

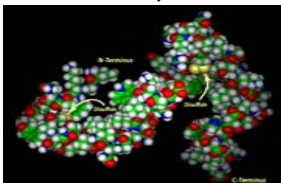
Advanced Preclinical Development of Cyanovirin-N as an Anti-HIV Vaginal Microbicide

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Cyanovirin-N (CV-N) is an 11 kDa protein with potent anti-HIV activity and is under development as a topical anti-HIV microbicide. CV-N was originally discovered in and purified from extracts of the cyanobacterium (blue-green alga) *Nostoc ellipsoides*. It has been reported that CV-N is extremely resistant to various methods used for physicochemical degradation. CV-N can withstand treatments with denaturants, detergents, organic solvents, and extreme temperatures without significant loss of antiviral activity. Low nanomolar concentrations of CV-N inactivate T-lymphocyte tropic, macrophage tropic, and dual-tropic primary isolates of HIV-1, as well as laboratory strains of HIV-1, HIV-2, SHV, and FIV. Moreover, CV-N is active against all international subtypes of HIV-1. CV-N binds irreversibly to the HIV surface envelope glycoprotein gp120, specifically interacting with the high mannose groups. This binding blocks the essential interactions between gp120 and receptors on various target cells, which leads to inhibition of attachment and fusion of the virus particle to the target cell, thereby inhibiting viral infection. We have expressed recombinant CV-N as inclusion bodies in the cytoplasm of *E. coli*. A purification scheme has been developed that exploits the physicochemical properties of this protein, in particular its stability in a harsh inclusion body purification scheme. Under the conditions developed, this method yields 140 mg of highly purified CV-N per L of high-density cell culture, which represents a 14-fold increase over the best recombinant CV-N yield reported to date. This purification scheme results in monomeric CV-N as analyzed by SDS-PAGE, isoelectric focusing, and reverse phase- and size exclusion-HPLC. This recombinantly expressed and refolded CV-N binds to gp120 with nanomolar affinity and retains its potent anti-HIV activities in cell-based assays. Our studies have also included evaluating the antiviral activity of a variety of excipients, alone and in combination with recombinant CV-N, as well as the *in vitro* activity of formulated drug product. Specifically, recombinant CV-N was formulated in hydroxyethylcellulose (HEC) or a co-polymer and tested for antiviral activity in Chinese rhesus macaques. The macaques were treated with Depo-Provera (20 mg), treated with 1.0 ml of CV-N gel vaginally and then challenged with 0.5 ml of SHV₁₀₂₆₃. In animals treated with 0.5% CV-N in HEC (n=5), no infections were noted up to 4 weeks post infection as measured by plasma viral RNA. Similarly, none of the treated animals had detectable anti-SHIV antibody at 40 days post-infection. The *in vitro* and *in vivo* results obtained in these evaluations suggest that CV-N formulated gel will be an effective vaginal microbicide. In addition to its chemical and physical properties, HEC placebo gel was analyzed for *in vitro* and *in vivo* effects on safety and efficacy. The results show that the HEC placebo has appropriate physical properties, is sufficiently stable as a vaginal gel formulation, and is safe for use in the clinical study of investigational microbicides. Finally, the unique properties of CV-N and its potent inactivation of HIV make this protein relevant for development as a topical anti-HIV microbicide, and advanced preclinical development is underway.

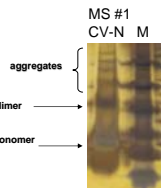
3D Structure of Cyanovirin-N



CV-N Purification: MS#1

CV-N protein was purified by unpurified methods and stored lyophilized. Monomeric protein purity was <60%.

12% SDS-PAGE shown here with silver staining.



Refined Purification

MS #2 CV-N

CV-N Purification: MS#2

CV-N protein was purified by a refined inclusion body method, followed by AEX-chromatography and lyophilization (as described by Collettori, DM, et al. Protein Exp Purif 39, 229-236). Monomeric protein purity was >90%.

monomer

HEC "universal" placebo and gel formulation

The "universal" placebo is comprised of 96.3% H₂O, 2.7% Natrosol 250 HX Pharm Hydroxyethylcellulose (Hercules Inc.), 0.85% NaCl, 0.1% sorbic acid, and 0 to 0.02% caramel color by weight. pH is adjusted to 4.4 with sodium hydroxide. The "universal" placebo is based on the gelling agent hydroxyethylcellulose (HEC), an unchanged linear polymer which does not impart anti-viral properties inherent in many charged polymers, nor the physical barrier protection of high-yield strength gelling agents. The gel has been designed to display negligible buffering capacity, thus avoiding acid-mediated microbicidal effects, even though it is adjusted to a low pH to match that of the healthy vagina. The gel is isotonic to prevent epithelial swelling or dehydration, and contains sorbic acid, a preservative without anti-HIV activity and which is considered one of the safest preservatives because it is readily broken down by cellular fatty acid metabolic pathways.

HEC placebo stability has been demonstrated by its viscosity profile and forced degradation data. Also, the efficacy of the placebo gel has been shown by *in vitro* and *in vivo* activity data. The safety of the gel has also been demonstrated in an RVI (no vaginal irritation).

See D Tien et al., ACC, submitted.

