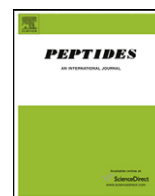




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Virucidal activity of a scorpion venom peptide variant mucroporin-M1 against measles, SARS-CoV and influenza H5N1 viruses

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ABSTRACT

Outbreaks of SARS-CoV, influenza A (H5N1, H1N1) and measles viruses in recent years have raised serious concerns about the measures available to control emerging and re-emerging infectious viral diseases. Effective antiviral agents are lacking that specifically target RNA viruses such as measles, SARS-CoV and influenza H5N1 viruses, and available vaccinations have demonstrated variable efficacy. Therefore, the development of novel antiviral agents is needed to close the vaccination gap and silence outbreaks. We previously identified mucroporin, a cationic host defense peptide from scorpion venom, which can effectively inhibit standard bacteria. The optimized mucroporin-M1 can inhibit gram-positive bacteria at low concentrations and antibiotic-resistant pathogens. In this investigation, we further tested mucroporin and the optimized mucroporin-M1 for their antiviral activity. Surprisingly, we found that the antiviral activities of mucroporin-M1 against measles, SARS-CoV and influenza H5N1 viruses were notably increased with an EC₅₀ of 7.15 µg/ml (3.52 µM) and a CC₅₀ of 70.46 µg/ml (34.70 µM) against measles virus, an EC₅₀ of 14.46 µg/ml (7.12 µM) against SARS-CoV and an EC₅₀ of 2.10 µg/ml (1.03 µM) against H5N1, while the original peptide mucroporin showed no antiviral activity against any of these three viruses. The inhibition model could be via a direct interaction with the virus envelope, thereby decreasing the infectivity of virus. This report provides evidence that host defense peptides from scorpion venom can be modified for antiviral activity by rational design and represents a practical approach for developing broad-spectrum antiviral agents, especially against RNA viruses.

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1. Introduction

Rapidly changing global landscapes and local environments, enormous increases in the human population and urbanization in many developing countries, and the advances in the speed and volume of global transportation have created increased opportunities for the emergence and re-emergence of viral diseases. Viruses with RNA as their genetic material can quickly adapt to and exploit these various conditions because of their high genetic variation rates (mutation, recombination and reassortment). Not surprisingly, many prominent recent examples of emerging or re-emerging diseases have been caused by RNA viruses, such as SARS-Coronavirus, H5N1 avian influenza viruses, H1N1, HIV, Han-

tavirus, and West Nile virus [10–12,15,21,23,32]. Outbreaks of measles continue, and it is still the leading cause of morbidity and mortality in children worldwide, despite the availability of live attenuated vaccines for more than 30 years [2,6]. Effective antiviral agents are lacking that specifically target RNA viruses such as measles, SARS-CoV and influenza H5N1. The current available therapies used in the clinic for measles virus infection treatment, which is administered orally or intravenously alone or in combination with immune serum globulin, have demonstrated variable efficacies and side effects such as high teratogenicity and anemia [2,30,31]. Many other strategies, such as antisense molecules, adenosine and guanosine nucleosides, brassinosteroids, coumarins, peptide inhibitors, and modulators of cholesterol synthesis, have also failed [2,25,26]. Moreover, approved or universally recommended therapies have been lacking for SARS-CoV and influenza H5N1 infections until now, even though more and more antiviral agents against SARS-CoV and influenza H5N1 have been reported [8,17,24]. Therefore, the development of new antiviral agents is needed to provide more options for managing cases of diseases caused by RNA viruses in both developed and developing countries.

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Cationic antimicrobial peptides (AMPs), which are found in all living species, are potential broad-spectrum antiviral agents [1,7,34]. Scorpions use venom as a weapon to subdue prey and to defend against predators. Scorpion venom is a cocktail of peptides and proteins with diverse bioactivities, thus representing an unexplored resource for use in peptide drug design and development [18,22,37]. Antimicrobial peptides from scorpion venom have attracted more attention due to their potential application [9,36]. Although the antibacterial and antifungal activities of AMPs have been the main focus of studies to date, some of these molecules have also been shown to be effective against viral pathogens such as Junin virus, herpes simplex virus, adenovirus, rotavirus, vaccinia virus, HCV and HIV [1,5,20,28,33].

Mucroporin is the first cationic host defense peptide from the venom of the scorpion *Lychas mucronatus*, which was cloned and shown to exhibit specific effects on inhibiting bacteria by our group recently [9]. We found that mucroporin can effectively inhibit standard bacteria, especially gram-positive bacteria. The optimized design of mucroporin-M1 by amino acid substitution resulted in inhibition of gram-positive bacteria at low concentrations and increased inhibition of antibiotic-resistant pathogens [9]. Recently, we also found a novel α -helical peptide Hp1090 from scorpion venom can inhibit HCV replication and prevents the initiation of HCV infection [33]. In this investigation, we tested mucroporin and its optimized peptide variant mucroporin-M1 for other bioactivities including antiviral function against measles, SARS-CoV and influenza H5N1 viruses. Surprisingly, we found that the virucidal activity of mucroporin-M1 was notably increased, whereas the original peptide mucroporin showed no virucidal activity at all. The results in this report indicate that scorpion venom-derived antimicrobial peptides are not only a natural resource for inhibiting antibiotic-resistant bacteria but also can be molecular templates for designing antiviral peptides and antiviral agents for measles, SARS, influenza and other emerging or re-emerging RNA viral diseases.

2. Materials and methods

2.1. Molecular design

Mucroporin is a 17-amino acid antimicrobial peptide from the venom of the scorpion *Lychas mucronatus*, which was recently characterized by our group. To enhance the net positive charge of the hydrophilic side, we designed mucroporin-M1 by substituting Glycine and Proline residues with Lysine or Arginine (G3R, P6K, G10K, and G11R) [9]. As controls, mucroporin-S1 and mucroporin-S2 were also designed by removing several N-terminal amino residues from mucroporin.

