ABSTRACT

**Aim:** A standard strain of HSV-1 (strain F) and Acyclovir (ACV)-resistant strains HSV-1 (strain 153/BLUE/106) were used to investigate the antiviral activity of Cyanovirin-N (CVN) and its derivatives (LCVN, mPEG10K-ALD-LCVN). Moreover, antiviral mode and mechanism of antiviral of LCVN was studied.

**Experimental:** HSV-1 (strain F) was maintained in our laboratory and propagated in Vero cells. HSV-1 (strain 153/BLUE/106) resistant strains were isolated from children with herpes labial in Guangzhou Children’s Hospital. Purified recombinant CVN, LCVN and the PEGylated product 10 K PEGALD-LCVN were prepared in-house by a process modified as reported previously. Vero cells were maintained in our laboratory and grown in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cytopathic effect (CPE) and MTT assays were used to evaluate the effect of CVN and its derivatives on HSV-1 in Vero cells. The number of copies of HSV-DNA was detected by real-time fluorescence quantitative PCR (FQ-PCR).

**Result and discussion:** The results showed that CVN, LCVN and mPEG10K-ALD-LCVN had a low cytotoxicity on Vero cells with a median lethal concentration (TC50) of 1.456 ± 0.340 μM, 1.747 ± 0.097 μM and 1.403 μM respectively; LCVN and mPEG10K-ALD-LCVN completely inhibit viral infection at the concentration of 0.1 μM and 2 μM respectively. The antiviral activity of CVN, LCVN and mPEG10K-ALD-LCVN were stronger than acyclovir significantly at Low micromolar. The effect of inhibiting viral attachment and penetration of LCVN were modest whereas the treatment after virus infection was more effective. The results showed that LCVN cannot inhibit the transcription of immediate early gene (UL54), but it can significantly inhibit transcription of the late gene (UL27), thereby inhibiting viral proliferation.

**Conclusion:** It is easy for the nucleoside analogue to develop drug-resistant strains following long period of treatment, whereas LCVN can inhibit HSV-1 and its ACV-resistant strains though multiple mechanisms. Thus, LCVN is an effective drug for HSV-1 and its ACV-resistant strains.

**KEYWORDS** Herpes simplex virus type I, cyanovirin-N, mode of action, resistant strains

INTRODUCTION

Herpes simplex virus type 1 (HSV-1), an enveloped DNA virus, causes a variety of infections in humans. Primary infection usually occurs during childhood and subsequent to the initial outbreak, the virus enters the peripheral nervous system, residing there permanently in a latent state of infection; it is reactivated by the proper stimulus and causes recurrence of symptoms. Serious infection with HSV-1 can also lead to life-threatening encephalitis and ocular infections that result in corneal inflammation and scarring.1,2

Currently, most of the treatments for HSV are based on nucleoside analogs of guanine, for example, acyclovir (ACV) is specifically phosphorylated by viral thymidine kinase in infected cells.3 However, widespread use of ACV has shown that HSV develops resistance to ACV through mutations in genes coding for thymidine kinase or for DNA polymerase.4–6 Thus, some immunocompromised patients and organ transplant recipients with recurrent HSV lesions develop resistance to ACV after repeated treatments.7,8 Therefore, it is important to develop new antiviral drugs with different mechanisms of action which can substitute for, or complement, acyclovir.
New type of antiviral agents from natural sources, especially those that possess high efficacy on resistant mutant viral strains and low toxicity to the host, are considered to be the most promising agent. One such candidate is cyanovirin-N (CVN), a 101-amino acid protein (11 kDa) with known three-dimensional structure, that was originally isolated from an aqueous extract of the cyanobacterium Nostoc ellipsosporum and later produced recombinantly in Escherichia coli as an active agent against HIV. The recombinant CVN is identical to natural CVN in structure and bioactivity. CVN contains two sequence repeats, 50 and 51 amino acids long, which exhibit significant similarity and equivalently positioned disulfide bonds. No similarity with any other proteins thus far deposited in published databases has been reported.

Linker-CVN (LCVN) was designed as a CVN derivative with a flexible and hydrophilic linker (Gly3Ser4) at the N-terminus. In our previous study, we have produced the purified recombinant LCVN protein in E. coli with high efficiency. The N-terminal a-amino of LCVN was PEGylated to create 10 K PEG–aldehyde (ALD)–LCVN. LCVN and 10 K PEG–ALD–LCVN retained the specificity and affinity of CVN for high mannose N-glycans. The linker-extended CVN and the mono-PEGylated derivative were determined to be promising candidates for the development of an anti-HIV-1 agent. In this report, we describe the activity of CVN, LCVN and 10 K PEG–ALD–LCVN against a standard strain HSV-1 (strain F) and ACV-resistant strains HSV-1 (strain 153/BLUE/106) in vitro.

