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Super short membrane-active lipopeptides inhibiting the entry of influenza A virus

Wenjiao Wu [1], Jingyu Wang [1], Dongguo Lin, Linqing Chen, Xiangkun Xie, Xintian Shen, Qingqing Yang, Qiuyi Wu, Jie Yang, Jian He *, Shuwen Liu *

School of Pharmaceutical Sciences, Southern Medical University, 1838 Guangzhou Avenue North, Guangzhou 510515, PR China

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ABSTRACT

Influenza A viruses (IAV) are significant pathogens that result in millions of human infections and impose a substantial health and economic burdens worldwide. Due to the limited anti-influenza A therapeutics available and the emergence of drug resistant viral strains, it is imperative to develop potent anti-IAV agents with different mode of action. In this study, by applying a pseudovirus based screening approach, two super short membrane-active lipopeptides of C12-KKWK and C12-OOWO were identified as effective anti-IAV agents with IC_{50} value of 7.30 ± 1.57 and 8.48 ± 0.74 mg/L against A/Puerto Rico/8/34 strain, and 6.14 ± 1.45 and 7.22 ± 0.67 mg/L against A/Aichi/2/68 strain, respectively. The mechanism study indicated that the anti-IAV activity of these peptides would result from the inhibition of virus entry by interacting with HA2 subunit of hemagglutinin (HA). Thus, these peptides may have potentials as lead peptides for the development of new anti-IAV therapeutics to block the entry of virus into host cells.

1. Introduction

Influenza A viruses are enveloped RNA viruses belonging to the family of *Orthomyxoviridae* [1]. They cause seasonal epidemics and reoccurring pandemics of influenza, which pose a significant and worldwide threat to human health. This threat has occurred periodically since the spread of the highly pathogenic H5N1 avian influenza in 1997 [2]. The morbidity caused by annual influenza epidemics is estimated to be in the range of 3 to 5 million cases, and the mortality is up to half a million [3], thus bringing into an urgent need for the new anti-influenza A viral agents to provide a first line of defense against such an outcome. Vaccination has been an effective approach in prevention of influenza A viral infection. However, due to the constant antigenic drift and the emergence of new subtypes of current virus, influenza A viruses remain to be persistent threats to mankind [4]. Particularly, in the face of a pandemic outbreak, vaccine production always lags a few months behind the emergence of the new virus [5]. Therefore, antiviral chemotherapy remains to play an important role in the prevention and treatment of influenza.

Currently, two classes of antivirals are clinically available to control human influenza virus infections. They are (a) neuraminidase (NA) inhibitors, such as oseltamivir, zanamivir and peramivir, which inhibit virus budding [5], and (b) viral matrix 2 ion protein (M2) inhibitors. The M2 ion channel blockers such as amantadine and rimantadine inhibit viral uncoating by blocking the proton channel activity of the influenza A viral M2 protein [6]. These drugs have been used for many years and the appearance of drug resistant strains has been reported [7]. In addition, adamantanes may be associated with neuropsychiatric side effects such as insomnia, confusion, and hallucinations, etc [8]. These drawbacks highlight the urgency to develop new antiviral drugs that effectively target other viral proteins or cellular factors involved in the influenza virus life cycle [6,9].

In regard to the influenza virus-life cycle, several events are involved and some of them have been reported as potential targets for chemotherapeutic intervention. These are including inhibition of the essential functions of the three viral surface glycoproteins: the hemagglutinin (HA), involved in cell entry and fusion; the M2 ion channel protein, involved in virus uncoating; and the neuraminidase (NA), related to the release of new virus particles [9]. Among them, virus attachment mediated by HA is the first step for viral infection [10,11]. The HA of influenza virus is a surface protein and is presented as a homo-trimer with each monomer consisting of two chains, HA1 and HA2, linked by a single disulfide bond. In the events of virus entry, the HA1 subunit is responsible for binding the virus to host cell of sialic acid-containing receptors, while the HA2 subunit is responsible for fusion [12,13]. Thus, the HA including HA1 and HA2 subunits is a potential target for antiviral drug to intervene thereby blocking the entry of viruses into host cells.

Based on this progress, in this work, we employed a pseudovirus based "entry inhibitor" screening approach to evaluate our membrane-active lipopeptide library for potent antiviral agents that

* Corresponding authors.
E-mail addresses: jianhe@smu.edu.cn (J. He), liusw@smu.edu.cn (S. Liu).
[1] These authors contributed equally.

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were able to block the entry of viruses (entry inhibitors). The pseudovirus was constructed as previously reported by employing the plasmids encoding hemagglutinin glycoprotein (HA) and neuraminidase protein (NA) into HIV backbone [14], while the lipopeptide library was designed by combining the knowledge that (a) arginine, lysine and tryptophan rich peptides always possess potent antimicrobial activities, and (b) lipopeptides are a class of antimicrobial peptides possessing high potencies [15]. As a consequence of initial screening, two very short lipopeptides of C12-KKWK and C12-OOWO being able to inhibit the infectivity of H5N1 influenza A pseudovirus were identified. Their antiviral activities were then confirmed by testing the viral strains of influenza A/Puerto Rico/8/34 (H1N1) and A/Aichi/2/68 (H3N2). Furthermore, the structures of C12-KKWK and C12-OOWO were optimized which led to the identification of more potent peptides of C20-KKWK and C20-OOWO as effective influenza A virus "entry inhibitors". In this paper, we report on the antiviral activity and the mode of action of these peptides.

