# Chapter 2

## Engineered cyanovirin-N oligomers show enhanced HIV neutralization

**Abstract**

Cyanovirin-N (CVN), a protein originally isolated from the cyanobacterium *Nostoc ellipsosporum*, has been shown to bind specifically to glycosylated gp120 on HIV particles, preventing viral fusion and neutralizing the virus. Here, we present dimeric and trimeric variants of CVN that display increased potency for neutralizing HIV-1. The molecules were expressed and purified to homogeneity and then assessed for their ability to prevent HIV-1 infection in a cell culture-based neutralization assay. We found that covalently linking two CVN monomers ($CVN_2$) through a flexible polypeptide linker decreased the concentration of protein at which 50% of the virus was neutralized ($IC_{50}$) up to ten-fold compared to wild-type CVN. The addition of a third CVN monomer ($CVN_3$), however, showed minimal further neutralization enhancement. Our data also suggest a linker-length dependence in the neutralization activity, although variability in the assays masks a definitive trend. In addition, CVN and dimeric variants displayed significant cross-clade and cross-strain reactivity against 33 strains of HIV-1 and neutralized most strains with decreased $IC_{50}$s compared to the most effective broadly neutralizing antibody tested.

The increased neutralization exhibited by these linked CVN variants provides a novel path to improve our understanding of how CVN prevents HIV infection, and the broad cross-strain reactivity holds promise for the future therapeutic utility of these and other engineered CVN variants.

**Introduction**

Treatment and prevention of HIV-1 have proven to be difficult and complex problems. Vaccines thus far have been unsuccessful in generating broadly reactive neutralizing antibodies that confer immunity to the virus, partly because of HIV-1's rapid mutation rate and partly because of the lack of epitopes on its envelope proteins. The HIV-1 envelope is composed mainly of two proteins: gp41 and gp120, which are products of a single precursor protein, gp160. gp41 contains a transmembrane region, which anchors the envelope protein to the membrane, as well as a region that interacts specifically with gp120; gp120 contains binding sites for the primary receptor, CD4, and coreceptors, CCR5 and CXCR4. These gp41-gp120 dimers form trimers on the surface of the virus, creating the envelope spikes.[1] While gp41 and gp120 by nature must contain some invariable regions, these conserved regions are typically masked or difficult to access. For example, the CD4 binding site is located in a cleft in the protein, allowing this region to evade the human immune system,[2,3] and the binding sites for the coreceptors are revealed only after a conformational change induced by CD4 binding.[4] Additionally, gp120 and gp41 are both heavily glycosylated, obscuring many potential epitopes. In fact, approximately 50% of the apparent molecular weight of gp120 is attributed to complex carbohydrates.[5] This “silent face” of the HIV envelope is fairly resistant to the human immune system because it is generated by the host and often recognized as “self.”

In addition to the lack of functional epitopes on the envelope proteins of HIV-1, HIV is a retrovirus, and therefore has a very high mutation rate due to the error-prone reverse-transcriptase. Because of this rapid mutation rate, there is a great deal of variation in HIV viruses within an individual, between individuals, and geographically. A

successful treatment or vaccine must therefore induce a broadly neutralizing response such that it affects many strains and many clades. Thus far, however, only a few broadly neutralizing antibodies have been isolated that are capable of neutralizing primary isolates of HIV-1.[6-10] To date, four broadly neutralizing antibodies have been discovered and extensively studied. The first, IgG1b12, recognizes a conserved, recessed area of gp120 that overlaps with the CD4 binding site.[3] IgG1b12 is capable of neutralizing approximately half of HIV-1 strains tested, including some strains from each clade.[6,7,9] The monoclonal antibodies 4E10 and 2F5 both recognize conserved areas of gp41, near the viral membrane,[11,12] but differ in their abilities to neutralize HIV-1. 4E10 is the most broadly reactive of these neutralizing antibodies, neutralizing all 93 strains tested from 12 different clades. Unfortunately, although it is able to neutralize broadly, it does so with only modest potency.[9] 2F5, on the other hand, is not capable of neutralizing most viruses from clade C and is effective against only approximately 50% of clade D viruses.[7,9] The final anti-HIV antibody is 2G12. 2G12 differs from the other broadly neutralizing antibodies in that it recognizes the carbohydrates on gp120 and not the actual protein itself.[13] In contrast to the standard "Y" structure of antibodies, the Fabs of 2G12 form a domain-swapped structure that allows it to bind two carbohydrate chains approximately 35 Å apart.[14] 2G12 is mostly effective against viruses from clade B and exhibits limited or no neutralization of viruses from other clades.[6,7,9]

Cyanovirin (CVN), a lectin from the cyanobacterium *Nostoc ellipsosporum*, is uniquely suited to play a role in HIV treatment and prevention.[15] CVN, like the 2G12 antibody, binds specifically to $\alpha$1-2 oligomannose molecules,[16] which are highly expressed on gp120, and neutralizes enveloped viruses including HIV,[15] Ebola,[17] and

influenza.[18] Also like 2G12, CVN contains two carbohydrate binding sites per molecule, indicating there is a potential avidity effect upon binding.[16] CVN is also distinctive in its small size. The 11-kDa protein is much smaller than even a single-chain Fv fragment (scFv) and therefore has the ability to bind to areas on gp120 that are sterically occluded from scFv, Fab, or IgG binding.[19] Additionally, unlike 2G12, which is specific to carbohydrates on specific residues,[20] CVN is specific only to the type of linkage and therefore less sensitive to escape mutations that eliminate a single glycosylation site. In fact, glycosylation on gp120 has been shown to increase over the course of infection[20] and act as a mechanism for escape from neutralizing antibodies.[21] CVN may be an optimal therapeutic in these cases due to its reaction to a broad range of high mannose carbohydrates.

We are interested in studying the effects of oligomerization on CVN to see whether increasing the number of binding sites and varying the distances between those sites increases the efficacy of neutralization for HIV-1 and other enveloped viruses. Previous studies of 2G12 indicated that higher order oligomers are more effective at neutralizing HIV.[22,23] West *et al.* showed that natural dimers of 2G12 are up to 80-fold more potent than the monomer,[22] and the oligomeric 2G12-IgM engineered antibody tested by Wolbank *et al.* exhibited up to 28-fold greater efficacy than 2G12-IgG.[23] Although CVN can also exist in a domain-swapped form,[24] it is unclear whether the domain-swapped dimer exhibits similar increases in neutralization to 2G12, since differing accounts have been published.[25,26] By dimerizing or trimerizing CVN, we not only increase the number of binding sites and therefore potentially affect the avidity of

binding, we can also effectively increase the separation of binding sites and therefore possibly crosslink glycosylation sites that are separated by a greater distance.

In addition to determining the effect of oligomerization on CVN efficacy, we are interested in testing these variants against a large number of HIV-1 strains to ascertain their cross-clade neutralization. Toward these goals, we have successfully created linked dimers ($CVN_2$) and trimers ($CVN_3$) of CVN and assayed their HIV neutralization function against 33 viruses from three clades. These variants contain a polypeptide linker of varying length (L0 to L20) connecting the C-terminus of one CVN to the N-terminus of another (Table 2-1, Figure 2-1).

**Methods**

*Construct generation.* The gene for wild-type (WT) CVN was constructed using a recursive PCR method with 40-mer synthesized oligos,[27] and cloned into the NdeI and BamHI sites of pET11a. The protein contained an N-terminal 6-histidine purification tag followed by a Factor Xa protease cleavage site. $CVN_2$ L5 and $CVN_2$ L10 were constructed using PCR-based cloning to insert a tandem repeat of the WT CVN gene into the WT plasmid. The $CVN_3$ L5 gene was created by inserting an *Escherichia coli*-optimized WT CVN DNA sequence between the two existing copies of the WT gene in $CVN_2$ L5. Other dimeric and trimeric genes of varying linker lengths were constructed using the QuikChange Site-Directed Mutagenesis Kit to insert or delete codons corresponding to linker amino acids (Stratagene). All constructs were verified through DNA sequencing and restriction analysis to ensure the correct sequence and number of CVN repeats.

