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Cyanovirin-N binds to the viral surface glycoprotein, $GP_{1,2}$ and inhibits infectivity of Ebola virus☆

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Abstract

Ebola virus (Ebo) causes severe hemorrhagic fever and high mortality in humans. There are currently no effective therapies. Here, we have explored potential anti-Ebo activity of the human immunodeficiency virus (HIV)-inactivating protein cyanovirin-N (CV-N). CV-N is known to potently inhibit the infectivity of a broad spectrum of HIV strains at the level of viral entry. This involves CV-N binding to N-linked high-mannose oligossacharides on the viral glycoprotein gp120. The Ebola envelope contains somewhat similar oligosaccharide constituents, suggesting possible susceptibility to inhibition by CV-N. Our initial results revealed that CV-N had both in vitro and in vivo antiviral activity against the Zaire strain of the Ebola virus (Ebo-Z). Addition of CV-N to the cell culture medium at the time of Ebo-Z infection inhibited the development of viral cytopathic effects (CPEs). CV-N also delayed the death of Ebo-Z-infected mice, both when given as a series of daily subcutaneous injections and when the virus was incubated ex vivo together with CV-N before inoculation into the mice. Furthermore, similar to earlier results with HIV gp120, CV-N bound with considerable affinity to the Ebola surface envelope glycoprotein, $GP_{1,2}$. Competition experiments with free oligosaccharides were consistent with the view that carbohydrate-mediated CV-N/$GP_{1,2}$ interactions involve oligosaccharides residing on the Ebola viral envelope. Overall, these studies broaden the range of viruses known to be inhibited by CV-N, and further implicate carbohydrate moieties on viral surface proteins as common viral molecular targets for this novel protein.

Keywords: Cyanovirin-N; Virucidal agent; HIV; Ebola; Filovirus; Enveloped viruses

1. Introduction

The Ebola virus (Ebo) is a biological safety level 4 (BSL-4) pathogen that causes sporadic outbreaks of severe hemorrhagic fever in central Africa, with a high mortality rate (Sanchez et al., 2001; Bray, 2002). No effective prophylactic or therapeutic agents are currently available (Bray and Paragas, 2002). Fortunately, experience has shown that outbreaks can be halted fairly rapidly by isolating infected individuals and rigorously enforcing barrier nursing methods. In recent years, some progress has been made towards the development of an effective Ebola vaccine (Vanderzanden et al., 1998; Xu et al., 1998; Rao et al., 1999; Sullivan et al., 2000; Wilson et al., 2001), and knowledge of the filovirus replication cycle has identified a number of

Abbreviations: CV-N, cyanovirin-N; HIV, human immunodeficiency virus; GP, glycoprotein; sGP, secreted glycoprotein; Man, oligomannose; ELISA, enzyme-linked immunosorbent assay; s.c., subcutaneous; i.p., intraperitoneal; EMEM, Eagle's minimal essential medium; MTD, mean time to death; pfu, plaque forming units

☆ Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditations of Laboratory Animal Care International.

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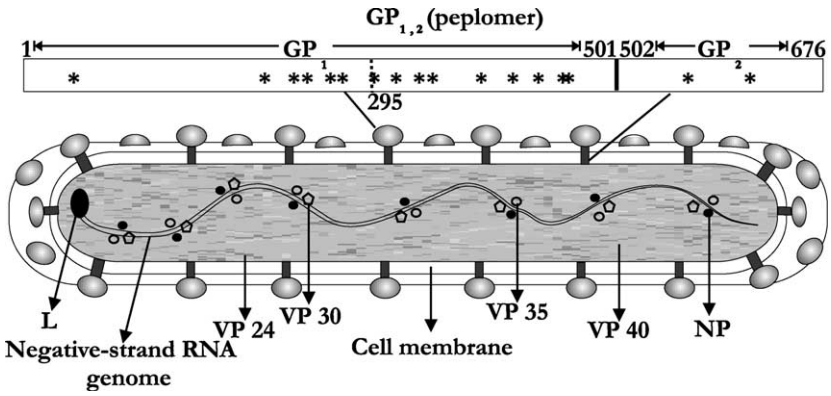

Fig. 1. Schematic illustration of Ebola virus. The virion encodes seven structural proteins with similar functions for the four known strains of Ebola: Ebola Zaire (Ebo-Z), Ebola Sudan (Ebo-S), Ebola Ivory Coast (Ebo-IC) and Ebola Reston (Ebo-R) (Kiley et al., 1980; Elliot et al., 1993; Sanchez et al., 1993). The nucleocapsid (NP), the polymerase (L), and the structural proteins VP30 and VP35 are associated with the linear non-segmented negative-stranded RNA genome. VP24 and VP40 are matrix proteins. The GP gene encodes two different products. The first product is the peplomer glycoprotein $GP_{1,2}$ that resides in the viral membrane and forms the spikes that are visible on the virion surface. $GP_{1,2}$ consists of the disulfide-linked subunits GP_1 and GP_2. The second product, sGP, is expressed by RNA editing of the same gene and is secreted by infected cells. The N-terminal 295 residues are identical for both $GP_{1,2}$ and sGP, and this is indicated by dotted lines in the $GP_{1,2}$ representation at the 295 position. sGP contains 69 amino acids at the C-terminus that is also highly glycosylated (not shown). Asterisks represent potential sites for N-glycosylation on the $GP_{1,2}$ molecule.

targets for pharmacologic intervention (Bray and Paragas, 2002). A group of adenosine analogs that inhibit a cellular enzyme, *S*-adenosylhomocysteine hydrolase, are highly active against Ebola virus both in vitro and in mice (Bray et al., 1998; Bray and Paragas, 2002). However, no antiviral compounds have been identified that interfere with Ebola infection by blocking virus binding to target cells.

