The Highly Specific Carbohydrate-Binding Protein Cyanovirin-N: Structure, Anti-HIV/Ebola Activity and Possibilities for Therapy

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Abstract: Cyanovirin-N (CV-N), a cyanobacterial lectin, is a potent viral entry inhibitor currently under development as a microbicide against a broad spectrum of enveloped viruses. CV-N was originally identified as a highly active anti-HIV agent and later, as a virucidal agent against other unrelated enveloped viruses such as Ebola, and possibly other viruses. CV-N's antiviral activity appears to involve unique recognition of N-linked high-mannose oligosaccharides, Man-8 and Man-9, on the viral surface glycoproteins. Due to its distinct mode of action and opportunities for harnessing the associated interaction for therapeutic intervention, a substantial body of research on CV-N has accumulated since its discovery in 1997. In this review we focus in particular on structural studies on CV-N and their relationship to biological activity.

Keywords: Cyanovirin-N, HIV; gp120, Ebola; $GP_{1,2}$, SARS, microbicidal agent, high-mannose oligosaccharides.

INTRODUCTION

Enveloped viruses are the causative agents for a large number of life-threatening diseases in humans. In the fight against infectious diseases scientists and clinicians are engaged in extensive efforts aimed at developing new and improved vaccines and new classes of drugs, such as antibiotics and other pharmacologically active agents. Despite remarkable progress and advances in our fights to reduce and eradicate diseases, today's challenges for biomedical science are still imposing: we are faced with outbreaks of unusual viral infections and the emergence of novel dangerous microbes. Three examples are (i) the global spread of human immunodeficiency virus (HIV, (Fig. **1**)), a level 2 pathogen that causes the acquired immune deficiency disease syndrome (AIDS) [1, 2]; (ii) the enigmatic outbreaks of Ebola virus (Fig. **1**), a level 4 pathogen (the most deadly human disease causing agent) that induces severe hemorrhagic fever [3-5]; and (iii) the sudden 2003 outbreak of a 'mystery pneumonia' caused by a novel coranovirus that induces severe acute respiratory syndrome (SARS) [6]. To date, there is no effective vaccine against HIV and the latest clinical trials of anti-HIV vaccines yielded disappointing results [7-9]. Even fewer avenues towards interfering with Ebola and SARS exist. In the case of HIV and Ebola, difficulties encountered are mainly caused by the lack of natural immunity to these viruses and no immune correlates of protection in humans exist [10-14], and similar problems may lie ahead for dealing with SARS. Therefore, the development of clinically useful vaccines against these types of pathogens remains a most pressing challenge in infectious diseases research.

Over the last 20 years we have witnessed an extensive and comprehensive research effort with respect to HIV, and indeed, HIV is perhaps one of the best-understood viruses at the present time. The fight against AIDS has yielded several effective therapies and drugs that target different phases of the viral life cycle [10, 15]. Treatment of HIV-infected individuals generally involves a combination, or 'cocktails', of several classes of drugs, directed specifically against at least two key HIV enzymes, protease and reverse transcriptase. This so called HAART therapy has proved highly successful and has dramatically reduced mortality. Overall, there is no doubt that remarkable progress has been achieved in combating AIDS and understanding the details of the viral life cycle. Despite these major advances, AIDS pandemics continue to pose serious global concerns. According to the latest UN estimates, over 40 million people around the world are thought to be infected with HIV and more than 60 million globally have been infected with the virus since AIDS was first reported more than 20 years ago. This sets the death toll at more than 20 million people, and each day an estimated 16, 000 people become newly infected with HIV. Most of the treatment successes occurred in the wealthy, western countries and the battle against the HIV/AIDS pandemic in the poorest nations in Africa and Asia is lacking far behind. In sub-Saharan Africa alone, more than 29 million HIV/AIDS cases exist. Therefore, novel strategies for vaccine design and programs to control the spread of the disease in these regions of the world remain an urgent priority.

Nearly a decade ago new initiatives directed at antiviral microbicides were encouraged. Such agents, when incorporated into vaginal and rectal gels, foams and suppositories for *ex vivo* or topical use, can decrease or prevent the sexual transmission of HIV [16-24]. Promising candidates for such barrier applications are substances that directly interact with HIV virions, thus preventing viral entry into and fusion with the target cells.

One such candidate is cyanovirin-N (CV-N), a 101 amino acid protein, that was originally isolated [25] from an aqueous extract of the cyanobacterium *Nostoc ellipsosporum* (Fig. **2A**) in a screen of material from the U.S. National Cancer Institute's (NCI) Natural Products Repository conducted in the Laboratory of Dr. Michael R. Boyd (NCI) for potential anti-HIV activity [26-27]. CV-N inactivated not only laboratory-adapted T-tropic, M-tropic, and dual-tropic

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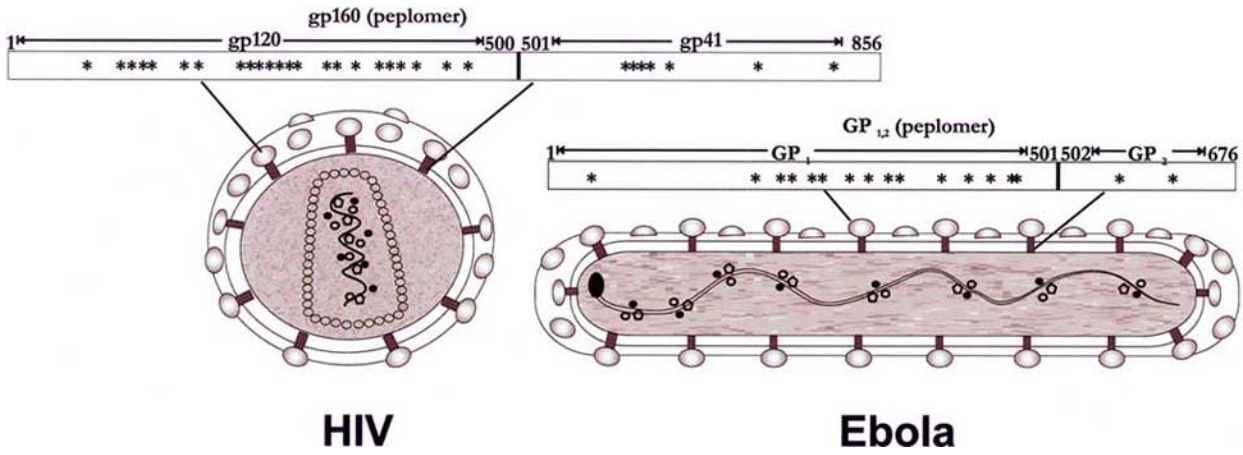

