An update of the recombinant protein expression systems of Cyanovirin-N and challenges of preclinical development

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Abstract

Introduction: Human immunodeficiency virus (HIV) is a debilitating challenge and concern worldwide. Accessibility to highly active antiretroviral drugs is little or none for developing countries. Production of cost-effective microbicides to prevent the infection with HIV is a requirement. Cyanovirin-N (CVN) is known as a promising cyanobacterial lectin, capable of inhibiting the HIV cell entry in a highly specific manner.

Methods: This review article presents an overview of attempts conducted on different expression systems for the recombinant production of CVN. We have also assessed the potential of the final recombinant product, as an effective anti-HIV microbicide, comparing prokaryotic and eukaryotic expression systems.

Results: Artificial production of CVN is a challenging task because the desirable anti-HIV activity (CVN-gp120 interaction) depends on the correct formation of disulfide bonds during recombinant production. Thus, inexpensive and functional production of rCVN requires an effective expression system which must be found among the bacteria, yeast, and transgenic plants, for the subsequent satisfying medical application. Moreover, the strong anti-HIV potential of CVN in trace concentrations (micromolar to picomolar) was reported for the in vitro and in vivo tests.

Conclusion: To produce pharmaceutically effective CVN, we first need to identify the best expression system, with Escherichia coli, Pichia pastoris, Lactic acid bacteria and transgenic plants being possible candidates. For this reason, heterologous production of this valuable protein is a serious challenge. Since different obstacles influence clinical trials on microbicides in the field of HIV prevention, these items should be considered for evaluating the CVN activity in pre-clinical and clinical studies.

Introduction

Until now, human immunodeficiency virus (HIV) infection or acquired immune deficiency syndrome (AIDS) remains a global health problem. The fight against HIV-1 has been going on with the FDA approved drugs designed for inhibiting the HIV fusion and its reverse transcriptase or protease activity. A combination therapy coupling antiretroviral treatment with chemical drugs has been shown to have positive effects on patients’ quality of life. Antiretroviral therapies usually fail due to HIV drug resistance, adverse effects and the long period of treatment. Continuous development of effective novel agents and safe anti-HIV drugs seems to be necessary. Valuable sources of natural compounds isolated from animals, plants, and marine microorganism offer new opportunities for identifying novel anti-HIV agents. For example, polysaccharides, sulfated sterols, terpene, peptides, and alkaloids could make possible treatments of choice for HIV. Among these various components, lectins - anti-carbohydrate-binding proteins - are the most interesting microbicides. This is mainly because of their non-immunoglobulin nature, recognition potential and reversible binding to complicated glycoconjugate moieties. Lectins are naturally occurring compounds found within a broad spectrum of organisms including algae, fungi, sea corals, higher plants, prokaryotes, actinomycete, worms, invertebrates, and vertebrates (Table 1). Four algae groups (rhodophyta, phaeophyta, chlorophyta, and...
cyanobacteria) can produce lectins, with cyanobacteria responsible for the production of a total 4.2%. Because of their potential to block the envelope glycoprotein 120 (HIV-gp120), algal lectins possess higher anti-HIV activity compared to plant lectins. Such a high activity at little concentrations (nanomolar to picomolar) subsequently leads to the inhibition of HIV infusion.

In addition to anti-HIV, antibacterial, and antifungal activities, these components could participate in many other biological processes like host-microbe interactions, cell targeting, and communication, apoptosis induction, differentiation and metastasis. Observations based on the recent studies confirm that several lectins not only have significant anti-HIV activities but also show activity against other enveloped viruses. So far, cyanovirin N (CVN), scytovirin (SVN), Microcystis viridis lectin (MVL), and griffithsin (GRFT) have been recognized as the most promising anti-HIV candidates from the algae-originated lectin family. The purpose of this study was to review the past, present, and future aspects of the artificial production of CVN, a commonly known antiviral cyanobacterium lectin, via both prokaryotic and eukaryotic expression systems. We have also compared the efficacy of the products obtained in each expression system.