2. Materials and methods

2.1. Organisms and cell culture

Madin Darby Canine Kidney (MDCK) cells and 293T cells were obtained from the American Type Culture Collection (ATCC) and cells were grown in Dulbecco's Modified Eagle Medium (DEME, Gibco) containing glutamine, supplemented with 10% fetal bovine serum (FBS). Influenza A/Puerto Rico/8/34 (H1N1) and A/Aichi/2/68 (H3N2) viruses stock were propagated in the allantoic cavities of 9-day-old embryonated hen eggs at 37 °C. The allantoic fluid was harvested, clarified by low-speed centrifugation and stored at −80 °C. The virus titer was determined through the analysis of the 50% tissue culture infective dose ($TCID_{50}$) on MDCK cells and evaluated by using the method developed by Reed-Muench formula [16].

2.2. Peptide synthesis

All methods for peptide synthesis in this study have been previously described [15]. Briefly, peptides were synthesized on an ABI 433A peptide synthesizer with 0.1 mmol scale by using standard 9-fluorenylmethoxy carbonyl (Fmoc) solid phase protocols on Rink Amide MHBA resin. The peptide chain assembly was catalyzed by using HBTU/HOBt coupling chemistry with four-fold excess, except for lipid chain, where ten-fold excess was employed. All peptides were cleaved from the resin by using cleavage cocktail containing 87.5% trifluoroacetic acid, 2.5% ethanedithiol, 5% thioanisole and 5% deionized water (2–3 h, room temperature). The molecular weight of each peptide was confirmed by electrospray ionization mass spectrometry (ESI-MS, Waters), and the purity of peptide was analyzed with Shimadazu 10A HPLC instrument on a C18 column (250 × 4.6 mm, Sepax Technologies, US). The HPLC is as follows: flow rate, 0.8 mL/min; mobile phase, solvent A: water (0.075% trifluoroacetic acid), B: acetonitrile/methonal (1:1 supplementary with 0.075% trifluoroacetic acid); gradient: 15% to 20% B (2 min), 20% to 60% B (10 min), 60% to 80% B (6 min), 80% to 90% B (6 min) with the final products purity of >90% (Fig. S1), otherwise purified with HPLC if necessary.

2.3. Measurement of the inhibitory activity on the entry of H5N1 pseudovirus

The plasmids encoding HA and NA of A/Thailand/Kan353/2004 were used to prepare H5N1 pseudovirus. The protocol was adopted as reported before [14]. Briefly, 293T cells (75–95% confluent) were co-transfected with 1 μg HA plasmid, 1 μg NA plasmid, and 3 μg HIV backbone plasmid (pNL4-3.luc.R^-E^-), which contains an Env and Vpr defective, luciferase-expressing HIV-1 genome, into 6-well plate, using polyethylenimine (PEI) as the transfection reagent. After transfection for 48 h, the culture supernatants were harvested and centrifuged at 2000 rpm for 5 min. Aliquots were stored at −80 °C. The titer of pseudovirus was quantitated by the luciferase assay.

To measure the inhibitory effects of tested peptides, MDCK cells (1×10^4/well) were seeded in 96-well plates and grown overnight. The peptides in various concentrations were incubated with pseudotyped particles for 30 min at 37 °C. Subsequently, the virus–peptide mixture was transferred to the cells and incubated for another 48 h. Cells were washed with PBS and lysed with the lysing reagent included in the luciferase kit (Promega, Madison, WI). Aliquots of the cell lysates were transferred to 96-well flat bottom luminometer plates, followed by the addition of luciferase substrate. The luciferase activity was measured in a microplate luminometer (Genios Pro Tecan, US). CL-385319 at 50 μM was used as a positive control, while wells without peptides as a negative control [14].

2.4. Virus titer reduction assay

Antiviral activities of peptides were further assessed by the virus titer reduction assay as reported previously with minor modifications [17,18]. Briefly, MDCK cells were seeded into a 96-well plate at 2×10^4 per well and incubated overnight until grown up to 90% confluence. Influenza A/Puerto Rico/8/34 (H1N1) and A/Aichi/2/68 (H3N2) viruses at 100 $TCID_{50}$ were respectively mixed with peptides at the indicated concentrations and incubated at 37 °C for 30 min. Subsequently, the virus–peptide mixtures were added to the cells and incubated for another 30 min. Then cells were washed twice with PBS to remove unabsorbed virus, followed by the addition of serum free DMEM supplemented with 1 mg/L TPCK-trypsin and 0.2% BSA. At 48 h post-infection, the inhibition of viral replication was determined by measuring the virus titer in the supernatant.

To measure the inhibition of viral replication, 50 μL supernatant was added to a 96-well black plate. Then added 20 μL H_2O and 30 μL 4-MUNANA substrate dissolved in dilute buffer (33 mM MES pH = 3.5 and 4 mM $CaCl_2$) at a final concentration of 20 μM, and the mixture was further incubated for 1 h at 37 °C in the dark. The reaction was terminated with 150 μL 14 mM NaOH (dissolved in 83% ethanol) and the fluorescence of the mixture was recorded at the excitation wavelength of 340 nm and emission wavelength of 440 nm with the multifunctional microplate reader (SpectraMax M5, US). The inhibition ratio was determined by using the equation:

$$\text{Inhibitory activity}(\%) = \frac{(F_{virus} - F_{sample})}{(F_{virus} - F_{substrate})} \times 100\%$$

where F_{virus} is the fluorescence of the supernatants of the virus control, $F_{substrate}$ is the fluorescence of the substrate control (buffer and substrate), and F_{sample} is the fluorescence of the supernatant of the infected cells that were treated with the peptide. Finally, IC_{50} was determined by extrapolation of the results from various doses tested using a linear equation. The experiment was repeated at least twice with a similar result each time.