Fig. (1). Schematic illustration of HIV and Ebola virions. Potential N-glycosylation sites on the respective viral surface glycoproteins gp160 and $GP_{1,2}$ are indicated by asterisks "*".

strains of HIV type 1 (HIV-1) and HIV type 2 (HIV-2), but also primary clinical isolates of HIV-1 [25]. It was active at low nanomolar concentrations and it was established that its specificity was directed at N-linked high mannose oligosaccharides Man-8 and Man-9 on the viral surface glycoprotein gp120 [28]. 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 [29-32]. The inhibitory activity of CV-N against HIV is at least 100-fold higher (EC_{50} = 1ng/ml; [25]), than the reported antiviral activities of other lectins (EC_{50} = 0.1-2μg/ml; [33, 34]) which often require calcium or additional cofactors for optimal binding. CV-N is unique in its high specificity towards the epitope Manα1→2Man. The novel and unsuspected recognition of carbohydrate by CV-N suggested testing for further possible antiviral activity. Indeed, additional antiviral activity has been reported against a number of other enveloped viruses [25, 35].

Ebola research is less advanced than HIV research, primarily because Ebola outbreaks are sporadic and emerge unpredictably. In addition, research on this devastating virus is extremely difficult and slow due to its high pathogenicity. Fortunately, experience shows that Ebola outbreaks can be curtailed fairly rapidly by isolating infected individuals and rigorously enforcing barrier-nursing methods [36, 37]. Thus, the virus death toll still stands below 2000 victims since the first outbreak was reported in Zaire in 1976. More recently, however, Ebola research has become a high priority, given the potential use of this agent in bio-terrorism and biological warfare. Efforts have concentrated on developing an effective Ebola vaccine [38-42] and several interesting targets and drug leads have become available [12, and references therein]. For example, inhibition of the cellular enzyme, S-adenosylhomocysteine (SAH) hydrolase by a group of adenosine analogs proved highly effective in protecting against Ebola virus both *in vitro* and in mice [43]. However, no antiviral compounds have been identified that interfere with virus binding to target cells in Ebola infection. Since Man-8 and Man-9 oligosaccharides are also found on the Ebola virus glycoprotein, $GP_{1,2}$, it was reasonable to assume that CV-N might have similar effects on Ebola as observed for HIV. The inhibitory effect of CV-N on Ebola infection both *in vitro* and *in vivo* was recently investigated [44], involving a collaboration between scientists from laboratories at the National Institutes of Health (NIH) and Ebola experts from the US Centers of Disease Control and Prevention (CDC) and the US Army Medical Research Institute of Infectious Diseases (USAMRIID) and will be further discussed below.

SARS research is new and progressing with amazing speed thanks to modern technologies and a well-orchestrated worldwide effort. Within a few months after SARS being linked to a novel coronavirus, the genome had been sequenced and made publicly available [45]. However, finding a vaccine for combating this new disease may take significantly longer. Since the SARS causing coranovirus also has a surface glycoprotein testing of CV-N against this virus seems an obvious choice.

Overall, CV-N can be considered a promising anti-viral candidate and this review summarizes and discusses the activity, biochemical, biophysical and structural data available to date.

FOLDING, STRUCTURE AND STABILITY OF CV-N

Structural studies are crucial for elucidating specific conformational determinants responsible for antiviral activity of CV-N. The initial biochemical characterization of CV-N yielded the primary amino acid sequence and disulfide bonding pattern of the protein isolated from the cyanobacterial extract [25, 46]. All further work was carried out on protein expressed from a synthetic gene over-expressed in *E. coli* [25, 47]. CV-N contains two sequence repeats, 50 and 51 amino acids long, that exhibit significant similarity and equivalently positioned disulfide bonds (Fig. **2B**) [46]. No similarity with any other proteins thus far deposited in public databases was noted. Structurally, several high-resolution three-dimensional structures of CV-N with and without bound carbohydrate have been determined by NMR and X-ray crystallography since its discovery [48-54]. These structures revealed the crucial determinants for high-mannose-mediated binding of CV-N to viral surface envelope glycoprotein [25, 28, 30-32, 44]. Two different quaternary structures of CV-N were observed. The original solution structure was a monomer [48] whereas the