In an effort to identify an antiviral agent of this type, we evaluated the activity of a natural product, cyanovirin-N (CV-N) that is currently in preclinical development as a topical (vaginal or rectal) microbicide to prevent sexual transmission of human immunodeficiency virus (HIV). CV-N is an 11 kDa protein of known three-dimensional structure (Bewley et al., 1998; Yang et al., 1999; Barrientos et al., 2002). It was originally isolated from aqueous extracts of the cultured cyanobacterium *Nostoc ellipsosporum* in a screen of natural product extracts aimed at identifying novel anti-HIV agents (Boyd et al., 1997). CV-N blocks HIV infection by binding to the surface envelope glycoprotein, gp120 (Boyd et al., 1997; Mariner et al., 1998; Esser et al., 1999; Dey et al., 2000; O'Keefe et al., 2000; Mori and Boyd, 2001). 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) (O'Keefe et al., 2000; Bolmstedt et al., 2001; Shenoy et al., 2001). We therefore suspected that CV-N might be active against certain other viruses, such as the Ebola virus, whose envelope proteins possess similar oligosaccharide structures.

Ebola virus expresses two types of glycoprotein (GP) molecule from a single gene. The primary gene product is a C-terminally truncated surface glycoprotein (sGP) that is released from infected cells (Sanchez et al., 1996, 1998). Its role in pathogenesis is still uncertain. The full-length form of GP, which is incorporated into the virion envelope, is expressed as the result of an "editing" event in the course of transcription (Feldmann et al., 1999). It consists of two subunits, GP_1 and the membrane-anchored GP_2, which are covalently linked by a disulfide bond as $GP_{1,2}$, which forms a spike on the virion surface (Fig. 1) (Sanchez et al., 1996, 1998). $GP_{1,2}$ plays a central role in viral entry into target cells by binding to an as yet unidentified receptor and mediating fusion (Feldmann et al., 1999; Weissenhorn et al., 1999). Nabel and colleagues recently reported that recombinantly expressed $GP_{1,2}$ is damaging to cells in culture, suggesting that it may also contribute directly to virulence (Yang et al., 2000).

$GP_{1,2}$ is heavily glycosylated, carrying both N-linked (including high-mannose oligosaccharides) and O-linked carbohydrates (Feldmann et al., 1999). Because $GP_{1,2}$ occupies the entire surface of the virion, we reasoned that CV-N might inhibit the binding of the Ebola virus to its target cells by binding to high-mannose oligosaccharides on the virion surface. We therefore examined the binding of CV-N to the Ebola $GP_{1,2}$ and screened for its ability to interfere with Ebola infection both in vitro and in vivo.

2. Methods

2.1. Chemicals and proteins

The CV-N used in this study was produced by recombinant methods. The DNA construct to produce recombinant CV-N, described in Mori et al. (1998), codes for 101 amino acids starting with L_1GKF at the N-terminus and ends in $LKYE_{101}$ at the C-terminus. The quantity of CV-N was determined using a Hitachi Model #L8800 amino acid

analyzer according to manufacture's protocols. Anti-CV-N polyclonal IgG antiserum was produced as described in Boyd et al. (1997). All chemicals used were analytical reagent grade.

2.2. Western blot analysis of CV-N/$GP_{1,2}$ interactions

Ebola virus isolates of the Zaire (Ebo-Z'76), Sudan (Ebo-S'00), Ivory Coast (Ebo-IC'94) and Reston (Ebo-R'89) species were grown in Vero E6 cells, from which virions were purified as previously described (Sanchez et al., 1998) and stored in a nitrogen vapor freezer. Virion lysate preparations were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5 and 10% gels and transferred to a nitrocellulose nylon membrane (Feldmann et al., 1994). Membranes were blocked with 5% milk in PBS + 0.1% Tween20 for 30 min. CV-N was diluted to 3 μg/ml in PBS and membranes were incubated with CV-N for 90 min. After washing three times with PBS-T (PBS containing 0.05% Tween20), the membranes were incubated for 1 h with rabbit-anti-CV-N polyclonal IgG antibodies (1:3000 dilution). A control membrane was incubated with PBS only. Both the CV-N treated and the control membranes were washed three times with PBS-T and treated with horseradish peroxidase-conjugated goat-anti-rabbit antibodies (Boehringer Mannhein, Indianapolis, IN) at a dilution of 1:7000 for 1 h. Finally, membranes were extensively washed with PBS-T, and the immunocomplexes were detected by chemiluminescence using ECL reagents according to manufacture's instructions (Amersham, Buckinghamshire, UK).

2.3. Preparation of GP_1-Z'76 from Ebola virion particles

GP_1-Z'76 was isolated from inactivated Ebo-Z'76 virion particles as described elsewhere (Sanchez et al., 1998). Briefly, virions were treated with non-ionic detergent, nucleocapsid particles removed by ultracentrifugation and the supernatant fluid was subjected to SDS-PAGE on a preparative 7.5% homogenous gel. The band containing GP_1 was excised and subjected to electroelution in buffer containing ammonium bicarbonate (100 mM) for 6 h at 10 °C and at constant voltage (40–50 V). The eluate was then extensively dialyzed against PBS buffer and stored at −80 °C. Yields and quantities of the purified protein were assessed by SDS-PAGE gels stained with silver.