2.2. Peptide synthesis, purification and characterization

Mucroporin (**LFGLIPSLIGGLVSAFK**), mucroporin-M1 (**LFRLIK-SLIKRLVSAFK**) and its C-terminal peptides mucroporin-S1 (**SLIG-GLVSAFK**) and mucroporin-S2 (**VSAFK**) corresponding to residues 7–17 and 13–17 of mucroporin, respectively, were synthesized on an Abimed AMS 422 synthesizer by Fmoc solid-phase peptide synthesis. All peptides were C-terminally amidated. Peptides were deprotected and released from the resin by trifluoroacetic acid (TFA) treatment. After precipitation by cold diethyl ether, the peptides were filtered (Millex-HV, 0.45 μ m, Millipore) and further purified on a C18 column (EliteHPLC, China, 10 mm \times 250 mm, 5 μ m, 300 A) using a linear gradient from 30% to 95% H₂O with 0.1% TFA over 40 min with a constant flow rate of 5 ml/min. Peaks were detected by their absorbance at 220 nm and collected manually. The peptides were lyophilized, and their purity was assessed by

high-performance liquid chromatography (HPLC) and mass spectrometry.

2.3. Cells and viruses

Vero C1008 (ATCC CRL 1587), MDCK, and Hela-ACE2 were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 37 °C under a 5% CO₂ atmosphere, which is hereafter referred to as "growth medium". DMEM supplemented with 2% FBS and 1% penicillin and streptomycin was used for virus attachment, which we refer to as "2% medium".

The Edmonston strain of measles virus (MeV), which was obtained from the American Type Culture Collection, was propagated in Vero C1008 cells, and the virus titer was determined by plaque assay also in Vero C1008 cells. We used the H5N1 pseudovirus with wild-type H5 (H5N1) and N1 (PR8) and the SARS-CoV pseudovirus in this study. We produced H5N1 pseudovirus with eGFP as a reporter by cotransfection of 293T cells with pHCMV-wtH5 (H5N1), pHCMV-N1(PR8), pMP71-eGFP-pre and pSV-Mo-MLVgagpol plasmids, which were kindly provided by Dr. Wang, Wuhan Institute of Virology, Chinese Academy of Sciences (CAS). The SARS-CoV pseudovirus with the luciferase assay system was generated by cotransfection of plasmids pNL4.3.Luc.E-R-(HIV-Luc) and pCMV2B-S with 293T cells as described previously [12,35].

2.4. Virus titration and antiviral effects of peptides

Virus titration of MeV was performed by plaque assay. Briefly, Vero cell monolayers in 24-well plates were inoculated with serially diluted MeV or experimental sample. The inoculums were replaced with 0.5% agarose solution in MEM supplemented with 3% FBS after absorption of the virus for 2 h. The cell monolayers were incubated at 37 °C for 5–6 days and then fixed and stained in a 4% formaldehyde solution with 0.5% crystal violet in PBS for 2–4 h at room temperature. The clear plaque number was visualized, and the virus titer in plaque-forming units (pfu) was calculated. The experiment was done in triplicate and repeated at least three times.

To test the peptides for their antiviral effect on MeV, we followed two protocols. First, we assayed for inhibition of MeV plaque formation by treating 1×10^3 pfu/ml of viruses with serially diluted peptides in 2% medium, incubating at 37 °C for 1 h and then conducting virus plaque assays as described above. Second, we assayed for suppression of MeV replication, in which 1×10^3 pfu/ml of viruses was treated with serially diluted peptides in 2% medium, incubated at 37 °C for 1 h, and then subject to a new round of infection on Vero cell monolayers. Finally, we assessed the titer of the newly yielded virus. Briefly, after absorption of the virus-peptide mixture on Vero cells for 1 h, the inoculum was removed, and fresh growth medium was added for subsequent culturing for 48 h. Then the culture supernatant and cell lysate were collected, freeze-thawed three times and centrifuged for 10 min at $2300 \times g$ to remove cellular debris. The newly yielded virus titer was assessed by plaque assay. The above experiments were repeated at least three times.

For titration of SARS-CoV pseudovirus, diluted SARS-CoV pseudovirus was added to Hela-ACE2 cells in 96-well plates for 6 h at 37 °C and then replaced by fresh growth medium. Sixty hours later, cells were lysed directly on the plates and immediately analyzed for luciferase activity with a luciferase assay system (Promega, Madison, WI) and a Modulus™ microplate multimode reader. The SARS-CoV pseudovirus titer is presented as the luciferase relative light units (RLU).

For assaying H5N1 pseudovirus, diluted H5N1 pseudovirus was added to MDCK cells in 96-well plates for 1 h at 37 °C. The inoculum was removed and replaced by fresh growth medium. Forty-eight hours later, eGFP fluorescence expressed in infected MDCK cells was examined and photographed by fluorescence microscopy. The number of fluorescent cells was counted and used to calculate the H5N1 pseudovirus titer.

To test the peptides for their antiviral effect on SARS-CoV pseudovirus and H5N1 pseudovirus, 2.0×10^4 RLU/ml of SARS-CoV pseudovirus or 1.0×10^4 pfu/ml of H5N1 pseudovirus was incubated with serially diluted peptides for 1 h in 2% medium and then subjected to the titer assay as described above. The antiviral effect experiments for SARS-CoV and H5N1 were repeated at least three times respectively.