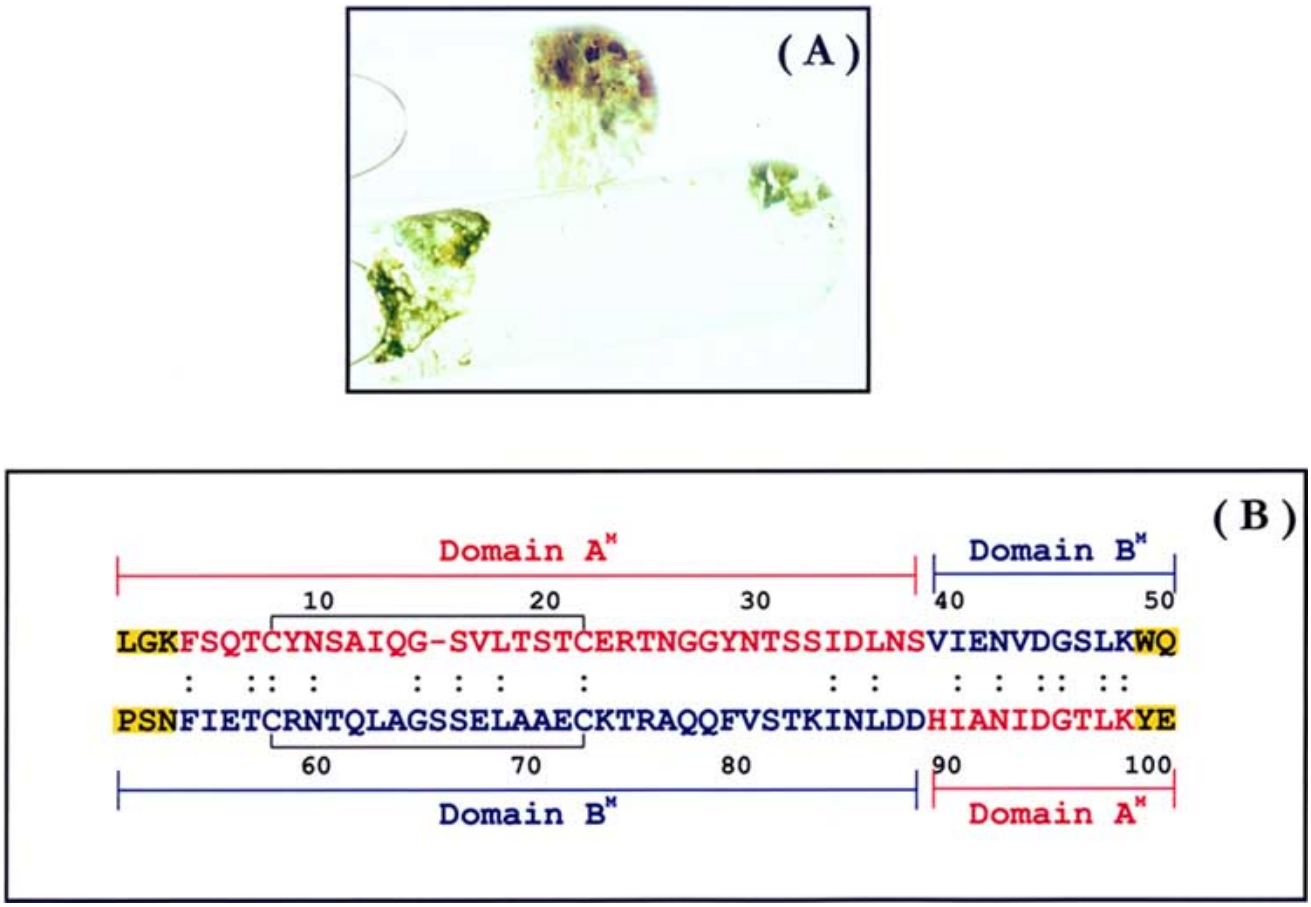

Fig. (2). (A) Laboratory cultures of Nostoc ellipsosporum. Aqueous extracts from one strain tested positive in screening assays for anti-HIV activity and the active component was identified as CV-N. (B) Amino acid sequence alignment illustrating the two sequential repeats in CV-N. For optimal alignment a one residue gap "-" was inserted between positions 15 and 16. Identical residues are denoted by ":"; residues marked in red and blue delineate the three-dimensional domain organization (A^M; red, B^M, blue) and yellow residues mark those residues that were not involved in the circular permutation of the CV-N variant; the two disulfide bonds are indicated by black brackets.

subsequently determined X-ray structure [49] consisted of a domain-swapped dimer (Fig. **3A**). Depending on experimental conditions, either form predominates and can be isolated for biophysical, structural and functional studies. [51].

In dilute solution, the major form of the protein is monomeric and its structure was solved by heteronuclear, multidimensional NMR. It comprises a compact, bilobal novel fold with pseudo-symmetry (Fig. **3A**) [48]. Interestingly, the amino acid sequence repeats of CV-N do not constitute the repeated structural domains (Figs. **2B** and **3A**). Rather, the two symmetrically related domains, A and B, are formed by strand exchange across the two sequence repeats. In the monomer, the pseudo-symmetric domains A^M and B^M consist of residues [1-38/90-101] and [39-89], respectively, each comprising a triple-stranded β-sheet with a β-hairpin packed on top. At the pseudo-twofold axis both domains are held together by a cluster of hydrophobic residues comprising V39, H90, W49 and Y100, residing in two helical turns and the tips of the two β-hairpins. In addition, the interaction between W49 and D89, involving both hydrophobic contacts and a hydrogen bond between the NεH atom of W49 and the Oδ atom of D89, contributes to this interface. In the middle of the sequence (residues 51-56) a helical linker is found in domain B^M whose counterpart in domain A^M consists of the N- and C-termini.

In the X-ray structure of CV-N the protein was found as a domain-swapped dimer (Fig. **3B**) [49] in which the monomers open up, and the two halves of the dimer contain essentially the same interactions as the monomer. Using the domain notation for the monomer introduced above, the domain-swapped dimer contains the following four domains: two of these are identical to domain A^M, although they contain residues from two different polypeptide chains. These are referred to below as domains A^D and $A^{D'}$, comprising residues 1-38 from monomer 1 (M) and 90'-101' from monomer 2 (M'), and residues 1'-38' from M' and 90-101 from M, respectively. The other two domains are similar, but not identical, to domain B^M. They are referred to as domains B^D and $B^{D'}$, comprising residues 39-50 and 51'-89' from M and M', and residues 39'-50' and 51-89 from M' and M, respectively. Both differ from domain B^M in the linker region that is helical in the monomer whereas it is essentially extended in the dimer. Only domain-swapped dimer structures are observed in all X-ray structures to date [49, 51, 53, 54]. Initially it was suggested that low pH