*Expression and purification.* WT CVN and all oligomeric variants were expressed in BL21(DE3) *E. coli* cells in LB including ampicillin. The cultures were induced with 1 mM ITPG when the cells reached mid-log and grown for an additional 3-5 hours at 37˚C. The harvested cells were lysed using an EmulsiFlex-C5 (Avestin, Inc.) and the insoluble fraction was resuspended in buffer containing 6 M GnHCl and 10 mM imidazole and centrifuged to remove debris. The solubilized CVN was then purified using a denatured protocol on a Ni-NTA gravity column (Qiagen). The protein was eluted in buffer containing 6 M GnHCl and 250 mM imidazole and transferred to dialysis tubing with a MWCO of 5,000 Da. WT CVN and all variants were refolded by dialyzing the Ni-NTA eluate against native buffer overnight at room temperature.[28] Following refolding, the solution was filtered to remove any precipitant and concentrated using Amicon 5000 MWCO centrifugal concentrators to approximately 0.5 mL (Millipore). The proteins were then additionally purified on a Superdex-75 column and eluted in 25 mM sodium phosphate pH 7.4, 150 mM NaCl. Pure protein was concentrated or stored as eluted at 4˚C. Attempts to cleave the His-tag using Factor Xa were unsuccessful under several conditions, so the tag was left intact for the studies described here.

Amino acid analysis was performed on WT CVN, $CVN_2$ L5, $CVN_2$ L10, $CVN_3$ L5 and $CVN_3$ L10 to determine extinction coefficients at 280 nm (Jinny Johnson, Texas A&M University). These experimentally determined extinction coefficients (WT: 10471 $M^{-1}cm^{-1}$, $CVN_2$s: 20800 $M^{-1}cm^{-1}$, $CVN_3$s: 32000 $M^{-1}cm^{-1}$) were used to calculate the protein concentration.

*Surface plasmon resonance (SPR) assays.* SPR (Biacore) experiments were performed on a T100 instrument (Biacore). Approximately 30 response units (RUs) of WT CVN were immobilized on flow cells 2 through 4 of a CM5 chip through standard amine coupling. Flow cell 1 was reserved as a control. All assays were conducted in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 0.0005% v/v Surfactant P20, 1 mM EDTA; Biacore). Various analytes were injected over the surface for 60 seconds at a flow rate of 30 μL/min. The chip was regenerated with two pulses of 50 mM NaOH. In some cases, complete regeneration was not achieved and a new chip was created. The data were analyzed for binding or lack of binding based on the sensorgram.

*Cell viability assays.* Cellular toxicity was assayed using an XTT cell proliferation kit (MD Biosciences). This assay is based on the observation that live cells can reduce XTT and other tetrazolium salts, resulting in a colored solution. Cells that are not metabolically active, however, are not able to reduce XTT and therefore there is no color change after the addition of the reagent. WT CVN or a variant protein was incubated with approximately 5000 Tzm-Bl cells in D-MEM high glucose medium with L-glutamine, sodium pyruvate, 50 μg/mL gentamicin, and 10% heat inactivated BSA at 37˚C in a $CO_2$ incubator for 48 hours. The prepared XTT reagent was added to each well and incubated for an additional two hours. The plates were shaken gently and the absorbance at 450 nm (the absorbance of the reduced XTT product) was measured on a Safire$^2$ plate reader (Tecan). Each protein was assayed in triplicate, and the average absorbance values were used to determine the percent of cells that were viable (*%Viable*) using Equation 2-1, where $A_{CVN}$ is the absorbance of the well containing cells and CVN, $A_{negative}$ is the average

absorbance of wells containing no cells or protein, and $A_{cell}$ is the average absorbance of wells containing cells but no CVN. The percent of viable cells was plotted as a function of the CVN concentration.

$$\%Viable = \frac{A_{CVN} - A_{negative}}{A_{cell} - A_{negative}} * 100 \quad (2\text{-}1)$$

*HIV neutralization assays.* HIV neutralization assays were performed by Priyanthi Peiris, a technician in Prof. Pamela Bjorkman's lab, according to the methods by Li *et al*.[6] The assays used Tzm-Bl cells, a HeLa cell line that expresses CD4 and the HIV-coreceptors CCR5 and CXCR4 and contains a viral Tat-induced luciferase reporter gene. Only when infected by the HIV-1 pseudovirus will these cells express luciferase, allowing a high-throughput measurement of neutralization. HIV-1 pseudovirus particles from pseudotyped primary virus strains were prepared as described.[6,21] The SC422661.8 strain (clade B) was used for all assays unless otherwise noted. One column of 8 wells in a 96-well plate contained cells but no virus and was used to determine the background level of luminescence (cell control). Additionally, one column contained cells and virus but no inhibitory compound, acting as both a positive control and a maximal signal of infection (viral control). In the remaining wells, approximately 250 $TCID_{50}$ of virus was incubated with varying amounts of CVN or CVN variant in triplicate for one hour at 37˚C. Each plate contained WT in triplicate as an internal control. Typically eight three-fold dilutions starting with 200 nM protein were tested to create a neutralization curve. Approximately 10,000 freshly trypsinized cells were added to each well and the plate was incubated for 48 hours. The cells were then lysed using Bright Glo Luciferase Assay

Buffer (Promega), which was diluted 4X. The lysate was then transferred to a new plate and the luminescence was measured on a Victor$^3$ Multilabel Counter (PerkinElmer, Inc.).

To determine the $IC_{50}$ of neutralization, the luminescence corresponding to a given protein concentration was first averaged across the three replicates, then the percent neutralization *(%Neutralization*) was calculated based on Equation 2-2, where *RLU* is the average relative luminescence for a given concentration, *CC* is the average luminescence from the cell control wells, and *VC* is the average luminescence from the viral control wells. The percent of virus neutralized was then plotted as a function of neutralizing protein in Kaleidograph (Synergy Software) and fitted to Equation 2-3, where $IC_{50}$ is the concentration of CVN at which 50% of the virus is neutralized and *C* is the concentration of CVN. The reported error is the error associated with the curve fit to the experimental data.

$$\%Neutralization = \left(1 - \frac{RLU - CC}{VC - CC}\right) * 100 \tag{2-2}$$

$$\%Neutralization = \frac{100}{1 + \frac{IC_{50}}{C}} \tag{2-3}$$

To minimize the plate-to-plate deviations between assays, we normalized each variant's $IC_{50}$ compared to WT $IC_{50}$ on the same plate. These data are presented as "$IC_{50}$: fold lower than WT" and were calculated by dividing the $IC_{50}$ for WT by the $IC_{50}$ for the variant. Each variant was independently tested between one and five times. Error bars were calculated by propagating the error from the WT and variant curve fits as well as multiple trials if applicable, according to standard methods.

In addition to the assays done here at Caltech, WT and two dimer variants were tested against multiple envelopes from various HIV-1 clades by Professor Michael Seaman's lab at Harvard Medical School through the Collaboration for AIDS Vaccine Discovery (CAVD) Neutralizing Antibody Laboratory. These assays were performed according to the same protocol described above, but 33 pseudoviruses from clades A, B, and C were tested to determine the cross-clade reactivity. The data were analyzed as described above.

