Cyanovirin-N potently inhibits human immunodeficiency virus type 1 infection in cellular and cervical explant models

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In the absence of a protective vaccine against human immunodeficiency virus (HIV), there is an urgent need for the development of effective topical microbicides to prevent HIV infection. Candidate vaginal microbicides should provide protection against circulating strains, be cheap, stable on storage, safe and easy to use. Here we describe a detailed study of the safety and efficacy of Cyanovirin-N (CV-N) in vitro, and in an ex vivo model of female genital tissue explants. CV-N demonstrated potent activity in the low nanomolar range against laboratory and primary isolates. Activity was related to the affinity of CV-N for binding to whole virions as determined by acoustic resonance. Potent activity was also observed against cell-associated HIV-1, although slightly reduced. CV-N activity in the presence of whole semen was reduced by 7–10-fold, although it remained in the low nanomolar range and was minimally modified by the presence of Candida albicans. Furthermore, CV-N potently inhibited infection of ectocervical explants and virus dissemination by tissue-emigrating cells. In peripheral blood mononuclear cell (PBMC) assays, CV-N was shown to have some mitogenic activity following 3 days exposure to compound, and this was associated with a modest increase in expression of gamma interferon, stromal cell-derived factor 1β and interleukin 4. However, 2 h exposure to CV-N had no effect on cytokine expression in PBMC or tissue explant culture over a 24 h period, suggesting that the potential for inflammation is low. Data presented here indicate that targeting HIV envelope glycoproteins may provide an effective strategy to prevent HIV-1 infection mediated by either cell-free virus or infected cells.

INTRODUCTION

The continuing human immunodeficiency virus (HIV)/AIDS epidemic highlights the urgent need for additional effective methods of prevention. More than 33 million people are living with HIV and the predominant mode of transmission is through heterosexual intercourse (UNAIDS/WHO, 2007). While condoms are highly effective at preventing HIV infection, they require partner consent and are contraceptive, therefore they are not an available option for many women at risk of HIV infection (Shattock & Solomon, 2004). Furthermore, women are biologically more susceptible to HIV and other sexually transmitted infections (European Study Group on Heterosexual Transmission of HIV, 1992), and young women appear particularly vulnerable, accounting for 75% of HIV infection in the 15–24 age group in sub-Saharan Africa (Quinn & Overbaugh, 2005). Therefore new prevention methods that can be initiated by women are desperately needed. In the absence of a protective vaccine, increased emphasis is being placed on the development of topical microbicides that can be applied vaginally or rectally to prevent HIV-1 transmission. These compounds need to be inexpensive, effective, cheap, stable, safe and acceptable in order to be widely used (Dhawan et al., 2006; Klasse et al., 2008; Shattock & Moore, 2003).

A range of different compounds are currently in development as potential microbicide candidates. The majority of these compounds target either virus attachment or post-entry steps of the HIV-1 replication cycle (Balzarini & Van Dammme, 2007; Klasse et al., 2008). Among the entry inhibitors under evaluation, some compounds target the viral particle, while others aim at blocking cellular co-receptors that mediate viral entry (Ketas et al., 2007). Carbohydrate-binding agents (CBAs) that specifically target HIV glycoproteins represent an important strategy that could be employed against HIV transmission. Cyanovirin-N (CV-N) directly targets HIV spike glycoproteins, preventing attachment and subsequent viral fusion, and effectively
renders the virus non-infectious. CV-N, an 11 kDa protein originally purified from the cyanobacterium *Nostoc ellipsosporum*, potently inactivates a wide spectrum of HIV-1 strains and primary isolates, as well as a number of other viruses including HIV-2, simian immunodeficiency virus, Ebola and hepatitis C virus (Barrientos & Gronenborn, 2005; Boyd et al., 1997; Helle et al., 2006). The mechanism of action of CV-N involves multiple binding to high-mannose oligosaccharides predominantly in the C2–C4 region of the HIV-1 gp120 protein (Hu et al., 2007; Shenoy et al., 2001). This binding then inhibits the conformational change required for virus–target cell attachment and subsequent fusion. Previous *in vivo* studies, with CV-N formulated as a gel, have shown promising efficacy when the compound was used topically as a rectal or vaginal microbicide in macaques challenged with the highly pathogenic SHIV89.6P virus (Tsai et al., 2003, 2004).