### Candidate microbicides against HIV-1

The candidate microbicides target four steps of the HIV-1 life, cycle, including (i) entry and fusion of the virus, (ii) reverse transcriptase activation, (iii) integration, and (iv) virus maturation that is done via proteolytic cleavage. The main selection criteria for microbicides concerns their safety and specific anti-HIV activity. Each anti-HIV drug can interfere with HIV replication cycle specifically. Vagina, rectum and male urethra are the initial virus infection targets in mucous membranes through which HIV can enter the bloodstream and infect the host. The microbicides could be formulated in different forms such as vagina gels/rings, creams, and lubricants or suppositories, delivering active ingredients slowly during coitus and daily or extended periods of time. The HIV infection can be blocked in the primary stage if exposed to high concentrations of topical active microbicides. In this case, the risk of infection in the healthy subjects may be reduced due to the toxicity induced by longtime exposure to microbicides. Events in initial infection through the mucosal tissues and the action of anti-HIV microbicides were reviewed by Haase in 2011 and are summarized in Table 2. CVN inhibits the first step of infection (viral entry and fusion into the host cell). HIV fusion into cells is mediated sequentially via the interaction of gp120-CD4 with co-receptors CCR5 or CXCR4. This connection induces the formation of non-covalently linked heterodimers (trimers) of gp120 and gp41. Finally, the viral-cellular membrane fusion allows the development of pores and viral genetic material (ssRNA), which can integrate into the cell genome to start the next viral replication cycle. Several strategies are involved in targeting the potential candidate microbicde as an entry inhibitor. The first one concerns binding of gp120 to CD4 which is inhibited when the CD4-binding

| Source | Lectins | Origin |
|---|---|---|
| Algae | Cyanovirin-N (CVN) | Nostoc ellipsosporum |
| | Scytovirin (SVN) | Scytonema varium |
| | Oscillatoria agardhii Agglutinin | Oscillatoria agardhii |
| | Microvirin | Microcystis viridis |
| | Agglutinin | Oscillatoria agardhii |
| | Boodlea coacta lectin | Boodlea coacta |
| | Griffithsin | Red alga Griffithsia |
| | Cyt-CVNH | Cyanobacterium Cyanotheca |
| | | (7424) |
| Plants | | |
| | Jacalin | Artocarpus heterophyllus (Jackfruit seed) |
| | Concanavalin A | Canavalia ensiformis (Jack bean) |
| | Musa acuminate lectin | Banana |
| | MH lectin | Myrianthus holstii |
| | NP Lectin | Narcissus pseudonarcissus (Lent lily) |
| | PCL lectin | Polygonatum cytonema |
| | LanF lectin | Musa acuminate and Musa balbisiana |
| Actinomycete | Actinohinv | Longispora albida |
| Worm | Polycheate lectin | Chaetopterus Variopedatus (Marine Worm) |
| | SV Lectin | Serula Vermicularis (Sea Worm) |
| Nematode | C-type lectin mermaid | Laxus Oneistus |

### Table 1. A list of lectins with anti-HIV activity

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|---|---|---|
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| | Scytovirin (SVN) | Scytonema varium |
| | Oscillatoria agardhii Agglutinin | Oscillatoria agardhii |
| | Microvirin | Microcystis viridis |
| | Agglutinin | Oscillatoria agardhii |
| | Boodlea coacta lectin | Boodlea coacta |
| | Griffithsin | Red alga Griffithsia |
| | Cyt-CVNH | Cyanobacterium Cyanotheca |
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Comparison of expression systems to recombinant production of CVN

Table 2. Initial infection events of HIV in the mucosal tissues

| Events in initial HIV-infection in vaginal lumen                          | Duration   | Candidates Microbicides                      |
|------------------------------------------------------------------------|------------|----------------------------------------------|
| Crossing the mucosal barrier and viral attachments                     | Minutes to hours | Entry/fusion inhibitors                      |
| Primary local propagation in initial targets (CD4+ T cells, CCR5 and CXCR4 co-receptors, Dendritic cells) / founder population | Hours/days   | Reverse transcriptase and integrase inhibitors |
| Second expansion influx as locally and activating extra target cells / dissemination of virus into draining lymph nodes | Days        | Protease inhibitors                          |
| Self-sustaining proliferation (in the regional lymph node) / diffusion as systematically (in a blood vessel) | Days-weeks  |                                              |

CVN has 2 similar domains: domain A spanning 1-38 and 90-101 residues and domain B which is formed by residues 39-89. It is determined that domain B has no terminal, but both terminals (C and N) exist in domain A (Fig. 1). The flexibility of the domain A is higher than that of domain B, which can affect the binding affinity of domains to gp120. It was found that domain-swapped dimers formed under physiological conditions in which proline residue plays an important role (Fig. 2).

CVN is resistant to different conditions, including 0.5% SDS detergents, denaturants of organic solvents (CH₃CN, MeOH, and dimethyl sulfoxide), and continuous freezing and thawing. Moreover, it can preserve the anti-HIV activity in high temperatures (at 100°C for 15 minutes). CVN slows the envelope-facilitated cell fusion process at low concentrations (nanomolar) by blocking the interaction of gp120-oligomannoses with CD4. The anti-HIV activity of CVN against various primary strains of HIV has been reported by various laboratories. Furthermore, its inhibitory effect has been investigated against several viruses (Ebola, influenza, simian immunodeficiency [SIV], feline immunodeficiency, hepatitis C, measles, and herpes simplex type-1,6). The high sensitivity of these viruses to CVN is attributed to the N-linked oligosaccharides with high mannose content. Moreover, the inhibitory activity of CVN suggests that it can be utilized not only as an anti-HIV topical microbicide but also for the improvement of a broad-spectrum of antiviral components. Apart from most of the other lectin candidates, only nanomoles of CVN and SVN can inhibit half of the HIV virus in the in vitro test. CVN also has a high affinity for binding strongly to glycoproteins (Mannose8 or Mannose9).