2.5. Cytotoxic assay

In vitro cytotoxicity testing of mucroporin and mucroporin-M1 was performed by the traditional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) procedure [16]. Briefly, Vero cells, MDCK cells or Hela-ACE2 cells were seeded into 96-well plates at a density of 2×10^4 cells/well and incubated at 37 °C in CO₂. After 12 h, culture medium was replaced by 100 µl serial dilutions of the peptides, and the cells were incubated for 24 h. Then 20 µl of a sterile-filtered MTT (Sigma) stock solution in phosphate buffered saline (PBS), pH 7.4 (5 mg/ml), was added to each well. After 4 h, unreacted dye was removed by aspiration, and the insoluble formazan crystals were dissolved in 150 µl/well isopropyl alcohol containing 0.1 mol/L HCl and measured spectrophotometrically in an ELISA reader (Thermo Labsystems) at a wavelength of 570 nm. As a positive control, we used 0.5% NP-40, a lysis reagent, which could kill all of the cells in 5 min, and the mean absorbance was subtracted from each test. The relative cell viability (%) related to control wells containing cell culture medium without peptide was calculated by $[A]_{\text{test}}/[A]_{\text{control}} \times 100$. The cytotoxicity of each peptide is expressed as the 50% cytotoxic concentration (CC₅₀), which is the concentration that inhibits growth of 50% of cells relative to non-treated control cells. This experiment was repeated at least three times.

2.6. Treatment of MeV-infected cells with mucroporin-M1

To analyze the inhibition of MeV-infected cells by mucroporin-M1 and to determine the possible effect on the viral replication cycle, Vero cell monolayers in 24-well plates were inoculated with 1×10^4 pfu/ml of MeV at 37 °C for 2 h. After washing three times with serum-free DMEM, the cells were treated with 20 µg/ml of mucroporin-M1 at different time intervals of 0–12 h, 12–24 h, 24–36 h, and 36–48 h p.i. (post infection). Infected monolayers without peptide treatment were used as controls. At 48 h p.i., the infected cells were harvested, and the yields of MeV were analyzed by plaque assay. This experiment was repeated at least three times.

Five more treatment groups were designed for further analysis of the effect of mucroporin-M1 on MeV infection *in vitro*: (1) Vero cells were pretreated with mucroporin-M1 and then infected with MeV; (2) MeV and mucroporin-M1 were mixed and incubated for 1 h, and then the mixture was inoculated in Vero cells; (3) MeV and mucroporin-M1 were mixed but without incubation, and the mixture was inoculated in Vero cells immediately; (4) Vero cells were infected with MeV first and then were treated with mucroporin-M1; and (5) As a control, Vero cells were infected with MeV without any treatment of mucroporin-M1. To compare the effects of mucroporin-M1 to viral infection, 20 µg/ml of mucroporin-M1 peptide and 1×10^3 pfu/ml of MeV were used in all groups, and the adsorption time of the virus and the peptide treatment time were 1 h in all groups. The cells were washed three times with serum-free

DMEM to remove unattached virus or free peptide after infection or treatment. The inhibitory effects of different groups were analyzed by plaque assay. This experiment was repeated three times.

2.7. Coupling of peptides to the CM5 biosensor chip

All interactions between measles virus and peptides were performed at 25 °C on a BIAcore 2000 instrument using a CM5 sensor chip (GE Healthcare Life Sciences). Peptides were attached to a chip surface followed by an 8-min activation of surface carboxyl groups using a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (1 M) and N-hydroxysuccinimide (0.25 M) at a flow rate of 5 ml/min. Peptides were diluted in 0.1 M sodium acetate to a final concentration of 200 µg/ml (pH 4.5) and then injected manually until the desired level of response units (RUs) was achieved. Following attachment, the remaining surface carboxyl groups were quenched with 35 ml of 1 M ethanolamine (pH 8.5) at a flow rate of 5 ml/min.

2.8. Analysis of measles virus binding to immobilized peptide on the CM5 biosensor chip

Binding experiments were performed in a HEPES-buffered saline solution (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween-20) at 25 °C with a flow rate of 5 µl/min. Aliquots (30 µl) of measles virus were injected onto the surfaces of the sensor chip at a flow rate of 5 µl/min. The sensor chip was regenerated by successive injections of 50 mM sodium hydroxide. Response curves were subtracted from the background signals generated from reference surfaces. Kinetic parameters were obtained by fitting the sensorgrams to a 1:1 (Langmuir) binding model using BIAevaluation 4.1 software. This experiment was repeated three times.

2.9. Statistical analysis

Statistical analysis was done by one-way ANOVA using Graph-Pad Prism software (version 4.02). Numerical results were one representative of at least three times repeated experiments expressed as mean ± standard deviation (S.D.) of three repeated samples from. The statistical significance for all tests was set at $p < 0.05$.

3. Results

3.1. Molecular design of Mucroporin-M1 and peptide synthesis, purification and characterization

Mucroporin-M1 (LFRLIKSLIKRLVSAFK) was designed based on the protein sequence of mucroporin to enhance the net positive charge of the hydrophilic side (Fig. 1) [9]. Glycine and Proline residues of mucroporin were replaced with Lysine or Arginine (G3R, P6K, G10K, and G11R). Mucroporin-M1 contains five positive charges, whereas mucroporin carries only one positive charge (Fig. 1). The secondary structures of the mucroporin and mucroporin-M1 peptides was predicted by the self-optimized prediction method (SOPM) [13] on the website (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_seccons.html), suggesting that mucroporin-M1 and mucroporin are composed of 94.12% and 76.47% α-helix, respectively. The helix of mucroporin-M1 can be divided into two parts, the hydrophobic and hydrophilic faces, which suggests that mucroporin-M1 is a good dipolar molecule. Mucroporin-S1 and mucroporin-S2 were designed by removing several N-terminal amino residues of mucroporin. These truncated peptides were used to test if the shortened, less

Mucroporin: LFGLIPSLIGGLVSAFK
Mucroporin-M1: LFRLIKSLIKRLVSAFK
Mucroporin-S1: SLIGGLVSAFK
Mucroporin-S2: VSAFK

Fig. 1. Peptide sequences and characteristics. Amino acid sequences and alignments of mucroporin, mucroporin-M1 and its C-terminal derivatives (mucroporin-S1 and mucroporin-S2). Four amino acid substitutions were made in mucroporin-M1 compared to mucroporin. The sequences of mucroporin-S1 and mucroporin-S2 correspond to residues 7–17 and 13–17 of mucroporin, respectively. Basic and polar residues are shown in red, nonpolar residues are indicated in blue and mutated residues are highlighted in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

expensive peptides have biological activities and were also used as controls in this study.