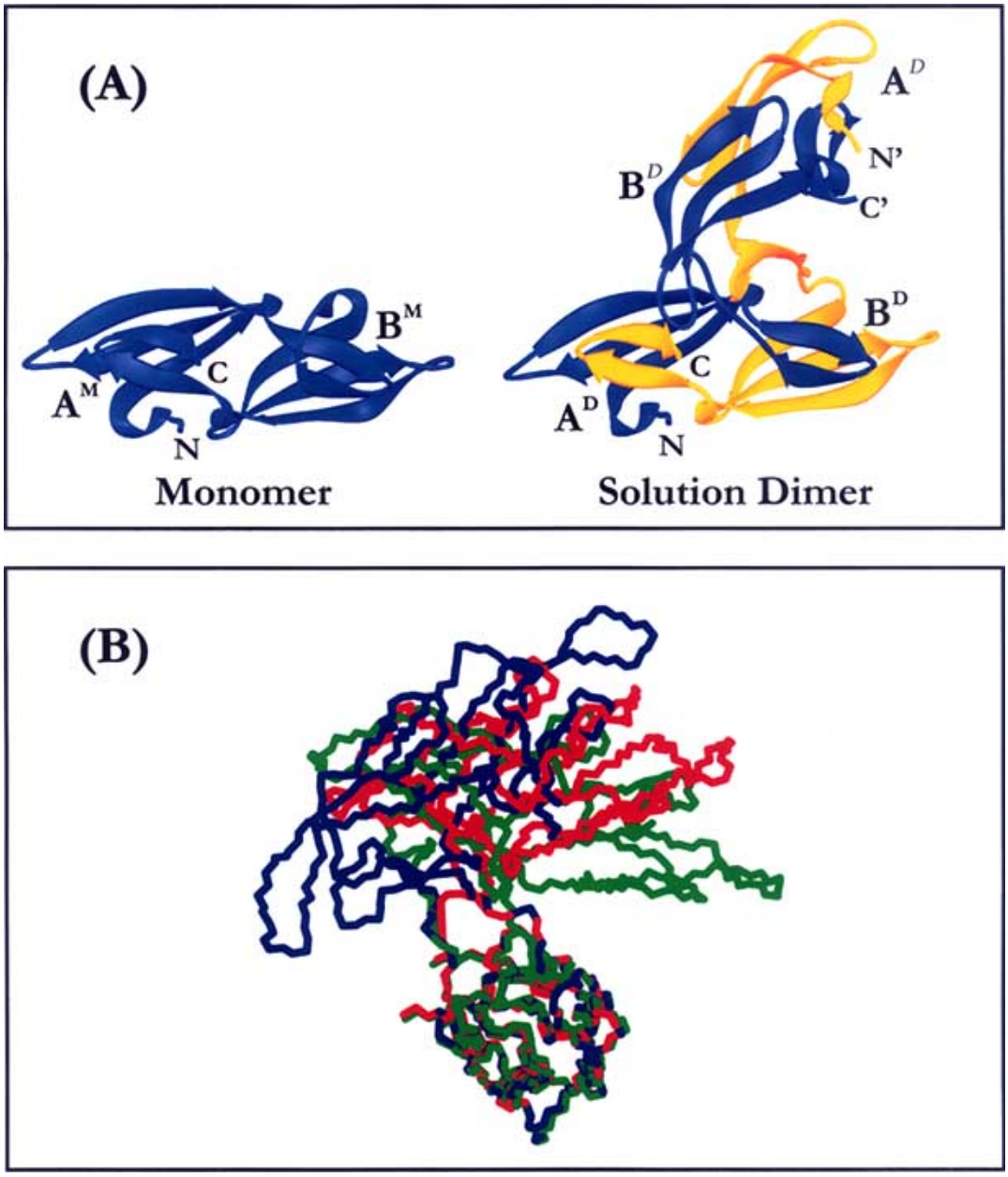

Fig. (3). (A) Ribbon representation of the solution structure of monomeric and domain-swapped dimer (pdb accession codes 2EZN and 1L5E, respectively). The different monomer units in the dimer are colored blue and yellow. The three-dimensional domains are labeled A^M/B^M in the monomer and $A^D/A^D/B^D/B^D$ in the dimer. The N- and C- termini are labeled N/C in the monomer and N/C/N'/C' in the dimer. (B) Superposition of the three domain-swapped dimer structures. The trigonal (3EZM) and tetragonal (1L5B) X-ray and the solution (1L5E) structures are shown in blue, green and red, respectively. A best-fit superposition for the backbone coordinates to the AB' half of the trigonal X-ray structure was carried out.

conditions were causing domain swapping [49, 55, 56], similar to the observation in several other systems [57, 58]. Further studies, however, demonstrated that the difference in quaternary structure (solution monomer versus domain-swapped dimer in the crystal) was not linked to the low pH in the crystal growth solutions [59], since a high pH tetragonal form [51] also contained domain-swapped dimer, both in the presence and in the absence of ligand [53]. In addition, a double mutant P51S/S52P also crystallizes as a domain-swapped dimer over a wide pH interval [54]. In solution, we prepared and isolated dimer at pH 4.0 or 8.0. Careful biophysical studies revealed that the domain-swapped dimer is a kinetically trapped folding intermediate at high protein concentrations that can be converted into the slightly more stable monomer form at physiological (>30ºC) temperature [51]. The most likely explanation for the exclusive presence of dimer in the diverse crystals is stabilization of this form by protein-protein contacts within the crystal lattice, tipping the balance in free energy toward the domain-swapped dimer [51].

Although the dimer undergoes conversion to monomer, its lifetime at room temperature or below is sufficiently long for biochemical and biophysical characterization [51]. The structure of dimeric CV-N was solved in solution (Figs. **3A** and **3B**) based on orientational NMR constraints derived

from residual dipolar couplings [51, 52]. This structure is virtually identical in inter-domain packing and overall folding to the structures in the trigonal and tetragonal crystals [49, 51], exhibiting solely a difference in the relative domain-domain orientations of the pseudo-monomeric halves (Fig. **3B**). There is a 8º/95º degree difference in θ/ϕ orientation between the two X-ray structures and a 39º/60º and 47º/35º degree difference between the solution and the trigonal and tetragonal structures, respectively. In solution, residues in the hinge of the dimeric species exhibit conformational mobility on the ms to s time scale, suggesting that reorientation around this hinge is easily possible [51]. A direct comparison of the three domain-swapped dimer structures is shown in (Fig. **3B**), clearly illustrating the variability in domain orientations between the X-ray and solution structures.

For both monomeric and domain-swapped dimeric CV-N to coexists in solution under identical conditions the free energies of folding for both quaternary states must be comparable and the kinetic barrier between the monomer and dimer must be significant [51]. The free energy difference between monomer and dimer can be altered by mutation of residues in the hinge region. Changing the pivotal proline in the hinge region to glycine results in stabilization of the monomeric P51G mutant by about 5.2 kcal/mol compared to wild-type. In addition, the energetic difference between the monomer and domain-swapped dimer is tipped more towards monomer for this mutant. Another interesting hinge region mutant is S52P [51, 60], which yields predominantly dimeric protein due to drastic destabilization of the monomer. Therefore, the removal or addition of a proline residue in the hinge region has a significant influence on the stability of the monomeric protein.

CV-N INHIBITION OF HIV VIRAL ENTRY

The combined experimental evidence from a large number of laboratories to date indicates that the virucidal activity of CV-N against HIV concerns early events in infection. In particular, CV-N blocks those interactions between the viral envelope glycoprotein and cellular receptors and co-receptors that are essential for viral entry and cell-to-cell fusion [25, 35, 61-63]. It was demonstrated that CV-N prevents both CD4-dependent and CD4-independent gp120 binding to target cells, blocks sCD4-induced gp120 binding to cell-associated co-receptors CXCR4 and CCR5, and induces sgp120 dissociation from target cells [35, 63].

Although the precise mechanism and delineation of details in the individual steps of CV-N's mode of action are still largely unknown, it was unequivocally demonstrated that recognition of N-linked high-mannose oligosaccharides (Man-8 and Man-9) on gp120 was intimately involved in viral inactivation of HIV [28, 30, 31]: (i) CV-N binds with high affinity to wild-type gp120 but not to deglycosylated or non-glycosylated recombinant gp120 [25, 28], (ii) CV-N alters or masks the interaction between gp120 and 2G12, a broadly neutralizing HIV antibody that recognizes a cluster of α1→2 mannose residues on the outer face of gp120 [62, 64], (iii) cell-fusion experiments [29] and ELISA [30, 44] demonstrated that Man-8 and Man-9 can directly compete with gp120 for CV-N binding, and finally (iv) the CV-N protein contains two binding sites for carbohydrate, exhibiting high-avidity interactions for Man-8 and Man-9 [29, 32]. The binding of CV-N to mannoses of the glycan-shield on gp120 that appears to be responsible for virus persistence and escape from neutralizing antibodies [65] is consistent with all available data and points to a unique position of this protein in the repertoire of possible entry inhibitors. Structural determinants for sugar binding and the structural basis for carbohydrate-mediated binding to gp120 are described in detail below.

