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Antiviral Activity of Recombinant Cyanovirin-N against HSV-1*

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Abstract: In this study, a standard strain of HSV-1 (strain SM_{44}) was used to investigate the antiviral activity of the recombinant Cyanovirin-N (CV-N) against Herpes simplex virus type 1 (HSV-1) *in vitro* and *in vivo*. Cytopathic effect (CPE) and MTT assays were used to evaluate the effect of CV-N on HSV-1 in Vero cells. The number of copies of HSV-DNA was detected by real-time fluorescence quantitative PCR (FQ-PCR). The results showed that CV-N had a low cytotoxicity on Vero cells with a CC_{50} of 359.03±0.56 μg/mL, and that it could not directly inactivate HSV-1 infectivity. CV-N not only reduced the CPE of HSV-1 when added before or after viral infection, with a 50% inhibitory concentration (IC_{50}) with 2.26 and 30.16μg/mL respectively, but it also decreased the copies of HSV-1 DNA in infected host cells. The encephalitis model for HSV-1 infection was conducted in Kunming mice, and treated with three dosages of CV-N (0.5, 5 & 10 mg/kg) which was administered intraperitoneally at 2h, 3d, 5d, 7d post infection. The duration for the appearance of symptoms of encephalitis and the survival days were recorded and brain tissue samples were obtained for pathological examination (HE staining). Compared with the untreated control group, in the 5mg/kg CV-N and 10mg/kg CV-N treated groups, the mice suffered light symptoms and the number of survival days were more than 9d and 14d respectively. HE staining also showed that in 5mg/kg CV-N and 10mg/kg CV-N treated groups, the brain cells did not show visible changes, except for a slight inflammation. Our results demonstrated that CV-N has pronounced antiviral activity against HSV-1 both *in vitro* and *in vivo*, and it would be a promising new candidate for anti-HSV therapeutics.

Key words: Recombinant cyanovirin-N; Herpes simplex virus type 1(HSV-1); Antiviral activity; Real-time FQ-PCR; Encephalitis

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 scarification [24,26].

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Immunocompromised individuals and the recipients of organ transplantations are at high risk for increased severity of HSV-1 infection [6,9]. In addition, HSV-1 has been shown to be a factor for spreading human immunodeficiency virus and causing severe diseases in AIDS patients [20,21].

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 [17,24]. However, widespread use of ACV has shown HSV develops resistance to ACV through mutations in genes coding for thymidine kinase or for DNA polymerase [4,10,11]. Thus, some immunocompromised patients and organ transplant recipients with recurrent HSV lesions develop resistance to ACV after repeated treatments [20,21]. Therefore, it is important to develop new antiviral drugs with different mechanisms of action which can substitute for, or complement, acyclovir.

New types 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. One such candidate is cyanovirin-N (CV-N), 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*[4] and later produced recombinantly in *Escherichia coli* [12]as an active agent against HIV. The recombinant CV-N is identical to natural CV-N in structure and bioactivity. CV-N 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 [2,3,13].

In our previous study, we have produced the purified and renatured recombinant CV-N protein in *Escherichia coli* with high efficiency[15]. In this report, we describe the activity of CV-N against HSV-1 *in vitro*, and further show the ability of CV-N to treat HSV-1 infection in mice.

MATERIALS AND METHODS

Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co.. The HSV DNA real-time FQ-PCR detection kit was purchased from Shenzhen PG Biotech, China. DMEM and fetal bovine serum (FBS) were purchased from GIBCO. All other chemicals used were of analytical reagent grade.

Preparation of recombinant CV-N

Purified recombinant CV-N, a unique 11-kDa cyanobacterial protein, was produced in *Escherichia coli* as reported previously [15]. In brief, the DNA coding sequence for CV-N was synthesized and amplified by PCR, the resulting PCR product was cloned into pET30a(+) vector and sequenced. The confirmed recombinant clone pET30a(+)-CV-N was transformed into *E.coli* BL21(DE3) and was induced to express proteins by IPTG. 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 11KDa and renatured successfully by the dilution method. After production the protein was stored at -80℃ until use.