Mucroporin, mucroporin-M1 and its C-terminal derivatives corresponding to residues 7–17 and 13–17 of mucroporin (Fig. 1), respectively, were successfully synthesized on an Abimed AMS 422 synthesizer by Fmoc solid-phase peptide synthesis. The purities of all above four peptides showed reliable quality of more than 95%. The molecule weights measured by mass spectrometry (MS) (1731.22 and 2031.57 Da) completely matched with the calculated molecule weights of the amidated mucroporin and mucroporin-M1 (1731.13 and 2031.58 Da).

3.2. Anti-MeV activity of mucroporin and its derivative peptides

We followed two protocols to screen the peptides quickly for their anti-MeV activities. First, we assayed for inhibition of MeV plaque formation; second, we assayed for suppression of MeV replication. Both protocols were used just after treating MeV by mixing 1×10^3 pfu/ml of MeV with different concentrations of mucroporin, mucroporin-M1, mucroporin-S1, or mucroporin-S2 and incubating for 1 h at 37 °C. We evaluated the direct effect of the peptides to MeV infectivity, as measured by plaque-forming units using the first protocol. At the same time, we evaluated the effect of peptides on MeV infectivity, as measured by the viral ability to further infect cells and produce viral progeny using the second protocol. We observed similar effects of the peptides on MeV infectivity by both protocols (Fig. 2). Only mucroporin-M1 showed a significant inhibitory effect on MeV infectivity, whereas the other peptides, including mucroporin, mucroporin-S1 and mucroporin-S2, did not show anti-MeV activity ($p < 0.001$). MeV infectivity could be inhibited almost completely by 10 µg/ml of mucroporin-M1 and was

Table 1

Antiviral activity and cytotoxicity of mucroporin and mucroporin-M1 peptides against MeV.

	EC ₅₀ (µg/ml)	CC ₅₀ (µg/ml)	SI
Mucroporin	I	>100	
Mucroporin-M1	7.15	70.46	9.85

EC₅₀: peptide concentration required to reduce virus infection by 50%; CC₅₀: peptide concentration required to reduce cell viability by 50%, as determined by the MTT method; SI (selectivity index) = CC₅₀/EC₅₀; I: inactive.

concentration dependent (Fig. 3A) with an EC₅₀ value of 7.15 µg/ml (3.52 µM) (Table 1). The anti-MeV activity of mucroporin-M1 was notably increased compared to that of the original peptide mucroporin ($p < 0.001$).

To determine the effective time needed for mucroporin-M1 to act on MeV, 1×10^3 pfu/ml of MeV was treated with 20 µg/ml of mucroporin-M1 or mucroporin for different times, and the viral infectivity was examined by the first protocol described above (Fig. 3B). The inhibitory effect of mucroporin-M1 peptide against MeV increased with a longer treatment time, and almost 100% inhibition of MeV infectivity occurred with 40 min of treatment. The anti-MeV activity of mucroporin-M1 depends on both the peptide concentration and treatment time. Altogether, these results suggest that the rationally designed peptide mucroporin-M1 can inactivate MeV or inhibit viral replication *in vitro*.

3.3. Cytotoxicity

To distinguish selective anti-viral activity from nonselective cytotoxicity, we tested the cytotoxicity of the peptides on mammalian cultured cells. Vero cell monolayers were treated with different concentrations of peptides for 24 h, and the viability of the cells was measured using the MTT assay. The CC₅₀ values, the peptide concentrations required to reduce cell viability by 50%, were calculated. As shown in Table 1, the CC₅₀ of mucroporin-M1 on Vero cells was 70.46 µg/ml (34.70 µM), which is almost 9.85 times the EC₅₀ value; therefore, the selective index (SI) is 9.85.

3.4. Time-course test of antiviral activity and action mode of mucroporin-M1 to MeV

To determine the relationship between the peptide antiviral activity and the MeV replication cycle, Vero cell monolayers in 24-well plates were infected with MeV and then treated with mucroporin-M1 at different time intervals of 0–12 h, 12–24 h, 24–36 h, and 36–48 h p.i. Our results revealed only minor dif-