CV-N BINDING TO HIGH-MANNOSE OLIGOSACCHARIDES: STRUCTURAL BASIS FOR CARBOHYDRATE RECOGNITION ON GP120.

Binding studies using isothermal titration calorimetry (ITC) and NMR with monomeric CV-N identified the Manα1→2Man moiety at the termini of the D1 and D3 arms as the consensus binding epitopes on the sugar and one carbohydrate binding pocket each on domains A^M and B^M, on the protein [29, 32]. Calorimetric titrations using various substructures of Man-9 revealed that sugar binding was largely driven by enthalpic contributions (negative ΔH values) and the larger sugars exhibited greater exothermic heats of binding. A negative ΔH of association suggested that favorable binding contacts such as polar/electrostatic, van der Waals and hydrogen bonds were mediated between CV-N and these oligosaccharides. Binding was however entropically disfavored for all of the CV-N-oligosaccharide interactions which meant that the sum total of binding entropy due to solvation effects, and to the rotational, translational, and conformational freedoms of CV-N and/or the oligosaccharides was greatly reduced as a result of binding. Such enthalpy-entropy compensation is commonly observed for protein-oligosaccharide interactions. The overall free energy of binding varied between –6 to –11 kcal/mol from the short to longer, branched oligomannoses and binding affinities for the individual sites are in the low micromolar range [32].

The sugar binding sites on CV-N consist of a semicircular cleft in domain A^M, comprising residues 1-7, 22-26 and 92-95, and a deeper pocket in domain B^M, comprising residues 41-44, 50-56 and 74-78. Both sites are separated by ~40 Å and are capable of recognizing the Manα1→2Man unit in the form of a dimannoside (Manα1→2Man) and a linear trimannoside (Manα1→2Manα1→2Man), either as individual small sugars or as the terminal arms of the higher Man-8 and Man-9 structures [29, 32, 50, 66].

The conformations of the sugars in the bound state and critical carbohydrate-protein interactions between the Manα1→2Man moiety and polar and charged amino acid residues on CV-N were delineated from the solution structure of monomeric CV-N, complexed with the Manα1→2Man dimannoside on domain B^M Fig. **4A** [50] and from the crystal structure of Man-9 and hexamannoside (HM) bound to domains A^D and A^D of the domain-swapped dimer (Figs. **4B** and **4C**) [53]. The Manα1→2Man unit bound in domain B^M has both mannopyranose rings in a chair conformation with the nonreducing pyranose ring (R2)

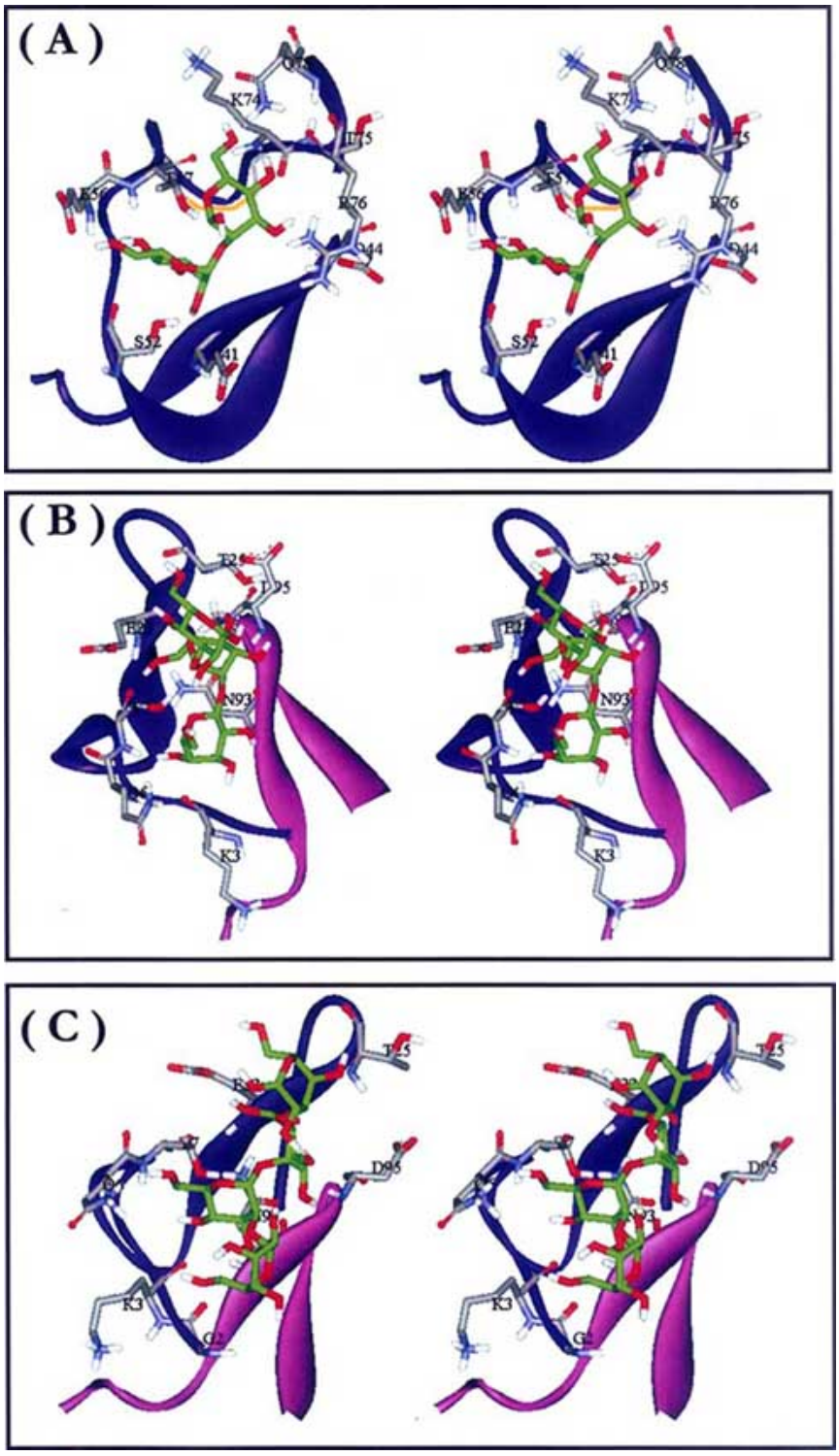

Fig. (4). Stereoview illustrating the interactions between CV-N and (A) a dimannoside in domain B^M, (B) a hexamannoside in domains A^D or A^D, and (C) Man-9 in domains A^D or A^D. (from structures 1IIY, 1M5 and 1M5M). Only those residues involved in protein-carbohydrate interactions are shown and labeled by amino acid type and number. The polypeptide backbones are represented in a ribbon representation. For (B) and (C), atoms residing in the same monomer are color coded blue and magenta. For clarity, only the mannopyrannose rings of the hexamannoside (B) and Man-9 (C) involved in protein-carbohydrate interactions are shown.

stacked over the reducing mannopyranose (R1) with ϕ/ψ values of 48° and +42°, as commonly observed for this disaccharide [67]. Both rings fit snugly into the site on domain B^M and direct protein-carbohydrate contacts are indicated in Fig. **4A**. The hydroxyl groups of the R2 and R1 rings interact with the side chains of polar and charged residues E41/S52/E56/T57 and D44/K74/T75/R76/Q78, respectively.

