



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 oligosaccharides 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.

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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 Accreditation of Laboratory Animal Care International.

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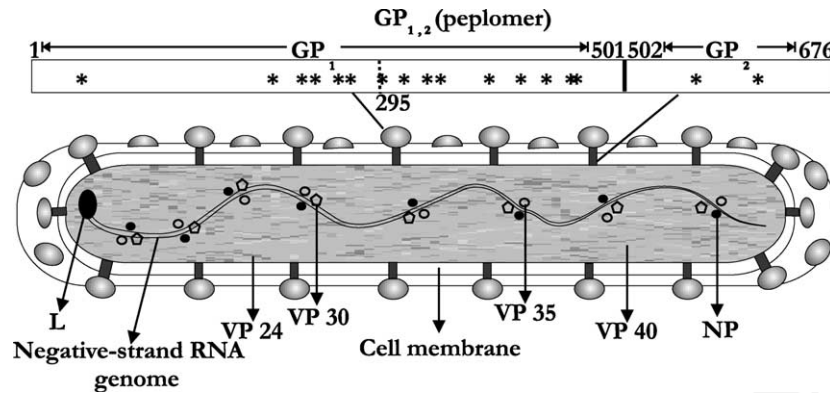


Fig. 1. Schematic illustration of Ebola virus. The virion encodes seven structural proteins with similar functions for the four known strains of Ebola: Ebola Zaire (Ebo-Z), Ebola Sudan (Ebo-S), Ebola Ivory Coast (Ebo-IC) and Ebola Reston (Ebo-R) (Kiley et al., 1980; Elliot et al., 1993; Sanchez et al., 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 GP₁ and GP₂. 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.

45 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.

52 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.

74 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

79 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, GP₁ and the membrane-anchored GP₂, 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).

92 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

103 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 L₁GKF at the N-terminus and ends in LKYE₁₀₁ at the C-terminus. The quantity of CV-N was determined using a Hitachi Model #L8800 amino acid

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

113 2.2. Western blot analysis of CV-N/GP_{1,2} interactions

114 Ebola virus isolates of the Zaire (Ebo-Z'76), Sudan (Ebo-
115 S'00), Ivory Coast (Ebo-IC'94) and Reston (Ebo-R'89)
116 species were grown in Vero E6 cells, from which viri-
117 ons were purified as previously described ([Sanchez et al.,](#)
118 [1998](#)) and stored in a nitrogen vapor freezer. Virion lysate
119 preparations were separated by sodium dodecylsulfate-
120 polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5 and
121 10% gels and transferred to a nitrocellulose nylon mem-
122 brane ([Feldmann et al., 1994](#)). Membranes were blocked
123 with 5% milk in PBS + 0.1% Tween20 for 30 min. CV-N
124 was diluted to 3 µg/ml in PBS and membranes were incu-
125 bated with CV-N for 90 min. After washing three times with
126 PBS-T (PBS containing 0.05% Tween20), the membranes
127 were incubated for 1 h with rabbit-anti-CV-N polyclonal
128 IgG antibodies (1:3000 dilution). A control membrane
129 was incubated with PBS only. Both the CV-N treated
130 and the control membranes were washed three times with
131 PBS-T and treated with horseradish peroxidase-conjugated
132 goat-anti-rabbit antibodies (Boehringer Mannheim, Indi-
133 anapolis, IN) at a dilution of 1:7000 for 1 h. Finally,
134 membranes were extensively washed with PBS-T, and the
135 immunocomplexes were detected by chemiluminescence
136 using ECL reagents according to manufacture's instructions
137 ([Amersham, Buckinghamshire, UK](#)).

138 2.3. Preparation of GP₁-Z'76 from Ebola 139 virion particles

140 GP₁-Z'76 was isolated from inactivated Ebo-Z'76 virion
141 particles as described elsewhere ([Sanchez et al., 1998](#)).
142 Briefly, virions were treated with non-ionic detergent, nu-
143 cleocapsid particles removed by ultracentrifugation and the
144 supernatant fluid was subjected to SDS-PAGE on a prepar-
145 ative 7.5% homogenous gel. The band containing GP₁ was
146 excised and subjected to electroelution in buffer contain-
147 ing ammonium bicarbonate (100 mM) for 6 h at 10 °C and
148 at constant voltage (40–50 V). The eluate was then exten-
149 sively dialyzed against PBS buffer and stored at –80 °C.
150 Yields and quantities of the purified protein were assessed
151 by SDS-PAGE gels stained with silver.