Cells and cell 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℃, in a humidified 5% CO_2 atmosphere.

Cytotoxicity assay (MTT assay)

The Cytotoxicity assay was performed as reported elsewhere [5]. Vero cells were trypsinized, and cell suspensions were seeded into 96-well plates (Falcon, San Mateo, CA, USA) at a concentration of 4.0×10^4 cells per well and incubated for 24h. The wells were then aspirated and the cells were treated with various concentrations of CV-N and incubated at 37℃, in a humidified 5% CO_2 atmosphere for 68-72h, and then 20μL MTT (5g/L) was added to each well. The plates were further incubated for 4h to allow the conversion of MTT to formazan by mitochondrial dehydrogenase, then the supernatant was aspirated, 150μL DMSO was added to each well to solubilize the formazan crystals. The absorbence (*A* value) at 570nm was recorded by an ELISA plate reader (Bio-Rad). 50% cytotoxic concentration (CC_{50}) was calculated by the Reed-Muench method.

The cytotoxic effect of CV-N on Vero cells was also determined by the trypan blue viability assay.

Virus and virus titration

HSV-1 SM_{44} strain was maintained in our laboratory and propagated in Vero cells. The virus was divided into aliquots and stored at -80℃ until use. Virus titers were calculated as 50% tissue culture infective dose ($TCID_{50}$) by cytopathic effect (CPE) assay.

Antiviral activity *in vitro*

Direct virucidal effect of CV-N. 100 μL of HSV-1 suspensions (100 $TCID_{50}$) were co-cultured with 100 μL serial twofold dilutions of CV-N at 37℃, in a humidified 5% CO_2 atmosphere for 2 h. The suspension was then added to a monolayer of Vero cells and further incubated for 2h. The supernatant was aspirated, the cells were rinsed carefully with MEM and further incubated with fresh DMEM medium containing 2% FBS. The $TCID_{50}$ was calculated by CPE assay.

CV-N treatment before virus infection. A monolayer of Vero cells in a 96-well plate were co-cultured with serial twofold dilutions of CV-N for 2 h at 37℃, in a humidified 5% CO_2 atmosphere, then the wells were aspirated and washed twice with PBS, and infected with 0.1mL 100 $TCID_{50}$/mL of HSV-1. After 2h incubation, the cells were washed twice with PBS and further incubated with fresh DMEM medium containing 2% FBS at 37℃, in a humidified 5% CO_2 atmosphere, until typical CPE was visible. The virus induced CPE was measured by inverted light microscopy and evaluated by the MTT assay.

CV-N treatment after virus infection. A monolayer of Vero cells in a 96-well plate were co-cultured with 0.1mL 100 $TCID_{50}$/mL of HSV-1 at 37℃, in a humidified 5% CO_2 atmosphere for 2 h, then the wells were aspirated, serial twofold dilutions of CV-N in fresh DMEM medium containing 2% FBS were added to Vero cells and further incubated at 37℃, in a humidified 5% CO_2 atmosphere, until typical CPE was visible. Viral infection was evaluated by CPE assay and MTT 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 six wells, and in at least three independent experiments.

*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℃, in a humidified 5% CO_2 atmosphere for 2 h, then the wells were aspirated,

CV-N in fresh DMEM medium containing 2% FBS was added to Vero cells and further incubated at 37℃, in a humidified 5% CO_2 atmosphere. The cells were harvested at 2h, 4h, 8h & 18h post-infection and stored at -80℃ until use. DNA was extracted and subjected to real-time FQ-PCR performed in a Light Cycler (Bio-rad Co.); 42 cycles of FQ-PCR were carried out in 40μL reaction mixtures using the following thermal cycle: 37℃ for 5min, 94℃ for 1min, 95℃ for 5sec and 60℃ for 30sec. The results were analyzed by the Light Cycler automatic analyzer.