2.4. Enzyme-linked immunosorbent assays (ELISA) protocols

GP_1-Z'76 (1 pmol per well) was bound to individual wells of 96-well Nunc-Immunosorp plates (Nagle Nunc International, Denmark) for 2 h at room temperature. The plates were washed with PBS-T (3×) and blocked overnight at 4 °C with 1% BSA (200 μl per well). Control wells were coated with 1% BSA only. Coated-plates were incubated with serial dilutions of CV-N (90.9–0.003 pmol per well) in quadruplicate for 1 h; rabbit-anti-CV-N polyclonal IgG antibodies were added for detection, followed by goat-anti-rabbit antibodies conjugated to alkaline phosphatase (Chemicon, Temecula, CA). After the addition of substrate buffer (10% diethanolamine, 17 mM *p*-nitrophenylphosphate, 1 mM $MgCl_2$, pH 9.2), the reaction was monitored by absorbance at 405 nm using a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA). Separate ELISAs were performed to analyze the capacity of the free high mannose oligosaccharide, Man-8 (Glyko Inc., Novato, CA), to block the CV-N/GP_1-Z'76 interaction. CV-N (1 pmol/ml) containing serial dilutions of Man-8 (10–0 pmol per well) were added to wells of the control plate coated with GP_1-Z'76 (1 pmol per well) and ELISAs were performed as described above.

The relative affinity of CV-N to GP_1-Z'76 and other glycosylated proteins was assessed using assays as described above, except that CV-N was added at a fixed concentration of 1 pmol per well to wells already coated with 1 pmol per well of the following proteins: GP_1-Z'76, recombinant gp120 (HIV-1) (Intracel Corp.), human serum albumin (HSA), α-acid glycoprotein (Sigma) and BSA.

2.5. In vitro testing of antiviral efficacy

All infectious material was handled in maximum-containment BSL-4 facilities. Laboratory personnel wore positive-pressure protective suits equipped with high-efficiency particulate air filters and supplied with umbilical-fed air. Vero clone E6 monkey kidney cells (Vero C1008, ATCC CRL 1586) were propagated in Eagle's minimal essential medium (EMEM) with Earle's salts, nonessential amino acids, 10% fetal bovine serum (FBS), glutamine, penicillin, and streptomycin at 37 °C in a 5% CO_2 atmosphere. Cells were seeded into 96-well polystyrene culture plates and used for antiviral drug testing once confluent. In replicate experiments, serial three-fold dilutions of CV-N in EMEM were dispensed into sequential columns of 96-well plates of Vero E6 cells, to give final concentrations ranging from 100 to 0.046 μM. EMEM without CV-N was dispensed into the final two columns. Ebola Zaire virus was then dispensed into the top three rows of each column to give a multiplicity of infection of approximately 0.01 plaque forming units (pfu) per cell, and EMEM without virus was dispensed into the bottom three rows. The plates were then returned to the incubator for 1 week. At that time, neutral red dye in EMEM at a concentration of 1.1 mg/ml was added to each well and the plates were incubated for a further 2 h. The wells were rinsed twice with phosphate-buffered saline solution, then 100 μl of a 1:1 mixture of 0.01 M ammonium phosphate buffer (pH 3.5) and ethanol was added to the wells. After 10 min of agitation on a rotary shaker, the optical density of the wells at 450 nm was determined using a 96-well plate reader (V-Max, Molecular Devices, Menlo Park, CA).

2.6. In vivo testing of antiviral efficacy

Adult female 6–8-week-old BALB/c mice were obtained from the National Cancer Institute (NCI), Frederick, MD, housed in filtertop microisolator cages and given commercial mouse chow and water ad libitum. The adaptation of Ebo-Z virus to adult, immunocompetent mice has been described (Bray et al., 1998). Briefly, a stock of Ebo-Z'76 (Mayinga) virus, previously passaged three times intracerebrally in suckling mice and twice in Vero cells, was passaged nine more times by subcutaneous (s.c.) or intraperitoneal (i.p.) inoculation in progressively older suckling BALB/c mice. Virus recovered from the liver of a moribund ninth-passage mouse was plaque-purified twice, amplified and aliquotted. This "mouse-adapted virus" is lethal for adult BALB/c and other mouse strains inoculated i.p., with a 50% lethal dose (LD_{50}) of 0.03 pfu.

Initial toxicity testing was performed by inoculating groups of five mice beneath the skin of the upper back with a range of daily doses of CV-N and monitoring them for appearance of illness (ruffled fur, diminished activity) and weight loss indicative of systemic toxicity. Once a dose of drug had been determined that was well tolerated, two types of experiments were performed. In the first, which evaluated the efficacy of CV-N treatment on Ebola infection, a cohort of mice was challenged by i.p. inoculation of 10 pfu (300 LD_{50}) of mouse-adapted virus suspended in EMEM. Subgroups of five mice were injected s.c. with CV-N dissolved in 0.1 ml of PBS, or with PBS only (placebo), beginning either the day before or within 1 h after infection and continuing daily through day 5 postinfection (experiment 1) or day 8 postinfection (experiment 2). All groups were held for daily observation of weight loss, illness, and death through day 21 postinfection. In the second type of experiment, mouse-adapted Ebola virus was pre-mixed with various concentrations of CV-N dissolved in EMEM, or with EMEM only (placebo), and incubated for 2–4 h before being injected i.p. into groups of mice, which were then observed daily as described.