152 2.4. Enzyme-linked immunosorbent assays (ELISA) 153 protocols

154 GP₁-Z'76 (1 pmol per well) was bound to individual wells
155 of 96-well Nunc-Immunsorp plates (Nagle Nunc Interna-
156 tional, Denmark) for 2 h at room temperature. The plates
157 were washed with PBS-T (3 ×) and blocked overnight at 4 °C
158 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 quadru-
plicate for 1 h; rabbit-anti-CV-N polyclonal IgG antibodies
were added for detection, followed by goat-anti-rabbit an-
tibodies conjugated to alkaline phosphatase (Chemicon,
Temecula, CA). After the addition of substrate buffer (10%
diethanolamine, 17 mM *p*-nitrophenylphosphate, 1 mM
MgCl₂, 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 oligosac-
charide, Man-8 (Glyko Inc., Novato, CA), to block the
CV-N/GP₁-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 GP₁-Z'76 (1 pmol
per well) and ELISAs were performed as described above.

The relative affinity of CV-N to GP₁-Z'76 and other gly-
cosylated proteins was assessed using assays as described
above, except that CV-N was added at a fixed concentra-
tion of 1 pmol per well to wells already coated with 1 pmol
per well of the following proteins: GP₁-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% CO₂ atmo-
sphere. 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 dis-
pensed 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 ammo-
nium 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).

213 2.6. *In vivo testing of antiviral efficacy*

214 Adult female 6–8-week-old BALB/c mice were obtained
 215 from the National Cancer Institute (NCI), Frederick, MD,
 216 housed in filtertop microisolator cages and given commercial
 217 mouse chow and water ad libitum. The adaptation of Ebo-Z
 218 virus to adult, immunocompetent mice has been described
 219 (Bray et al., 1998). Briefly, a stock of Ebo-Z'76 (Mayinga)
 220 virus, previously passaged three times intracerebrally in
 221 suckling mice and twice in Vero cells, was passaged nine
 222 more times by subcutaneous (s.c.) or intraperitoneal (i.p.)
 223 inoculation in progressively older suckling BALB/c mice.
 224 Virus recovered from the liver of a moribund ninth-passage
 225 mouse was plaque-purified twice, amplified and aliquotted.
 226 This “mouse-adapted virus” is lethal for adult BALB/c and
 227 other mouse strains inoculated i.p., with a 50% lethal dose
 228 (LD₅₀) of 0.03 pfu.

229 Initial toxicity testing was performed by inoculating
 230 groups of five mice beneath the skin of the upper back with
 231 a range of daily doses of CV-N and monitoring them for
 232 appearance of illness (ruffled fur, diminished activity) and
 233 weight loss indicative of systemic toxicity. Once a dose of
 234 drug had been determined that was well tolerated, two types
 235 of experiments were performed. In the first, which evalu-
 236 ated the efficacy of CV-N treatment on Ebola infection, a
 237 cohort of mice was challenged by i.p. inoculation of 10 pfu
 238 (300 LD₅₀) of mouse-adapted virus suspended in EMEM.
 239 Subgroups of five mice were injected s.c. with CV-N

240 dissolved in 0.1 ml of PBS, or with PBS only (placebo),
 241 beginning either the day before or within 1 h after infection
 242 and continuing daily through day 5 postinfection (exper-
 243 iment 1) or day 8 postinfection (experiment 2). All groups
 244 were held for daily observation of weight loss, illness, and
 245 death through day 21 postinfection. In the second type of
 246 experiment, mouse-adapted Ebola virus was pre-mixed with
 247 various concentrations of CV-N dissolved in EMEM, or
 248 with EMEM only (placebo), and incubated for 2–4 h before
 249 being injected i.p. into groups of mice, which were then
 250 observed daily as described.