Thus potent antiviral activity, lack of toxicity, resistance to physico-chemical denaturation as well as the unusual high genetic barrier to resistance (Balzarini et al., 2006; Hu et al., 2007; Witvrouw et al., 2005) suggest that CV-N is a promising anti-HIV molecule for use as a topical microbicide (Boyd et al., 1997; Esser et al., 1999).

Here we describe a detailed preclinical evaluation of CV-N, using both *in vitro* cell-based assays and an *ex vivo* tissue model. These studies expand and build on our early demonstration of compound efficacy in tissue and animal models (Tsai et al., 2004). In this study, we have evaluated multiple aspects of CV-N activity including: the ability to block cell-free and cell-to-cell HIV-1 transmission; its activity in the presence of semen; efficacy in the presence of *Candida albicans* and activity in human cervical explant cultures (Fletcher et al., 2005; Greenhead et al., 2000; Hu et al., 2004). The potential mitogenic effects of CV-N have been assessed in peripheral blood mononuclear cell (PBMC) cultures, while the potential induction of inflammation has been assessed by measuring changes in production in a panel of 23 cytokines in both PBMC and cervical tissue explants.

**METHODS**

**Cell and virus culture.** PM1 T cells (kindly provided by the NIH AIDS Research & Reference Reagent Program) and PBMCs were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 2 mM l-glutamine. TZM-bl cells (kindly provided by the NIH AIDS Research & Reference Reagent Program) were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented as above. The different HIV-1 strains were grown in phytohaemagglutinin (PHA)-stimulated PBMCs as previously described (Greenhead et al., 2000). Infection was monitored by p24 ELISA assay [AIDS Vaccine Program, National Cancer Institute (NCI) at Frederick, MD, USA]. The TCID₅₀ was determined in PHA-activated PBMCs. Chronically infected PM1 cells were established following infection with HIV-1BR, HIV-1LAI and HIV-1GR strains. Cyanovirin-N was obtained from Biosyn.

**CV-N activity against different HIV-1 primary isolates.** Primary HIV-1 clinical isolates, belonging to different clades, were kindly provided by the NIH AIDS Research & Reference Reagent Program. The following isolates were tested in this study: RW/92/016 (clade A), BR/92/003 (clade B), ZA/97 (clade C), UG/92/035 (clade D), TH/93/073 (clade E) and BC6/01 (clade O). In order to test the antiviral activity of CV-N, PBMCs were isolated from single donor buffy coats with Ficoll-Hypaque and activated for 2 days with 5 μg PHA ml⁻¹. After stimulation, cells were seeded in triplicate at a concentration of 2 × 10⁶ per well and infected with the different isolates, at an m.o.i. of 1 in the presence or absence of CV-N at different concentrations. After 7 days of culture infection was evaluated by p24 ELISA (NCI) and IC₅₀ was determined.

**Cell-to-cell transmission assay.** TZM-bl adherent indicator cells, which can be infected by both R5 and X4 HIV-1 and enable quantitative analysis of HIV-1 infection using either β-galactosidase or luciferase as reporter genes (Li et al., 2005; Wei et al., 2002), were used in these experiments.

TZM-bl cells were seeded at a concentration of 5 × 10⁶ per well in 96-well microplates. The following day, chronically infected PM1 cells (cell numbers corresponding to 100 TCID₅₀) were incubated with CV-N at different concentrations for 1 h at 37 °C. Medium was then removed from TZM-bl cells and infected PM1 cells, with or without compound, were added to wells in triplicate. After 1 h of incubation, wells were washed once to remove PM1 cells, and TZM-bl cells were incubated in complete DMEM for 24 h. The following day, cells were washed with PBS and lysed and luciferase expression was assessed after addition of substrate (Luciferase Assay System, Promega) as previously described (Wei et al., 2002).

**Proliferation assay.** PBMCs were seeded at a concentration of 2 × 10⁵ cells per well in triplicate and incubated with CV-N at different concentrations for either 2 h or 3 days. Positive control cells were incubated with 2 μg PHA ml⁻¹. After 3 days the cultures were pulsed for 16–18 h with 1 μCi (37 kBq) [³H]thymidine per well and then harvested as described above. Cellular DNA was captured on a filter membrane and dried and incorporated radioactivity was measured by scintillation counter (Beckman). The stimulation index was calculated by dividing the mean c.p.m. value of stimulated samples by the mean c.p.m. of unstimulated ones.