Antiviral activity *in vivo*

48 specific-pathogen-free Kunming mice (4 weeks of age, 17-19g in weight, 1:1 ratio of male and female) were purchased from Qingdao Laboratory Animal Center, certification No was SCXK(lu) 20030010. The mice were randomly divided into the following four groups with 12 mice per group: HSV-1 control group, high dose of CV-N (10mg/kg) treated group, medium dose of CV-N(5mg/kg) treated group, low dose of CV-N (0.5mg/kg) treated group. Preparation of herpes simplex encephalitis model was performed as described elsewhere[27]. Briefly, the mice were infected with HSV-1 (10^{-4} $TCID_{50}$/mL) by intracerebral injection, drugs were administered intraperitoneally in a 500μL inoculum volume at 2h, 3d, 5d and 7d post infection respectively. Control animals were treated with normal saline instead of CV-N. The mice were held for two weeks and observed daily; the duration for the appearance of symptoms of encephalitis and the survival days and survival rates were recorded.

The mice of each group were sacrificed when they began to die or at the end of the experiment, brain tissue samples were obtained for pathological examination (HE staining).

Statistical analysis

Data was expressed as $X\pm s$ and analyzed by F and q test by the SPSS10.0 software package. $P<0.05$ was considered to be statistically significant.

RESULT

Effects of CV-N on HSV-1 *in vitro*

The MTT assay showed that CV-N had little cytotoxic effect on the proliferation of Vero cells, with a CC_{50} value of 359.03±0.56 μg/mL. The trypan blue exclusion assay indicated the viable cell numbers in the CV-N treated and untreated cells were the same, confirming that the CV-N had little toxic effects on the cells.

To explore whether the antiviral activity of CV-N was due to virucidal activity, we evaluated the $TCID_{50}$ of HSV-1 after treatment with various concentrations of CV-N for 2 h. The result indicated the $TCID_{50}$ of HSV-1 was the same in all groups treated with various concentrations of CV-N (0μg/mL, 6.25μg/mL, 12.50 μg/mL, 25μg/mL, 50μg/mL, 100μg/mL, 200μg/mL), all the cells showed typical CPE of HSV-1, suggesting that CV-N had no virucidal effect on HSV-1.

When CV-N were added after infection, CV-N showed significant inhibitory effect on HSV-1, with a treatment index (TI) of 158.86, and mild inhibitory effect on HSV-1 when added before infection, with a TI of 11.90 (Table 1).

To further confirm whether CV-N could repress HSV-1 DNA replication, the number of copies of HSV-

Table 1. Mode of action of CV-N on HSV-1

	IC_{50}(μg/mL)	TI
Drug added before infection	30.16 ±1.11	11.90
Drug added after infection	2.26 ±1.02	158.86

TI is expressed as the ratio of CC_{50} / IC_{50}

Table 2. The inhibition of CV-N on HSV-1 DNA replication

Time(h)	n	HSV control	6.25μg/mL CV-N	
		Log(copies/mL)	Log(copies/mL)	Inhibition rate(%)
2	9	4.55±0.23	3.63±0.22[a]	18.15±2.17
4	9	5.35±0.35	3.89±0.17[a]	26.85±1.76
8	9	6.46±0.31	4.55±0.24[a]	29.91±1.83
18	9	7.29±0.27	4.62±0.19[b]	36.89±1.58

Compared with virus control [b] $p<0.01$, [a] $P<0.05$. "n" refers to the number of seeded cell wells in the 6-well plates.

DNA was detected by real- time FQ-PCR. The results showed that the copies of HSV-1 DNA significantly decreased with the treatment of CV-N from 2 h to 18 h and the inhibitory rate on HSV-DNA increased in a time-dependent manner (Table 2).