Cyanovirin-N as a promising anti-HIV candidate

This anti-HIV candidate was isolated from cyanobacterium (Nostoc elliposporum) by Boyd et al. CVN consists of 101-amino acids with two carbohydrate binding domains.

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**Fig. 1.** The sequence of the amino acid corresponding to wild-type CVN, reported by Boyd et al in 1997. The proline 51 highlighted in green color is responsible for swapping of domains and aggregation of monomers form of CVN. Two disulfide bonds (C8-C22 and C58-C73) formed by four cysteine residues (colored with red).
safety. Meanwhile, PEGylation of CVN may reduce its immunogenic and mitogenic potentials.\(^{52}\)

Chimeric CVN designed with Pseudomonas exotoxin PE38 demonstrated increased cytotoxic effects on H9 cells (HIV-infected gp120-expressing cells).\(^{53}\) In addition, another recombinant chimera composed of CVN and 20 residues of MPER (gp41 membrane-proximal external region) was engineered to inhibit viral entry through dual-activity.\(^{54}\)

**Mechanism of CVN action**

Interaction of gp120 with CD4 T cells and CXCR4 and CCR5 – associated with HIV entry process - is illustrated in Fig 3.\(^{55}\) The HIV-gp120 molecules contain several variables (V1-V5) and conserved domains (C1-C5) with 25 N-linked (asparagine sites) glycosylation site following the motif: asparagine-X-(serine or threonine), where X could be any other amino acid except proline.\(^{56}\) The glycosylation spike has the high-mannose or hybrid type of oligosaccharides. The Man9 and Man8 are common ends for the high-mannose oligosaccharides on gp120. CVN can bind at a nanomolar level to Man9GlcNAc2 (Manα1→2Manα1→6Manα).\(^{57}\) Studies have confirmed that CVN contains two binding sites for carbohydrate in each domain with low and high affinities, separated by a distance of ~ 40 Å. Bewley et al revealed that domain B and domain A have a high and low affinity for dimannose binding, respectively\(^{57}\) with a 10 fold difference in affinity. The nine key residues at the high-affinity binding sites (i.e., glutamic acid41, serine52, asparagine53, glutamic acid56, threonine57, lysine74, threonine75, arginine76 and glutamine78) can interact with dimannose through hydrogen and electrostatic bonds. In addition, five residues (i.e., lysine3, glutamine6, threonine7, glutamic acid23 and threonine25) also play an important role in the interactions occurring at low-affinity site (Fig. 4).\(^{58}\)

Furthermore, it was determined that the nested dimer of CVN might lead to an increase in activity compared to wild-type CVN in anti-HIV cellular and fusion assays.\(^{59}\)

**Application of different expression systems for recombinant production of CVN (rCVN)**

The interesting properties of CVN include poor toxicity, resistance to various denaturation conditions, and most importantly, selective action on HIV-1. Therefore, CVN is compatible with the host immune system\(^{60}\) and can be considered for development of topical microbicides (vaginal or rectal). Besides, because highly pure protein is required, the recombinant production of CVN becomes important. CVN has already been artificially expressed in numerous heterologous expression systems including bacteria, yeast, and transgenic plants.\(^{61}\)

Based on macaque studies, 5 mg of rCVN could be administered twice a week (as an effective dose) and it is required to products of 5000 kg per year to supply needs of 10 million women.\(^{62}\) Such a large scale production of rCVN with high efficiency and low cost could be practicable only through expression systems.

**Bacterial expression systems**

*Escherichia coli* is an organism which is broadly used for the production of different recombinant proteins.\(^{63,64}\) The advantages of this expression system are its remarkable genetic and physiological properties, short time of generation, ease of handling, known fermentation process and finally the high specific yields. CVN as a monomeric protein holding two necessary disulfide bonds with no glycosylation, was first artificially expressed by Boyd et al in *E. coli* at periplasmic spaces (using the expression...
vector pFLAG-1). They showed that low nanomolar concentrations of either natural or recombinant CVN irreversibly inactivate HIV-1, HIV-2, and SIVs in different ranges (0.1 to 7.8 nM of EC50 (50% effective concentration)).7 Because of membrane structure, low level of chaperones or foldases and the high concentration of periplasmic proteases, the final yield of CVN was considerably low.