**Inhibition of infection in the presence of semen.** Semen was obtained from five different donors with written consent (according to the local research ethics committee) and pooled before use. An aliquot of semen was heat-inactivated (HI) for 30 min at 56 °C. Virus, either HIV-1BR or HIV-1BF was then incubated with fresh semen, used at a concentration of 25%, HI semen or media for 1 h at 37 °C as previously described (Bouhlah et al., 2002). TZM-bl were seeded as described above and exposed to various concentrations of CV-N for 1 h prior to infection. Virus, in the presence or absence of semen and HI semen, was then added to TZM-bl cells, in the presence of CV-N, for 24 h. The final concentration of semen was 12.5% (v/v), a concentration determined by MTT assay to be non-toxic for TZM-bl cells CC₅₀ [50 % cellular cytotoxicity (of the pooled semen corresponding to 57%)], this concentration of semen did not affect any of the assay parameters and did not cause any detectable enhancement of infection. The following day luciferase expression was measured as described above.

**Inhibition of infection in the presence of *C. albicans*.** TZM-bl cells were exposed to various concentrations of CV-N for 1 h prior to infection in the presence of different dilutions of *C. albicans* (strain SC5314, kindly provided by Professor C. Kelly, King’s College, London, UK). Then either HIV-1BR or HIV-1BF (100 × TCID₅₀) was
added to the cell cultures and incubated overnight. The following day luciferase was measured as described above.

**Purification of HIV-1 particles and biosensor binding assay.**
Viral stocks HIV-1<sub>BaL</sub> and HIV-1<sub>1RF</sub> were inactivated with 1 mM aldrithiol-2 (AT-2) for 1 h at 37 °C, clarified, layered on top of a 20% sucrose gradient and centrifuged at 100 000 g for 18 h at 4 °C, as previously described (Segura et al., 2006). Viral pellets were resuspended and subsequently incubated with CD45-conjugated microbeads (Miltenyi Biotec) for 3 h, after which microparticles, rich in CD45, were magnetically removed, leaving purified functional viral particles as previously described (Esser et al., 2001; Trubey et al., 2003). CD45-depleted viral particles were then repelled and subsequently resuspended in degassed HEPES buffer for functional binding experiments.

In order to assess the binding of CV-N to HIV-1, an acoustic biosensor was used. The automated four channel RAP-ID platform (Akubio) was used in this study according to a protocol described previously (Li et al., 2006). CV-N was first covalently bound to a quartz crystal chip, and used in this study according to a protocol described previously (Li et al., 2006; Greenhead et al., 2000; Hu et al., 2004). Tissue was obtained from women undergoing planned therapeutic hysterectomy at St George’s and Kingston Hospitals (London, UK) with written consent (according to the local research ethics committee). Cervical tissue, including both epithelium and stroma, was cut into 3 mm<sup>3</sup> explants (according to the local research ethics committee). Cervical tissue, including both epithelium and stroma, was cut into 3 mm<sup>3</sup> explants and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U penicillin ml<sup>-1</sup>, 100 μg streptomycin ml<sup>-1</sup> and 2 mM l-glutamine.

Explants were pretreated for 2 h with CV-N at different concentrations before exposure to HIV-1<sub>BaL</sub> (5 × 10<sup>4</sup> TCID<sub>50</sub>) for 2 h at 37 °C. Subsequently, explants were washed four times with PBS and cultured overnight in 96-well microplates without readdition of the compound. The following day, explants were transferred to a new microplate and cultured in medium only for 11 days, with supernatant harvests every 3 days.

Migratory cells, present in the overnight culture plate, were washed with PBS and co-cultured with 4 × 10<sup>5</sup> PM1 cells, in order to assess virus transfer. Supernatants were harvested every 3 days. HIV-1 infection was determined by p24 ELISA in both culture supernatants. The high-sensitivity INNOTEST p24 ELISA kit (Innogenetics Group; lower detection limit 5 pg ml<sup>-1</sup>) was used for explant supernatants, while the ELISA from NCI was used for the migratory-cell culture, with higher levels of p24 are produced.

**Cytokine detection by multiplex bead immunoassay.**
Tissue explants or PBMCs were exposed to different concentrations of CV-N. Explants were exposed to compound for 2 h, then washed and cultured overnight with medium only. Supernatants were collected both after 2 h exposure and after overnight incubation. PBMCs were exposed to CV-N for either 2 h or 3 days, and cytokine release was assayed after 3 days of culture, in parallel with the proliferation assay.