The crystal structures of the CV-N dimer complexed with Man-9 and hexamannoside revealed two carbohydrate binding sites on opposite ends of the molecule in domains A^D and A^D. No carbohydrate was observed in domains B^D and B^D [53]. The density for the sugars fit two stacked α1→2-linked mannose rings for hexamannoside and three stacked mannose rings for Man-9, with the remainder of the oligosaccharide protruding into solution. In the complex with hexamannoside, ϕ/ψ values of –3.6º and +10.7º are observed for the R2/R1 rings and each pyranose ring is in direct contact with residues K3/Q6/T7/N93 and E23/T25/D95/G96, respectively (Fig. **4B**). In the complex with Man-9, the density unequivocally identifies the D1 arm as the only binding moiety. Here, the protein-carbohydrate contacts involve residues G2/K3/E101, K3/Q6/T7/N93 and E23/T25/D95 interacting with R3 (the nonreducing mannopyranose ring in Man-9), R2 and R1 rings, respectively (Fig. **4C**). Interestingly, residues involved in protein-carbohydrate contacts in domain A^D/A^D coincide with being the top ranking surface hydrophobic cluster, comprising residues L1, G2, K3, Q6, T7, T25, N26, N93, I94 and D95 on domain A^M using the high-resolution structure of the monomer [48]. The three stacked rings of the D1 arm of Man-9 exhibit ϕ/ψ values of –4.5º and +22.7º and –39.4º and +45.9º for the R3/R2 and R2/R1 ring orientations, respectively (Fig. **4C**). Chemical shift mapping with hexamannoside and nonamannoside demonstrated that all four sugar-binding sites on domain A^D/A^D and B^D/B^D in the swapped-dimer of CV-N are capable of interacting with carbohydrate in solution, suggesting that the lack of bound sugar in the crystal in domains B^D/B^D is most likely caused by the particulars of crystal packing [68].

Comparing all three complexes (Fig. **4A-C**) reveals that there is not a defined set of specific residues responsible for recognition of the Manα1→2Man sequence: two chemically distinct binding sites are capable of recognizing the Manα1→2Man epitope. For example, three key polar residues in domain B^M, E41, S52 and Q78, are replaced by non-polar residues in domain A^M, namely A92, G2 and G27. It therefore seems likely that the topology of the binding sites is crucial for protein-carbohydrate recognition. The unique protein scaffold may provide compact pockets for optimal orientation and packing of the critical Manα1→2Man residues. A comparison between binding sites on CV-N [53] and the human antibody 2G12 [69] complexed with Man-9 does not reveal any common sequence requirement for sugar recognition.

Two different models have been proposed for gp120 binding by CV-N, involving binding to the high-mannose sugars on the envelope glycoprotein *via* one site on CV-N or both sites simultaneously (Fig. **5**). Although a given individual binding site on CV-N appears to be able to discriminate between a disaccharide and trisaccharide molecule, the binding affinities of domains A^M and B^M are nearly identical for larger, branched oligosaccharides, such as nonamannose and Man-9 [32]. Therefore, they are equally available for binding to carbohydrates on the surface of gp120. Whether indeed both sites on CV-N bind simultaneously to the high mannoses on gp120 has not been clearly established and can only be answered rigorously by completely abolishing of one of the sites.

More than one plausible mechanistic proposal for HIV inactivation exist: (i) CV-N masks the carbohydrates that play a role in the early stages of viral-cell fusion; (ii) CV-N binding to gp120 attached oligosaccharides lock the glycoprotein into a fusion-incompetent state, thereby rendering it unable to undergo the crucial conformational changes necessary for interaction with the co-receptors.

As to the question whether monomeric or dimeric CV-N contains antiviral activity, it is worth pointing out that identical activity and binding affinity towards gp120 was observed when pure monomeric or dimeric protein was used as the reagent in cellular assays [51], consistent with the fact that, under assay conditions (38ºC) essentially all dimer will convert to monomer within the time course of the

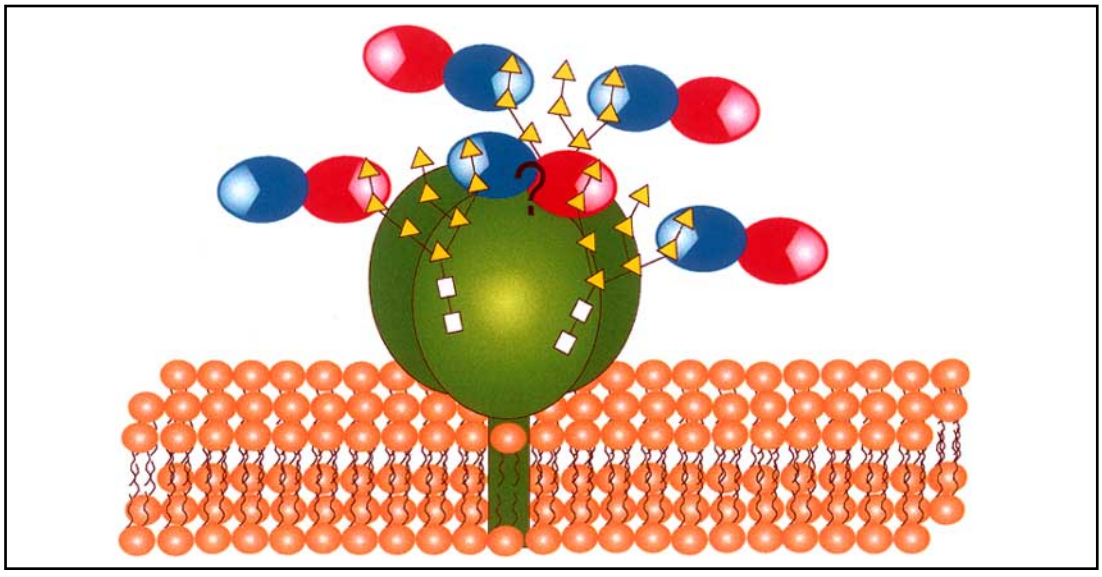

Fig. (5). Schematic model for the interaction between cyanovirin-N and high-mannose oligosaccharides (Man-9 for illustration purposes) on a viral surface envelope glycoprotein (i.e. HIV gp120 or Ebola $GP_{1,2}$). The trimeric glycoprotein is colored green and the mannopyranose and GlcNAc rings of Man-9 are represented as yellow triangles and white squares, respectively. The pseudo-symmetry of cyanovirin is indicated by the bilobal shape and the N- and C-terminal-binding pockets are colored blue and red.

experiment. In addition, for pure protein, a dimerization constant of 2.5 mM (50°C) was determined, making it highly unlikely that a dimeric species of wild-type CV-N will ever exist under physiological conditions.