In 2005 Colleluori et al used a method to increase the yield of rCVN, which was subsequently followed by the expression of inclusion bodies (IB) within the cytoplasm of E. coli. Under the harsh purification conditions, physicochemical properties of CVN remained stable. Also, the final yield of rCVN was 14 fold higher than the previous reports.66 Refolding and purification were performed via conventional methods and ion exchange chromatography, respectively, while the anti-HIV potential of rCVN was retained at nanomolar level. In addition, the results showed that IC50 (50% inhibitory concentration) for cell-cell fusion, virus-cell attachment and PBMC (human peripheral blood mononuclear cells) infection were 15 ng/mL, 3.2 ng/mL, and 0.11 pg/mL, respectively. Finally, approximately 40 mg/L and 140 mg/40 g wet cell/L of rCVN were produced inside shaking flasks and fed-batch high cell density culture, respectively. But, heterogenic isomers of rCVN (including full-length monomeric, dimeric and N-terminal deleted residue) were produced too, indicating the weakness of IB production form.

An artificial expression of rCVN in E. coli has been tried using numerous vectors, while the low level of final yield and IB formation were unacceptable; and at this point, the chaperone-fusion expression system came into the play. Fortunately, in this system hexa-histidine and small ubiquitin-related modifier (SUMO) tags were used for the production of soluble rCVN in the cytoplasm of E. coli. This construct, SUMO-CVN, helps in rapid purification of intact and native rCVN as a soluble and biologically active homogeneous form. Finally, rCVN activity was evaluated via WST-1 (water-soluble tetrazolium salt (WST)-1) method. The study reported significant activity of rCVN against HIV-1/IIIB. The inhibition levels were 71% (concentration: 56.25 nM) and 62% (concentration: 28.13 nM) compared to AZT (control) which showed a 64.62% inhibitory function (concentration: 0.11 pg/mL). Finally, approximately 40 mg/L and 140 mg/40 g wet cell/L of rCVN were produced inside shaking flasks and fed-batch high cell density culture, respectively. But, heterogenic isomers of rCVN (including full-length monomeric, dimeric and N-terminal deleted residue) were produced too, indicating the weakness of IB production form.

Lactococcus lactis and Lactobacillus plantarum are other attractive commensal bacteria considered for the delivery of rCVN into the vaginal channel.69 Pusch et al in 2005 bioengineered strains of lactic acid bacteria (LAB), L. lactis MG1363, and Lactobacillus plantarum NCIMB8826 to express rCVN.

The antiviral activity was confirmed in lymphocytic H9 cells infected with HIV-1 NL4-3 in a dose-dependent manner. It was reported that 83% of the inhibition of viral infection resulted using 10 µg cell extract of transformed L. lactis (with pTSV1-CVN) while a complete inhibition was achieved with 30 µg of cell extract. The comparison of the expressed rCVN level between the 2 bacteria demonstrated that the production level in L. plantarum was higher than that of L. lactis, due to the higher growth rate of the former. The construct containing Usp45 leader sequence (pTSV2-D-CVN) increased the secretion of CVN (about 6-8 fold), with a dose-dependent anti-HIV1 activity in contrast to the control CVN (1.5 to 150 nM). The obtained anti-HIV activity of pTSV2-D-CVN was the same as that of a control CVN (1.5 nM; EC50). In addition, 15 nM purified rCVN from pTSV2-D-CVN suppressed viral activity, subsequently leading to a 10-fold increase in secretion efficiency of CVN. Also, inhibitory concentrations of lactococcal-derived rCVN compared with E. coli-derived rCVN certified its biological activity.68 Li et al in 2011 incorporated the rCVN secretion into food by bioengineered lactic acid bacteria (LAB). They found that CVN was expressed in the rectal vault after feeding pigtail macaques with yogurt containing bioengineered LAB as a starter culture. Moreover, the peak viral burden in the experimental groups was significantly lower than that of the control animals. They concluded that the formulation of CVN in LAB for an oral administration could be a feasible approach for the mucosal delivery of interesting microbicides.71 Lotti et al in 2017 used response
surface methodology (RSM) to optimize the expression of CVN homology gene found in the indigenous strain of *Nostoc ellipsosporum* LZN. RSM analysis suggested the optimum condition of the protein expression for three parameters (0.6 mM IPTG concentration, 29°C growth temperature and 12h induction time). The CVN homology protein was expressed in periplasmic fractions using pET22b vector in *E. coli* (BL21).\(^\text{72}\)

In summary, the production of the soluble form of rCVN in the cytoplasmic and periplasmic space of *E. coli* needs additional downstream processes which make this system suitable for use.

**Eukaryotes expression systems: yeast and transgenic plants**

*Pichia pastoris* is another host of valuable properties, it can be cultured cheaply and rapidly, represents post-translational modification (PTM) pathways, secretes recombinant proteins more efficiently, produces recombinant proteins, and most importantly doesn’t require intense processing technology. Based on the codon usage bias of *P. pastoris*, rCVN could be expressed in this system.