**Results**

*Dimer and trimer variants.* To directly assay the effects of dimerization and trimerization on the activity of CVN, we generated proteins consisting of two or three tandem repeats of CVN (Figure 2-1). The resulting proteins had one copy of the protein linked through its C-terminus to the N-terminus of the next copy through a flexible polypeptide linker encoded in the gene. In this study, we tested dimers ($CVN_2$s) with 14 different linkers ranging from 0 to 20 amino acids (all Gly or Ser) for their ability to neutralize HIV in a cell-based assay. In addition, we assayed trimers ($CVN_3$s) with three linkers comprised of 0, 5, or 10 amino acids (Table 2-1).

*Expression and purification.* WT and all CVN variants were expressed into inclusion bodies at 37˚C using standard *E. coli* expression protocols. After solubilizing the proteins in 6 M GnHCl and running a Ni-NTA purification step, the proteins were refolded by dialyzing against native buffer. Most of the refolded protein solutions, including those for WT, $CVN_2$ L0, $CVN_2$ L10, and $CVN_3$ variants, had little or no

precipitation after dialysis, indicating the conditions were sufficient for refolding without favoring aggregation. Some variants, however, including $CVN_2$ L1 and $CVN_2$ L3, experienced significant precipitation during the refolding step. These solutions were filtered before continuing with the purification protocol. Gel filtration was performed on the refolded proteins to separate the desired species (WT: monomer; $CVN_2$s: dimer; $CVN_3$s: trimer) from higher molecular weight species including domain-swapped dimers or tetramers and aggregates. WT CVN eluted at approximately 0.59 CV, $CVN_2$s eluted at approximately 0.54 CV, and $CVN_3$s eluted at approximately 0.50 on the gel filtration column. Reinjection of purified sample indicated the protein was stable in its purified oligomeric state for weeks to months when stored at 4˚C.

*Surface plasmon resonance (Biacore) assays.* To assess the efficacy of the refolding protocol, WT CVN was assayed for its ability to bind gp120. WT CVN was immobilized to a Biacore chip and 100 nM gp120HxBc2 was flowed over. Significant binding was observed at all surface densities tested and the binding was virtually irreversible (data not shown). Various regeneration conditions including low pH, high pH, and high concentrations of NaCl were used, but the chip was never fully regenerated. We therefore concluded that WT CVN was properly folded and able to bind specifically to gp120, but kinetic and binding constants could not be obtained using this method.

We also tested for domain-swapping and aggregation on the surface. WT CVN was immobilized and WT and various $CVN_2$ proteins were analyzed for binding. No binding was observed for WT, $CVN_2$ L0, $CVN_2$ L1 or $CVN_2$ L10 (data not shown).

These proteins did not aggregate on the surface or bind to WT CVN. We were not able to observe any intermolecular domain-swapping under these conditions.

*HIV neutralization assays.* WT CVN was tested for its ability to neutralize HIV pseudovirus in cell culture. The $IC_{50}$ of CVN is reported to be in the low nanomolar range.[25,29-31] In our assays, WT CVN neutralized HIV envelope SC422661.8 with $IC_{50}$s between 2 and 14 nM over 16 independent trials, consistent with published values. Because of the variation we saw in these experiments and to minimize any plate-to-plate deviations due to incubation conditions, viral particle preparation, or other systematic differences, we report all variant data relative to the WT $IC_{50}$ from the same 96-well plate.

Dimeric $CVN_2$ proteins were also tested for their ability to neutralize HIV strain SC422661.8 (Figure 2-2). All variants displayed $IC_{50}$s lower than WT CVN, showing enhanced neutralizing compared to WT. For $CVN_2$ L0 and $CVN_2$ L10, the increase in efficacy is nearly ten-fold. While there appears to be a linker-length dependence to the data, this may be an artifact of the complicated biological assay and not a reflection of actual differences in activities.

To test the hypothesis that more binding sites make better neutralizing variants, we created and assayed trimeric $CVN_3$ molecules. Similar to the $CVN_2$ results, these variants were significantly more effective at HIV neutralization than WT. However, compared to the $CVN_2$ variant, adding an additional CVN repeat did not increase the efficacy of HIV neutralization, and the three variants tested ($CVN_3$ L0, $CVN_3$ L5, and

$CVN_3$ L10) did not have significantly different activities from their $CVN_2$ counterparts (Figure 2-3).

After identifying $CVN_2$ L0 and $CVN_2$ L10 as the best performing oligomeric variants against the SC422661.8 strain, we assayed these proteins as well as WT for their cross-clade reactivity. The proteins were tested against a total of 33 viruses from three clades of HIV (Table 2-2). One of the most difficult obstacles in developing HIV neutralizing monoclonal antibodies (NAbs) is their lack of cross-clade reactivity. Most NAbs effectively neutralize viruses from one or two clades, but often are not effective against other clades. It is important for a potential therapeutic to be effective against as broad a range of viruses as possible. In the case of WT CVN and the dimeric mutants, all 33 of the HIV pseudoviruses were neutralized with $IC_{50}$s less than 300 nM (Figure 2-4, Table 2-2). Only the 4E10 NAb was as cross-clade reactive, while 2G12 and 2F5 were not effective at neutralizing clade C viruses, and IgG1b12 was not effective against clade A viruses.

In addition to the broad cross-clade reactivity of WT CVN and the $CVN_2$ variants, we were interested in the overall efficacy of the CVN proteins as compared to the NAbs. To simplify the analysis, we chose the NAb with the lowest $IC_{50}$ for each individual envelope and compared WT CVN, $CVN_2$ L0, and $CVN_2$ L10 to that variant (Figure 2-5). This comparison therefore indicates how effective the CVNs are against the best broadly neutralizing antibody for each strain. For many strains, WT CVN is less effective than the best NAb, as indicated by bars with negative values. However, by dimerizing the protein, we increased the efficacy of neutralization and generated variants that exhibit better neutralization than the best NAb against a given HIV strain. In fact, $CVN_2$ L0 is better

than the best NAb for every virus tested except for 4 out of 31 cases (2 of the 33 viruses did not have corresponding NAb data). Although it is only moderately more effective against some strains, $CVN_2$ L0 is at least 5-fold better than the best NAb against 19 out of 31 envelopes and at least 10-fold better against 11 envelopes. Additionally, $CVN_2$ L0 has an $IC_{50}$ 215-fold lower than the NAbs in one case (clade B, TRJO4551.58). Through dimerization, we have created a variant that is not only broadly cross-clade reactive, but is also more effective at neutralizing HIV-1 than the commonly studied NAbs.

Similarly to the results from neutralization assays on SC422661.8, the cross-clade data indicate that the dimerized variants are significantly more effective at neutralizing various strains of HIV-1 than WT CVN (Figure 2-6). Specifically, $CVN_2$ L0 neutralized with a lower $IC_{50}$ than $CVN_2$ L10 in 32 out of 33 cases, and $CVN_2$ L10 neutralized with a lower $IC_{50}$ than WT in all 33 cases.

*Cell viability assays.* An alternative explanation to our HIV neutralization assay data is that CVN is not in fact neutralizing HIV, but instead killing the host cell. In this case, the cell would not express luciferase upon infection because its cellular machinery would be nonfunctional. To test this hypothesis, we checked WT CVN, $CVN_2$ L5, and $CVN_2$ L10 for toxicity against Tzm-Bl cells using an XTT cell proliferation assay. We tested concentrations up to 25-fold higher than the highest concentration used in the HIV neutralization assays. Our data indicate that at the concentrations relevant for the neutralization assays, CVN and the $CVN_2$ variants are not toxic (Figure 2-7). We did, however, see limited toxicity both by the XTT assay as well as visual inspection of the cells at protein concentrations above 1 μM. This result is consistent with published

reports[15,32] and indicates the activity we see against HIV is in fact neutralization of the virus and not toxicity to the host cell.

