Inhibitory effect of PRO 2000, a candidate microbicide, on dendritic cell-mediated HIV transfer

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Running title: PRO 2000 inhibits DC-mediated transfer

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Abstract

Without an effective vaccine against HIV infection, topical microbicide development has become a priority. The sulfonated polyanion PRO 2000, a candidate topical microbicide now in Phase II/III clinical trials, blocks HIV infection of cervical tissue in vitro. Dendritic cells (DC) are among the first cell types to contact HIV in the genital tract and facilitate the spread of the virus. Thus, interfering with virus-DC interactions is a desirable characteristic of topical microbicides as long as that does not interfere with the normal function of DC. PRO 2000 present during capture of replication-defective HIV JrFL reporter virus or replication-competent HIV BaL by monocyte-derived DC (MDDC) inhibited subsequent HIV transfer to target cells. Continuous exposure to PRO 2000 during MDDC-target cell co-culture effectively inhibited HIV infection of target cells. PRO 2000 inhibited HIV capture by MDDC. In addition, the compound blocked R5 and X4 HIV envelope-mediated cell-cell fusion. Interestingly, simultaneous exposure to PRO 2000 and LPS attenuated the cytokine production in response to stimulation, suggesting that the compound altered DC function. While efficient block of MDDC-mediated virus transfer and infection in the highly permissive MDDC-T cell environment reinforces the potential value of PRO 2000 as a topical microbicide against HIV, the impact of PRO 2000 on immune cell functions warrants careful evaluation.
Introduction

Heterosexual transmission is the leading mode of HIV transmission worldwide. Almost half of the people living with HIV/AIDS are women and the vulnerability of women to acquiring HIV infection requires the development of effective and acceptable female-controlled methods to reduce HIV transmission such as microbicides (NIH Fiscal Year 2005 Plan for HIV-related research).

Observations derived from the macaque model using either SIV or SHIV have provided important insights into local events that occur at the time of infection (31) (1) (30) (42). In particular, tracking vaginal SIV infection in macaques has shown that dendritic cells (DC) get infected with SIV and then migrate to draining lymph nodes within hours (20) where they can efficiently transmit virus to T cells.

DC represent immune system sentinels that engulf pathogens in the peripheral tissues including the genital tract and process them for antigen presentation on MHC class I and II molecules (35) (43) (25) (26). DC express CD4, dendritic-cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN) and other C-type lectin receptors (CLRs) that may facilitate capture and transfer of HIV (13, 14) (45). In addition to facilitating the transfer of virus to permissive T cells (trans infection) without de novo infection, the CLRs can enhance CD4/CCR5 mediated de novo infection of DC or “cis” infection (45). The importance of antigen presenting cells in the initial infection of cervical epithelium has also been demonstrated in human cervical tissue explant model developed by Shattock et al. (16) (21). The authors demonstrated that DC migrated out of the tissue and were able to transmit HIV to susceptible target cells. The rapid transfer of the virus to lymphoid tissue by DC underscores the need to develop strategies to interfere with HIV-DC interactions.
early during infection. Thus, interfering with virus-DC interactions represents a unique task for topical microbicides.

The largest class of topical microbicide candidates that has moved forward to clinical trials is negatively charged sulfated or sulfonated agents including PRO 2000 (6), polystyrene sulfonate (PSS) (19) (6), dextrin 2-sulfate (22), cellulose sulfate (CS) (2) (6) and carrageenan (47). These compounds block HIV and other STIs including HSV, C. trachomatis and N. gonorrhoeae to varying degrees in vitro. PRO 2000, CS and carrageenan have been formulated and are undergoing testing in Phase II/III clinical trials (reviewed in (8)). However, a recent phase III trial of CS was prematurely stopped due to safety concerns. Preliminary results at some trial sites indicated that use of CS was associated with an increased risk of HIV infection (www.aidsinfo.nih.gov), however the final analysis of that trial has not been completed. These compounds inhibit HIV and HSV entry by interacting directly with viral glycoproteins (6, 40).

The polyanion PRO 2000 was reported to block binding of HIV gp120 to its primary receptor CD4 (38). Polyanions have been reported to bind to gp120 with high affinity and inhibit binding of gp120 V3-directed Mab (4) (17) (36) (40). Importantly, cervical and vaginal lavage fluid from women who received a single application of PRO 2000 had significant HIV and HSV inhibitory activity in vitro with no apparent acute inflammatory response (28) (23). In the cervical tissue explant model PRO 2000 was effective against both HIV-1 X4 and R5 viruses (16) (11) and inhibited dissemination of virus by migratory dendritic cells (11). Partial protection (50-75%) in macaques treated with PRO 2000 and challenged with SHIV has been reported as well (46).
In order to understand the potential limitations of these compounds it is important to evaluate their interactions with mucosal cells involved in HIV transfer. In this study we tested the effect of PRO 2000 on HIV transfer using monocyte-derived DC (MDDC), which are phenotypically similar (HLA-DR⁺MR⁺DC-SIGN⁺) to DC mediating HIV transfer in the cervical explant model. We also assessed the effect of PRO 2000 exposure on MDDC cytokine production.
Material and methods

Reagents

Recombinant IL-2, -4 were purchased from R&D Systems Inc. (Minneapolis, MN) and GM-CSF from Berlex Laboratories, Inc. (Montville, NJ). Reagents for tissue culture were purchased from GIBCO Life Technologies (Grand island, NY). AB human serum was from MP Biomedicals (Solon, OH). LPS and PHA were from Sigma (St. Louis, Mo). PRO 2000 (sulfonated polymer) was obtained from Indevus Pharmaceuticals, Inc. (Lexington, MA). The fusion inhibitor T20 was obtained from the NIH AIDS Research & Reference Reagent Program (ARRRP).