Culture supernatants were simultaneously assessed for the presence of the following cytokines: granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), beta interferon (IFN-β), IFN-γ, interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12, IL-15, IL-16, IP-10, monocyte chemotactic protein (MCP)-1, MCP-2, monokine induced by IFN-γ (MIG), macrophage-inflammatory protein (MIP)-1α, MIP-1β, RANTES, stromal cell-derived factor (SDF)-1β, transforming growth factor (TGF)-β and tumour necrosis factor (TNF)-α.

**RESULTS**

**Antiviral activity of CV-N against laboratory and primary isolates**
To confirm the antiviral activity of CV-N used in these studies, it was first tested against three clade B HIV-1 laboratory strains, as examples of R5 and X4 viruses. The activity of CV-N was tested in TZM-bl against cell-free HIV-1<sub>BaL</sub> (R5), HIV-1<sub>1RF</sub> (X4) and HIV-1<sub>IIIB</sub> (X4). CV-N was used at non-toxic concentrations (CC<sub>50</sub> value of 0.75 mM). The IC<sub>50</sub> values observed were 0.005, 0.001 and 0.002 μM, respectively. The anti-HIV activity of CV-N was then assayed against different HIV-1 primary isolates, representative of clades A, B, C, D, E and O, in PBMC and monitored by detection of p24 antigen release. CV-N demonstrated potent activity against all isolates, with IC<sub>50</sub> values ranging from 0.002 to 0.031 μM (Table 1), in agreement with previous studies (Balzarini et al., 2006; Boyd et al., 1997).

**Analysis of the binding kinetics of CV-N to HIV-1**
To further understand differences in CV-N activity against the different isolates tested, the kinetics of CV-N binding to viral particles was examined using an acoustic biosensor.

**Table 1. CV-N activity against different HIV-1 primary isolates**
PBMCs were infected with the different clinical isolates and IC<sub>50</sub> were determined.

<table>
<thead>
<tr>
<th>HIV-1 primary isolate</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Coreceptor usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW/92/016 (clade A)</td>
<td>3.9</td>
<td>R5</td>
</tr>
<tr>
<td>BR/92/003 (clade B)</td>
<td>3.9</td>
<td>R5</td>
</tr>
<tr>
<td>ZA/97 (clade C)</td>
<td>15.6</td>
<td>R5</td>
</tr>
<tr>
<td>UG/92/035 (clade D)</td>
<td>7.8</td>
<td>R5</td>
</tr>
<tr>
<td>TH/93/073 (clade E)</td>
<td>2.0</td>
<td>R5</td>
</tr>
<tr>
<td>BCF01 (clade O)</td>
<td>31</td>
<td>R5</td>
</tr>
</tbody>
</table>
(Rapid 4; Akubio). In this system CV-N was first immobilized to the surface of a quartz crystal chip, AT-2 inactivated viral particles were then flowed over the surface. The variation in resonant frequency observed, a result of HIV-1 binding to the compound, allows determination of the kinetic parameters of the interaction. In these experiments HIV-1Bal and HIV-1RF were studied, along with a CV-N-resistant isolate of HIV-1HIB generated in vitro (Hu et al., 2007). HIV-1RF demonstrated potent binding to CV-N (Fig. 1b), with association rate constant (ka) and dissociation constant (Kd) values of 1.4819 \times 10^{5} M^{-1} s^{-1} and 33 nM, respectively. For HIV-1Bal (Fig. 1a) k_a = 9.295 \times 10^{4} M^{-1} s^{-1} and K_d = 0.59 \mu M. These values show that HIV-1RF binds faster to CV-N and dissociates more slowly than HIV-1Bal and are in agreement with the difference in the IC_{50} values for the two viruses shown above. The CV-N resistant isolate was also analysed (Fig. 1c) and the K_d observed was the highest of the viruses examined (18 \mu M), with a binding affinity that was 8% of that for HIV-1RF and 9% of that for HIV-1Bal. PHA was tested as a non-specific control; this lectin did not show any appreciable binding of any of the viruses tested (Fig. 1d, e and f).