3. Results

3.1. CV-N/$GP_{1,2}$ interaction by immunoblotting

Ebola virion lysates derived from Ebo-Z'76, Ebo-S'00, Ebo-IC'94 and Ebo-R'89 species were prepared for immunoblot analyses to assess the binding specificity of CV-N for Ebola proteins. A schematic illustration of an Ebola virion indicating the different viral proteins is provided in Fig. 1. For all four strains of Ebola, immunoblot analyses demonstrated that CV-N binds primarily to glycoprotein $GP_{1,2}$ and its reduced byproducts GP_1 and GP_2 (Fig. 2B and C). The differences in observed band intensity in Fig. 2C is mostly due to differential protein transfer efficiency of large molecular weight proteins during blotting. In Fig. 2B,

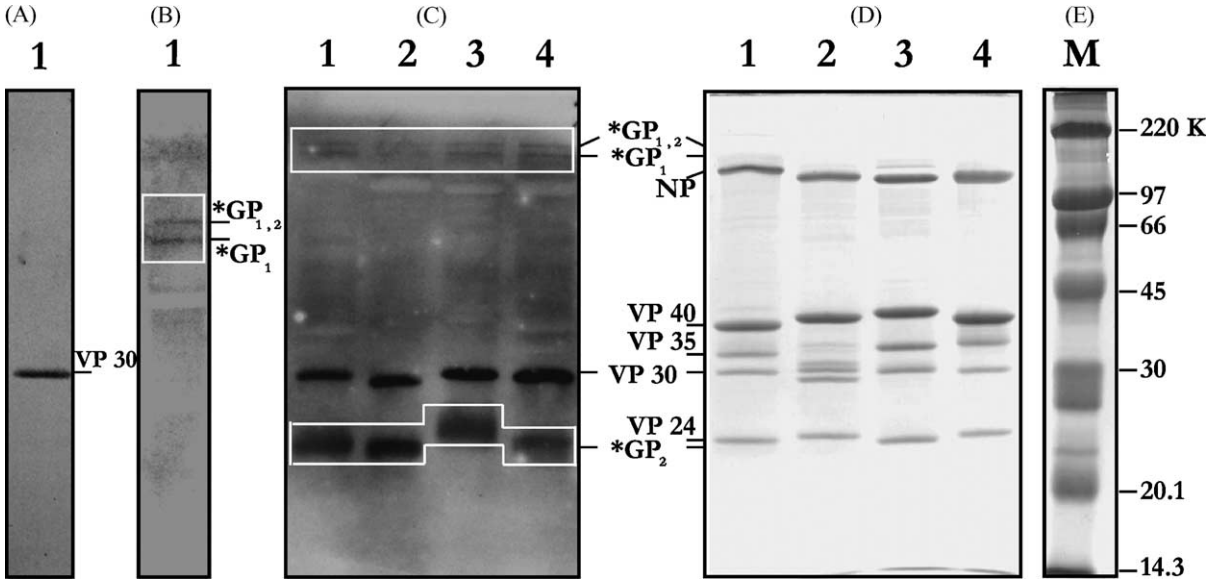

Fig. 2. Immunoblot analysis of CV-N binding to viral lysates from Ebo-Z'76 (lane 1), Ebo-S'00 (lane 2), Ebo-IC'94 (lane 3) and Ebo-R'89 (lane 4) virions. Lysates were subjected to SDS-PAGE on 10% (A, C, D, and E) and 7.5% (B) gels and blotted onto NC membranes. (A) A control blot was incubated with PBS, secondary antibody and reagents for ECL detection only; the band visible is due to non-specific binding of the secondary antibody to VP30. (B) The blot was incubated with CV-N, relevant primary and secondary antibodies and ECL reagents; proteins of 40 kDa and higher separate on a 7.5% gel. (C) Identical conditions as in (B); proteins from 14 kDa and higher separate on a 10% gel. (D) Coomassie blue-stained gel showing the position of all Ebola proteins; the exact positions of the proteins separated from Ebo-Z'76 lysate are indicated in the margin; similar position are seen for the other strains of Ebola; proteins marked by an asterisk do not stain with Coomassie blue, but are visible with Ponceau S stain (not shown). (E) Lane M, Coomassie blue-stained molecular weight markers; molecular masses (in kilodaltons) are shown at the right of the gel. (A) and (B) show results for Ebo-Z'76 (lane 1); similar results were obtained for the other strains of Ebola (lanes not shown).

the proteins were separated on a 7.5% gel, and the binding of CV-N to $GP_{1,2}$ and GP_1 was readily apparent. Strong binding was also observed for VP30 (Fig. 2C). As demonstrated with a control blot (Fig. 2A), this binding, however, is caused by the secondary antibody rather than CV-N or the primary antibodies. A Coomassie blue-stained gel with molecular weight markers was run in parallel and protein location was consistent with previous results (Fig. 2D and E; Kiley et al., 1980; Feldmann et al., 1993, 1994; Sanchez et al., 1998).

3.2. CV-N binding to $GP_{1,2}$ by ELISA

Further indication for a direct interaction between CV-N and $GP_{1,2}$ was obtained using ELISA experiments. The first experiment was conducted to compare the relative binding of the GP_1 fragment with HIV-1 gp120 (GP_2 was not tested) (Fig. 3). CV-N bound readily to HIV-1 gp120 (midpoint at 0.1 pmol; Boyd et al., 1997), as well as to GP_1. The control proteins HSA, α-acid glycoprotein and BSA showed little or no detectable interaction with CV-N (Fig. 3).

In an additional ELISA, the GP_1 fragment was bound to the microtiter plate wells and CV-N binding was tested in a concentration-dependent manner. The results are displayed in Fig. 4A. The response curve indicates saturable binding interaction with a midpoint at approximately 1 pmol.