INTERFERENCE WITH EBOLA VIRUS BINDING TO TARGET CELLS AND THE EFFECT ON INFECTIVITY

The US Centers for Disease Control and Prevention (CDC) classified Ebola as a Category A bio-warfare agent based on its high virulence, demonstrated aerosol infectivity and capacity for inducing fear and anxiety (http://www.cdc.gov). The quest for anti-Ebola agents has become an urgent priority, and to date, no antiviral drug currently in clinical use appears to provide any protection against Ebola. Based on results from HIV the pursuit of viral entry inhibitors like CV-N seems a promising avenue of investigation. Apparent similarities in both the fusion mechanisms [70] and glycosylation patterns [71, 72] of HIV and Ebola (Fig. **1**), prompted a test of CV-N binding to Ebola $GP_{1,2}$ and its interference with Ebola infection. *In vitro* as well as *in vivo* antiviral activity against the Zaire strain of the Ebola virus (Ebo-Z) was noted [44]. 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 administered in a series of daily subcutaneous injections or when inoculation of the mice was carried out after virus and protein were mixed *ex vivo*. Furthermore, similar to the results with HIV gp120, CV-N bound with considerable affinity to the surface envelope glycoprotein $GP_{1,2}$ of all strains of Ebola (Zaire '76; Reston '89; Ivory-Coast 94'; and Sudan '00). Since no protein sequence similarities exist between gp120 and $GP_{1,2}$, carbohydrate moieties on viral surface protein are the most likely molecular targets for CV-N in this case as well. The *in vitro* potency of CV-N against Ebo-Z (EC_{50} ~ 100 nM) was less than observed for HIV (EC_{50}s are generally in the 0.1-5 nM range) and could be the result of fewer high mannose sugars on the Ebola viral glycoprotein (Fig. **1**). Alternatively, these CV-N binding motifs may not mediate blockage of key sites on $GP_{1,2}$ involved with virus/cell interactions. To the best of our knowledge CV-N is the first known molecule proposed to bind Ebola virions and block cell entry, and thereby provides proof of concept for virion surface glycoprotein interactions as new avenues for Ebola therapeutic research. It is hoped that further research will help clarify the individual steps involved in Ebola infection and that this knowledge may lead to useful therapies in the future.

REDESIGN OF CV-N FOR IMPROVED PROPERTIES

A series of CV-N mutants was initially tested in crude extracts for gp120 binding and anti-HIV activity [73]. None of these mutants that exhibited anti-HIV activity failed to bind gp120. For those mutants that were devoid of activity, no data as to the structural integrity of the respective proteins were available, leaving the loss of activity ambiguous. As with all loss-of-function mutants, either the active site could have been destroyed or overall loss of structure could have occurred. The three-dimensional CV-N structures, both free and ligand-bound, provide the basis for a more rational approach to mutational design and analyses. The ensuing effects on conformation, stability and biological activity of variants of this interesting molecule can now be carried out in light of three-dimensional data.

The [P51G]CV-N Mutant

Practically, the most successful mutant reported to date is [P51G]CV-N [51]. Mutation of the single proline residue in the hinge region to glycine dramatically reduced conformational and oligomeric heterogeneity, rendering the P51G mutant predominantly monomeric. In addition, this mutation yielded a dramatic gain in thermodynamic stability of the protein, both in its monomeric and dimeric forms. A net stabilization of 5.7 kcal/mol is observed for the monomer. In this context it may be interesting to point out that, despite considerable effort, the P51G protein so far proved refractory towards crystallization, most likely because it exists preferentially in the very stable, monomeric form.

Further mutations were introduced into the [P51G]CV-N background: the potential glycosylation-susceptible asparagines residue at position 30 was replaced by A, Q or V [74]. All these P51G variants exhibited anti-HIV activity comparable to wtCV-N and retained the stability of [P51G]CV-N. These glycosylation-resistant, functional cyanovirins should be amenable to large-scale production in eukaryotic hosts.

Stable Domain-Swapped Dimers

Two stable domain-swapped dimers of CV-N have been reported so far: [S52P]CV-N [51, 60] and [ΔQ50]CV-N [56]. The former was found in a phage-display CV-N mutant library [60]. Mutation of the hinge loop serine residue to proline (S52P) dramatically promotes swapped dimer formation, caused by multimerization of the monomers due to significantly lowering the entropic cost of intermolecular association [51]. The dimer exhibits similar thermodynamic stability and anti-HIV activity as monomeric [P51G]CV-N [75].

Deletion of the hinge residue Q50 also resulted in exclusive domain-swapping [56]. [ΔQ50]CV-N was reported to be more potent in a HIV fusion assay than wtCV-N (by 3.5 fold) and this enhanced activity was attributed to the increased valency of the dimer, compared to the monomer [56]. However, recent data for this variant shows that identical antiviral activity exists for this dimeric protein and monomeric [P51G]CV-N [75].

The [P51S/S52P] Mutant

In this mutant the positions of the proline and its neighboring serine residue in the hinge region were exchanged. [P51S/S52P]CV-N exhibits biochemical and biophysical properties very similar to wtCV-N. It also exists as a monomer and domain-swapped dimer and the X-ray structure of the dimer resembles that of the dimeric form of wtCV-N, but shows a domain-domain orientation independent of pH [54].