**Inhibition of cell-associated HIV-1 transmission by CV-N**

Having determined the activity of CV-N against cell-free virus, its activity against cell-associated HIV-1 was evaluated using the same laboratory isolates. Chronically infected PM1 T cells were incubated with TZM-bl cells for 1 h, in the presence or absence of CV-N. After overnight culture, inhibition of infection was measured by luciferase assay. CV-N demonstrated potent activity against cell-associated infection with each of the three evaluated viruses, HIV-1Bal, HIV-1RF and HIV-1HIB, with IC_{50} values of 0.046, 0.004 and 0.025 \mu M, respectively (Fig. 2).

**CV-N activity in the presence of semen**

Since semen represents a natural vehicle for HIV-1 transmission, preservation of activity in its presence is an important parameter for microbicide candidate selection. Inhibition of HIV-1Bal and HIV-1RF by CV-N was tested using TZM-bl cells, in the presence or absence of whole semen [used at final concentration of 12.5% (v/v)]. HI of semen was performed in order to assess if any of the complement components had an effect on CV-N efficacy, since it is known that virus opsonization can enhance virus infectivity (Bouhlal et al., 2002).

Dose response curves for activity against HIV-1Bal suggest that semen had some effect on CV-N activity with the IC_{50} increasing from 0.005 \mu M in the absence of semen to 0.036 \mu M in the presence of semen (Fig. 3a). This effect appeared to be complement-independent, as the dose...
response curves for semen and HI-semen show a similar trend, and the IC\textsubscript{50} values are similar (0.036 and 0.029 \textmu M, respectively). Similarly, for HIV-1RF there was an increase in the IC\textsubscript{50} value in the presence of semen (from 0.001 to 0.01 \textmu M), whilst no significant differences were observed between semen and HI-semen (IC\textsubscript{50} values of 0.01 and 0.025 \textmu M) with ratios to the control (without semen) of 10 and 8.3, respectively (Fig. 3b). The maximal 10-fold increase in IC\textsubscript{50} values in the presence of semen observed in this study is markedly lower than that reported for other microbicide candidates (Neurath \textit{et al.}, 2006; Patel \textit{et al.}, 2007).

**CV-N activity in the presence of C. \textit{albicans}**

The ability of CV-N to prevent HIV-1 infection was determined in the presence of \textit{C. albicans}, a common genital infection that expresses high levels of mannan residues, in different linkages and that can be recognized by a number of carbohydrate-binding lectins (Lillegard \textit{et al.}, 2006). Here target cells were pretreated with CV-N in the presence of \textit{Candida}, at different dilutions, then exposed to either HIV-1\textsubscript{Bal} or HIV-1\textsubscript{RF}. The anti-HIV activity of CV-N was not impaired by the presence of \textit{Candida}, at any of the concentrations tested and for both viruses. The IC\textsubscript{50} values were very similar [0.005 \textmu M for HIV-1\textsubscript{Bal} (Fig. 4a) and 0.001 \textmu M for HIV-1\textsubscript{RF} (Fig. 4b)], with no significant shifts in the dose response curves.

**Inhibition of HIV-1 infection in human cervical tissue and blockade of virus propagation by migratory cells**

The ability of CV-N to prevent infection of the female genital tract was examined using ectocervical explants cultured in a non-polarized manner (Fletcher \textit{et al.}, 2005; Greenhead \textit{et al.}, 2000; Hu \textit{et al.}, 2004). Since R5 viruses predominate in the early stages of HIV-1 infection (Zhu \textit{et al.}, 1993) we selected the isolate HIV-1\textsubscript{BaL} for these experiments. CV-N was previously tested for its potential toxicity on tissue explants by MTT viability assay as described (CC\textsubscript{50}>0.45 mM, data not shown). Explants were pre-treated with a dilution series of CV-N for 2 h.
then exposed to HIV-1BaL (2 h) before washing in order to remove unbound virus and CV-N. Infection was monitored by p24 release following 11 days in culture. CV-N prevented infection of cervical tissue explants, in a dose-dependent fashion, with an IC₅₀ of 0.02 μM (Fig. 5a). Moreover, a 1 μM dose of CV-N was sufficient to provide 90% protection against HIV-1BaL infection.