**MATERIALS AND METHODS**

**Chemicals, reagents and media**

All the chemicals and reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated. All the media and supplements, including fetal bovine serum (FBS), were purchased from Invitrogen (New York, NY, USA) unless otherwise stated.

**Preparation of recombinant CVN, LCVN and 10 K PEG–ALD–LCVN**

Purified recombinant CVN, LCVN and the PEGylated product 10 K PEG–ALD–LCVN were prepared in-house by a process modified as reported previously. In brief, the DNA coding sequence for CVN or LCVN was synthesized and amplified by PCR, the resulting PCR product was cloned into pET3c(+) vector and sequenced. The confirmed recombinant clone pET3c(+)–CVN or pET3c(+)–LCVN was transformed into E. coli BL21(DE3) and was induced to express proteins by isopropyl-β-D-thiogalactoside. The expression of the protein was analyzed by SDS-PAGE and Western blot, and subsequently purified by Ni Sepharose column. The purified protein was found to be 11 kDa. After production the protein was stored at −80°C until use.

**Cells and cells culture**

Vero cells were maintained in our laboratory and grown in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. In the antiviral assay, the DMEM medium was supplemented with 2% FBS. All the cells were incubated at 37°C in a humidified 5% CO2 atmosphere.

**Cytotoxicity assay (MTT assay)**

The cytotoxicity assay was performed as reported elsewhere. Vero cells were trypsinized, and cell suspensions were seeded into 96-well plates (Falcon, San Mateo, CA, USA) at a concentration of 4.0 × 104 cells per well and incubated for 24 h. The wells were then aspirated and the cells were treated with various concentrations of CVN and incubated at 37°C, in a humidified 5% CO2 atmosphere for 68–72 h, and then 2.0 μL MTT (5 g/L) was added to each well. The plates were further incubated for 4 h to allow the conversion of MTT to formazan by mitochondrial dehydrogenase, then the supernatant was aspirated, 150 μL Dimethyl Sulphoxide was added to each well to solubilize the formazan crystals. The absorbence (A-value) at 570 nm was recorded by an ELISA plate reader (Bio-Rad). About 50% of cytocidal concentration (CC50) was calculated by the Reed–Muench method.

**Viruses and virus titration**

Herpes simplex virus type 1 (strain F) and ACV-resistant strains HSV-1 (strain 153/BLUE/106) were maintained in our laboratory and propagated in Vero cells. The virus was divided into aliquots and stored at −80°C until use. Virus titers were calculated as 50% tissue culture infective dose (TCID50) by cytopathic effect (CPE) assay.

**Antiviral activity in vitro**

Comprehensive antiviral effect of LCVN

A monolayer of Vero cells in a 24-well plate were co-cultured with serial twofold dilutions of LCVN for 2 h at 37°C, in a humidified 5% CO2 atmosphere, then the wells were aspirated and washed twice with PBS. About 100 μL of HSV-1 suspensions (100 TCID50) and 100 μL serial twofold dilutions of LCVN were added, in a humidified 5% CO2 atmosphere for 2 h at 37°C, then the wells were aspirated, serial twofold dilutions of LCVN in fresh DMEM medium containing 2% FBS were added to Vero cells and further incubated at 37°C, in a humidified 5% CO2 atmosphere, until typical CPE was visible. Viral infection was evaluated by CPE assay as described above.

**Virucidal effect of LCVN**

About 100 μL of HSV-1 suspensions (100 TCID50) were co-cultured with 100 μL serial twofold dilutions of LCVN at 37°C, in a humidified 5% CO2 atmosphere for...
Treatment after virus infection of LCVN

A monolayer of Vero cells in a 24-well plate were co-cultured with 0.1 mL 100 TCID₅₀/mL of HSV-1 at 37°C, in a humidified 5% CO₂ atmosphere for 2 h, then the wells were aspirated, serial twofold dilutions of LCVN in fresh DMEM medium containing 2% FBS were added to Vero cells and further incubated at 37°C, in a humidified 5% CO₂ atmosphere, until typical CPE was visible. Viral infection was evaluated by CPE assay as described above. All the controls were cultured under the same conditions with comparable media throughout the experiment. For all of the assays, each concentration was performed in three wells, and in at least three independent experiments.