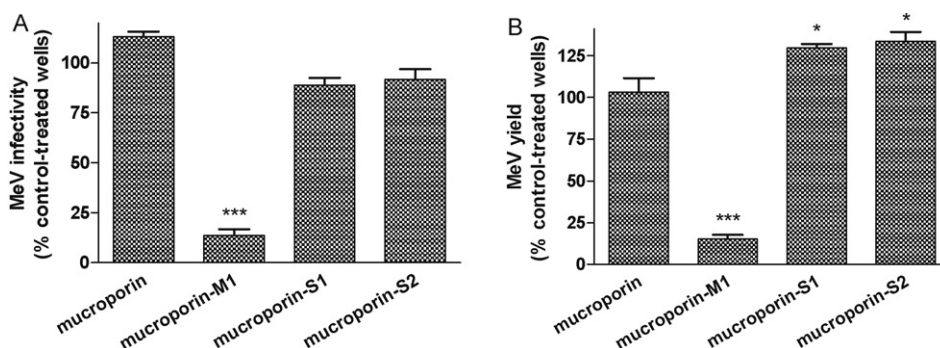


Fig. 2. Anti-MeV activity of mucroporin, mucroporin-M1, mucroporin-S1 and mucroporin-S2. The data are representative of four experiments, which showed average values for three parallel wells and were expressed as percentage of the virus titer relative to the control group treated without any peptide. The error bars indicate standard error of the mean. * means virus titers were significantly lower ($P \leq 0.05$) than those from cells exposed to no peptide, while ** means $P \leq 0.01$, *** means $P \leq 0.001$. (A) Inhibition of MeV plaque formation: 1×10^3 pfu/ml of viruses were treated with 10 µg/ml of peptides in 2% medium, incubated at 37 °C for 1 h and then subjected directly to virus plaque assay. (B) Suppression of MeV replication: 1×10^3 pfu/ml of viruses were treated with 10 µg/ml of peptides in 2% medium, incubated at 37 °C for 1 h, and then subjected to a new round of infection on Vero cell monolayers. The newly yielded virus titer was assessed by plaque assay.

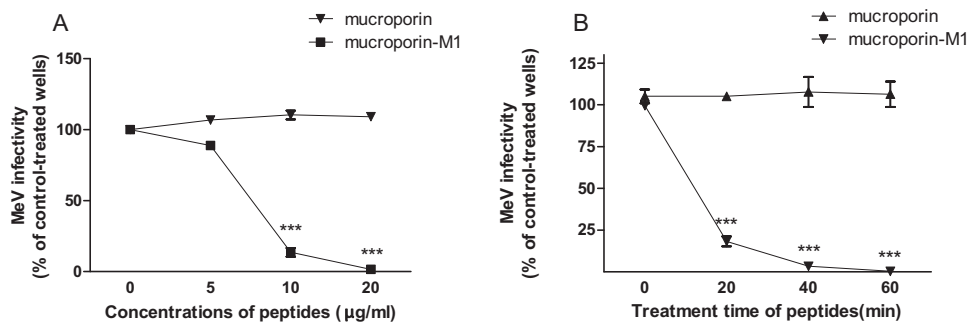


Fig. 3. Concentration- and time-dependent effects of mucroporin-M1 against MeV. The data are representative of three experiments, which showed average values for three parallel wells and were expressed as percentage of the virus titer relative to the control group treated without any peptide. The error bars indicate standard error of the mean. * means virus titers were significantly lower ($P \leq 0.05$) than those from cells exposed to no peptide, while ** means $P \leq 0.01$, *** means $P \leq 0.001$. (A) 1×10^3 pfu/ml of viruses were treated with different concentrations of peptides in 2% medium, incubated at 37°C for 1 h and then subjected directly to virus plaque assays. The group treated without any peptide was used as the control. (B) 1×10^3 pfu/ml of viruses was treated with $20 \mu\text{g/ml}$ of peptides in 2% medium for different time durations and then directly subjected to a virus plaque assay. The group treated without any peptide for the corresponding treatment time duration served as the control.

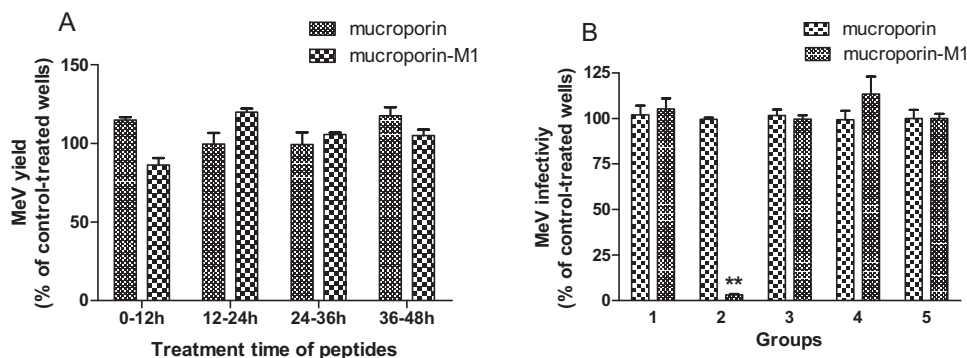


Fig. 4. Mechanism of mucroporin-M1 against MeV. The data are representative of three experiments, which showed average values for three parallel wells and were expressed as percentage of the virus titer relative to the control group treated without any peptide. The error bars indicate standard error of the mean. * means virus titers were significantly lower ($P \leq 0.05$) than those from cells exposed to no peptide, while ** means $P \leq 0.01$, *** means $P \leq 0.001$. (A) Inhibitory effects of mucroporin-M1 on the replication cycle of MeV. Vero cell monolayers in 24-well plates were infected with MeV and then were treated with $20 \mu\text{g/ml}$ of mucroporin-M1 at different time intervals of 0–12 h, 12–24 h, 24–36 h, and 36–48 h p.i. Infected monolayers without peptide treatment were used as controls. At 48 h p.i., the infected cells were harvested, and the yields of MeV were analyzed by plaque assay. (B) The inactivating effect by different treatments of mucroporin-M1 on MeV. Group 1, Vero cells were pretreated with mucroporin-M1 and then infected with MeV; Group 2, MeV and mucroporin-M1 were mixed and incubated for 1 h, and then the mixture was inoculated in Vero cells; Group 3, MeV and mucroporin-M1 were mixed but without incubation and the mixture was inoculated in Vero cells immediately; Group 4, Vero cells were infected with MeV and then treated with mucroporin-M1; and Group 5, Vero cells were infected with MeV but without mucroporin-M1 as a control. We used $20 \mu\text{g/ml}$ of mucroporin-M1 peptide and 1×10^3 pfu/ml MeV in all five groups and an adsorption time of virus or peptide treatment time of 1 h in all groups. The cells were washed three times with serum-free DMEM to remove unattached virus or free peptide after infection or treatment. The inhibitory effects of different groups were analyzed by plaque assay.

ferences between the different treatments. Mucroporin-M1 only showed about 20% inhibition of MeV infectivity with 0–12 h treatment and no discernable inhibition with 12–24 h, 24–36 h, and 36–48 h treatments (Fig. 4A). Therefore, after viral attachment, the peptide is unable to interfere with the following steps of virus replication.