We have previously shown spontaneous migration of CD4⁺ dendritic cells (DC) from cervical explant tissue during overnight culture, a population of cells able to bind virus via mannose C-type lectin receptors (MCLR) and/or CD4 (Hu et al., 2004). Migratory cells were harvested from explant cultures (exposed to compound and virus as described above) following overnight culture, washed to eliminate cell-free virus, and cocultured with permissive PM1 T cells. CV-N inhibited the transfer of virus from migratory cells as well. The concentration of 1 μM provided a maximum of 70% protection against viral dissemination after 7 days of cell culture (Fig. 5b).

Analysis of potential mitogenicity

As CV-N is a carbohydrate-binding agent (CBA), it was important to determine whether it could stimulate T-cell proliferation. The mitogenic properties of CV-N were assayed in PBMCs, after 3 days of culture by measurement of [³H]thymidine incorporation. The compound was previously tested on PBMCs for its cytotoxic effects by a MTT dye reduction assay (CC₅₀ value 1.63 mM, data not shown) and used at non-toxic concentrations. As previously demonstrated (Balzarini et al., 2006), CV-N has some mitogenic activity after 3 days of incubation with PBMCs, with a maximal stimulation index (SI) of 5 (Fig. 6a). We also tested proliferation after a 2 h exposure to the compound, followed by 3 days of culture, and the rate of proliferation was drastically reduced under these conditions with a maximal SI of 4 observed at the highest concentration tested (1.8 μM). For the other concentrations tested the SI was always below 2. The positive control PHA, used at a concentration corresponding to one tenth of the highest point tested for CV-N, showed SI values above 10 (Fig. 6a).

In parallel with the [³H]thymidine incorporation assay, culture supernatants were also tested for the presence of cytokines by Luminex assay. For cultures exposed to CV-N for 2 h no variation in the expression profile of 23 cytokines was detected, while those exposed to CV-N for 3 days demonstrated statistically significant (P<0.05) increases in the amounts of SDF-1β, IL-4 and IFN-γ released (Fig. 6b).

**Fig. 5.** CV-N inhibition of HIV-1BaL infection of cervical explants and dissemination by migratory cells. Infection of cervical explants was determined at day 3, 7 and 11 by detection of p24 antigen in the tissue culture supernatants (a). Migratory cells were cocultured with PM1 cells and infection was monitored by p24 ELISA at the same time points (b). CV-N was tested in tissue derived from three donors; each condition was tested in triplicate. Data represent the mean ± SEM.

**Fig. 6.** Proliferative effects and cytokine expression upon exposure to CV-N. Proliferation was assayed in PBMCs after 3 days of culture. Cells were exposed to CV-N at different concentrations for 2 h or 3 days (a). The SI was calculated by dividing the mean c.p.m. value of stimulated samples by the mean c.p.m. of unstimulated ones. Supernatants were then tested for cytokine release by Luminex and a significant increase (*, P<0.05) in the production of IFN-γ, IL-4 and SDF-1β was observed at the highest concentrations tested (b). Data represent the mean ± SEM of three independent experiments; each condition was tested in triplicate.
Effects of CV-N on cytokine expression in cervical tissue

In order to determine whether exposure of cervical tissue to CV-N would induce an inflammatory response, culture supernatants were also tested to determine any modulation of the secreted cytokine profile. Previous work validated this detection method and showed that compounds such as PRO2000 and dextran sulphate can induce significant increases of cytokines in cervical explants (Fletcher et al., 2006).

In these experiments explants were exposed to CV-N for 2 h then washed and cultured overnight. Supernatants were collected after 24 h and analysed by multiplex bead immunoassay. Untreated explants produced detectable levels of a wide range of cytokines (Table 2). Treatment with CV-N did not alter the production of the majority of the cytokines, and whilst there was a slight increase in G-CSF, IL-8 and IP-10 release, this did not reach statistical significance.

DISCUSSION

In this study we have performed a detailed preclinical evaluation of CV-N as a potential HIV-1 microbicide. The activity of CV-N against a broad range of primary isolates from different clades confirms previous studies and validates the activity of the CV-N used in this study (Balzarini et al., 2006; Boyd et al., 1997). Interestingly, activity seemed higher against primary strains than against laboratory isolates, a positive trait for a potential microbicide candidate. The ability of this candidate to inactivate a wide spectrum of laboratory strains and clinical primary isolates underlines the conserved nature of CV-N binding sites on gp120 and the high genetic barrier of this compound, already demonstrated with drug-resistance selection studies (Balzarini et al., 2006; Hu et al., 2007; Witvrouw et al., 2005).