2.5. Quantitative real-time PCR assay

The inhibition of viral matrix gene replication of the peptides was detected by quantitative real-time PCR as reported previously [19,20]. Briefly, influenza A/Puerto Rico/8/34(H1N1) virus at 100 $TCID_{50}$ was incubated with peptides at 37 °C for 30 min. Then the virus–peptide mixture was added to the cells in 6-well plate and absorbed for another 30 min. After absorption, cells were washed twice with PBS to remove unabsorbed virus, followed by the addition of serum free DMEM supplemented with 1 mg/L TPCK-trypsin and 0.2% BSA. At 24 h post-infection, the total RNA was extracted with TRIzol reagent (Invitrogen), RNA quality and quantity were determined by the UV spectrophotometer (Merinton SMA1000, US). The total RNA was reverse transcribed into

cDNA using PrimeScript RT reagent kit. Real-time PCR was performed in an ABI7500 PCR instrument (Applied Biosystems, US) with the SYBR Premix Ex Taq as the manufacturer's instruction. Influenza A Matrix protein gene expression was normalized to GAPDH gene, which stably expressed in MDCK cells. Fold changes in gene expression were calculated using a classical $2^{-\Delta\Delta CT}$ method. Ribavirin was employed as a positive control which was added at the concentration of 25 mg/L post-infection and the real-time PCR assay was conducted simultaneously to the cell treated with the peptides as described above. All samples were run in triplicate. The primer sequences used for specific genes are listed in supplementary material of Table 1. All experiments were carried out in triplicate. Statistical significance of the data was determined by one-way ANOVA method using SPSS software. Statistical significance was defined as $p < 0.05$ ($^{*}p < 0.01$, $^{**}p < 0.001$).

2.6. MTT assays

The cytotoxicity of the peptides on MDCK cells was measured using the MTT assay. Cells (1×10^4/well) were seeded in 96-well plates and grown overnight, then the medium was replaced with fresh medium containing serially diluted peptides and the cells were further incubated at 37 °C with 5% CO_2 for 48 h. The culture medium was removed and replaced with 100 μL 0.5 mg/mL 3-(4,5-dimethylthiozol-2-yl)-3,5-dipheryl tetrazolium bromide solution and incubated at 37 °C for 4 h. Then the supernatants were discarded and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The stained formazan product was determined spectrophotometrically at 570 nm on a GENios Pro microplate reader (Genios Pro Tecan, US).

2.7. Measurement of the inhibitory activity on the entry of VSVG pseudovirus

The VSV pseudovirus was constructed by using VSV-glycoprotein encoded plasmid and HIV backbone plasmid (pNL4-3.luc.R^-E^-). The protocol is similar to that of influenza A pseudovirus. To test the inhibitory activity of these peptides against VSVG pseudovirus, MDCK cells (1×10^4/well) were seeded in 96-well plates and grown overnight. The peptides were serially two-fold diluted from 100 to 3.12 mg/L in culture medium and then incubated with equal volume of pseudotyped particles at 37 °C for 30 min. Subsequently, the virus–peptide mixture was transferred into the MDCK cells and incubated for 48 h at 37 °C before performing luciferase assay as described above.

2.8. Hemagglutination inhibition (HI) assay

The HI assay was employed to evaluate the inhibitory effects of the peptides on viral adsorption into target cells. HI tests were performed using 4 times of the HA units (HAU) of H5 standard antigen per well (25 μL) in V-bottomed 96-well micro plates. An equal volume (25 μL) of peptides started from 200 mg/L of two-fold serial dilution in PBS was then added into the plate. Subsequently, 50 μL of chicken erythrocytes (0.5% v/v in PBS) was added to each well. An equal volume of HA1 antiserum (25 μL, 4HAU) was used as a positive control, while PBS as a negative control. The hemagglutination reaction results were read after incubation for 1 h at room temperature. The experiment was independently repeated three times, and each time with four parallel measurements. Both H5 standard antigen and antiserum were provided by Haerbin Veterinary Research Institute, China.

Table 1
IC_{50} of peptides against influenza A viral strains (mg/L).

Peptide	H1N1[a]	H3N2[a]	H5N1[b]
C12-OOWO	8.48 ± 0.74	7.22 ± 0.67	9.03 ± 0.63
C12-KKWK	7.30 ± 1.57	6.14 ± 1.45	12.57 ± 3.77
Ribavirin[c]	3.23 ± 0.77	4.20 ± 2.42	NT[d]
CL-385319	NT	NT	0.37 ± 0.12

[a] A/Puerto Rico/8/34 (H1N1) and Aichi/2/68 (H3N2). Data were acquired from the virus titer reduction assay.
[b] H5N1 pseudovirus. Data were obtained from the luciferase assay.
[c] Ribavirin and CL-385319 were used as positive controls.
[d] Not test (NT).