To help elucidate the inhibition mechanism, we varied the treatment time and order of addition of mucroporin-M1. Only group 2, in which MeV was treated directly with mucroporin-M1 by mixing together and incubating for 1 h in advance of infecting cells, showed almost 100% inhibition; the other four groups showed no inhibition at all (Fig. 4B). Group 3, in which MeV and mucroporin-M1 were mixed but without incubation, and group 1, in which Vero cells were pretreated with mucroporin-M1, showed no inhibition. We conclude from these experiments that the lethal step to MeV occurs when mucroporin-M1 interacts directly with the virus particle itself, thereby decreasing the virus infectivity. Therefore, mucroporin-M1 has virucidal activity against MeV.

3.5. Binding study of peptides to MeV

To confirm the direct interaction between mucroporin-M1 and MeV particles, we used the real-time nature of the biosensor, which

could measure the peptide-viral particle interaction. We made serial dilutions of the viruses in HEPES buffer. Aliquots ($30 \mu\text{l}$) of virus were injected onto the surfaces of the sensor chip at a flow rate of $5 \mu\text{l/min}$. To determine the association (K_a) and dissociation (K_d) constants between measles virus and peptides, sensorgrams were obtained with different concentrations of the viruses and different peptides. In this binding study, we used the 1:1 (Langmuir) binding model of BIAevaluation software to analyze the binding curves obtained from peptide-immobilized flow cells with virus. The relative association and dissociation constants for three independent experiments on each test solution are summarized in Table 2.

The signal resonance increased in a dose-dependent manner with increased virus concentration. MeV has a significantly higher affinity for mucroporin-M1 than mucroporin, as indicated by about

Table 2

Summary of equilibrated rate constants of mucroporin and mucroporin-M1 peptides to MeV.

	K_a (1/Ms)	K_d (1/s)	KA
Mucroporin	4.67×10^7	1.04×10^{-3}	4.49×10^{10}
Mucroporin-M1	1.09×10^9	1.32×10^{-3}	8.23×10^{11}

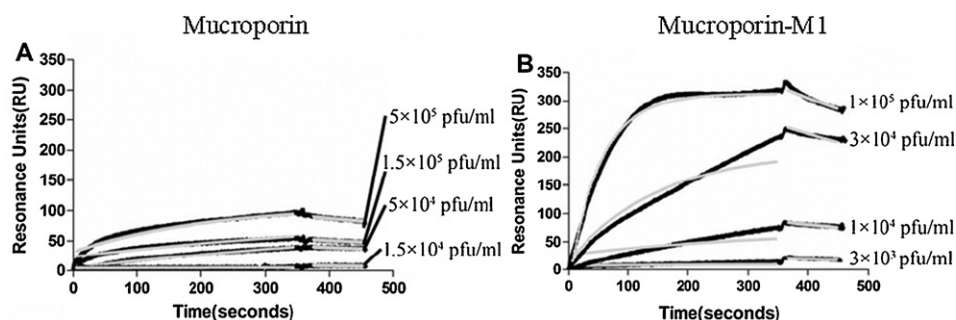


Fig. 5. Direct interaction between mucroporin (A) or mucroporin-M1 (B) and MeV by Biocore analysis. Viruses were diluted to concentrations of 3×10^3 , 10^4 , 3×10^4 , and 10^5 pfu/ml in HEPES buffer. Next, aliquots ($30 \mu\text{l}$) of virus were injected at a flow rate of $5 \mu\text{l}/\text{min}$ onto the surfaces of the sensor chip. The binding curves obtained from peptide-immobilized flow cells with both viruses were computationally fitted to the 1:1 (Langmuir) binding model using BIAevaluation software. Separate fitting of the data using the 1:1 model yielded a χ^2 value of <130 .

10-fold greater association rate constant (K_a) (Fig. 5A and B). These results support a significant and specific interaction between mucroporin-M1 and MeV.

3.6. Virucidal activity of mucroporin-M1 against SARS-CoV and influenza H5N1 viruses

With the virucidal activity of mucroporin-M1 on MeV, we hypothesized that it may have more broad virucidal activities against other viruses. Therefore, we further examined the effects of mucroporin-M1 on SARS-CoV and H5N1 viruses. SARS-CoV and influenza H5N1 pseudoviruses were used for this study, which are best safe alternatives at present. We incubated 2.0×10^4 RLU/ml of SARS-CoV pseudovirus or 1.0×10^4 pfu/ml H5N1 pseudovirus with serially diluted mucroporin-M1 for 1 h in 2% medium and then conducted the titer assay. Mucroporin-M1, but not mucroporin, showed significant inhibitory effects against both SARS-CoV pseudovirus (Fig. 6A) and H5N1 pseudovirus (Fig. 6B and C) in a concentration-dependent manner. Indeed, $20 \mu\text{g}/\text{ml}$ and $5 \mu\text{g}/\text{ml}$ of mucroporin-M1 could almost completely inhibit the infection of SARS-CoV pseudovirus and H5N1 pseudovirus, respectively (Fig. 6). The EC_{50} values of mucroporin-M1 to SARS-CoV and H5N1 pseudoviruses were $14.46 \mu\text{g}/\text{ml}$ ($7.12 \mu\text{M}$) and $2.10 \mu\text{g}/\text{ml}$ ($1.03 \mu\text{M}$), respectively. Meanwhile, cytotoxicity of mucroporin-M1 on HeLa-ACE2 and MDCK cells were also conducted, and the CC_{50} of mucroporin-M1 on HeLa-ACE2 cells and MDCK cells were $61.58 \mu\text{g}/\text{ml}$ ($30.31 \mu\text{M}$) and $83.35 \mu\text{g}/\text{ml}$ ($41.03 \mu\text{M}$) respectively. Thus, the selective indexes (SI) of mucroporin-M1 to SARS-CoV and H5N1 pseudoviruses are 4.26 and 39.69, respectively. Therefore, mucroporin-M1 possesses broad-spectrum virucidal activity.