In order to further understand the differences in IC₅₀ values observed in TZM-bl for HIV-1₅₄₀ and HIV-1₉₅₅ (0.005 and 0.001 µM, respectively), CV-N was tested for binding to these viruses with an acoustic biosensor (Akubio) that provides information about the kinetics of binding. HIV-1₅₅₅ bound to CV-N more rapidly and dissociated more slowly than HIV-1₅₄₀, with binding percentages of 100 and 85%, respectively. A CV-N-resistant virus (Hu et al., 2007) was used as a control and showed only 8–9% of the binding to HIV-1₅₅₅ and HIV-1₅₄₀. Furthermore, the control lectin PHA failed to bind virus. These results confirmed that differences in activity are related to CV-N affinity and may reflect differences in the amount of potential glycosylation sites between these two viruses (data not shown).

Semen is a natural vehicle for male-to-female and male-to-male transmission of HIV-1 and is generally not included in preclinical challenge studies performed in monkeys. However, previous studies (Neurath et al., 2006; Patel et al., 2007) have demonstrated that the activity of some compounds can be markedly reduced in the presence of semen, and that this is an important consideration when selecting candidates for microbicide development. In this study, inclusion of semen had a modest effect on its antiviral activity (Fig. 3), with a maximum tenfold increase in the IC₅₀ value. This reduction in efficacy is far less than that seen for compounds already in phase III clinical trials and may be insignificant when considering the high concentration of CV-N (1 and 2 % gels, corresponding to 0.9 and 1.8 mM) used to prevent vaginal transmission in the macaque challenge model (Tsai et al., 2003). It has yet to be determined whether the reduction in activity reflects masking of high-mannose residues on the virus by seminal plasma components or binding of CV-N to the components themselves (Sabatté et al., 2007).

The antiviral activity of CV-N was also evaluated in the presence of C. albicans, a yeast that displays on its cell-wall several mannose residues that can be recognized by lectins (Lillegard et al., 2006). This is a common cause of genital infections in women, particularly in immunocompromised individuals (Dupont et al., 1992; Fichtenbaum & Powderly 1998). Our studies demonstrate that CV-N retains potent activity against HIV-1 in the presence of C. albicans without an increase in the IC₅₀ values (Fig. 4). While we cannot exclude the possibility that other C. species or isolates might bind CV-N, these data suggest it is unlikely to be common to the species as a whole.

As well as inhibiting transmission of cell-free HIV-1, CV-N was able to block cell-associated virus, regardless of the R5

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Table 2. Profile of cytokine expression in tissue explants upon exposure to CV-N

Cytokine amounts (expressed in ng ml⁻¹) were detected by Lumines. Data indicated represent the mean values obtained from three tissue donors.

<table>
<thead>
<tr>
<th>CV-N (µM)</th>
<th>Control</th>
<th>2</th>
<th>1</th>
<th>0.5</th>
<th>0.25</th>
<th>0.125</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>65.56</td>
<td>80.83</td>
<td>79.72</td>
<td>83.99</td>
<td>88.03</td>
<td>51.90</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.03</td>
<td>0.04</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-1α</td>
<td>0.19</td>
<td>0.16</td>
<td>0.14</td>
<td>0.09</td>
<td>0.08</td>
<td>0.12</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.24</td>
<td>0.17</td>
<td>0.13</td>
<td>0.08</td>
<td>0.04</td>
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<tr>
<td>IL-6</td>
<td>37.40</td>
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<td>34.58</td>
<td>55.53</td>
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<td>33.45</td>
</tr>
<tr>
<td>IL-8</td>
<td>60.11</td>
<td>54.92</td>
<td>99.73</td>
<td>55.46</td>
<td>46.20</td>
<td>47.90</td>
</tr>
<tr>
<td>IL-16</td>
<td>0.71</td>
<td>0.82</td>
<td>0.82</td>
<td>0.90</td>
<td>1.56</td>
<td>0.28</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.85</td>
<td>1.84</td>
<td>2.88</td>
<td>0.63</td>
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<tr>
<td>MCP-1</td>
<td>13.50</td>
<td>13.94</td>
<td>11.46</td>
<td>9.73</td>
<td>8.39</td>
<td>9.64</td>
</tr>
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<td>MCP-2</td>
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<td>0.12</td>
<td>0.10</td>
<td>0.07</td>
<td>0.11</td>
</tr>
<tr>
<td>MIG</td>
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<td>0.44</td>
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<td>0.18</td>
<td>0.13</td>
<td>0.12</td>
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<tr>
<td>MIP-1β</td>
<td>0.32</td>
<td>0.32</td>
<td>0.17</td>
<td>0.28</td>
<td>0.20</td>
<td>0.14</td>
</tr>
<tr>
<td>MIP-1β</td>
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<td>0.09</td>
<td>0.06</td>
<td>0.07</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>RANTES</td>
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<td>0.06</td>
<td>0.06</td>
<td>0.08</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>SDF-1β</td>
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<td>0.10</td>
<td>0.06</td>
<td>0.08</td>
<td>0.06</td>
<td>0.05</td>
</tr>
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</table>
or X4 tropism of the strains examined (Fig. 2), although in the main this required a tenfold increase in concentration. In this respect, its activity was more potent than a range of neutralizing monoclonal antibodies currently being evaluated in this model (data not shown).