In Vitro Evaluation of WT CV-N and HEC Used in Monkey Study #1

Sample	Inhibition of Attachment IC ₅₀ (µg/mL)	Inhibition of Cell/Cell Fusion IC ₅₀ (µg/mL)	Inhibition of HIV Replication in CEM-SS Cells IC ₅₀ (µg/mL)	Inhibition of HIV Replication in PBMCs IC ₅₀ (µg/mL)
WT CV-N	0.011	0.014	0.17	0.015
0.5% CV-N in HEC	0.008	0.028	0.005	0.005
0.1% CV-N in HEC	0.014	0.038	0.008	0.02
0.02% CV-N in HEC	0.012	0.055	ND	0.04
HEC Alone	>1:80 dilution	>1:20 dilution	>1:500 dilution	>1:10 dilution

In Vitro Evaluation of WT CV-N, BSN5320 and Excipients Used in Monkey Study #2

Sample	Inhibition of Attachment IC ₅₀ (µg/mL)	Inhibition of Cell/Cell Fusion IC ₅₀ (µg/mL)	Inhibition of HIV Replication in CEM-SS Cells IC ₅₀ (µg/mL)	Inhibition of HIV Replication in PBMCs IC ₅₀ (µg/mL)
WT CV-N	0.003	0.017	0.055	0.028
BSN5302 CV-N	0.013	0.017	0.91	0.067
0.5% WT CV-N in Poloxamer	0.009	0.021	0.13	0.026
0.1% WT CV-N in Poloxamer	0.008	0.019	0.09	0.028
0.5% BSN5302 CV-N in Poloxamer	0.01	0.047	0.14	0.015
0.5% WT CV-N in HEC	0.01	0.057	0.16	0.041
HEC Alone	>1:444 dilution	>1:444 dilution	>1:888 dilution	>1:888 dilution
Poloxamer Alone	>1:500 dilution	>1:1000 dilution	>1:500	>1:1000 dilution

Summary and Conclusions

- Current process for CV-N purification yields highly purified, highly active material
- 0.5% CV-N in HEC from each monkey study appears to be active in the nM range in *in vitro* activity assays
- 0.5% CV-N in HEC using highly pure protein is efficacious in the *in vivo* monkey model for vaginal transmission of SHIV
- Physical, functional, and safety assessments conducted *in vitro* and *in vivo* reveal that the HEC placebo formulation is appropriate for use in the clinical evaluation of investigational microbicide formulations.

Future Studies

- Additional dose ranging studies *in vivo* (between 0.5% and 0.1%)
- Formulation development, geared toward properties of CV-N (ie. optimal pH of 4-5)
- Preclinical animal toxicology studies, such as vaginal absorption studies and RVI
- IND filing and initiation of Phase I human safety studies

Methodology – In Vitro Evaluations

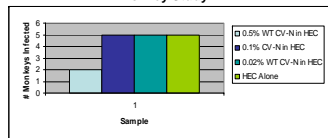
Anti-HIV Cytopathic Effect Assay
Following a six day acute infection of CEM-SS cells with HIV-1RF in the presence of compound, cell viability was measured spectrophotometrically (450/650 nm absorbance) using XTT dye reduction.

Attachment Assay
Compound and virus (at a pre-determined titer) were added to HeLa-LTR-β-Gal cells that had been plated in a 96-well flat-bottomed plate 24 hours prior to assay initiation. Cells, compound and virus were allowed to incubate for 2 hours at 37°C/5% CO₂. Following the incubation, the CV-N cells were allowed to remove any unbound virus and compound. Following addition of media to the wells and a 48 hour incubation, the cells were lysed and evaluated for β-gal expression using Gal-Screen (Tropix).

PBMC Assay
Peripheral blood mononuclear cells were isolated by ficoll hypaque gradient centrifugation from whole blood and activated with PHA. Following a seven day incubation, supernatant reverse transcriptase (RT) activity or p24 antigen was measured to quantify virus replication. Cell viability was determined by XTT dye reduction.

Fusion Assay
Compound was incubated with HeLa-LTR-β-Gal cells in a 96-well flat-bottom plate for 1 hour at 37°C/5% CO₂ before being co-cultured with HL2/3 cells for 48 hours. Following the incubation, the cells were lysed and evaluated for β-galactosidase expression using Gal-Screen (Tropix).

In Vivo Evaluation of WT CV-N and HEC Monkey Study #1

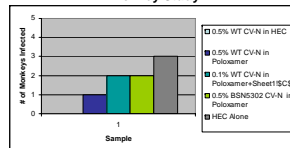


Methodology – In Vivo Evaluations

In the first study (n=20) animals were conditioned with Depo-Provera (20 mg) for four weeks prior to challenge. On day of challenge, animals were sedated, treated with 1 mL of gel vaginally, and after 15 minutes, challenged with 0.5 mL of SHV₁₀₂₆₃. Animals were followed for 8 weeks post-challenge and tested for viral load.

In the second study (n=23), two forms of CV-N were evaluated in two formulations. The difference between the two was that the resulting purified material much purer (90% by RP-HPLC).

In Vivo Evaluation of WT CV-N, BSN5302 CV-N and Excipients Monkey Study #2



*This work was supported in part by the Integrated Preclinical/Clinical Program for HIV Topical Microbicide Grant U19AI051650 within the NIAID at the National Institutes of Health; and with funds from the International Partnership for Microbicides, CONRAD, and USAID.

*The views of the authors do not necessarily represent those of the funding agencies.