The effect of pre-incubating CV-N with Man-8 was characterized in a separate ELISA. Man-8 binding reduced the CV-N/GP_1 response by up to 40% (Fig. 4B). Our results are consistent with recent reports suggesting that high mannose oligosaccharides (Man-8 and Man-9) components of HIV envelope glycoprotein are important for the binding and antiviral activity of CV-N, and similar competition assays indicated 50 and 20% inhibition of the CV-N/gp120 binding interaction with Man-8 and Man-9, respectively (O'Keefe et al., 2000; Bolmstedt et al., 2001; Shenoy et al., 2001).

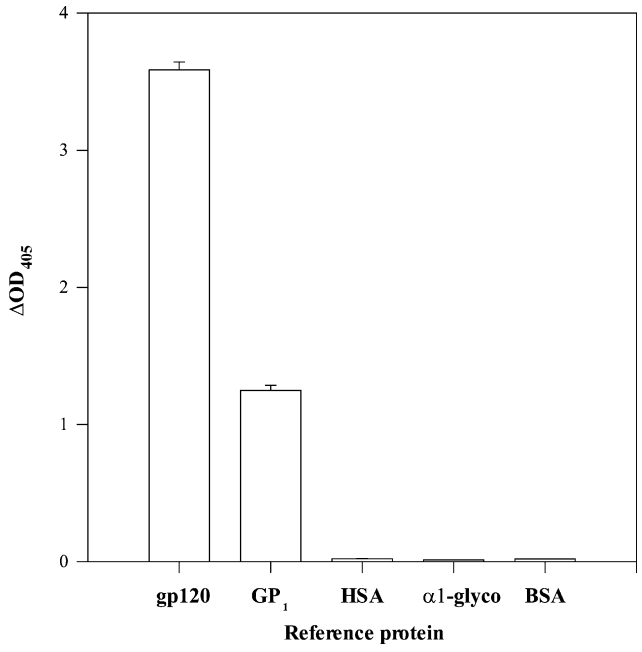

Fig. 3. ELISA comparing binding of CV-N to HIV-1 gp120 Ebola glycoprotein GP_1, and unrelated proteins from human serum (α1-acid glycoprotein and HSA) and BSA. All data are corrected for background antibody absorption in the absence of captured protein (typically <0.1 OD_{405}). Results are the average OD_{405} (±S.D.) from triplicate wells.

3.3. In vitro antiviral activity

The presence of CV-N in the medium of Vero E6 cells at the time of Ebola virus infection produced a range of concentration-dependent effects on the degree of virus-induced CPE measured 7 days later. A concentration of 0.04 μM or lower did not prevent the development of CPE (Fig. 5). By contrast, cells treated with 0.12 μM CV-N took up neutral red almost as well as uninfected cells treated with the same amount of drug. A further three-fold increase in concentration resulted in a toxic effect on both uninfected and infected cells, manifested as the loss of ability to take up neutral red. Identical results were obtained in two independent experiments (Fig. 5).

3.4. Treatment of Ebola-infected mice with CV-N

Initial toxicity testing showed that daily doses of 14 mg/kg of CV-N caused weight loss in mice, but doses of 4.8 mg/kg and lower did not cause any signs of illness. Two therapy experiments were performed in which groups of five Ebola-infected mice were treated daily with a range of doses of CV-N. All placebo-treated mice died from infection, with a mean time to death (MTD) of 6.5 and 6.6 days (Table 1). Nearly all mice in the groups treated with daily doses of CV-N ranging from 0.48 to 5.6 mg/kg also died, but the MTDs of all eight treated groups were greater than the MTDs of the two placebo groups (Table 1; Fig. 6). In two cases, the increase in MTD relative to the placebo group was statistically significant ($P < 0.05$), whereas for others the small group size ($n = 5$) prevented achievement of statistical significance. In the second experiment, the MTD tended to increase with increasing drug dose. The effect of treatment was the same if begun on day −1 or 0. CV-N-treated mice began to lose weight on day 3 or 4 postinfection, while still receiving medication, but treatment delayed the onset and slowed the rate of weight loss, compared to the placebo group (not shown). Increasing the duration of treatment from a total of 5 or 6 days in experiment 1, to 9 or 10 days in experiment 2 did not result in a greater duration of illness or in improved survival.

3.5. Result of mixing Ebola virus with CV-N before infection

In four experiments, groups of five mice were inoculated with virus pre-incubated with CV-N in EMEM, or with virus incubated with EMEM alone (placebo). The quantities