HSV-1 infection in mice

The symptoms of mice herpes encephalitis appeared 3-5d post infection varying greatly depending on the dosages of drugs. In the untreated virus control group, the mice showed a tendency to restless, convulsions, tremor, behavior changes and weight loss; even more drowsiness and paralysis were observed 2-3d post infection. On d5, animals of this group began to die, and by d7, all of them had died. In the 0.5mg/kg CV-N treated group, the mice began to show the typical symptoms 3-4 d post infection. On 5 d, animals in this group began to die, and by 8 d, all of them had died. In the 5mg/kg CV-N treated group, the mice suffered light symptoms and the survival days were more than 9d. However 10mg/kg CV-N treated mice had very light symptoms and all survived more than 14d. Compared with the untreated control group, 10 mg/kg CV-N was effective in treating encephalitis in mice (Fig. 1).

As shown microscopically, in the viral control group and the 0.5mg/kg CV-N treated group, the brain cells showed typical herpes simplex encephalitis pathological changes, such as nerve cell swelling, pyknosis of the nucleolus, edema of the perivascular space and intraventricular hemorrhaging. In the 5mg/kg CV-N treated group and 10mg/kg CV-N treated group however, the brain cells did not have visible changes, except for slight inflammation (Fig. 2).

DISCUSSION

CV-N was originally identified as an active agent against human immunodeficiency virus (HIV) and later as an antiviral agent against some other enveloped viruses. It was shown that CV-N inactivated not only laboratory-adapted T-tropic, M-tropic, and dual-tropic strains of HIV-1and HIV-2, but also primary clinical isolates of HIV-1, simian immunodeficiency virus and feline immunodeficiency virus. CV-N blocks HIV infection by binding to the surface envelope glycoprotein

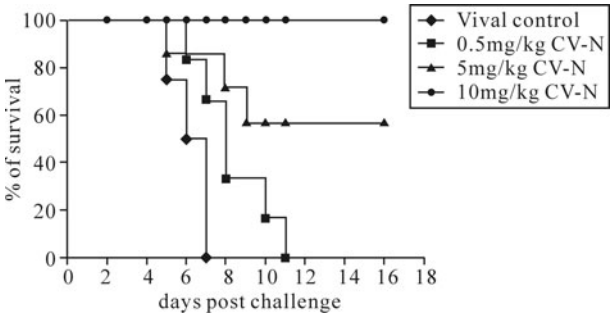

Fig. 1. Treatment of CV-N on herpes encephalitis in mice. Kunming mice were infected intracerebrally with HSV-1 (10^{-4} $TCID_{50}$/mL). CV-N were administered intraperitoneally in a 500μL inoculum volume at 2h, 3d, 5d & 7d post infection respectively. Control animals were treated with normal saline instead of CV-N.