However, N30 and P51 residues (responsible for dimer formation) of CVN were glycosylated in this system, resulting in loss of anti-HIV potential.\(^\text{73}\) Moreover, only 10 mg/L of rCVN in *P. pastoris* was produced, which was a very low yield compared to cytoplasmic expression in *E. coli*. Besides, dimeric aggregate forms of rCVN were also produced that complicated the efficient, large-scale production of pure monomeric rCVN. They also assayed rCVN homologs (Asn30 substituted with Ala, Gln, or Val, and Pro51 with Gly) to exclude heterogeneous conformational potential. All homologs showed an anti-HIV activity in contrast to wild-type CVN, while only one homolog (Pro51Gly) had a considerably more stable structure. The activity of CVN (as a control) and its homologs varied in different clinical and laboratory viral strains and targeted cells. Three CVN homologs showed lower EC50 than that of CVN against RoJo (in PBMC cells), Ba-L and ADA strains (in monocyes). However, these homologs had high EC50 (about 5.4 to 10.9 nM) compared to CVN (2.1 nM) in PBMC cells against WeJo strain. The Pro51Gly and its homologs exhibited lower levels of EC50 than CVN. The lowest EC50 was observed against RF strain in CEM-SS cells (0.1 vs. 0.49 nM). In conclusion, these functional rCVN homologs that are resistant to glycosylation could be more amenable for use in large-scale production via bacterial expression system or in eukaryotic hosts.\(^\text{73}\)

Another feasible eukaryotic system for the expression of rCVN is the transgenic plants. The production of rCVN should be efficient and cost-effective. Thus, it is best if the disadvantages (aggregation and heterogeneous production of rCVN) of fermentor-based systems (bacteria and yeast) could be eliminated. Hence, the large-scale rCVN production could be made possible through transgenic plants since they are easily prepared for the production and economically scaled up.

Sexton et al in 2006 explored proof of concept for the production of rCVN by transformation of *Nicotiana tabacum*. In this system, produced rCVN was about 130 ng/mg (of fresh leaf tissue), which stood for a minimum of 0.85% of the total soluble protein of the plant. Moreover, western blot analysis confirmed the production of preferred monomeric isomers of rCVN able to bind functionally to HIV-gp120. On the other hand, 0.64 μg/mL rCVN as rhizo-secretion was produced in the hydroponic media within 24 days. Based on these finding, they suggested the potential of transgenic plants to develop new strategies for large-scale production of microbicides.\(^\text{74}\) However, extensive optimization is needed for commercial viability which is undertaken to promote the rCVN yield in the transgenic plant.\(^\text{75,76}\) Following successful-expression of rCVN in the transgenic plant, a recombinant fusion protein including CVN along with HIV-neutralized monoclonal antibody (b12) was also manufactured. Also, the anti-HIV potency of this fusion was higher compared to that of b12 or the rCVN alone.\(^\text{77}\) The fused protein addresses the options available for the production of a combination of microbicide drugs in transgenic plants. This expression system can solve the economic related concerns of scale-up for most of the developing countries. Seeking a way for high-level rCVN production in plastid plants,\(^\text{77}\) Elghabi et al. in 2011 investigated the possibilities for the expression of rCVN in chloroplasts along with the green fluorescent protein (GFP) and PlyGBS. They discussed two challenges in their study, including the low stability of the mRNA and the protein produced which made sense when they observed a lack of detectable rCVN in the chloroplasts. Consequently, plastid expression of rCVN has been optimized through N-terminal fusions into rCVN coding sequence.\(^\text{78}\)