**Discussion**

We successfully created dimeric and trimeric CVN variants that have enhanced anti-HIV activity compared to WT CVN. These variants show excellent cross-clade and cross-strain reactivity and are more effective at neutralizing HIV than the most broadly neutralizing HIV antibodies.

Although the dimeric and trimeric variants show significant improvement in HIV neutralization, the mechanism responsible for the enhancement has thus far proved elusive. We hypothesize that differences in domain swapping may lead to an increase in efficacy. Previous reports are divided about whether the domain-swapped form of WT CVN is more active than the monomeric form.[25,26] However, because of the meta-stable state of domain-swapped WT, it is difficult to assay the dimerized form in current biological assays. Our variants, however, because they are covalently linked at their termini, have a much higher local concentration of CVN and therefore may be more stable as a domain-swapped dimer, even at physiological temperatures. Additionally, for variants with short linker lengths, the link may force a domain-swapped structure and may sterically hinder a monomeric-like form. Because the tandem CVN repeats are covalently attached, however, it is difficult to determine whether the molecule is in a monomeric-like dimer form or a domain-swapped dimer (Figure 2-1). We can effectively remove any inter-molecularly domain-swapped protein using the gel filtration purification step, but intra-molecularly domain-swapped variants are difficult to

distinguish using standard purification protocols. We plan to investigate the domain-swapped nature of the variants to determine whether this may play a role in the increased activity. NMR-based experiments may allow us to see long-range interactions that are consistent with domain-swapping. Alternatively, we could introduce a protease cleavage site in the linker and determine whether after cleavage the protein is still dimeric or shifts to a monomeric molecular weight.

In addition to potential differences in domain swapping, the simple increase in carbohydrate binding sites may increase the avidity of the CVN-gp120 interaction. WT CVN itself has a very high affinity for gp120,[15,29] but an increase in avidity in the $CVN_2$ variants may provide an extra force to prevent possible dissociation and escape of the virus. An alternate mechanism for increased neutralization is that the $CVN_2$s, with binding sites that are further apart than in WT CVN, are able to crosslink glycosylation sites on a single gp120 or crosslink multiple gp120 subunits on an envelope spike or, less likely, multiple spikes. This crosslinking would sterically hinder more gp120 subunits from binding to CD4 than would be blocked by WT CVN, thus decreasing the $IC_{50}$. An interesting note is that in the domain-swapped structure of WT CVN, every pair of carbohydrate binding sites is approximately 30 to 40 Å apart (Figure 2-8). The neutralizing antibody 2G12, which also binds the glycosylation site on gp120 and is also domain-swapped, has carbohydrate binding sites that are also approximately 35 Å apart.[14] Perhaps by stabilizing the domain-swapped structure of CVN, the carbohydrate binding sites of the $CVN_2$ variants are optimally positioned to interact with gp120 and neutralize the virus.

While addition of a second CVN molecule increases the efficacy of HIV neutralization significantly, the addition of a third CVN repeat ($CVN_3$) does not significantly increase it further. Although the mechanism for enhanced activity is not yet understood, if increased domain-swapping is involved, an unpaired, third CVN may not significantly increase the neutralization. To test this hypothesis, tetrameric CVN molecules can be engineered and tested for increased HIV neutralization. Alternatively, due to the close proximity of the N- and C-termini in the WT structure and their proximity to the low affinity carbohydrate binding site, the third CVN molecule may sterically prohibit access to some of the carbohydrate binding sites in the molecule, rendering those sites nonfunctional and therefore not conveying any additional effect.

WT CVN and the $CVN_2$ molecules show excellent cross-clade and cross-strain reactivity. This property is promising for the development of these or other variants for therapeutic use as they can be used potentially throughout the world. In addition, CVN variants could be used in combination therapy to direct gp120 evolution toward decreased glycosylation. Glycosylation itself has been shown to be important in the folding and function of viral glycoproteins[33] and in the case of HIV, deglycosylation of gp120 diminishes the binding to CD4, making the virus less infective.[34,35] Alternatively, deglycosylation of gp120 could merely reveal more protein epitopes that can be recognized by the adaptive immune system, allowing our own bodies to fight off infection more effectively.

Although these initial results are promising, more work needs to be done to elucidate a mechanism for the increased efficacy that we see for the dimeric and trimeric variants. It is probable that multiple mechanisms are at work to affect the neutralization

activity. Toward this goal, we have solved crystal structures of $CVN_2$ L0, $CVN_2$ L1, and $CVN_2$ L10 to determine whether any structural differences can account for changes in activity (Chapter 3 of this thesis). We also hope to employ a biophysical-based assay such as surface plasmon resonance or isothermal calorimetry to determine whether there are specific differences in the carbohydrate binding affinities between the $CVN_2$ variants and WT that explain the differences in HIV neutralization.

Additionally, we are testing our variants against other enveloped viruses including influenza, Andes virus (a highly lethal hantavirus), and vaccinia virus (a small pox model) to determine whether the increase in efficacy for the dimeric variants applies to all or most enveloped viruses or is specific to HIV.

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**Table 2-1.** $CVN_2$ and $CVN_3$ linker sequences.

| Variant | Linker Sequence |
|---|---|
| $CVN_2$ L0 | N/A |
| $CVN_2$ L1 | G |
| $CVN_2$ L3 | GSG |
| $CVN_2$ L5 | GGSGG |
| $CVN_2$ L6 | GSGGSG |
| $CVN_2$ L7 | $(GGS)_2G$ |
| $CVN_2$ L8 | $(GGS)_2GG$ |
| $CVN_2$ L9 | GGSGGGSGG |
| $CVN_2$ L10 | $(GGSGG)_2$ |
| $CVN_2$ L11 | $(GGS)_3GG$ |
| $CVN_2$ L13 | $GGS(GGGS)_2GG$ |
| $CVN_2$ L15 | $(GGSGG)_3$ |
| $CVN_2$ L17 | $GGS(GGGS)_3GG$ |
| $CVN_2$ L20 | $(GGSGG)_4$ |
| | |
| $CVN_3$ L0 | N/A |
| $CVN_3$ L5 | GGSGG |
| $CVN_3$ L10 | $(GGSGG)_2$ |

**Table 2-2.** $IC_{50}$s (nM) of CVN and HIV neutralizing antibodies[a] against various envelopes in HIV clades A, B, and C.