To determine the effective virucidal time needed for mucroporin-M1 to act on the pseudoviruses, different peptide-virus mixtures and incubation treating times were tested with an efficient concentration of mucroporin-M1. Our results showed that the inhibitory effects of mucroporin-M1 against both of the pseudoviruses were positively correlated with direct treatment time and that 100% inhibition of virus infectivity could be reached in no more than 40 min (data not shown).

In brief, the scorpion venom peptide variant mucroporin-M1 has broad-spectrum virucidal activity against measles, SARS-CoV and influenza H5N1 viruses. These results suggest that the rational modification of scorpion venom peptide may change the property of the peptide to endow it with new biological activities.

4. Discussion

Despite vaccination, measles remains a burden in both developed and developing countries. Currently used antiviral treatments

for MeV infections are limited to ribavirin and/or immune serum globulin [4,27], which show variable efficacy against different MeV strains. Furthermore, the IC_{50} value of ribavirin varies from 40 to $500 \mu\text{M}$ *in vitro* [3,14], and therapeutic use of ribavirin is also limited by its side effect such as anemia [2]. Development of novel antiviral agents is needed to close the vaccination gap and treat increasing outbreaks, which urged us to test our scorpion venom-derived peptides for anti-MeV activity and more. In previous work, we isolated different kinds of cationic antimicrobial peptides from a cDNA library of the venomous gland of scorpion and identified their potent growth-inhibitory activity against antibiotic-resistant gram-positive pathogens [9,18]. Mucroporin is the first cationic host defense peptide that has been isolated from the venom of the scorpion *Lychas mucronatus*; we recently cloned it and demonstrated its specific effects on inhibiting bacteria [9]. We found that mucroporin can effectively inhibit standard bacteria, especially gram-positive bacteria. Moreover, the mucroporin-M1 optimized by amino acid substitution resulted in inhibition of gram-positive bacteria at low concentrations and inhibition of antibiotic-resistant pathogens [9]. The main aim of this investigation was to further test mucroporin and mucroporin-M1 for their anti-viral activity. We found that the anti-MeV activity of mucroporin-M1 was notably increased by the molecular modifications, while the original peptide mucroporin showed no anti-MeV activity at all. In the optimized modification, the Glycine and Proline residues of mucroporin were replaced with Lysine or Arginine, thereby enhancing the net positive charge of the hydrophilic side and changing the secondary structure of the peptide. The modifications enabled the peptide to interact directly with MeV particles with increased affinity (Fig. 5). The anti-MeV activity of mucroporin-M1 presented an EC_{50} value of $7.15 \mu\text{g}/\text{ml}$ ($3.52 \mu\text{M}$), while the original peptide mucroporin showed no anti-MeV activity at all. Moreover, we found that mucroporin-M1 could also inactivate two other enveloped RNA viruses: SARS CoV, with an EC_{50} of $14.46 \mu\text{g}/\text{ml}$ ($7.12 \mu\text{M}$), and influenza H5N1, with an EC_{50} of $2.10 \mu\text{g}/\text{ml}$ ($1.03 \mu\text{M}$). Although the selective indexes (SI) of mucroporin-M1 to SARS-CoV and H5N1 pseudoviruses shows big diversity (4.26 and 39.69, respectively), our results provide evidence that host defense peptides from scorpion can be modified for antiviral activity by rational design and represent a template approach for developing broad-spectrum antiviral agents.

The molecular design of antiviral peptides is becoming a more important and attractive strategy for developing new antiviral drugs. Viral entry is an early key stage of viral infection. Many antiviral peptides have been designed to target the virus and cell interaction, such as competitive fusion inhibitors based on the template of the interacting domains of viral ligands and cellular receptors. One particular example of rational drug design is enfuvirtide, an HIV-1 fusion inhibitor (T-20), which functions by