Inhibition of virus adsorption of LCVN

A monolayer of Vero cells in a 24-well plate were precooled for 2 h at 4°C, then the wells were aspirated and washed twice with PBS, and infected with 0.1 mL 100 TCID₅₀/mL of HSV-1. After 2 h incubation, the cells were washed twice with PBS and further incubated with fresh DMEM medium containing 2% FBS at 37°C, in a humidified 5% CO₂ atmosphere, until typical CPE was visible. The virus induced CPE was measured by an inverted light microscopy and inhibition ratio was calculated by CPE assay.

Inhibition of virus puncture of LCVN

A monolayer of Vero cells in a 24-well plate were precooled for 2 h at 4°C, then the wells were aspirated and washed twice with PBS, and infected with 0.1 mL 100 TCID₅₀/mL of precooled HSV-1 suspensions (100 TCID₅₀) and 100 μL precooled serial twofold dilutions of LCVN at 37°C, in a humidified 5% CO₂ atmosphere for 2 h. After 2 h incubation, the cells were washed twice with PBS and further incubated with fresh DMEM medium containing 2% FBS at 37°C, in a humidified 5% CO₂ atmosphere, until typical CPE was visible. The virus induced CPE was measured by an inverted light microscopy and inhibition ratio was calculated by CPE assay.

Preventive effect of LCVN before virus infection

A monolayer of Vero cells in a 24-well plate were cocultured with serial twofold dilutions of LCVN for 2 h at 37°C, in a humidified 5% CO₂ atmosphere, then the wells were aspirated and washed twice with PBS, and further incubated with fresh DMEM medium containing 2% FBS at 37°C, in a humidified 5% CO₂ atmosphere, until typical CPE was visible. Viral infection was evaluated by CPE assay.

Real time Fq-PCR detection of viral DNA

A monolayer of Vero cells in a 6-well plate were co-cultured with HSV-1 at 37°C, in a humidified 5% CO₂ atmosphere for 2 h, then the wells were aspirated, LCVN in fresh DMEM medium containing 2% FBS was added to Vero cells and further incubated at 37°C, in a humidified 5% CO₂ atmosphere. The cells were harvested at 3, 6 and 9 h post-infection and stored at −80°C until use. DNA was extracted and subjected to real-time Fq-PCR performed in a LightCycler (Bio-Rad Co.); 42 cycles of Fq-PCR were performed in 40 μL reaction mixtures using the following thermal cycle: 37°C for 5 min, 94°C for 1 min, 95°C for 5 s and 60°C for 30 s. The results were analyzed by the Light Cycler automatic analyzer.

RESULT

Cytotoxic effect of LCVN/CVN/mPEG10K–ALD–LCVN on Vero Cells

The MTT assay showed that LCVN/CVN/mPEG10K–ALD–LCVN had little cytotoxic effect on the proliferation of Vero cells, with a CC₅₀ value of 1.747 ± 0.097, 1.456 ± 0.340 and 9.48 ± 1.403 μM respectively. Therefore, the concentration of LCVN/CVN/mPEG10K–ALD–LCVN should be less than

![Fig. 1 Cytotoxic effect of CVN and its derivatives on Vero cells.](image-url)
0.31 μM, and the concentration of mPEG10K–ALD–LCVN should be less than 2.5 μM (Fig. 1).

**Comprehensive antiviral effect of LCVN on HSV-1 153/HSV-1 BLUE/HSV-1 106 and HSV-1 F**

With the increase of concentration of LCVN, the inhibitory rate of LCVN on HSV-1 and its ACV-resistant strains increased with a dose-effect relationship. When the concentration of LCVN is less than or equal to 50 nM, the comprehensive antiviral effect of LCVN on HSV-1 153, HSV-1 BLUE and HSV-1 F were significantly stronger than on HSV-106. LCVN’s EC$_{50}$ on HSV-1 153, HSV-1 BLUE, HSV-1 106, HSV-1 F is 7, 5, 22, 20 nM respectively (Fig. 2).