| Clade | Envelope | 4 E10 | 2G12 | 2F5 | IgG1b12 | CV-N | $CVN_2$ L0 | $CVN_2$ L10 |
|---|---|---|---|---|---|---|---|---|
| **Clade A**[b] | | | | | | | | |
| | **DJ263.8** | N/A | N/A | N/A | N/A | 7.48 | 0.46 | 0.48 |
| | **Q23.17** | 108.75 | >300 | 46.25 | >300 | 15.4 | 1.35 | 2.59 |
| | **Q842.d12** | 87.5 | >300 | 53.75 | >300 | 19.1 | 2.83 | 3.34 |
| | **Q259.d2.17** | 89.375 | >300 | 66.25 | >300 | 162.6 | 13.79 | 41.1 |
| | **3718.v3.c11** | 71.875 | >300 | 21.25 | >300 | 64.33 | 4.01 | 8.89 |
| | **0330.v4.c3** | 36.25 | 4.375 | 59.375 | >300 | 2.69 | 0.11 | 0.25 |
| | **3415.v1.c1** | 146.875 | 13.125 | 227.5 | 156.25 | 5.69 | 0.28 | 0.44 |
| **Clade B**[c] | | | | | | | | |
| | **SF163.LS** | 1.875 | 3.75 | 0.625 | 0.0625 | 16.43 | 1.05 | 1.69 |
| | **PV0.4** | 40.625 | 7.5 | >300 | >300 | 4.09 | 0.23 | 0.49 |
| | **CAAN5342.A2** | 16.875 | >300 | 22.5 | >300 | 34.29 | 9.49 | 14.61 |
| | **WITO4160.33** | 1.875 | 6.875 | 3.75 | 19.375 | 1.85 | 0.1 | 0.12 |
| | **AC10.2.29** | 1.875 | >300 | 8.125 | 11.875 | 5.01 | 0.27 | 0.79 |
| | **SC422661.8** | 5.625 | 13.125 | 4.375 | 1.25 | 4.21 | 0.24 | 0.42 |
| | **6535.3** | 1.25 | 12.5 | 11.875 | 8.75 | 18.26 | 1.35 | 2.56 |
| | **THRO4156.18** | 1.875 | >300 | >300 | 3.125 | 7.75 | 0.59 | 0.74 |
| | **REJO4541.67** | 4.375 | >300 | 3.75 | 4.375 | 11.48 | 0.5 | 0.68 |
| | **TRJO4551.58** | 28.125 | >300 | >300 | >300 | 4.48 | 0.13 | 0.35 |
| | **QH0692.42** | 8.75 | 17.5 | 6.25 | 1.875 | 14.32 | 1.02 | 2.39 |
| | **TRO.11** | 1.875 | 2.5 | >300 | >300 | 9.57 | 0.53 | 0.85 |
| | **RHPA4259.7** | 43.125 | >300 | 75 | 0.625 | 11.57 | 1.14 | 2.04 |
| **Clade C**[d] | | | | | | | | |
| | **MW965.26** | N/A | N/A | N/A | N/A | 7.06 | 0.4 | 0.89 |
| | **ZM197M.PB7** | 3.125 | >300 | 76.875 | 124.375 | 4.34 | 0.41 | 0.49 |
| | **ZM249.PL1** | 13.125 | >300 | >300 | 20 | 22.93 | 1.93 | 2.31 |
| | **ZM53M.PB12** | 43.75 | >300 | >300 | 161.875 | 19.24 | 1.41 | 3.42 |
| | **ZM214M.PL15** | 25 | >300 | >300 | 18.75 | 29.35 | 1.46 | 2.59 |
| | **Du156.12** | 1.25 | >300 | >300 | 5 | 24.32 | 1.99 | 3.8 |
| | **Du442.1** | 4.375 | >300 | >300 | 1.25 | 5.02 | 0.28 | 0.48 |
| | **Du172.17** | 1.875 | >300 | >300 | 6.25 | 3.31 | 0.33 | 0.38 |
| | **CAP45.2.00.G3** | 16.25 | >300 | >300 | 4.375 | 1.21 | 0.17 | 0.41 |
| | **CAP210.2.00.E8** | 7.5 | >300 | >300 | 127.5 | 16.75 | 1.43 | 1.03 |
| | **ZM233M.PB6** | 7.5 | >300 | >300 | >300 | 4.56 | 0.24 | 0.29 |
| | **ZM109F.PB4** | 3.75 | >300 | >300 | >300 | 18 | 2.77 | 5.49 |
| | **ZM135M.PL10a** | 3.75 | >300 | >300 | >300 | 16.79 | 1.89 | 3.08 |

[a] A molecular weight of 160,000 g/mol was used to convert neutralizing antibody data (4E10, 2G12, 2F5, IgG1b12) from μg/mL to nM.
[b] Clade A neutralizing antibody data, personal communication, Prof. Michael Seaman.[36]
[c] Clade B neutralizing antibody data from Li *et al.,* 2005.[6]
[d] Clade C neutralizing antibody data from *Li et al.*, 2006.[7]

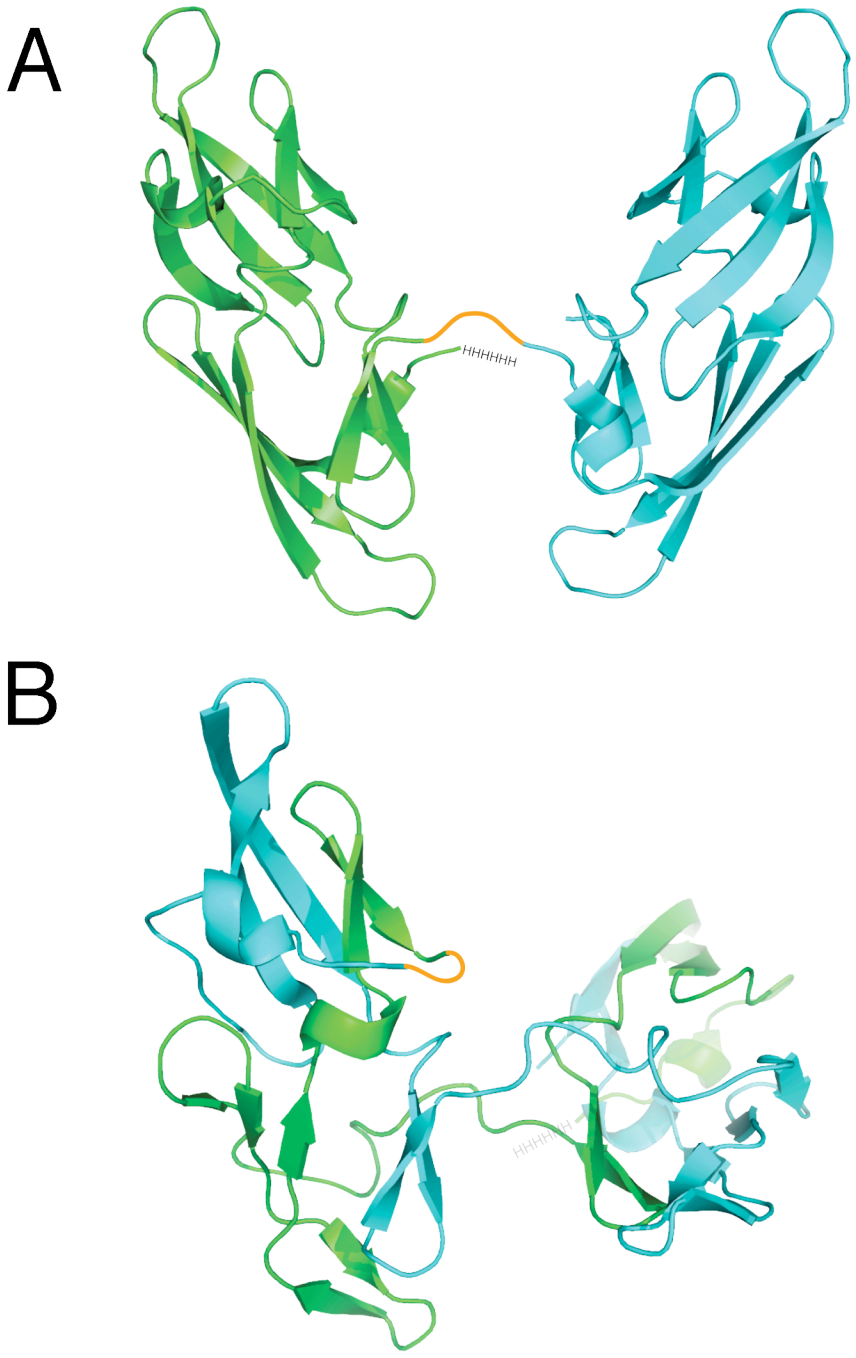


**Figure 2-1.** Model of generic $CVN_2$ protein. The CVN repeats are shown in green and cyan and the flexible polypeptide linker is shown in orange. The N-terminal His-tag is depicted as HHHHHH. The $CVN_2$ structures may adopt a linked monomer structure (A) or a linked domain-swapped structure (B). These representations were created using solved WT CVN structures[16,24] and are not based on any structural data (see Chapter 3 for structural information).