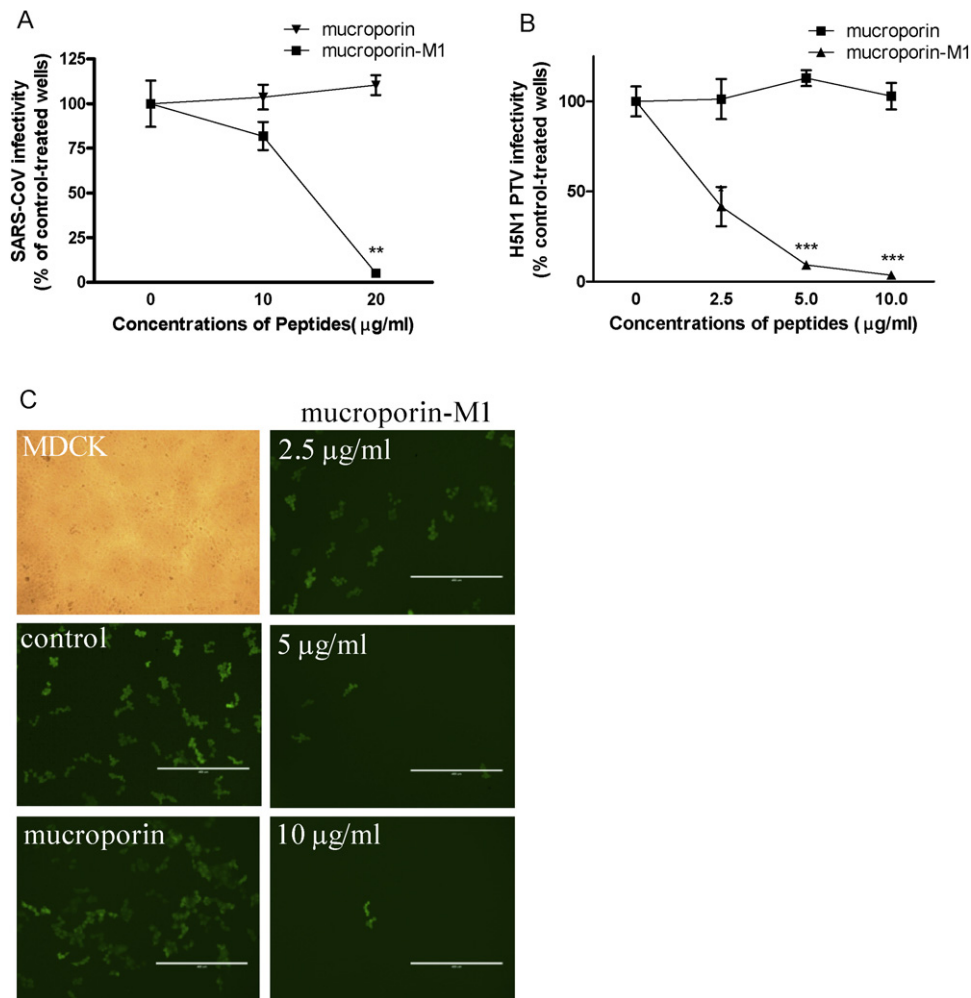


Fig. 6. Virucidal activity of mucroporin-M1 against SARS-CoV (A) and influenza H5N1 viruses (B and C). (A and B) 2.0×10^4 RLU/ml of SARS-CoV pseudovirus or 1.0×10^4 pfu/ml of H5N1 pseudovirus was incubated with serially diluted mucroporin-M1 or mucroporin for 1 h in 2% medium and then subjected to the titer assay. The groups treated without any peptide were used as controls. The data are representative of three experiments, which showed average values for three parallel wells and were expressed as percentage of the virus titer relative to the control group. The error bars indicate standard error of the mean. * means virus titers were significantly lower ($P \leq 0.05$) than those from cells exposed to no peptide, while ** means $P \leq 0.01$, *** means $P \leq 0.001$. (C) Fluorescent microscopy of the MDCK cells infected by the peptide-treated and untreated H5N1 pseudovirus. H5N1 pseudoviruses were incubated with serially diluted mucroporin-M1 or mucroporin for 1 h and then were added to MDCK cells for examination of their infectious ability. The upper left panel displays the MDCK monolayer cells by light microscopy. The middle left panel shows the untreated control group. The lower left panel shows the group treated with 10 μg/ml of mucroporin. The right panel shows the groups treated with mucroporin-1 at different concentrations. Bar, 400 μm.

disrupting the HIV-1 molecular machinery at the final stage of fusion with the target cell, thereby preventing uninfected cells from becoming infected [19]. Recently, some AMPs from natural resources attracted attention because some of these molecules have been shown to be effective against viral pathogens [1,5,28]. A peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans showed very broad-spectrum of antiviral activities against herpes simplex viruses HSV-1 and HSV-2, polio virus and measles virus in addition to its anti-bacterial activities [29]. More natural resources should be screened for their potential antiviral activity. Scorpion venom is a cocktail of peptides and proteins with a diversity of special bioactivities, thus representing an unexplored resource for use in drug design and development [18,22,37]. We believe scorpion venom is also a valuable resource for antiviral drug development and tried testing mucroporin-M1 and its original molecular template mucroporin for their anti-MeV activity. We found that mucroporin-M1 can also inactivate two other enveloped RNA viruses, SARS-CoV and influenza H5N1.

Mucroporin was reported to kill pathogens by breaking the bacterial membrane [9], but had no anti-measles virus activity. When

the Glycine and Proline residues of mucroporin were replaced with Lysine or Arginine, which resulted in mucroporin-M1 with a more net positive charge of the hydrophilic side, mucroporin-M1 could inactivate MeV effectively. Inactivation of MeV was enhanced as the concentrations and treated time of mucroporin-M1 increased, revealing that the mechanism of action of mucroporin-M1 is at least partly due to direct interaction with the measles virus surface. Further studies on the relationship between peptide inhibition and the MeV replication cycle suggest that once the viral attachment step achieved, the peptide is unable to interfere with the following steps of virus replication. Experiments testing various treatment times and orders of addition of mucroporin-M1 revealed that MeV treatment with mucroporin-M1 by mixing together and incubating for 1 h in advance of infecting cells showed the most effective inhibition. Taken together, we speculate that mucroporin-M1 inhibits MeV, SARS-CoV and influenza H5N1 by direct virucidal action. Mucroporin-M1 may bind to the virus envelope by surface charge interactions and drastically decrease the infectivity of the above three viruses. However, mucroporin has no inhibitory activity against vaccinia virus, an enveloped DNA virus (data not shown). Therefore, whether the mucroporin-M1 functions against the viral

surface protein, or just surface lipid, or both need to be investigated further.

In summary, we identified a rationally designed scorpion venom peptide variant mucroporin-M1 and described its dual antimicrobial activities against viruses and bacteria, which may be considered as a lead compound for treating co-infections of viruses and bacteria. The peptide with dual antimicrobial activities can be also considered as a good additive to antiseptic mouthwashes and handwashes. All in all, the discovery of bioactive mucroporin-M1 suggests that cationic host defense peptides from scorpion venom might be good templates for antiviral peptide design and shows their potential for multifunctional antiviral agents.

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