infection. Each data point in the figure represents mean ± standard deviation of three independent experiments.

In conclusion, the Sophora quinolizidine alkaloids are a class of structurally unique natural products compared with other anti-IAV agents. Aloperine was reported to have anti-inflammatory and anticancer activities.[24,25] It will be interesting to study if aloperine derivatives reported here also possess anti-inflammatory activity, as flu virus infection often causes inflammation of the respiratory system. The anti-IAV mechanism of action of the aloperine derivative is clearly different from that of oseltamivir. Oseltamivir does not prevent the accumulation of NP in infected MDCK cells, but **17** strongly inhibits the expression of NP in the cells. Abrogation of the antiviral activity of **17** by rec-NP suggests that the compound may interact with NP. However, further studies are needed to determine if NP is indeed the target of **17**.

In summary, this study has identified a class of quinolizidine alkaloids as new anti-IAV agents. Dihydroaloperine exhibited the most potent anti-IAV activity among the tested quinolizidine alkaloids. The anti-IAV potency of the quinolizidine alkaloids could be improved by chemical modifications on the N12 and C16 positions of the aloperine scaffold. These compounds are effective against the PR8 virus that is resistant to oseltamivir and amantadine. These alkaloids have relatively low molecular mass and optimal physicochemical properties (Mass = 232; Log P = 1.27 for aloperine) for

dx.doi.org/10.1021/ml500236n | ACS Med. Chem. Lett. 2014, 5, 942−946

further drug development. Thus, the quinolizidine alkaloids or their derivatives may have potential to be developed into useful anti-IAV therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

Experimental section, including materials and methods for biological assays, synthesis, structural determination, and spectroscopic data of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by an NIAID Grant AI65310 (C.-H.C.) and an NIDA grant DA024589 (L.H.).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

IAV, influenza A virus; NP, nucleoprotein; HA, hemagglutinin; NA, neuraminidase; DAPI, diamidino-2-phenylindole

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