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

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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 protein 2 (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 [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

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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

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