251 3. Results

252 3.1. *CV-N/GP_{1,2} interaction by immunoblotting*

253 Ebola virion lysates derived from Ebo-Z'76, Ebo-S'00,
 254 Ebo-IC'94 and Ebo-R'89 species were prepared for im-
 255 munoblot analyses to assess the binding specificity of CV-N
 256 for Ebola proteins. A schematic illustration of an Ebola
 257 virion indicating the different viral proteins is provided in
 258 Fig. 1. For all four strains of Ebola, immunoblot analy-
 259 ses demonstrated that CV-N binds primarily to glycoprotein
 260 GP_{1,2} and its reduced byproducts GP₁ and GP₂ (Fig. 2B
 261 and C). The differences in observed band intensity in Fig. 2C
 262 is mostly due to differential protein transfer efficiency of
 large molecular weight proteins during blotting. In Fig. 2B,

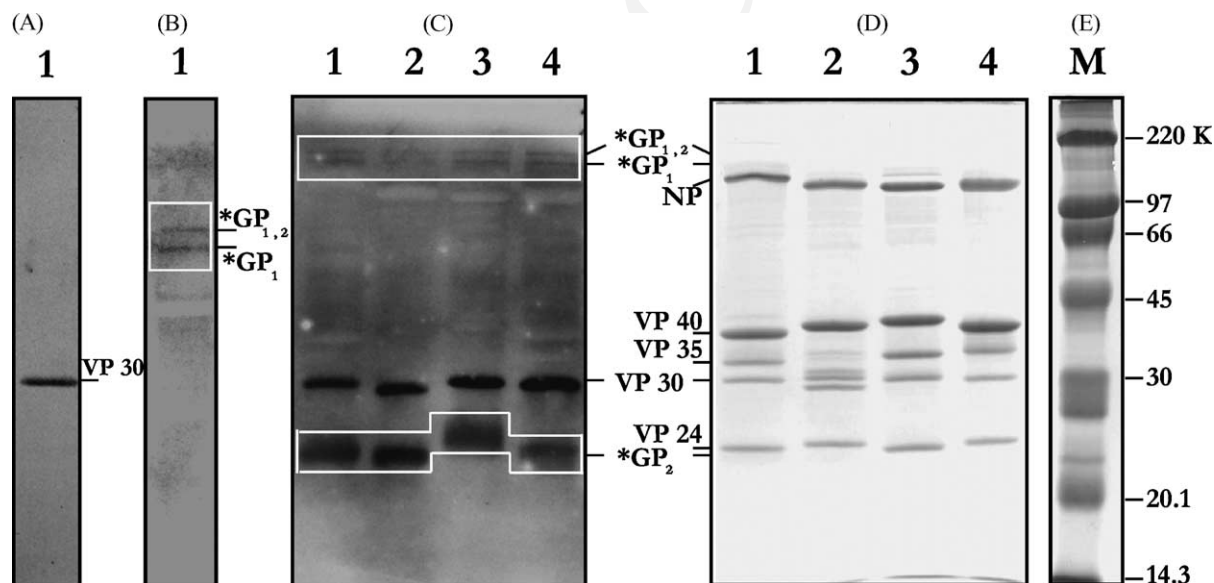


Fig. 2. Immunoblot analysis of CV-N binding to viral lysates from Ebo-Z'76 (lane 1), Ebo-S'00 (lane 2), Ebo-IC'94 (lane 3) and Ebo-R'89 (lane 4) virions. Lysates were subjected to SDS-PAGE on 10% (A, C, D, and E) and 7.5% (B) gels and blotted onto NC membranes. (A) A control blot was incubated with PBS, 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 GP₁ 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 GP₁ fragment with HIV-1 gp120 (GP₂ 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 GP₁. 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 GP₁ 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/GP₁ response by up to 40% (Fig. 4B). Our results are