Drake et al, in 2013, generated a wild type of marshmallow plant (*Althaea officinalis* L.) with transgenic roots by *Agrobacterium rhizogenes* for rCVN expression. After a seven-day culture in liquid medium, the mass of the wild-type and CVN root lines was increased by 49% and 19%, respectively. The concentration of CVN in the root tissue was 2.4 µg/g fresh weights, whilst the concentration level in the culture media was 0.02 µg/mL during a period of 24 hours.\(^\text{79}\) Murad et al in 2014 used transgenic soybean seeds to produce CVN. In this study, the bombardment of 1000 somatic embryonic axes was done via two vectors. The first vector, pbCong, included CVN coding sequence, the p-conglycinin gene (signal peptide) and also, CaMV35s as a terminator. The second vector, pAC321, contained Imazapyr herbicide gene as a resistance marker gene. Characterization of CVN expression levels in the R1 seeds was performed using ELISA-gp120. The anti-HIV activity was investigated by semi-purified recombinant CVN. As a result, only
8 transgenic plants from a total 20 herbicide selected plants had CVN gene, with 2 transgenic plants able to HIV-gp120. The expression level was reported 1.5% after the analysis of total soluble protein by NanoUPLC-MS. Moreover, semi-puriﬁed CVN from soybeans showed a 10-fold weaker anti-HIV activity than that of the control (expressed in E. coli). A major limitation of this system was dilute recombinant yields with other proteins in soybeans such as b-conglycinin, because CVN has a more binding affiinity to such proteins. They suggested pure CVN should be produced as a ﬁnal yield. In another study, O’keefe et al, in 2015, produced biologically active rCVN in genetically modiﬁed soybean (350 µg/g per dry seed weight). Moreover, rCVN at 0.82-2.7 nM managed to inhibit HIV (EC50) in contrast to E. coli-derived rCVN of 0.45-1.8 nM. It was determined that harvesting soybean oils according to the standard industrial processing would not reduce the rCVN antiviral potential. On the other hand, in this process, both the soybean oil and rCVN met the criteria to be considered anti-HIV microbicides. The endosperm of rice, a new platform for rCVN production, reported by Vamvaka et al in 2016, yielded a ﬁnal product which effectively prevented HIV-1BLA infection. They preferred rice endosperm mainly due to suitable storage and transport form of dried seeds. Additionally, crude extract could be prepared locally and was applicable as a microbiocide drug without any additional puriﬁcation process. The crude extract volume of rCVN in the rice seeds was more than 10 µg rCVN/gram dry seed weight, with a dose-dependent gp120-binding activity (EC50 = 1.8 nM). The results conﬁrmed that rCVN had solubility and correct foldings as well. Furthermore, this platform could be directly used as topical microbiocides due to its safety, and non-toxic effects in the human cells.

Moderia et al in 2016 optimized the production of rCVN via rhizosecretion platform and focused to simplify downstream processing. Within a week of culture, the production in hydroponic medium reached to 20 µg/mL. After concentration of rCVN, it was determined that the semi-puriﬁed rCVN could neutralize HIVBa-L strain with an IC50 of about 6 nM.

As a ﬁnal conclusion, the lower yield of rCVN was produced in P. pastoris than E. coli system. In N. tabacum a higher level of rhizosecretion was reported and rCVN was produced as a monomeric form with functional anti-HIV activities (Table 3). It is important to note that the transgenic expression system can be optimized to obtain higher levels of rCVN.

Preclinical test of CVN against HIV

Buckheit et al, in 2012, comprehensively reviewed 5 priority aspects of topical anti-HIV microbiocides to obtain successful clinical trials (in large-scale) in the near future as follows:

(i) the role of vaginal and rectal environments (physiological and biological) that can modify microbiocide functionality,
(ii) the application of models (in vivo, ex vivo, in vitro) to orientate pharmacokinetics (distribution rate, absorption and retention level in tissue and body ﬂuids) and pharmacodynamics properties (biological activity) of microbiocides,
(iii) determining the ﬁnal dose of microbiocides and recognizing the effect of different doses, formulation and a suitable delivery system to alter pharmacokinetics and pharmacodynamics properties,
(iv) focusing on formulations and delivery into vaginal or rectal or both,
(v) using multi-purpose prevention approaches (targeting several infections and also contraceptive products at the same time).

The effectiveness and safety of different candidates for the HIV prevention have been investigated via pre-clinical developments which are brieﬂy indicated in Table 4.

In an in vivo study, the efﬁcacy of rCVN topical gel was evaluated in both male and female macaques (Macaca fascicularis) infected rectally and vaginally with SHIV89.6P virus (chimeric SIV/HIV1). It was found that rCVN had no cytotoxicity or any clinical adverse effects. None of the macaques treated with 1%-2% CVN gel showed any sign of SHIV89.6P infection and side effects after using the gel rectally. The results conﬁrmed that the topical rCVN gel could inhibit the rectal transmission of SHIV in macaque models. These ﬁndings provide some clinical insights to use rCVN as a topical microbiocide for targeting sexual transmission of HIV in humans.