2.9. Neuraminidase inhibition assay

Influenza virus NA activity was assayed by quantifying the fluorescent product resulting from the cleavage of the substrate of [4-MU-NANA; 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium] by neuraminidase of the virus. The NA inhibition assay was conducted using NA from influenza A/Puerto Rico/8/34(H1N1) virus as described previously [21]. Briefly, 30 μL influenza virus solutions was added to a 96-well black plate and mixed with 10 μL two-fold diluted peptides ranging from 200 to 0.39 mg/L to react for 15 min at room temperature. Then added 30 μL substrate dissolved in dilute buffer (33 mmol/L MES (pH 3.5) and 4 mmol/L $CaCl_2$) at a final concentration of 20 μM and 30 μL H_2O, and the mixtures were further incubated for 1 h at 37 °C in the dark. The reaction was terminated with 150 μL 34 mM NaOH (83% ethanol) and the fluorescence of the mixture was recorded at the excitation wavelength of 340 nm and emission wavelength of 440 nm.

$$\text{Inhibitory activity}(\%) = \frac{(F_{virus} - F_{sample})}{(F_{virus} - F_{substrate})} \times 100\%$$

where F_{virus} is the fluorescence of the influenza virus control (virus, buffer and substrate), $F_{substrate}$ is the fluorescence of the substrate control (buffer and substrate), and F_{sample} is the fluorescence of the tested samples (virus, sample solution and substrate). Finally, IC_{50} was determined by extrapolation of the results from various doses tested using a linear equation. The experiment was repeated at least twice with a similar data each time.

2.10. Hemolysis inhibition assay

Chicken red blood cells (cRBCs) were washed twice with PBS and re-suspended to make a 2% (v/v) suspension in PBS, which was stored at 4 °C until use. 100 μL of compound diluted in PBS was mixed with an equal volume of the influenza virus A/PR/8/34 (H1N1) strain (10^7 $TCID_{50}$/mL) in a 96-deepwell plate. After incubating the virus–compound mixture at room temperature for 30 min, 200 μL of 2% chicken erythrocytes pre-warmed at 37 °C was added. The mixture was incubated at 37 °C for another 30 min. To trigger hemolysis, 100 μL of sodium acetate (0.5 M; pH 5.0) was added and mixed well with the erythrocyte suspension and incubated at 37 °C for 15 min. To separate nonlysed erythrocytes, plates were centrifuged at the end of incubation at 3000 rpm for 5 min. 200 μL of supernatant was transferred to another flat-bottom 96-well plate. The OD540 was read on a microtiter plate reader.

2.11. The antiviral activity of the peptides against H274Y mutation virus

The procedure was adopted as the same as abovementioned. Briefly, MDCK cells were seeded into a 96-well plate at 2×10^4 per well and incubated overnight until grown up to confluent. Influenza strain of A/Puerto Rico/8/34 (H1N1) with H274Y mutation virus at 100 $TCID_{50}$ was mixed with peptides in 2-fold dilution and incubated at 37 °C for 30 min. Subsequently, the virus–peptide mixtures were added to the cells and incubated for another 30 min. Then cells were washed twice with PBS to remove unabsorbed virus, followed by the addition of DMEM supplemented with 1 mg/L TPCK-trypsin and 0.2% BSA. At 24 h post-infection, the cells were immobilized with 80% pre-cooled acetone for 10 min and then viral replication was determined by measuring the levels of influenza virus nucleoprotein (NP) using an

influenza A virus NCP ELISA kit (Photometric; Virusys Corp). Percent protection was calculated as [1 − (mean OD450compound − mean OD450medium) / (mean OD450DMSO − mean OD450medium)] × 100%, where mean OD450compound, mean OD450medium, and mean OD450DMSO refer to the absorbance (optical density at 450 nm [OD450]) of compound- and virus-containing samples, the absorbance of no-virus control samples, and the absorbance of DMSO- and virus-containing. Oseltamivir (Tamiflu) was used as a positive control with IC_{50} value of 0.33 ± 0.037 mg/L, while the IC50 value for WT of Influenza strain of A/Puerto Rico/8/34 (H1N1) was 0.017 ± 0.002 mg/L.

2.12. Statistical analysis

Graph Pad Prism 5 (San Diego, CA) was used to determine the half cytotoxic concentration (CC_{50}) and half inhibitory concentration (IC_{50}) values of peptides. All IC_{50} and CC_{50} values were calculated as the means ± standard error of the mean (SEM) from triplicate assay from at least three separate experiments. Statistical significance of the data was determined by independent test or one-way ANOVA method using SPSS software.

3. Results

3.1. Peptide C12-KKWK and C12-OOWO display an antiviral activity toward pseudo-influenza A virus

The "entry inhibitor" screening program was initiated by using a pseudovirus based approach, of which the H5N1 influenza A pseudovirus was constructed by employing the plasmids encoding hemagglutinin glycoprotein (HA) and neuraminidase protein (NA) of A/Thailand/Kan353/2004, and the antiviral effect was evaluated by measuring the inhibitory effect on the infection of H5N1 pseudovirus [14]. Upon screening our previously designed membrane-active lipopeptide library [15], two "hits" of C12-KKWK and C12-OOWO possessing the antiviral activity were identified. The preliminary results in Table 1 showed that both C12-KKWK and C12-OOWO displayed a good inhibitory effect with IC_{50} values of 12.57 ± 3.77 and 9.03 ± 0.63 mg/L, respectively.