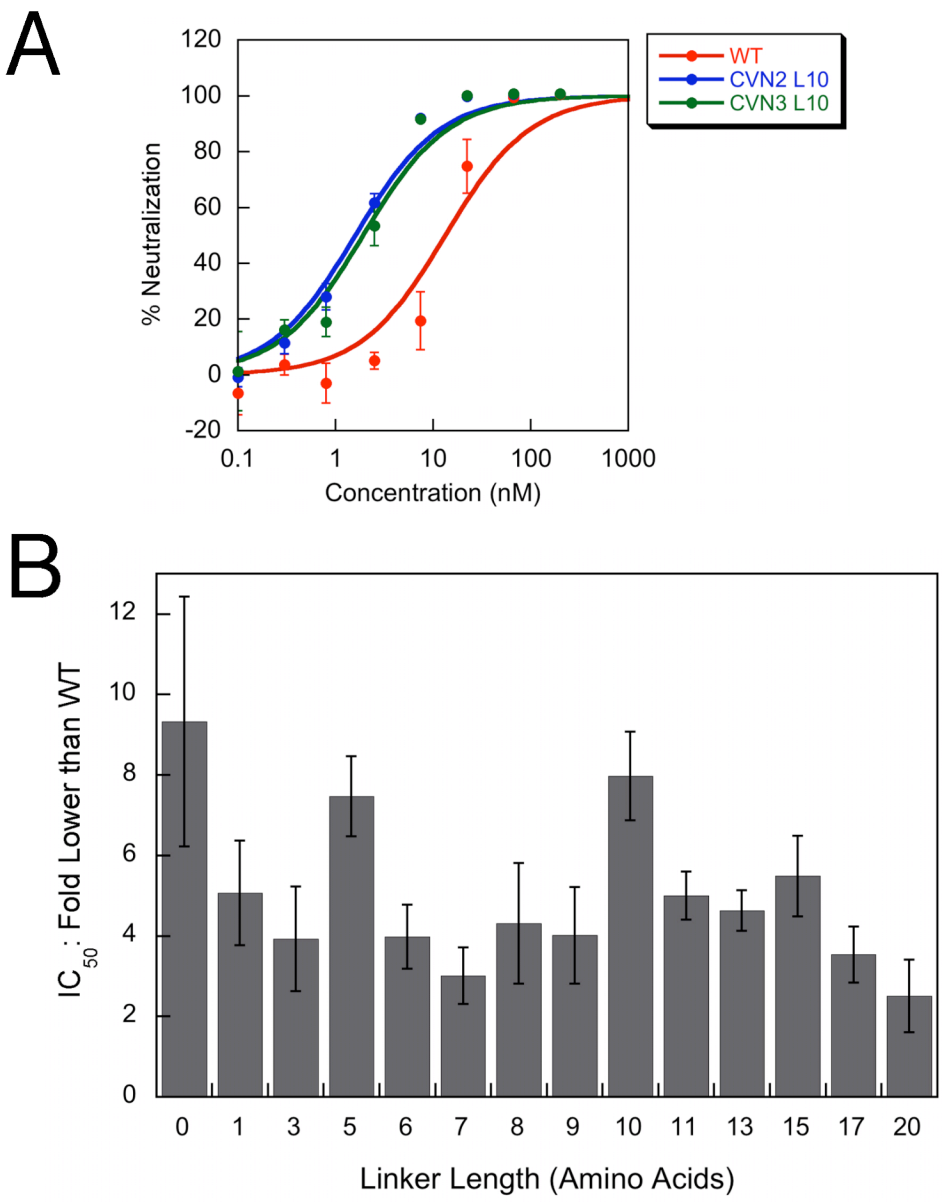


**Figure 2-2.** HIV neutralization assay results. (A) Typical neutralization data for WT CVN and two variants run on the same plate. The data are analyzed and fit as described in the methods. (B) Summary of $IC_{50}$s from various $CVN_2$s of differing linker lengths as compared to WT on the same plate. $CVN_2$ L0, $CVN_2$ L5, and $CVN_2$ L10 show the largest increase in efficacy over WT. All linked dimers, however, are at least two-fold more effective than WT.

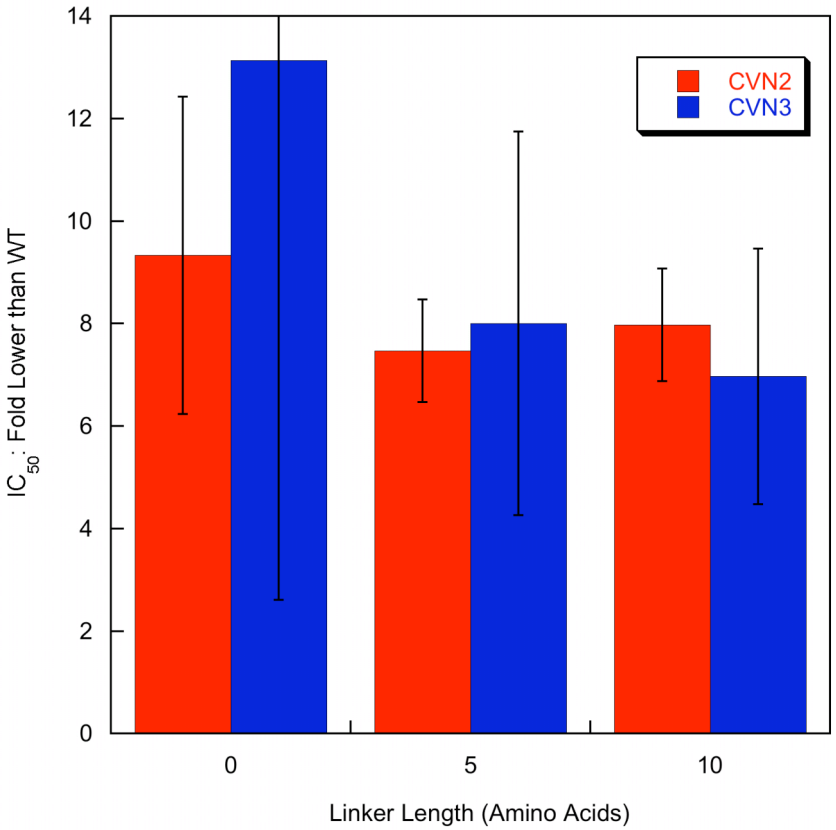


**Figure 2-3.** $CVN_3$ HIV neutralization data. The $CVN_3$ variants are all significantly more effective at neutralizing HIV than WT, but there is no significant difference between $CVN_2$s (red bars) and $CVN_3$s (blue bars) of these linker lengths.