Results obtained in our ex vivo tissue explant model demonstrate that CV-N was able to inhibit HIV-1 isolation in cervical explants, with an IC₃₀ of 1 µM. Moreover, this concentration inhibited 70% of virus dissemination by dendritic cells that spontaneously migrate out of the tissue explants. These data are in keeping with previous observations of activity against cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)-mediated transmission in cellular models (Balzarini et al., 2007; Turville et al., 2005), suggesting that CV-N may be active against in cis and in trans cell-mediated infection. The higher IC₅₀ value (0.36 µM) observed in our migratory cell model (compared with previous studies) might be due to differences between experimental protocols, such as the shorter time of exposure to CV-N used here and the lack of readoption of the compound after infection (Balzarini et al., 2007). The 2 h exposure time chosen for this study takes into consideration that a woman would most probably apply a microbicide based on CV-N in a coital dependent fashion and that the concentration would rapidly diminish following intercourse through associated dilution due to vaginal fluid, ejaculated semen and probable post-coital cleansing by the woman. It is hard to anticipate the level of compound that would be retained for a prolonged period after intercourse; however, our observation that a 2 h exposure to CV-N can inhibit infection is encouraging even if levels can be maintained for a prolonged period following intercourse through appropriate formulation.

While compound activity under physiological conditions is a critical parameter for microbicide selection, compound safety is equally important. This has been thrown into sharp relief by recent safety concerns over the prematurely halted phase III trial of cellulose sulphate (Honey, 2007) and previous studies of nonoxynol-9 products (Van Damme et al., 2002). Safety is of paramount importance for microicides as they will be used by at risk populations and possibly several times a day. In this study no tissue toxicity was observed with the highest concentrations of CV-N tested (0.45 mM). However, in common with other lectins, a previous study (Balzarini et al., 2006) indicated that CV-N has some mitogenic properties for PBMCs, raising some safety concerns over its in vivo use. In this study, 3 days exposure of PBMC to CV-N did induce low levels of T-cell proliferation, with a maximum stimulation index of 5.4 at 0.36 µM and increased levels of SDF-1β, IL-4 and IFN-γ release (Fig. 6b). However, proliferation was greatly reduced when exposure to CV-N was reduced to 2 h followed by 3 days in culture (Fig. 6a), and no significant alteration in the normal pattern of cytokine expression was observed at 24 h. In agreement with these findings, analysis of cytokine production by cervical tissue explants exposed to CV-N for 2 h demonstrated no significant alterations of the normal profile of cytokine expression. It is unclear whether the low mitogenic activity seen with PBMC cultures exposed to CV-N for 3 days would be representative of responses to topically applied CV-N in vivo. Indeed, no adverse effects were seen in macaques following vaginal or rectal application of 5 mg formulated in HMC gel (Tsai et al., 2003). Nevertheless, repeat application studies would be required to exclude any chronic inflammatory potential.

In conclusion, CV-N demonstrates potent cross-clade activity, retains its activity in the presence of semen and C. albicans and is able to block infection in cellular and ex vivo human genital explant models. While potential mitogenic properties raise some safety concerns, their in vivo relevance could be assessed in non-human-primate studies. Furthermore, discovery of additional carbohydrate-binding agent candidates, such as Griffithsin, that do not show any mitogenic activity in PBMCs (data not shown) adds weight to the concept that targeting HIV glycoproteins will provide an important strategy in preventing HIV transmission.

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REFERENCES