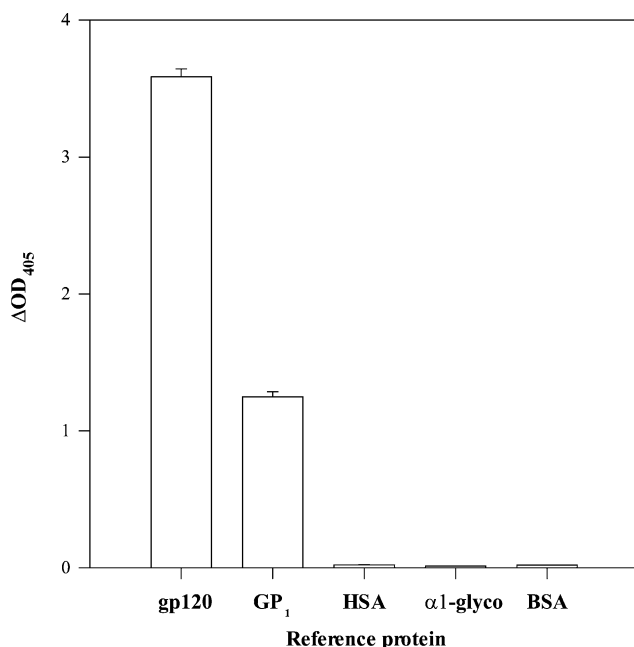


Fig. 3. ELISA comparing binding of CV-N to HIV-1 gp120 Ebola glycoprotein GP₁, and unrelated proteins from human serum (α 1-acid glycoprotein and HSA) and BSA. All data are corrected for background antibody absorption in the absence of captured protein (typically <0.1 OD₄₀₅). Results are the average OD₄₀₅ (\pm S.D.) from triplicate wells.

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).

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 μ M or lower did not prevent the development of CPE (Fig. 5). By contrast, cells treated with 0.12 μ M 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