3.2. C12-KKWK shows a potent antiviral activity against influenza A viral strains

The antiviral activity of C12-KKWK and C12-OOWO was further assessed by testing the viral strains of influenza A/Puerto Rico/8/34 (H1N1) and A/Aichi/2/68 (H3N2) with a virus titer reduction assay [22]. The inhibition of viral replication was evaluated by measuring the enzymatic activity of neuraminidase, a method employed for determining the virus titer in the supernatant [23]. The resultant fluorescence of the mixture was recorded at the excitation wavelength of 340 nm and emission wavelength of 440 nm. As a result, the IC_{50} values of C12-KKWK and C12-OOWO were 7.30 ± 1.57 and 8.48 ± 0.74 mg/L respectively against A/Puerto Rico/8/34 strain, and 6.14 ± 1.45 and 7.22 ± 0.67 mg/L against A/Aichi/2/68 (H3N2) strain (Table 1).

In parallel to the measurement of the enzymatic activity of neuraminidase, the antiviral activity against influenza A/Puerto Rico/8/34 (H1N1) was further confirmed by using a quantitative RT-PCR (Fig. 1). As reported, influenza matrix protein directly reveals to virus replication [24], we then inspected the mRNA levels of M protein after treatment with peptides. The results displayed a dramatically reduced level of mRNA expression on treatment with both C12-KKWK and C12-OOWO (Fig. 1), consistent with the results observed from the neuraminidase activity assay.

The initial results inspired us for a further speculation that whether these peptides would be able to inhibit the replication of drug-resistant viral strain. We then tested the antiviral activities of these peptides against influenza strain of A/Puerto Rico/8/34 (H1N1) with H274Y mutation, an influenza A (H1N1) virus with H274Y neuraminidase mutation which is known for its resistance to neuraminidase inhibitor of Oseltamivir [25]. As a result, by treatment of virus with peptides similar to that in CPE inhibition assay, the levels of influenza virus nucleoprotein (NP) were measured by using enzyme-linked immunosorbent assay (ELISA) to determine the antiviral effect. The IC_{50} values for each peptide were 8.692 ± 1.125 mg/L (C12-KKWK), 3.945 ± 0.374 mg/L (C18-KKWK), and 3.075 ± 1.094 mg/L (C20-KKWK) respectively, which are similar to the results obtained from other assays.

3.3. C12-KKWK is unable to inhibit the entry of vesicular stomatitis (VSV) pseudovirus

Next we then investigated how the C12-KKWK exerted its inhibitory effects on IAV. In this study, the pseudo-influenza A virus was constructed by encoding HA and NA plasmids of IAV into HIV backbone. Hence, the antiviral activity of these peptides may result from the interaction with either HA, NA glycoprotein of influenza A virus or HIV backbone. Given the same rout of endocytosis in their steps of infections, we employed vesicular stomatitis virus (VSV) as a control to study the possible target of these lipopeptides. The vesicular stomatitis pseudovirus was prepared by encoding VSV-glycoprotein plasmid into HIV backbone similar to that of IAV pseudovirus, and then was employed to evaluate the inhibitory effect of C12-KKWK.

The results in Fig. 2 showed that C12-KKWK was unable to significantly reduce the infectivity of VSV pseudovirus on MDCK cells, indicating the unlikeness of the HIV backbone as the target of C12-KKWK. Thus, the anti-IAV of these peptides may result from the interaction with HA or NA by which to exert the anti-influenza A virus effect.

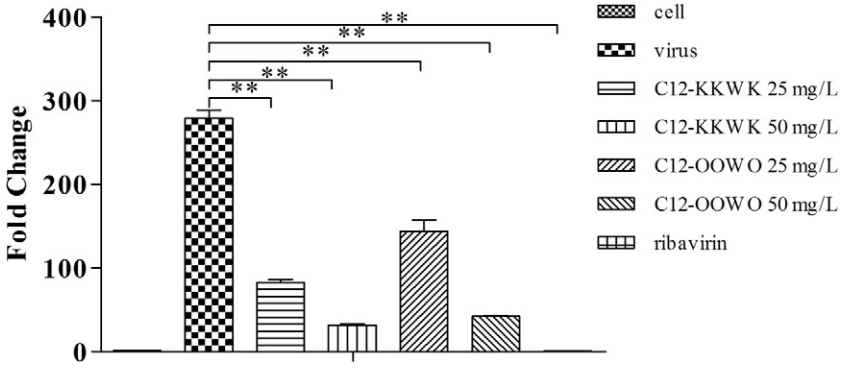

Fig. 1. The antiviral activity of C12-KKWK and C12-OOWO against influenza A/Puerto Rico/8/34(H1N1) determined by quantitative real-time PCR. The peptides were pre-incubated with A/Puerto Rico/8/34 virus at 100 $TCID_{50}$ for 30 min, and subsequently, the virus–peptide mixture was transferred to the cells for another 30 min. Then the cells were washed twice with PBS and added serum free DMEM medium supplemented with 1 mg/L TPCK trypsin and 0.2% BSA. At 24 h post-infection, the matrix gene was detected by quantitative real-time PCR.

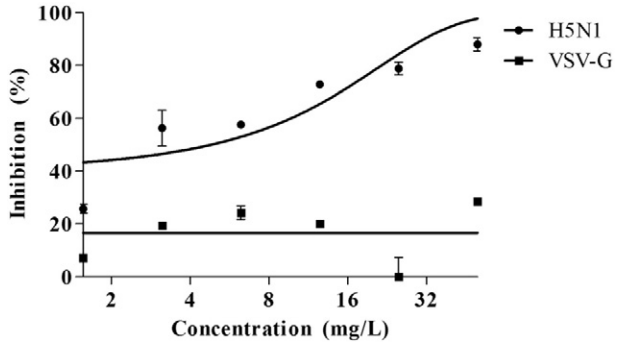

Fig. 2. Inhibition of the infectivity of VSVG pseudoviruses by C12-KKWK. The VSV pseudovirus was constructed by using VSV-glycoprotein encoded plasmid similar to that of H5N1 pseudovirus.[2] The peptides were serially two-fold diluted from 50 to 1.56 mg/L in culture medium and then incubated with equal volume of pseudo-typed particles at 37 °C for 30 min prior to transferring into the MDCK cells and subsequently incubated for 48 h at 37 °C before performing luciferase assay as described previously.