Circular-Permuted CV-N

Circular permuted proteins provide a different perspective for studying structure-function relationships that cannot be achieved through classical single-site or deletion mutagenesis. For CV-N, the circular permuted variant (cpCV-N) was constructed by covalently linking the N- and C-termini of the protein and creating new termini at different points in the amino acid sequence [76, 77]. The sequence of cpCV-N is shown in Fig. **2A** and residues at the N (-1, 1, 2, 3) and C termini (100, 101) as well as the loop residues (50-54) that were not involved in the swap are colored yellow. The overall architecture of cpCV-N was initially assessed by NMR using residual dipolar couplings and was found to be very similar to that of wtCV-N [76]. The mutant is less stable than the wild-type protein ($\Delta\Delta G \sim 2.2$ kcal/mol), exhibits lower binding affinity for gp120 (~10 fold) and shows significantly reduced anti-HIV activity (~1000 fold) [77]. In order to explain these findings in detail, a high-resolution solution structure was determined and compared to wtCV-N [78]. This comparison revealed that the observed loss in stability of cpCV-N is caused by less favorable packing of several residues at the pseudo twofold axis, constituting the interface responsible for holding the two halves of the molecule together. In particular, both N- and C-terminal residues exhibit conformational mobility, resulting in fewer and less favorable contacts between them. The important hydrophobic and hydrogen-bonding network between residues W49, D89, H90, Y100 and E101 that was observed in wtCV-N is no longer present. In particular, Y100 and E101 are flexible and the tryptophan side-chain is in a different conformation compared to the wild-type protein. In addition, the binding of high-mannose type oligosaccharides was investigated. As for wtCV-N, two carbohydrate-binding sites were identified on the protein and the Manα1$\rightarrow$2Man linked moieties on the sugar were delineated as binding epitopes. Unlike in wtCV-N, however, both binding sites on cpCV-N exhibit very similar surfaces, despite their chemically different nature. Thus, comparable binding affinities for the respective sugars are observed and both sites are equally available for binding to carbohydrates on the surface of gp120. Based on the detailed comparison of cpCV-N with wtCV-N it is now possible to optimize and redesign CV-N for improved activity.

Other Mutants

Several variants were constructed with the aim to only retain one of the two sugar binding sites on CV-N and thereby reducing the binding avidity. Interestingly, all mutants reported to date exhibited activity indistinguishable from wild-type CV-N with respect to blocking env-mediated fusion [79].

POTENTIAL CLINICAL APPLICATIONS OF CV-N

CV-N has considerable potential for the development as a prophylactic, topical anti-HIV microbicide to prevent sexual transmission of HIV. Possible formulations for CV-N may be creams, foams or vaginal and rectal suppositories creating a simple physical barrier agent against the spread of viral diseases [15, 25, 34, 80]. The main attributes that make CV-N a particularly attractive candidate for virucidal use are: (i) the broad spectrum of activities against different immunodeficiency retroviruses; (ii) the early action in viral entry events *via* the protein's ability to interfere with viral attachment and/or crucial receptor interactions; (iii) the possibility to prevent fusion and transmission of HIV-1 between infected and uninfected cells [25]; (iv) the remarkable robustness against physicochemical degradation; (v) the exceptional oligosaccharide binding specificity; and (vi) the low toxicity in cell cultures (>1 μM) and benign behavior in a rabbit vaginal and rectal macaque transmission models [81, 82].

Further potential clinical applications could be based on using CV-N as a chimeric molecule with Pseudomonas exotoxin A [83]. In this chimera, CV-N serves as the targeting moiety homing on HIV- infected cells that express gp120. In this context it was shown that the hybrid toxin-CV-N molecule displays enhanced cytotoxicity for HIV-infected, compared with uninfected H9 cells, indicating its potential use to selectively target and destroy HIV-infected host cells. Other applications explore the feasibility of expressing CV-N in Streptococcus gordonii or Lactobacillus for local delivery of CV-N to prevent sexual transmission of HIV [84].

In the context of Ebola therapeutics, a recent *in vivo* study showed that CV-N was able to reach systemic circulation and exhibited measurable activity [44], although the distribution patterns are still unknown. The apparent ability of CV-N to traverse the varied physiological compartments after subcutaneous injection was intriguing, and further pharmacokinetic analysis of CV-N in animal models after various routes of administration are required.

The application of CV-N to other enveloped viruses will depend primarily on the mechanism of infectivity for a particular virus. In general, CV-N will no doubt serve as an invaluable reagent to further examine and dissect details in the early steps of virion to cell binding and fusion for a variety of virus/host systems. Initial methods for CV-N's use in *ex vivo* inactivation of HIV in blood or plasma [85] can be extended to include solution- or solid-phase CV-N compositions, matrix-anchored CV-N, or other CV-N based technologies to target and/or remove infectious virus from medical equipment, supplies, or other blood products and tissues or cells.

CONCLUSIONS

CV-N represents a new class of lectin with unprecedented oligosaccharide binding specificity over other, previously described lectins. Structural studies by NMR and X-ray crystallography on both monomeric and dimeric CV-N, free or sugar-bound, revealed that the Manα1$\rightarrow$2Man unit on the external arms of Man-9 and Man-8 is the binding epitope on the sugar. Protein-carbohydrate recognition occurs with high-specificity and the binding constant is in the low micromolar range, similar to that observed for other lectins. There is no obvious sequence requirement for the binding site on the protein; it appears that the protein surface structure and topology is the main determinant for binding. Despite considerable structural knowledge on the molecular basis of oligosaccharide interaction, the exact mechanism for CV-N antiviral activity still needs further elucidation. In this

context we would like to draw attention to a recent structural study on the neutralizing human antibody 2G12 in complex with Man-9 [69]. The authors show elegantly that this antibody adopts a domain-swapped dimer structure capable of multivalent interactions with the oligomannoses on the surface of gp120. Like with CV-N, multivalency leads to high avidity and potency at the nanomolar level, even if individual sugar binding exhibits micromolar affinity.

Given the fact that the primary binding epitope for CV-N on gp120 was the carbohydrate, studies of CV-N's activity against other enveloped viruses were pursued. All the viruses for which antiviral activity has been reported to date [25, 35, 44, 86, 87] bear heavily-glycosylated viral surface glycoproteins containing high-mannose oligosaccharides. It therefore could well be that CV-N may display inhibitory effects towards other enveloped viruses such as the novel human coranovirus that caused the outbreak of SARS [6]. As pointed out earlier, the effectiveness of CV-N against any individual virus will depend on this virus's mode of infection. Nevertheless, CV-N can function as an invaluable tool to assess events at the level of viral entry in all these cases.

As for immediate developments, structure-guided design of CV-N homologues with greater affinity and *in vivo* efficacy are now possible based on the current wealth of available structural information. In addition, screening for CV-N like molecules using CV-N/glycoprotein based high-throughput assays may also yield new and surprising results [88, 89]. Likewise, investigating other lectins for targeting alternative carbohydrate structures present on viral glycoproteins may yet represent another avenue in the search for novel antiviral agents.

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