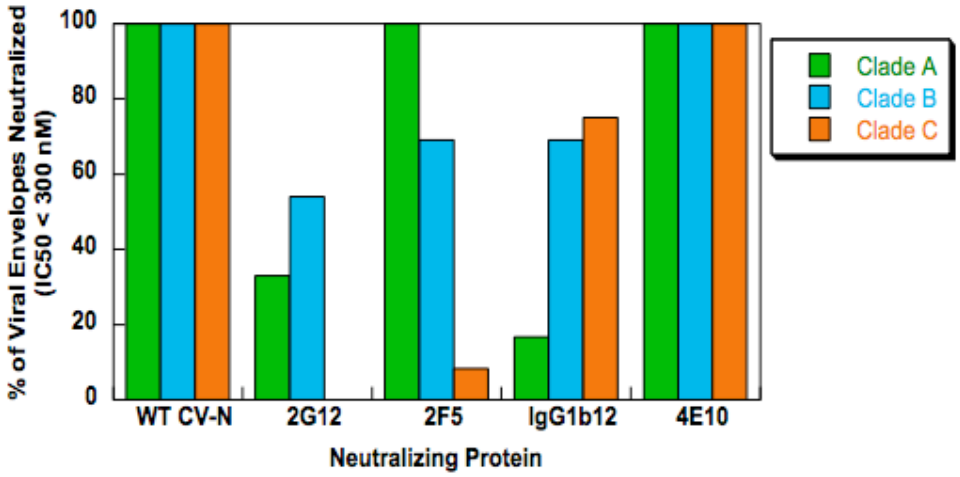


**Figure 2-4.** WT CVN cross-clade reactivity compared to broadly HIV neutralizing antibodies. WT CVN effectively neutralized all of the HIV pseudoviruses from clades A, B, and C. The 2G12 HIV neutralizing antibody neutralized some clade A and clade B viruses, but is not effective against any clade C viruses. The 2F5 neutralizing antibody works against only a few clade C envelopes and the IgG1B12 antibody is not fully effective against clade A envelopes. 4E10 is the only HIV neutralizing antibody with comparative cross-clade reactivity to WT CVN, neutralizing all viruses tested.

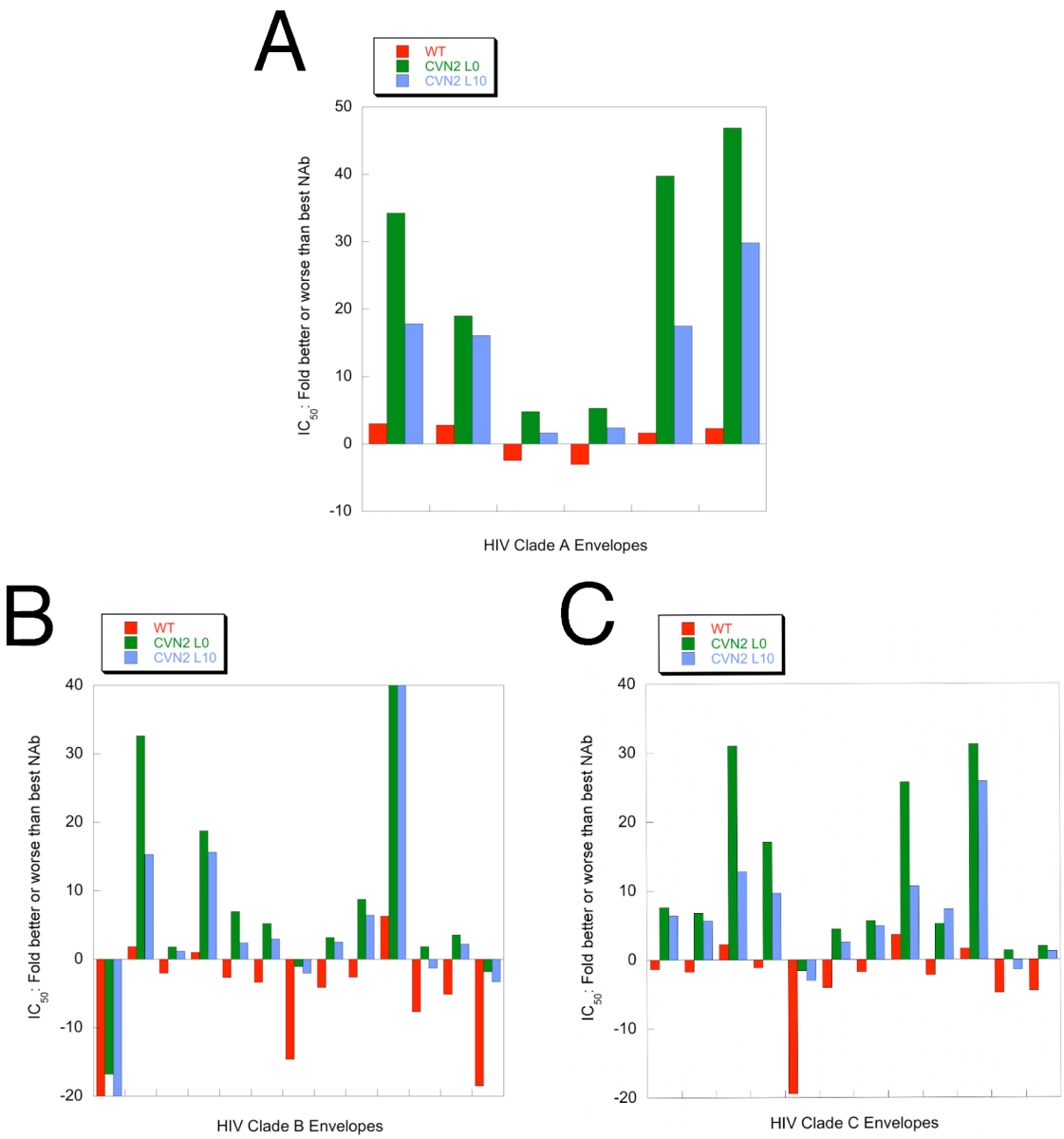


**Figure 2-5.** Engineered $CVN_2$ variants neutralize most HIV pseudoviruses with a lower $IC_{50}$ compared to the most effective broadly neutralizing antibody (NAb). For each envelope, the neutralizing antibody with the lowest $IC_{50}$ was chosen for comparison (see Table 2-2). CVN, $CVN_2$ L0, and $CVN_2$ L10 were evaluated against this best NAb from each envelope. CVN $IC_{50}$s that were lower than the NAb $IC_{50}$ were treated as described in the methods ("Fold lower than WT"). For variants with higher $IC_{50}$s than the NAb, the "Fold worse than best NAb" is the negative inverse of "Fold lower than WT" to provide clarity in the plot. Variants that have $IC_{50}$s lower than the best NAb are shown with positive bars and those with $IC_{50}$s higher than the best NAb are shown with negative bars. (A) Clade A envelopes. (B) Clade B envelopes. (C) Clade C envelopes.

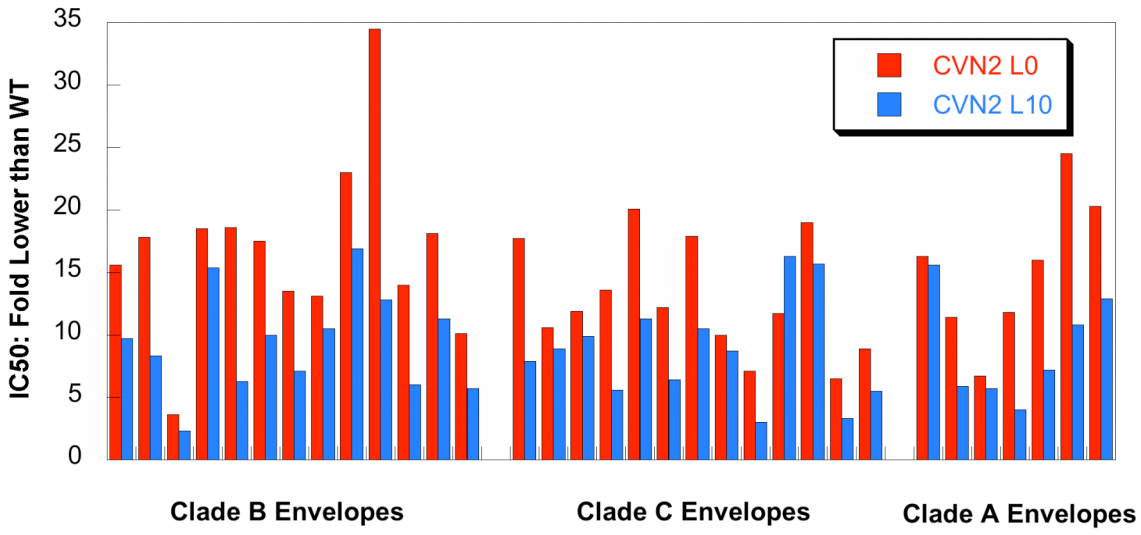


**Figure 2-6.** Engineered CVN variants are more effective at neutralizing various HIV pseudoviruses than WT CVN. For every virus tested, $CVN_2$ L10 neutralized with a lower $IC_{50}$ than WT CVN. For all viruses except one (a clade C envelope), $CVN_2$ L0 neutralized with a lower $IC_{50}$ that $CVN_2$ L10. $CVN_2$ L0 was up to 35-fold better at neutralizing HIV as compared to WT. $CVN_2$ L10 was at most 15-fold better neutralizing than WT.