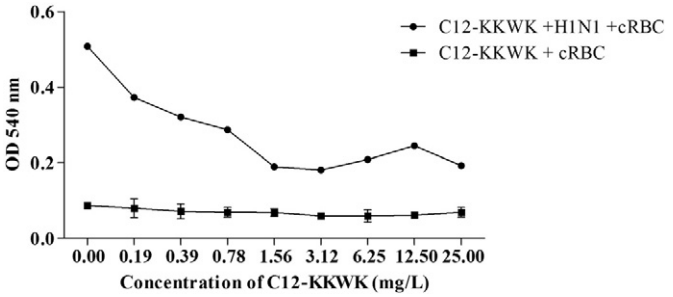

Fig. 3. Hemolysis inhibition assay. C12-KKWK in PBS was mixed with an equal volume of the influenza virus A/PR/8/34 (H1N1) strain (10^7 $TCID_{50}$/mL) in a 96-deepwell plate, and 200 μL of 2% chicken erythrocytes was then added. The mixture was incubated at 37 °C for another 30 min, following addition of 100 μL of sodium acetate (0.5 M; pH 5.0) to trigger hemolysis. The mixture was incubated at 37 °C for 30 min for HA acidification and hemolysis, and subsequently, the plates were centrifuged and the supernatant was monitored at OD540 on a microtiter plate reader.

3.4. C12-KKWK inhibits the hemolysis of chicken red blood cells (cRBCs) under the low pH condition

A critical step for virus entry is the membrane fusion induced by low pH. To investigate whether C2-KKWK inhibits the conformational changes of HA2 subunit, thereby blocking the fusion of viral-cell membrane, we then carried out a hemolysis inhibition assay under the acidic condition. As shown in Fig. 3, when exposure the mixture of chicken red blood cells (cRBCs) with influenza virus strain of A/PR/8/34 (H1N1) (10^7 $TCID_{50}$/mL) to the twofold serial dilutions of C12-KKWK in acidic buffer (pH = 5), the hemolysis of cRBCs was apparently inhibited in a dose-dependent manner, suggesting that interruption of the conformational changes of HA2 triggered by low pH would be the possible mechanism of C12-KKWK, by which to block the entry of virus (Fig. 3).

3.5. C12-KKWK is unable to inhibit the activity of neuraminidase (NA)

According to the influenza A pseudovirus construction model in this study, it was reasonably deduced that the antiviral activity of C12-KKWK may result from the interactions with HA or NA, or both. To explore the possible target, a neuraminidase inhibition assay was applied to evaluate the antiviral effect and to determine the possible target of C12-KKWK. The assay was carried out by quantifying the fluorescent products resulting from the cleavage of the substrate of [4-MUNANA; 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium] by NA [26]. The NA was derived from influenza A/Puerto Rico/8/34 (H1N1) virus as described previously [14]. The results in Fig. 4 showed that only less than 20% inhibitory effects were observed, suggesting that C12-KKWK was unable to inhibit the NA activity significantly. Thus, the most possible target of C12-KKWK would be the hemagglutinin (HA) by which to block the entry of virus into host cells.

3.6. Possible interactions between C12-KKWK and receptor-binding site of HA

The HA is located on the surface of the virus as a homotrimer with each monomer consisting of two subunits, HA1 and HA2. In the events of virus entry, the HA1 subunit is responsible for binding the virus to host cell of sialic acid-containing receptors, while the HA2 subunit is responsible for fusion [27]. To demonstrate the interactions between C12-KKWK and the possible receptor-binding site of HA, we then carried out a hemagglutination inhibition (HI) assay by observing the inhibitory effect of agglutination of chicken erythrocytes and to determine whether HA1 would be the potential target of these lipopeptides [21]. The experiment procedure was adopted from previously reported paper [21], and the results showed that C12-KKWK was unable to inhibit the agglutination of red blood cells [data not shown], implying the unlikeness of sialic acid binding site on HA1 subunit as the binding of C12-KKWK binding site.

To further assess the potential target of C12-KKWK, we then performed a docking simulation by using Sybyl 2 software. The HA structure used was that of 4CQP (H5N1) obtained from the Protein Data Bank (PDB). The total score of the interaction between C12-KKWK and HA2 was 8.7277, which is higher than 6.2342 of the interaction between C12-KKWK and HA1. As shown in the possible interaction of the HA2-C12-KKWK complex (Fig. 5), the residues of Gln 62, Leu 89, Thr 93 and Thr 107 form strong intramolecular hydrogen bonding with lysins of C12-KKWK. In addition, the residues of Ile 56 and Ile 55 are observed to be close to the lipid chain where hydrophobic interactions would occur.

3.7. The antiviral activity of C12-KKWK is improved by structure and activity relationship study

Next, we studied the relationships between peptide structures and the antiviral activities of these peptides. Considering the critical role of lipophilicity in the generation of antimicrobial peptides, we then varied the lengths of lipid tail to search for more potent viral entry blockers.