Further, several agents may inﬂuence the efﬁcacy of topical microbiocides including vaginal ﬂuid, semen, personal hygiene habits, and commensal bacteria. These factors can dilute or neutralize topical drugs. Therefore, inﬂuencing factors should be evaluated both in vitro and ex vivo conditions of female genital tissue explants. In preclinical trials, the complexity of vaginal and rectal physiology anatomy, vaginal ﬂuids, the effect of semen after coitus, and pH of vaginal, rectal and microflora thereof should be under evaluation. It was proved that the rCVN preserved its activity in the presence of semen in a low nanomolar concentration and was not much inﬂuenced by Candida albicans. Additionally, CVN effectively prevented ectocervical explants’ infection and viral-dissemination by tissue-emigrating cells. In another preclinical test, rCVN, PRO 2000, and tenofovir as PMPA gel were assessed in an explant model of penile tissue. According to the results, 11 µg/mL CVN prevented infection with HIV-1 about 95%. As a control test, total protection using PRO 2000 and PMPA was obtained with 10-100 fold higher dose than that of rCVN. CVN had no cytotoxic effects on genital tissue explants (male or female), however, up-regulation of cytokine was detected after 2-hour exposure to CVN. Finally, these ﬁndings propose CVN as a worthy candidate for more assaying in non-human primates followed by human clinical
| Expression systems for producing rCVN | Anti-HIV assay |
|--------------------------------------|---------------|
| **Microorganism** | **Yield** | **Strain** | **Cells** | **IC50** |
| **Bacterial** | | | | |
| E. coli: BL21(DE3), BL21(DE3)plysS, Origami(DE3), Origami(DE3)plysS, B834(DE3), B834(DE3)plysS | pFLAG-1 (periplasmic) | - | HIV-1: G1, 205, SK1, 214, G910-6, MN, 11B, RF, A17 | MT-2, U937, CEM-SS | 0.1 to 7.8 nM |
| | pB57(-)-ompA-CVN (periplasmic) | 10 mg/liter of cell culture | HIV-1: G1, 205, SK1, 214, G910-6, MN, 11B, RF, A17 | MT-2, U937, CEM-SS | 2.3 to 7.6 nM |
| | pET26b(+)-pelB-CVN (periplasmic) | 40 mg/L and 140 mg/40g wet cell/L | HIV-1 clade B | PBMC | 0.026 ± 0.007 µM |
| | pET-SUMO-CVN | | HIV-1/IIIB | MT-4 cells | 22.3 ± 3.74 nM (IC50) |
| Streptococcus gordonii, two-host (GP1307, GP1305) | M6-CVN | 0 to 36800 ng/well (used in ELISA) | HIV-1RF | - | Concentration-dependent |
| Lactobacillus jensenii | pOSEL175(Pε-APVT-CVN (PS1G)) | 0.10 ± 0.05 to 5.02 ± 0.35 µg/mL (extracellular CVN) | CCR5-tropic HIV (Bal) | CEM-SS or HeLa-X4-LTR-β-gal cells | 0.3 nM (IC50) |
| Lactobacillus lactis, lactobacillus plantarum | pTSV2, pTSV1 | 250 ng/mL | HIV-1/IIIB | H9 cells, MT-4 Cells, PBMC | 15 nM |
| **Yeast** | **Pinus pastoris** | pP52A | 10 mg/L | Rolo, Ba-L, ADA strains | PBMC | 5.4 to 10.9 nM |
| Pichia pastoris | pL32-CVN | 2.4 µg/g (in root tissue), rhizosecretion 0.02 µg/ml/24 h | HIV-III B | - | - |
| | pCR4-TDOPO-CVN | 0.3% TSP | - | - | - |
| | pL32-CVN | 130 ng/mg (in leaf tissue), rhizosecretion 0.6 µg/ml/24 h, 0.85% TSP | HIV-1/IIIB | CEM-SS cells | 0.82-2.7 nM |
| | pβCong1CVN | 350 µg/g (in dry seed) | HIV-1RF | CEM-SS cells | 19.7 µg/ml |
| | pβCongCVN | 1.5% | - | - | - |
| **Plants** | **Althaea officinalis** | pL32-CVN | 2.4 µg/g (in root tissue), rhizosecretion 0.02 µg/ml/24 h | HIV-III B | - | - |
| Nicotiana tabacum | pCR4-TDOPO-CVN | 0.3% TSP | - | - | - |
| | pL32-CVN | 130 ng/mg (in leaf tissue), rhizosecretion 0.6 µg/ml/24 h, 0.85% TSP | HIV-1/IIIB | CEM-SS cells | 0.82-2.7 nM |
| Soya been seeds | pβCong1CVN | 350 µg/g (in dry seed) | HIV-1RF | CEM-SS cells | 19.7 µg/ml |
| | pβCongCVN | 1.5% | - | - | - |
| **rice endosperm** | pRPS | 2.4-0.8 µg/g dry seed weight | HIV-1/IIIB | CEM-SS cells | 19.7 µg/ml |
Comparison of expression systems to recombinant production of CVN

**Table 4. A list of some HIV/AIDS clinical trials on candidate microbicides, antiretroviral treatments and HIV vaccines**