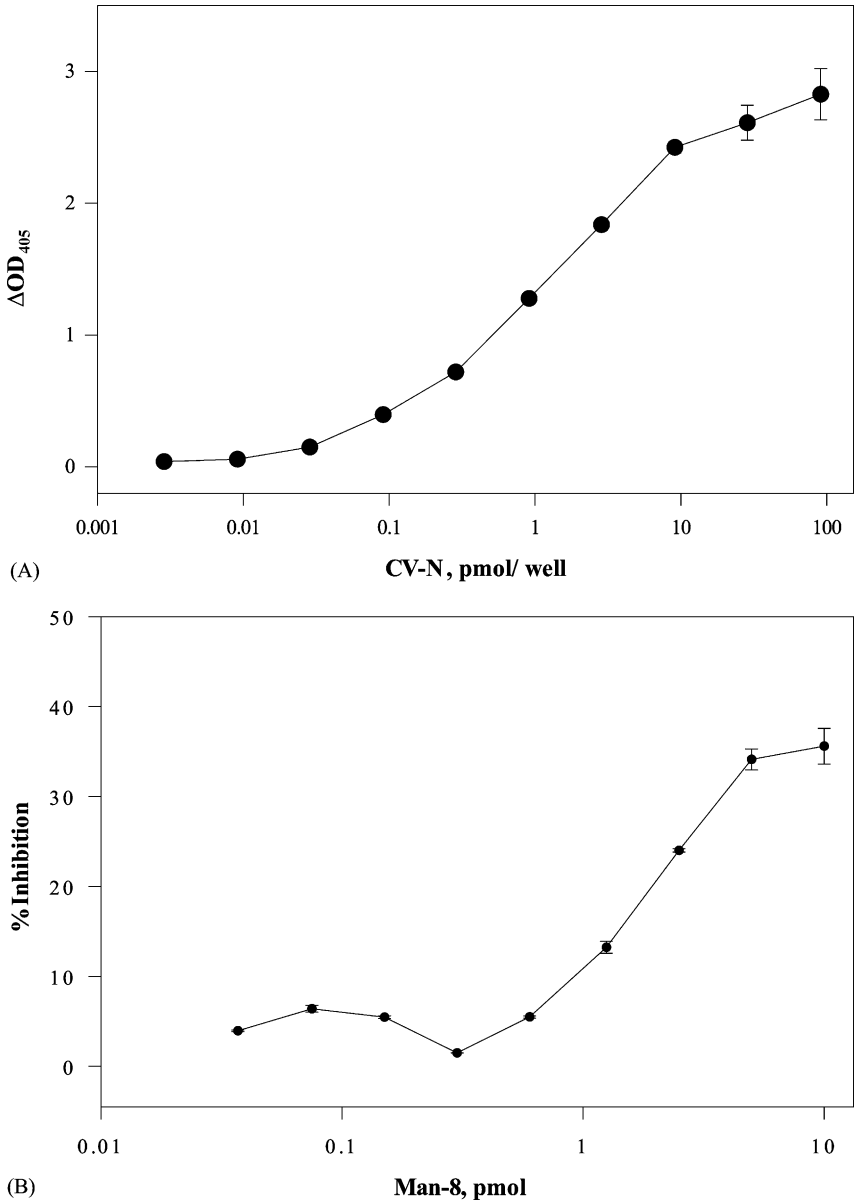

Fig. 4. (A) ELISA of CV-N binding to Ebola surface glycoprotein GP_1. CV-N logarithmic serial dilutions were added either to 1 pmol per well GP_1 or to BSA-coated control wells. Anti-CV-N polyclonal antibodies were used to detect bound protein with subsequent detection by alkaline phosphatase conjugated goat-anti-rabbit antibodies. The enzymatic reaction was followed by measuring the absorbance at 405 nm. Points are averages (±S.D.) of quadruplet determinations. Background was subtracted and is typically <0.1 OD_{405}. (B) Inhibition of GP_1 binding to CV-N by free oligomannose-8 by ELISA. CV-N was pre-incubated with or without increasing concentrations of Man-8 before adding the CV-N to GP_1-coated wells. Error bars are standard deviations based on at least triplicate determinations.

of CV-N injected were within the tolerated limit defined by toxicity testing. Table 2 shows representative data from these experiments for concentrations of CV-N in the range of 0.1–10 μM. All 11 groups of mice challenged with virus pre-incubated with concentrations of CV-N greater than 1 μM had MTDs greater than those of the placebo groups. For 8 of these 11 groups, the increase in MTD was statistically significant ($P < 0.05$). Increasing the concentration of CV-N above 10 μM did not result in a further prolongation of survival (data not shown). In contrast, only one of the six groups challenged with virus pre-incubated with a concentration of CV-N lower than 1 μM had an MTD greater than its placebo group.

In addition to prolonging the mean time to death, pre-incubation of virus with CV-N also delayed the onset of illness, as indicated by loss of body weight. Fig. 7 illustrates this effect for experiment 4. In this experiment, protection against the onset of weight loss was seen only when the CV-N concentration was 4.6 or 0.46 μM, but not for lower concentrations.

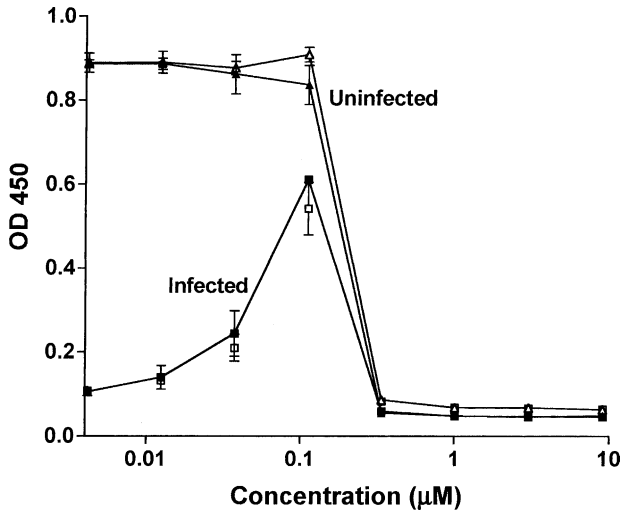

Fig. 5. Results of two experiments in which antiviral activity was measured by neutral red uptake 7 days postinfection of Vero E6 cells infected with Ebo-Z virus in the presence of increasing concentrations of CV-N. Open symbols, experiment 1; closed symbols, experiment 2.