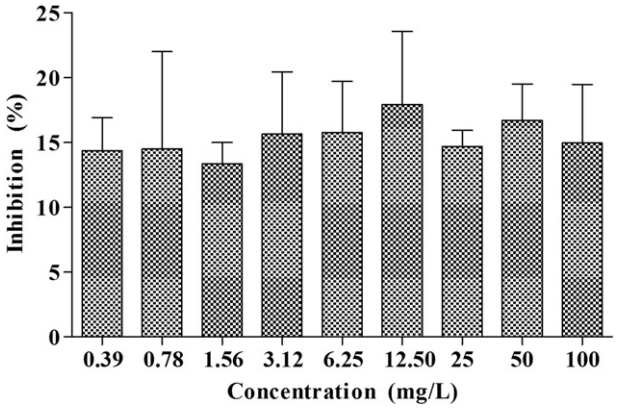

Fig. 4. Neuraminidase inhibition assay of C12-KKWK. Influenza virus NA activity was assayed by quantifying the fluorescent product resulting from the cleavage of the substrate of [4-MU-NANA; 2-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid sodium] by NA. After reaction for 1 h at 37 °C in the dark, the fluorescence was recorded at the excitation wavelength of 340 nm and emission wavelength of 440 nm. The experiment was independently repeated at least twice. Peramivir was used as a positive control, which showed 86.7% inhibition at 3.9 mg/L.

[2] Peptide C12-KKWK that inhibits the infectivity of H5N1 pseudovirus at various concentrations was employed as a comparison. Each data was expressed as the mean of three independent replicates. CL-385319 at 50 μM was used as a positive control, while wells without peptides as a negative control.

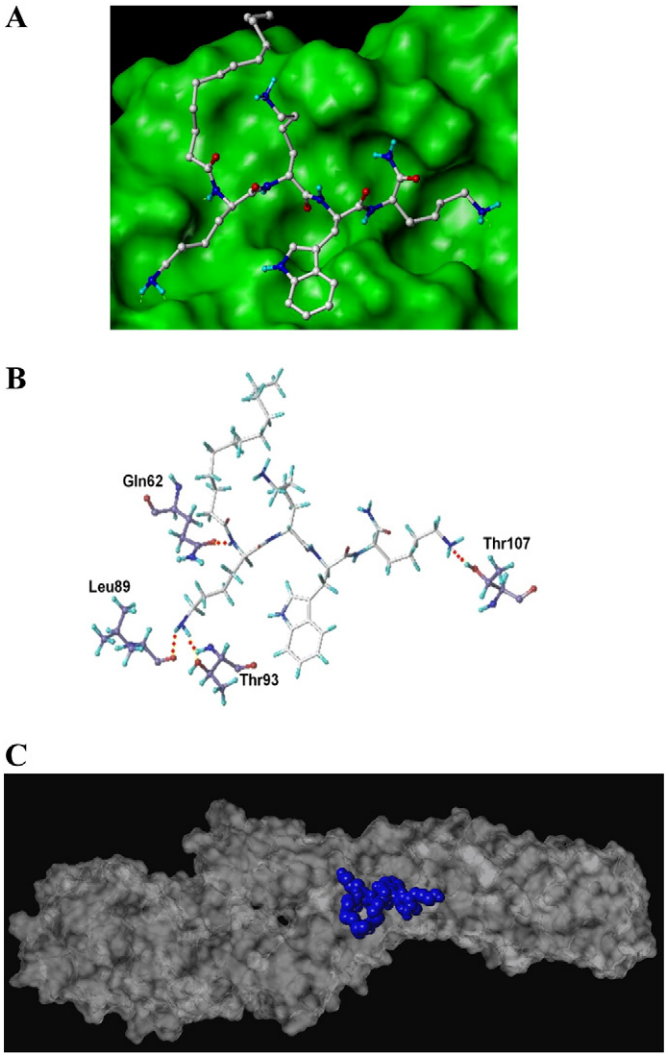

Fig. 5. The docking simulation was performed in Sybyl 2.0 software. The HA structure used was that of 4CQP (H5) obtained from the Protein Data Bank (PDB). As a consequence of docking, the total score was 8.7277 between the interaction of C12-KKWK and HA2, while of 6.2342 was obtained between C12-KKWK and HA1. A and B show a possible interaction of the HA2-C12-KKWK complex, where C12-KKWK is recognized by the active pocket of HA2. C is the 3D structure of the whole HA molecule (gray) and the binding site where the lipopeptide (blue) is located.

The sequences and activities are listed in Table 2. As a result, peptides C20-KKWK and C20-OOWO were proved to be the most active peptides with IC_{50} values of 2.72 ± 0.30 and 2.14 ± 0.10 mg/L, respectively.

3.8. The cytotoxicities of these peptides are associated with the lengths of lipid chains

A significant concern in the development of peptide drug is the toxicities toward mammalian cells. To assess the discrimination ability of these peptides between mammalian cells and pathogenic viruses, MTT assay was employed to test the cytotoxicities of selected peptides against MDCK cells [14]. The data in Table 2 showed that the cellular toxicities of these peptides were highly associated with the lengths of lipid tails, of which C20 fatty acid substituted peptides appeared to be the most toxic peptides, while the peptides consisting of shorter lipid chains were less toxic. Therefore, C20-OOWO and C20-KKWK were represented as the most toxic peptides with CC_{50} values of 47.31 ± 0.57 and 53.20 ± 0.66 mg/L, respectively. Overall, these results were consistent with our previous observations that a higher hydrophobicity is sometimes accompanied with a higher cellular toxicity (Table 2) [15].