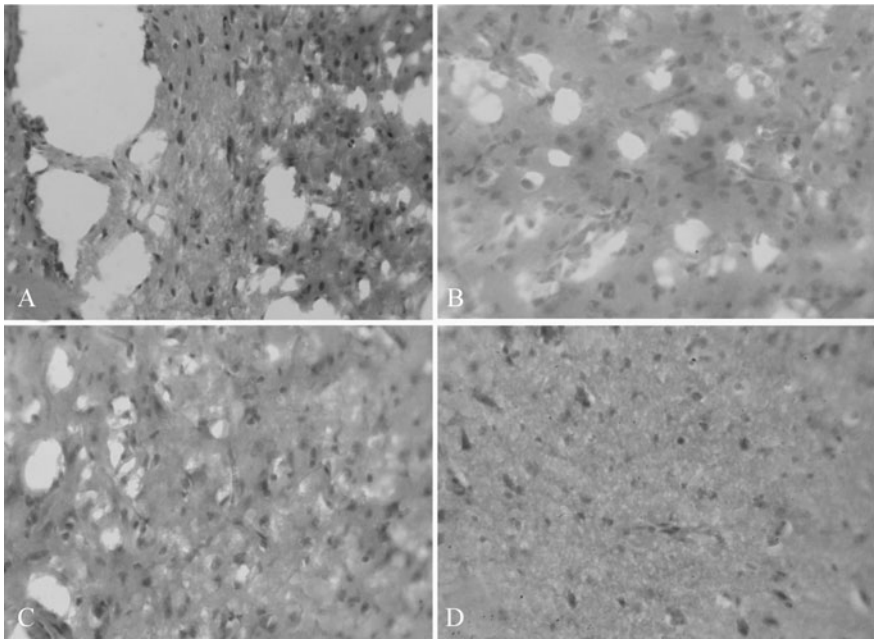

Fig. 2. Pathological changes in brain tissue of herpes encephalitis in mice (HE staining). A: HSV-1 infected mice treated with normal saline: severe swelling of the nerve cells, pyknosis nucleolus and perivascular edema. B: HSV-1 infected mice treated with low dose of CV-N(0.5mg/kg): mild swelling of the nerve cells and perivascular edema. C: HSV-1 infected mice treated with median dose of CV-N (5mg/kg): cells have no visible changes, except slight inflammation. D: HSV-1 infected mice treated with high dose of CV-N (10mg/kg): cells have no visible changes, except slight inflammation.

gp120 [8,14,18]. Further study of the mechanism of CV-N/gp120 interaction revealed that CV-N bound to high-mannose oligosaccharides on gp120, specifically oligomannose-8 (Man-8) and oligomannose-9 (Man-9) [3,8,18]. By binding to these sugars, CV-N interferes with pivotal interactions between viral and target cell receptors, preventing viral entry and cell-to-cell-fusion. This unique affinity for high mannose oligosaccharides might explain the activity of CV-N against additional viruses with similar structured constituents. More recently, CV-N has been shown to be highly active against a wide spectrum of influenza A and B virus strains, including clinical isolates and an *in vitro* derived neuraminidase inhibitor-resistant strain [19,22]. CV-N also has both *in vitro* and *in vivo* moderate activity against the Zaire strain of the Ebola virus (Ebo-Z) by inhibiting glycoprotein-mediated virus entry into the cell[1].

Previous reports had indicated modest *in vitro* activity of CV-N against HSV-1[19,23] 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 CV-N on the replication of HSV-1 DNA in cell cultures, also there are possible differences in the virus-inhibitory effects of CV-N depending on differences amongst cell lines, virus strains and quality of CV-N. To elucidate the mode of action of CV-N against HSV-1 we first assessed the antiviral activities of CV-N against HSV-1 *in vitro*. A standard strain of HSV-1 (SM_{44} strain) grown in Vero cells was used in this assay. The results demonstrated that CV-N had low cytotoxicity to Vero cells. Furthermore, while CV-N could not directly inactivate HSV-1

infectivity, it could prevent the CPE of HSV-1 when added before or after viral infection. To confirm this result, the number of copies of HSV-DNA were evaluated by real-time FQ-PCR which showed that the inhibition rates on HSV-DNA increased in a time-dependent manner.

An important question is whether or not CV-N has antiviral effect *in vivo*. In the context of an experimental infection of mice with Ebola virus, CV-N has been shown to reach the systemic circulation and exhibit measurable activity, suggesting that this drug can cross several physiological compartments after subcutaneous injection[1]. Prior to our present investigation, treatment of HSV infections *in vivo* with CV-N has not been reported. To definitively answer this question, a mouse encephalitis model for HSV-1 infection was conducted. CV-N demonstrated antiviral activity in mice by causing reductions in mortality and herpes encephalitis, suggesting CV-N has potential application for treatment of HSV-1 infections in mammals. The usefulness of the mice model for the assessment of the HSV-1 virus susceptibility to drug candidates is well recognized, and the results of our studies provide proof-of-principal of the antiherpes virus potential of CV-N in this animal model. Taken together, this work provides an important step forward in the development of CV-N as an antiviral microbicide.

In summary, given the fact that CV-N exhibited low toxicity, high resistance to physicochemical denaturation[4,12,16] and pronounced antiviral activities against HSV-1, it would be a promising new candidate for anti-HSV therapeutics. But the exact target of CV-N inside the host cell remains to be elucidated. Current experiments are in the process of investigating this question.

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