4. Discussion

Recently, the quest for anti-Ebola viral agents has become an urgent priority and several interesting targets and avenues have become available (reviewed by Bray and Paragas, 2002, and references therein). Since CV-N exhibited an exceptional range of anti-HIV activity against both laboratory-adapted and primary clinical isolates of HIV-1 and HIV-2 that was shown to be carbohydrate-mediated, interest in this compound has widened in scope. In fact, CV-N has been reported to inactivate other enveloped viruses such as feline immunodeficiency virus, and measles virus, but not vaccinia virus, human herpesvirus 1 (HHV-1), cytomegalovirus, or adenovirus type 5 viruses (Boyd et al., 1997; Dey et al., 2000). Due to the similarities in both the fusion mechanism and glycosylation spectrum of HIV and the Ebola virus (Biller et al., 1998; Feldmann et al., 1994), it seemed logical to test whether CV-N could also be active against the latter highly lethal pathogen.

Despite their similar fusogenic mechanisms, no direct protein sequence similarities exist between gp120 and $GP_{1,2}$ (Sanchez et al., 1996). Both envelope proteins have been reported, however, to carry high mannose oligossacharides (Biller et al., 1998; Feldmann et al., 1994). At the present time, unlike gp120, no complete carbohydrate analysis is available for $GP_{1,2}$, although the primary amino acid sequence does contain 17 predicted N-glycosylation sites (Fig. 1) (Feldmann et al., 1993, 1994). As shown herein, CV-N does indeed bind with high specificity, relative to unrelated proteins, to the Ebola surface glycoprotein $GP_{1,2}$. Both in immunoblots from whole-virus lysates of Ebola treated with CV-N and in ELISA experiments with purified GP_1, measurable binding of CV-N to virus-free GP_1 was clearly demonstrated. Furthermore, the dependence of this binding on CV-N/oligosaccharide interactions was indicated by the inhibition of CV-N/GP_1 binding in the presence of the Man-8. In additional experiments with soluble Ebola GP (sGP) produced in a recombinant baculovirus expression system, which can only glycosylate proteins with high mannose oligosaccharides, CV-N bound to the recombinant Ebola glycoprotein (data not shown) to a greater extent than to the Vero cell produced GP_1. As GP_1 and sGP have identical N-termini (Fig. 1; Sanchez et al., 1996), this provided further evidence that CV-N/GP_1 interactions are oligosaccharide mediated.

The CV-N/$GP_{1,2}$ interactions were predictive of both in vitro and in vivo efficacy of CV-N against Ebo-Z. The in vitro potency of CV-N against Ebola (EC_{50} ~100 nM) was somewhat weaker than that against HIV (where EC_{50}s are generally in the 0.1–5 nM range). This might result if there are fewer Man-8 and Man-9 constituents on the Ebola viral glycoprotein, or if these CV-N binding motifs do not necessarily comprise key sites on $GP_{1,2}$ involved with virus/cell interactions. Notwithstanding the relatively modest antiviral potency of CV-N against the Ebo-Z strain, we proceeded with some in vivo screening experiments.

Table 1
Treatment experiments 1 and 2

Experiment	Days treated	Daily dose (mg/kg)	Survival[a]	MTD[b]	Significance vs. placebo[c] (*P*)
1	−1 to 5	5.6	1/4	12.7 ± 3.5	0.095
	0 to 5	5.6	1/5	8.5 ± 0.58	0.0027
	0 to 5	Placebo	0/5	6.5 ± 0.58	–
2	−1 to 8	4.8	2/5	9.3 ± 1.53	0.075
	−1 to 8	1.4	0/5	7.8 ± 0.84	0.10
	−1 to 8	0.48	0/5	7.6 ± 0.55	0.14
	0 to 8	4.8	1/5	10.0 ± 1.83	0.031
	0 to 8	1.4	0/5	7.2 ± 0.45	0.32
	0 to 8	0.48	2/5	7.3 ± 0.58	0.28
	0 to 8	Placebo	0/5	6.6 ± 1.14	–

Effect of daily s.c. inoculation of cyanovirin-N on the survival of mice challenged with i.p. injection of mouse-adapted Ebo-Z virus.
[a] Total number of mice surviving at day 21 postinfection.
[b] Mean time to death and standard deviation (days) of those mice that died.
[c] By two-tailed *t*-test.

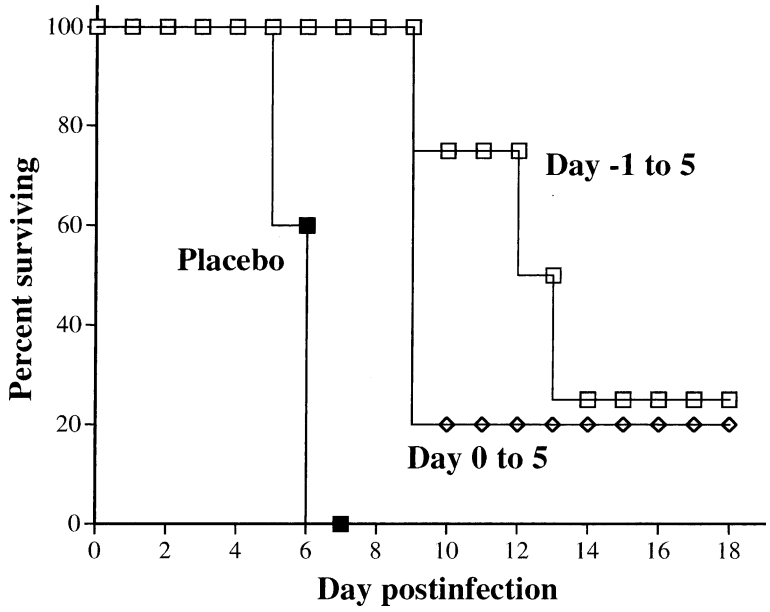

Fig. 6. Survival of BALB/c mice inoculated i.p. with 10 pfu (30 LD_{50}) of mouse-adapted Ebo-Z virus and treated daily, beginning on the indicated day, with subcutaneous injections of 5.6 mg/kg of CV-N, or with placebo (treatment experiment 1). (■): Placebo group; (□): treated from day −1 to 5; (◇): treated from day 0 to 5.