**Comprehensive antiviral effect of mPEG10K–ALD–LCVN on HSV-1 153/HSV-1 BLUE/HSV-1 106 and HSV-1 F**

With the increase of concentration of mPEG10K–ALD–LCVN, the inhibitory rate of mPEG10K–ALD–LCVN on HSV-1 F and its ACV-resistant strains increased with a dose-effect relationship. When the concentration of mPEG10K–ALD–LCVN is less than or equal to 1000 nM, the comprehensive antiviral effect of mPEG10K–ALD–LCVN on HSV-1 153, HSV-1 BLUE and HSV-1 F were significantly stronger than on HSV-106. EC$_{50}$ of mPEG10K–ALD–LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106, HSV-1 F is 181, 218, 642 and 186 nM respectively (Fig. 3).

**Comprehensive antiviral effect of CVN on HSV-1 153/HSV-1 BLUE/HSV-1 106 and HSV-1 F**

With the increase of concentration of CVN, the inhibitory rate of CVN on HSV-1 and its ACV-resistant strains increased with a dose-effect relationship. When the concentration of CVN is less than or equal to 100 nM, the comprehensive antiviral effect of CVN on HSV-1 153, HSV-1 BLUE were significantly stronger than on HSV-106 and HSV-1 F. EC$_{50}$ of CVN on HSV-1 153, HSV-1 BLUE, HSV-1 106, HSV-1 F is 14, 10, 30 and 44 nM respectively (Fig 4).

**Comprehensive antiviral effect of ACV on HSV-1 153/HSV-1 BLUE/HSV-1 106 and HSV-1 F**

With the increase of concentration of ACV, the inhibitory rate of ACV on HSV-1 and its resistant strains increased
Antiviral activity and its mechanism of LCVN

Inhibition of virus adsorption of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F

Linker-cyanovirin-N showed mild inhibition of virus adsorption on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F. With the increase of concentration of LCVN, the inhibitory rate of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F increased with a dose-effect relationship. When the concentration of LCVN was 100 nM, the inhibition rate of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F were 36.41, 43.29, 29.11 and 20.33% respectively (Fig. 8).

Inhibition of virus puncture of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F

Linker-cyanovirin-N showed mild inhibition of virus puncture on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F. With the increase of concentration of LCVN, the inhibitory rate of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F increased with a dose-effect relationship. When the concentration of LCVN was 100 nM, the inhibition rate of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F were 46.34, 42.86, 54.35 and 37.50% respectively (Fig. 9).

Inhibitory effect of LCVN on HSV-1 153/HSV-1 BLUE/HSV-1 106 and HSV-1 F after infection

When CVN were added 2–9 h after infection, CVN showed significant inhibitory effect on HSV-1 and its resistant strains with a dose-effect relationship. The comprehensive antiviral effect of CVN on HSV-1 153, HSV-1 BLUE and HSV-1 106 was significantly stronger than on HSV-1 F. EC$_{50}$ of CVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F is 118.34, 10, 30 and 44 nM respectively (Fig. 5).

Virucidal activity of LCVN on HSV-1 F and HSV-1 153/HSV-1 BLUE/HSV-1 106 and HSV-1 F

To explore whether the antiviral activity of LCVN was due to virucidal activity, we evaluated the inhibition rate of HSV-1 F and ACV-resistant strains of HSV-1 after treatment with various concentrations of CVN for 2 h. The result indicated the inhibition rate of HSV-1 and ACV-resistant strains of HSV-1 were the same in all groups treated with various concentrations of CVN, all the cells showed typical CPE of HSV-1, suggesting that LCVN had no virucidal effect on HSV-1 (Fig. 6).

Preventive effect of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F

Linker-cyanovirin-N showed mild preventive effect on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F. When the concentration of LCVN was 100 nM, the inhibition rate of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F were 36.07, 35.94, 34.46 and 13.19% respectively (Fig. 7).
strains, and mild inhibitory effect on HSV-1 and its resistant strains when added 13 h after infection (Fig. 10).

**Effect of LCVN on gene expression of HSV-1 153/HSV-1 BLUE/HSV-1 106 and HSV-1 F at various stages**

To further confirm whether LCVN could repress HSV-1 DNA replication, the number of copies of HSV DNA was detected by real-time Fq-PCR. According to the characteristics of HSV-1 gene expression, immediate early gene UL54, early gene UL52 and late gene UL27 were detected at 3, 6 and 9 h respectively after infection. The results showed that LCVN has no effect on the RNA synthesis of the immediate early gene (UL54). LCVN slightly inhibited the RNA synthesis of virus early gene (UL52), in addition, LCVN significantly inhibited the RNA synthesis of late viral gene (UL27) (Figs. 11–13).