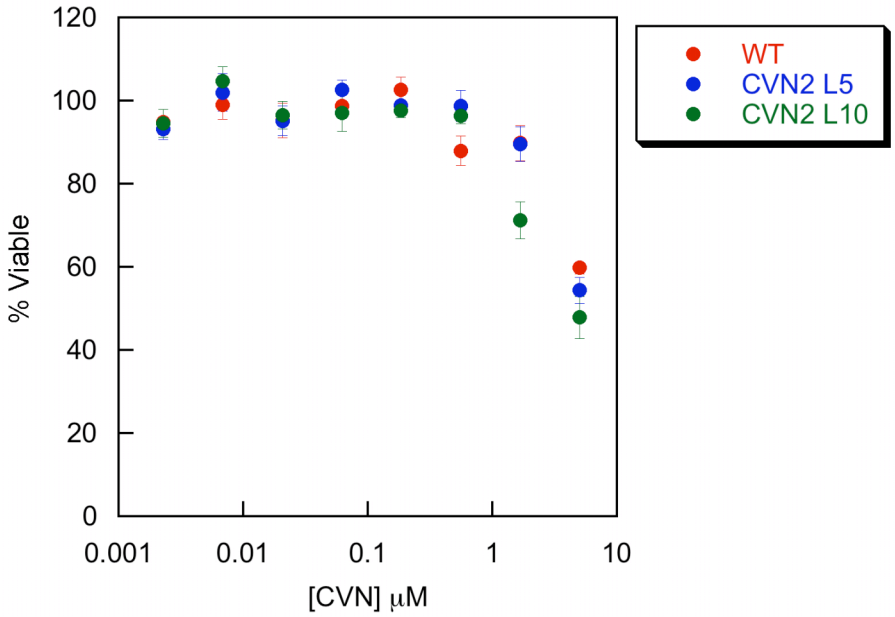


**Figure 2-7.** Cellular toxicity assay of CVN and $CVN_2$s. An XTT-based assay was used to determine whether CVN and $CVN_2$ variants are toxic to Tzm-Bl cells in culture. The CVNs are not toxic at the concentrations used in the HIV neutralization assay (up to 200 nM), but some toxicity was observed at higher concentrations.

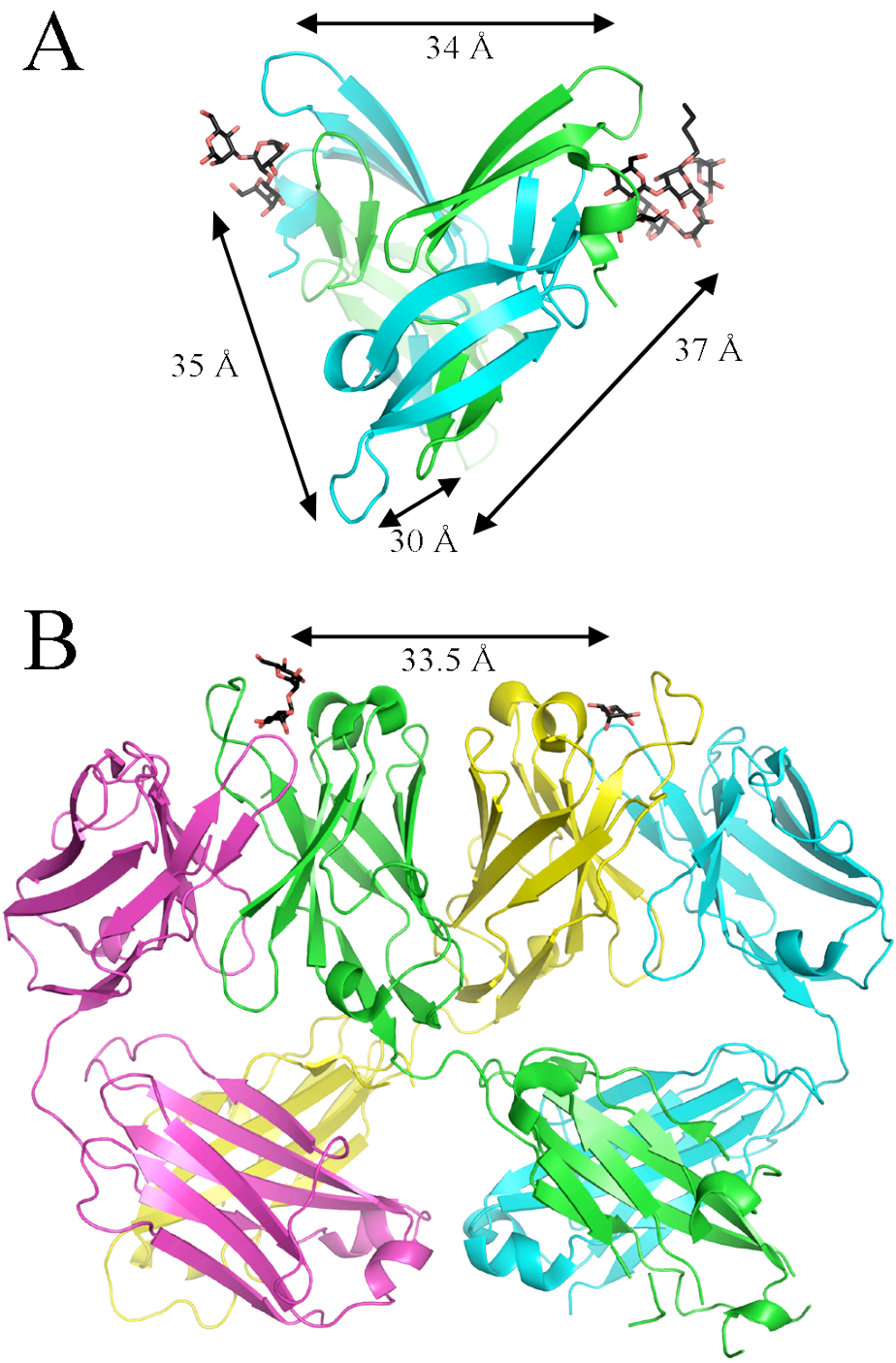


**Figure 2-8.** Carbohydrate binding site spacing in CVN and the 2G12 anti-HIV Fab. (A) Each of the four carbohydrate binding sites in the WT CVN crystal structure ($P4_12_12$ space group)[37] is approximately 30 to 40 Å from the other sites. (B) The 2G12 Fab, which is specific to carbohydrates on gp120 and is broadly neutralizing, has an unusual domain-swapped form in the crystal structure.[14] This domain-swapping rigidifies the carbohydrate binding sites with respect to each other and holds them approximately 35 Å apart.