Table 2
Inhibitory effect of designed lipopeptides.

Sequence	IC_{50} (mg/L) [a]	CC_{50} (mg/L) [b]	SI [c]
C6-OOWO	>50	NT [d]	NA [d]
C8-OOWO	>50	NT	NA
C10-OOWO	57.81 ± 0.29	NT	NA
C12-OOWO	9.03 ± 0.63	124.42 ± 1.26	13.77
C14-OOWO	8.21 ± 0.17	105.43 ± 1.84	12.84
C16-OOWO	7.35 ± 0.90	101.40 ± 0.92	13.79
C18-OOWO	4.18 ± 0.20	64.30 ± 2.08	15.38
C20-OOWO	2.14 ± 0.10	47.31 ± 0.57	22.10
C6-KKWK	>50	>200	NA
C8-KKWK	>50	>200	NA
C10-KKWK	56.18 ± 11.90	>200	NA
C12-KKWK	12.57 ± 3.77	137.00 ± 4.10	10.89
C14-KKWK	9.96 ± 2.59	115.90 ± 0.66	11.63
C16-KKWK	8.81 ± 2.95	103.50 ± 1.39	11.74
C18-KKWK	4.68 ± 0.90	74.73 ± 5.49	15.93
C20-KKWK	2.72 ± 0.30	53.20 ± 0.66	19.53

[a] Inhibitory activities against A/Thailand/Kan353/2004 H5N1 influenza A pseudovirus infection of MDCK cells. Data was acquired from the luciferase assay.
[b] Cytotoxicities against MDCK cells tested with MTT assays.
[c] Selective index was acquired by using CC_{50} divided by IC_{50}.
[d] Not test (NT).; not available (NA).

4. Discussion

The rapid emergence of new antiviral resistance strains to currently available drugs such as adamantanes or neuraminidase inhibitors has created an urgent need for new antiviral targets and new therapeutics for the prevention and treatment of influenza virus infections. In this paper, we identified a super short lipopeptide of C12-KKWK and its derivatives effectively inhibiting the entry of influenza A virus into host cells. The lipopeptides being selected as viral "entry blockers" are mainly based on the following facts that (a) in addition to antimicrobial activities, many peptides are also able to inhibit the replication of viruses, thereby making them potential candidates as potent antivirals [28,29]; (b) many hydrophobic lipids or polymer-containing agents such as sialic acid-containing lipids [30], polymers [31], sialic acid-conjugated dendritic polymers [32,33], and fatty acid-containing sialic acid mimics [34] show antiviral activities that have been developed as viral entry blockers; and (c) the clinically used HIV entry inhibitor of enfuvirtide (Fuzeon) has shown the powerful potential of peptide drugs in the prevention of viral infections [35]. In addition, the super small size, less immune response and easy to access of these peptides also intrigued us for this study.

The glycoproteins of hemagglutinin (HA) and neuraminidase (NA) are found on the influenza A viral particle surface: HA involves binding of the virion to target cells and subsequently injecting the viral genomic material into the target cell, while NA is associated with the release of progeny virions from infected cells [36]. These proteins are usually the targets for antiviral drugs to intervene. By employing a viral entry inhibition assay, we identified that C12-KKWK and its derivatives were able to block the entry of IAV, while unable to block the entry of VSV-G. The mechanism-of-action study indicated that the antiviral effects of these peptides may result from the interaction with HA, but not NA. However, the detailed mechanisms by which to block the viral entry are not yet to be fully understood. Our preliminary studies suggested that C12-KKWK may not act on sialic acid binding site of HA1, instead, it might interact with HA2 subunit to inhibit the conformational changes of HA2 thereby blocking the process of membrane fusion. However, this study cannot exclude the possibility that C12-KKWK may also act on a non-specific receptor on HA thereby inhibiting the entry of viruses [37].

The structure and activity relationship study showed that both the antiviral activity and cytotoxicity were enhanced alongside the increase of lipid chain, as well as the selective indices, indicating a useful strategy to optimizing the structures of these peptides in our future research.

In conclusion, by employing a pseudovirus based screening approach, in this study, we identified a group of IAV "entry blockers" with only four amino acid residues. Although the anti-IAV potencies of these peptides are in the range of micro-molar concentrations, their structures could be improved by chemical modifications. More importantly, these compounds possess a different mechanism from current anti-IAV drugs in clinical practice which will encourage us for further investigations.

Abbreviations

HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	Hydroxybenzotriazole
DMF	Dimethylformamide
PBS	Phosphate Buffer Saline
Fmoc	9-fluorenylmethoxy carbonyl
ESI-MS	electrospray ionization mass spectrometry
IAV	Influenza A viruse
NA	neuraminidase
HA	hemagglutinin
MDCK	Madin Darby Canine Kidney
IC_{50}	the half maximal inhibitory concentration
CC_{50}	the half maximal cytoxic concentration
$TCID_{50}$	the 50% tissue culture infective dose
MTT	3-(4,5-di methyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
4-MUNANA	2-(4-methyl umbelliferyl)-α-D-N-acetylneuraminic acid sodium
PDB	the Protein Data Bank
FBS	fetal bovine serum
ELISA	enzyme-linked immunosorbent assay

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.06.015.

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