| Intervention                      | Clinical trials | ClinicalTrials.gov identifier |
|-----------------------------------|-----------------|-------------------------------|
| Anti-HIV microbicides             |                 |                               |
| nonoxynol-9                       | Phase 3         | NCT0000926                    |
| Cellulose sulfate (6%)            | Phase 3         | NCT00153777                   |
| PRO 2000 gel                      | Phase 2         | NCT00074425                   |
| Tenofovir gel                     | Phase 2         | NCT00441298                   |
| Dapivirine (TMC120)               | Phase 1         | NCT00304642                   |
| UC-781                            | Phase 1         | NCT00132444                   |
| CD4-IgG2                          | Phase 1         | NCT0000876                    |
| Antiretroviral treatment          |                 |                               |
| Raltegravir, tenofovir/emtricitabine | Phase 4     | NCT01025427                   |
| Combivir+Kaletra                  | Phase 4         | NCT00385645                   |
| Lopinavir/Ritonavir               | Phase 4         | NCT00234975                   |
| Truvada/ Emtricitabine            | Phase 4         | NCT00362687                   |
| Maraviroc                         | Phase 4         | NCT01049204                   |
| Ritonavir boosted Atazanavir      | Phase 4         | NCT01829802                   |
| HIV Vaccines                      |                 |                               |
| EHVAT01                           | Phase 2         | NCT02972450                   |
| rMVA-HIV/ rFPV-HIV                | Phase 1         | NCT00107549                   |
| rSV                               | Phase 1         | NCT01859325                   |
| VAC-3S                            | Phase1/2        | NCT01549119                   |
| Dendritic cell vaccine            | Phase1/2        | NCT00402142                   |

Studies as well as investigation of its safety and mitogenic properties in the future studies.94

Beside *in vivo* evaluations (in non-human primates, humanized mice and sheep), *ex vivo* models provide unmet data of microbicide pharmacokinetics, pharmacodynamics, and effective dose concentration prior to human clinical trials.96,97

In addition, another model like *in vitro* transwell can be engaged to assay the permeability of final formulated drugs and the potential for transport across vaginal and rectal epithelial cell layers.98

**Concluding remarks**

The anti-HIV potency of cyanobacterial lectins, e.g. CVN, promotes the development of novel microbicides to be deployed at the first line of defense for the prevention of sexual transmission of the HIV. On the other hand, the high-level production of rCVN via different expression systems remains a primary challenge because CVN is susceptible to form disulfide bonds and side chain modifications. Comparison of rCVN functionality isolated from prokaryote and eukaryote systems confirmed that the transgenic plant (as a molecular farming99) offers the most complicated system paving the way for the future production of this drug candidate commercially. It can provide safe and cheap approaches to produce recombinant valuable proteins through the development of transgenic plants in the field. In addition, transgenic plants accumulated recombinant proteins in specific organs which were replete with interesting proteins for long period storage. The advantages of producing pharmaceutical proteins, vaccines and antibodies in plants include economical production system, favorable large-scale production, harvesting and storage, elimination of purification process by allocation of plant tissue for recombinant protein (edible vaccines), use of intracellular environment of plant cells (chloroplast or plastid) to direct production of protein, scaling-up as industrial level and lack of harmful pathogens and pyrogen substances.100

Different non-specific agents, (Buffergel, Carraguard, and SAVVY) like the first generation of HIV microbicides, led to unfavorable clinical trial outcomes, except for Pro 2000 0.5% gel (the results were pending from phase III clinical trials).101,102 In addition, the second generation of HIV microbicides was introduced, which led CVN and griffithsin to be placed in the lectin categories. It was indicated that various factors could influence the CVN activity in clinical trials such as the presence of semen, vagina and rectum flora (predominantly *Lactobacillus* sp.), type of formulation, dose level, delivery system, infection in the reproductive tract and the time of administration following sexual events. The remarkable progress in developing an HIV vaccine103 raised an urge for the involvement of a combinational technology. Therefore, nanotechnology methods are considered to be engaged in the formulation of multiple microbicides to increase the half-life of CVN, HIV vaccines, and pre-exposure prophylaxis.

Further studies confirmed that the gel formulation of CVN demonstrated strong efficacy in *in vivo* models. Furthermore, the antiviral effect of CVN was stronger than that of PRO2000 according to a number of *ex vivo* experiments.94,95 Nonetheless, testing all the new compounds, combinations, and different formulations are not feasible in large trials. Thus, preclinical assays are critically required to understand the relative potential of rCVN in selecting the best expression system. However, all preclinical tests seem to have some limitations, and
their advantages in predicting clinical efficacy are still unclear. Therefore, product prioritization should be based on some criteria, including in vitro drug capacity, data on animal efficacy, product development stages, cost of goods and materials, safety, and last but not the least, ability to measure the pharmacokinetic/pharmacodynamic properties in clinical trials. Aside from the evaluation of CVN and other microbicides in in vitro, ex vivo and in vivo models, and application of antiretroviral therapy, it is suggested that the effect of friendly microorganisms, like probiotics, should be noted. In 2016, Miller et al in a systematic review concluded that probiotics could actually improve the CD4 count in HIV patients when consumed daily for a long time. In another study people with HIV-1 were treated with probiotics, Saccharomyces boulardii. The study found that microbial translocation and IL-6 were reduced in patients, which could cover the safety concerns of CVN.

Ethical approval
The present article presents no study with human participants or animals performed by any of the authors.

Competing interests
There is no conflict of interests to be reported.

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