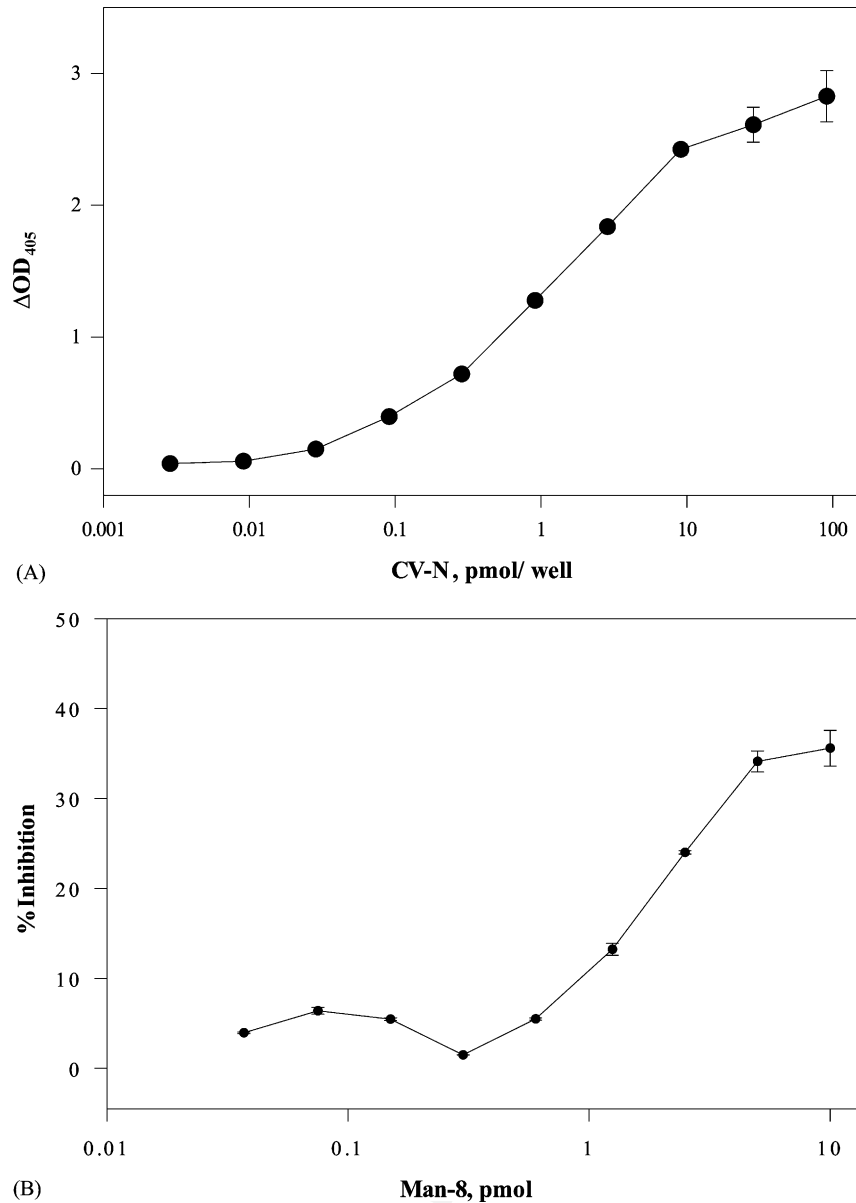


Fig. 4. (A) ELISA of CV-N binding to Ebola surface glycoprotein GP₁. CV-N logarithmic serial dilutions were added either to 1 pmol per well GP₁ or to BSA-coated control wells. Anti-CV-N polyclonal antibodies were used to detect bound protein with subsequent detection by alkaline phosphatase conjugated goat-anti-rabbit antibodies. The enzymatic reaction was followed by measuring the absorbance at 405 nm. Points are averages (\pm S.D.) of quadruplet determinations. Background was subtracted and is typically $<0.1 OD_{405}$. (B) Inhibition of GP₁ 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 GP₁-coated wells. Error bars are standard deviations based on at least triplicate determinations.

340 of CV-N injected were within the tolerated limit defined
 341 by toxicity testing. Table 2 shows representative data from
 342 these experiments for concentrations of CV-N in the range
 343 of 0.1–10 μ M. All 11 groups of mice challenged with virus
 344 pre-incubated with concentrations of CV-N greater than
 345 1 μ M had MTDs greater than those of the placebo groups.
 346 For 8 of these 11 groups, the increase in MTD was statisti-
 347 cally significant ($P < 0.05$). Increasing the concentration
 348 of CV-N above 10 μ M did not result in a further prolon-
 349 gation 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 μ M 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 il-
 lustrates 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 μ M, but not
 for lower concentrations.

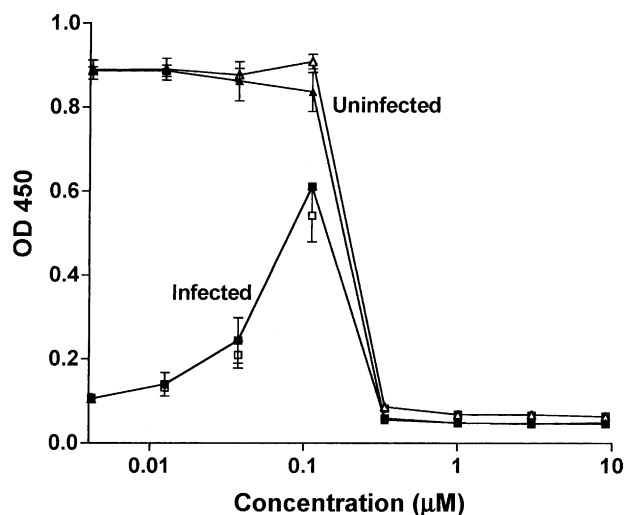


Fig. 5. Results of two experiments in which antiviral activity was measured by neutral red uptake 7 days postinfection of Vero E6 cells infected with Ebo-Z virus in the presence of increasing concentrations of CV-N. Open symbols, experiment 1; closed symbols, experiment 2.

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 oligosaccharides (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 GP₁, measurable binding of CV-N to virus-free GP₁ was clearly demonstrated. Furthermore, the dependence of this binding on CV-N/oligosaccharide interactions was indicated by the inhibition of CV-N/GP₁ 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 GP₁. As GP₁ and sGP have identical N-termini (Fig. 1; Sanchez et al., 1996), this provided further evidence that CV-N/GP₁ 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 (EC₅₀ ~100 nM) was somewhat weaker than that against HIV (where EC₅₀s 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

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 (days) of those mice that died.

^c By two-tailed *t*-test.