In vivo studies using a lethal mouse model of Ebo-Z infection showed that a series of a daily subcutaneous injections of approximately 5 mg/kg of CV-N, begun the day before virus infection, resulted in a significant delay in the onset of visible illness and weight loss and in the time of death, compared to placebo-treated control mice (Table 1). A three-fold lower dose of drug was less effective, and increasing the drug dose three-fold, to approximately 15 mg/kg, resulted in visible illness and weight loss in the absence of viral infection. The narrow "therapeutic index" of CV-N in this in vivo model appears to mirror the relatively small difference (three-fold) between the EC_{50} and IC_{50} of CV-N in the in vitro model used in this investigation. It is significant to note however that s.c. administration of CV-N appeared to inhibit infection by Ebo-Z. The apparent ability of CV-N, an 11 kDa protein, to traverse the varied physiological compartments necessary to show efficacy after s.c. injection was intriguing. To confirm that CV-N was

Table 2
Representative results from pre-mixing experiments 1–4

Experiment	Concentration (μM)	Dose (mg/kg)	Survival[a]	MTD[b]	Significance vs. placebo[c] (*P*)
1	6.1	0.64	0/5	8.2 ± 0.45	0.039
	1.8	0.19	0/5	7.2 ± 0.45	0.55
	Placebo	–	0/5	7.4 ± 0.55	–
2	9.1	0.67	0/5	8.4 ± 0.55	0.0027
	4.6	0.33	0/5	8.2 ± 0.45	0.0033
	Placebo	–	0/5	6.2 ± 0.84	–
3	9.1	1.0	1/5	8.0 ± 0.00	0.009
	4.6	0.5	0/5	7.8 ± 0.45	0.01
	Placebo	–	0/5	5.6 ± 1.14	–
4	4.6	0.5	0/5	7.8 ± 0.45	0.23
	0.46	0.05	1/5	7.5 ± 1.0	0.26
	0.046	0.005	0/5	6.6 ± 0.55	0.53
	Placebo	–	0/5	7.0 ± 1.2	–

Effect of pre-incubating mouse-adapted Ebo-Z with various concentrations of cyanovirin-N dissolved in EMEM, or with EMEM only, on the survival of mice after challenge. Dose: quantity of cyanovirin delivered to each mouse.

[a] Total number of mice surviving at day 21 postinfection.

[b] Mean time to death and standard deviation (days) of those mice that died.

[c] By two-tailed *t*-test.

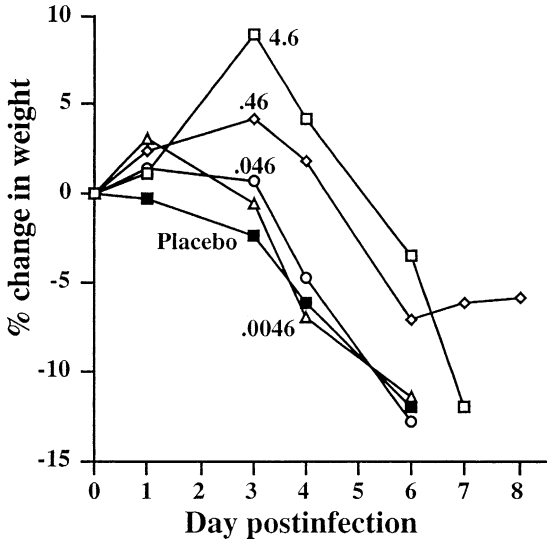

Fig. 7. Percent change in weight of BALB/c mice inoculated i.p. with 10 pfu of mouse-adapted Ebo-Z virus pre-mixed with the given concentration of CV-N (pre-mixing experiment 4). (■): Placebo group; (□): cyanovirin concentration 4.6 μM; (◇): 0.46 μM; (○): 0.046 μM; (△): 0.0046 μM.

indeed able to reach systemic circulation, pharmacokinetic analysis of CV-N in mice after various routes of administration is underway, and will be reported elsewhere in due course.

Additional in vivo experiments provided further evidence that CV-N interacts directly with the Ebola virus. Pre-incubation of Ebo-Z virions with various concentrations of CV-N prior to i.p. administration of the treated virus prolonged MTD in a concentration dependent manner (Table 2). Taken together with the other in vivo and earlier ELISA studies, it appears that CV-N interacts with the envelope glycoproteins on the surface of the Ebola virus in such a way to inhibit the infection of susceptible host cells and that this interaction can take place across significant physiological barriers.

The in vitro and in vivo antiviral results with CV-N were encouraging. To our knowledge CV-N is the first known molecule exhibiting anti-Ebola activity presumably by inhibiting glycoprotein-mediated virus entry into the cell. CV-N may thus be a useful tool to further probe the molecular details of Ebola attachment and entry into cells. Carbohydrates are well known to be associated with many pathogen/host interactions, and CV-N's interactions with specific higher order carbohydrate structures encourages new studies of novel antiviral mechanisms. Furthermore, CV-N's apparent masking of specific sugar side chains of Ebola virus $GP_{1,2}$ may suggest new avenues Ebola therapeutic research, for which a comprehensive carbohydrate analysis on $GP_{1,2}$ is necessary. Finally, the design or discovery of CV-N mimetics or homologues with greater affinity and in vivo efficacy may shed new light on the Ebola viral entry mechanism as well as new leads for drug development.

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