**DISCUSSION**

Referring to the HSV-1 growth curve\(^ {17} \), it was found that the virus titer extracellular was basically stable after inoculation of virus after 1 h, and it did not begin to rise until 24 h, indicating that the virus had been absorbed after 1 h. The titer of the intracellular virus began to rise after 2 h, indicating that the virus began to replicate at this time, and the virus titer extracellular increased gradually after 24 h, indicating that the virus began to release. From 72 to 96 h, the virus titer intracellular/extracellular reached the peak and began to decline, due to the death of the cells and the inactivation of the virus.

Previous reports had indicated modest in vitro activity of CVN against HSV-1\(^ {18} \) by inhibiting HSV-1 entry into natural target cells of human ocular origin and impairing the viral glycoprotein induced cell-to-cell fusion. However, no reports have shown the effect of CVN on the replication of HSV-1 RNA in cell cultures, also there are possible differences in the virus-inhibitory effects of CVN depending on differences amongst virus strains. To elucidate the mode of action of CVN against HSV-1, we first assessed the antiviral activities of CVN against HSV-1 in vitro.

A standard strain of HSV-1 (strain F) and ACV-resistant strains of HSV-1 (strain 153/BLUE/106) grown in Vero cells were used in this assay. The results demonstrated that LCVN had low cytotoxicity to Vero cells. Furthermore, while LCVN could not directly inactivate HSV-1 (strain 153/BLUE/106/F) infectivity, it could prevent the CPE of HSV-1 (strain 153/BLUE/106/F) when added before or after viral infection. LCVN has preventive effect on HSV-1 (strain F) and HSV-1 (strain 153/BLUE/106/F) and inhibiting viral adsorption and penetration. To confirm this result, the number of copies of HSV-RNA (strain HSV-1 153/BLUE/106/F) at various stages were evaluated by real-time Fq-PCR which showed that after LCVN treatment, the expression level of immediate early gene UL54 was not obviously down, and the expression of early gene UL52 and late gene UL27 was obviously inhibited. Proliferation of HSV-1 in intracellular is highly programmed. After virus infection, immediate early gene began to express.
Under the action of its activation, the early gene began to express. Under the action of immediate early and early gene expression products, the late gene was further transcribed and finally reached the peak of expression. Our results showed that the under treatment of LCVN, the expression level of UL27 (HSV-1 153, HSV-1 BLUE, HSV-1 106, HSV-1 F) decreased significantly, and there were two possible reasons for this result: (1) LCVN inhibits the expression of key genes of viral replication, including immediate early genes. Because only one immediate early gene expression level is detected, we cannot exclude that LCVN inhibits the expression of other key genes and inhibits virus biosynthesis. (2) LCVN may inhibit the replication process of virus DNA. In either way, the result is the inhibition of the proliferation of the virus. Therefore, the specific mechanism of LCVN on HSV-1 153, HSV-1 BLUE, HSV-1 106, HSV-1 F remains to be elucidated.

By studying the efficacy of LCVN at different time, we found that LCVN could inhibit the infection of HSV-1 153, HSV-1 BLUE, HSV-1 106 and HSV-1 F from the beginning of the virus infection and 20 h after virus infection. According to our results, it is speculated that the inhibitory effect of LCVN on HSV-1 infection is due to its inhibiton of virus adsorption, penetration and the inhibition of virus proliferation intracellular. In summary, LCVN inhibits HSV-1 (strain F) and ACV-resistant strains of HSV-1 (strain153/BLUE/106) by multi-mechanisms extracellular/intracellular.

Cyanovirin-N, LCVN and mPEG10K–ALD–LCVN had a good antiviral effect on HSV-1 (strain F) and ACV-resistant strains of HSV-1 (strain153/BLUE/106), and the inhibitory effect on ACV-resistant strains of HSV-1 (strain153/BLUE/106) were significantly stronger than that of the positive drug ACV at micromolar. LCVN has the functions of preventing cell infection, inhibiting virus adsorption, inhibiting virus puncture and therapeutic effect. The most notable antiviral effect is the inhibiting effect after viral infection. LCVN can significantly inhibit the transcription of the late gene UL27 of HSV-1 (strain153/BLUE/106/F). In this paper, the activities of CVN and its derivatives (LCVN and mPEG10K–ALD–LCVN) against standard strain HSV-1 (strain F) and ACV-resistant strains of HSV-1 (strain153/BLUE/106) were studied, and the antiviral mechanism of LCVN was preliminarily studied. As a non-nucleoside inhibitor, LCVN has the potential to be developed into a new anti HSV-1 drug.

REFERENCES