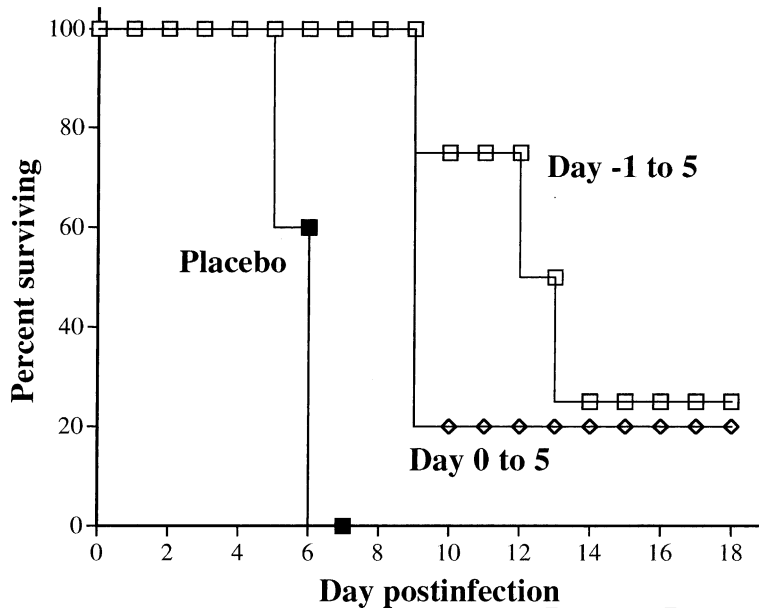


Fig. 6. Survival of BALB/c mice inoculated i.p. with 10 pfu (30LD₅₀) of mouse-adapted Ebo-Z virus and treated daily, beginning on the indicated day, with subcutaneous injections of 5.6 mg/kg of CV-N, or with placebo (treatment experiment 1).

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. 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 EC₅₀ and IC₅₀ 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

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 (days) of those mice that died.
^c By two-tailed *t*-test.

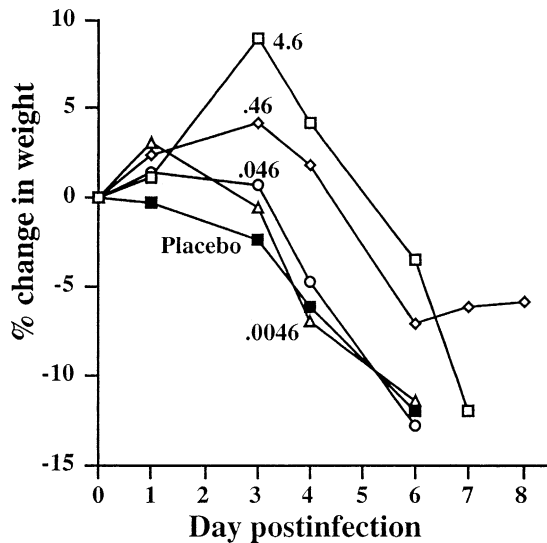


Fig. 7. Percent change in weight of BALB/c mice inoculated i.p. with 10 pfu of mouse-adapted Ebo-Z virus pre-mixed with the given concentration of CV-N (pre-mixing experiment 4).

431 physiological compartments necessary to show efficacy af-
 432 ter s.c. injection was intriguing. To confirm that CV-N was
 433 indeed able to reach systemic circulation, pharmacokinetic
 434 analysis of CV-N in mice after various routes of adminis-
 435 tration is underway, and will be reported elsewhere in due
 436 course.

437 Additional in vivo experiments provided further evi-
 438 dence that CV-N interacts directly with the Ebola virus.
 439 Pre-incubation of Ebo-Z virions with various concentra-
 440 tions of CV-N prior to i.p. administration of the treated
 441 virus prolonged MTD in a concentration dependent manner
 442 (Table 2). Taken together with the other in vivo and earlier
 443 ELISA studies, it appears that CV-N interacts with the en-
 444 velope glycoproteins on the surface of the Ebola virus in
 445 such a way to inhibit the infection of susceptible host cells
 446 and that this interaction can take place across significant
 447 physiological barriers.

448 The in vitro and in vivo antiviral results with CV-N
 449 were encouraging. To our knowledge CV-N is the first
 450 known molecule exhibiting anti-Ebola activity presumably
 451 by inhibiting glycoprotein-mediated virus entry into the
 452 cell. CV-N may thus be a useful tool to further probe the
 453 molecular details of Ebola attachment and entry into cells.
 454 Carbohydrates are well known to be associated with many
 455 pathogen/host interactions, and CV-N's interactions with
 456 specific higher order carbohydrate structures encourages
 457 new studies of novel antiviral mechanisms. Furthermore,
 458 CV-N's apparent masking of specific sugar side chains of
 459 Ebola virus GP_{1,2} may suggest new avenues Ebola thera-
 460 peutic research, for which a comprehensive carbohydrate
 461 analysis on GP_{1,2} is necessary. Finally, the design or dis-
 462 covery of CV-N mimetics or homologues with greater
 463 affinity and in vivo efficacy may shed new light on the

Ebola viral entry mechanism as well as new leads for drug 464
 development. 465

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