Cell preparation and culture

Human immature monocyte-derived DC (MDDC) were generated from CD14⁺ monocytes in the presence of IL-4 (100U/ml) and GM-CSF (1000U/ml). Briefly, PBMCs were isolated from buffy coats of anonymous donors using a Ficoll-Hypaque density gradient centrifugation. CD14⁺ monocytes were isolated using CD14 magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s protocol with modifications and cultured as previously described (12) in the presence of 1% AB human serum. To characterize MDDC phenotype, cells were stained with FITC-conjugated mAbs against HLA-DR (G46-6) used in combination with PE-conjugated mAbs against DC-SIGN (120507; R&D Systems), MR (19.2), CD80 (L307.4), CD83 (HB15e), CD86 (IT2.2) (all from BD PharMingen). Fluorescence was detected by a FACSscan flow cytometer (BD) and analyzed using FlowJo software. Background fluorescence was defined with irrelevant mouse IgGs. To generate mature DC (mMDDC), LPS (100ng/ml) was added to the cells on Day 6 of culture. After 48h LPS stimulation mMDDC had an
intermediate/mature phenotype with increased CD80, CD83, CD86 levels and decreased DC-SIGN and MR expression (not shown).

CD4+ T cells were isolated from PBMCs (which were first depleted of CD14+ to generate MDDC) using a CD4+ T Cell isolation kit according to the manufacturer’s protocol (Miltenyi Biotec) and cultured in RPMI supplemented with 10% FBS, antibiotics and IL-2 (50 U/ml). CD4+ T cells were stimulated with PHA (5µg/ml) for 1-2 days at 37°C prior to use.

HeLa-CD4+CCR5+ cells expressing endogenous CXCR4 and stably transfected with CD4 and CCR5 were a gift from Shawn E. Kuhmann (34). Cells were maintained in DMEM containing 10% FBS and glutamine.

**Effect of PRO 2000 present during HIV capture on MDDC-mediated HIV transfer**

Replication-defective HIV pseudotyped with JRFL envelope (RD HIVJRFL) was used in a single-cycle infection assay. RD HIVJRFL was prepared in 293T cells, using a previously described 3-plasmid co-transfection system: the packaging construct, pCMV delta R8.2 (32); the HIV reporter construct, pNL4-3.Luc.R-E (obtained from Dr. Nathaniel Landau through the ARRRP) (7) (18); and the envelope-expressing construct, pJRFL expressing an R5 envelope (gift from D. Littman, Skirball Institute, New York University, New York). Transfections were done using Lipofectamine 2000 (Invitrogen).

Replication competent R5 strain HIVBal (Advanced Biotechnologies Inc., Columbia, MD) was used in a multiple-round infection assay. For comparison purposes both viruses were titrated in CD4+CCR5+CXCR4+ TZM-bl cells (from ARRRP; gift of Drs J.C. Kappes, and X. Wu) and comparable doses (~63 and ~100 TCID50/1x10^6 cells, respectively) were used in the study.
To determine the effect of PRO 2000 on HIV transfer by MDDC, the compound was present only during HIV capture. In all experiments viruses were pre-incubated for 1h at 37°C with PRO 2000 or with medium before adding to the cells. MDDC were incubated with RD HIV$_{JRFL}$ or HIV$_{BaL}$ for 2h at 37°C in the presence or absence of PRO 2000. Then cells were washed 4x and plated with target cells (5x10$^5$ MDDC:5x10$^4$ HeLa CD4$^+$CCR5$^+$ cells or 1.25 - 2.5x10$^5$ MDDC:1x10$^6$ PHA-activated autologous CD4$^+$ T cells). Unbound HIV was efficiently removed before co-culture as cell-free final washes from virus-exposed MDDC failed to infect target cells (not shown). However, we cannot exclude the presence of bound residual PRO 2000 after washes. As a control, MDDC were cultured in the absence of target cells after exposure to the virus. To investigate the effect of PRO 2000 on direct infection of target cells, HeLa CD4$^+$CCR5$^+$ and CD4$^+$ T cells were infected with cell-free HIV in the presence or absence of PRO 2000 for 2h at 37°C, washed and cultured with or without the compound.

Luciferase activity of the lysates in the single-cycle infection assay was quantitated after 72h culture using a luciferase assay system (Promega) according to the manufacturer’s instructions. Luciferase activity (in relative light units [RLUs]) was measured on an EG&G Berthold MiniLumat LB 9506 luminometer (Berthold Technologies, Bad Wildbad, Germany).

In the multiple round infection assay, supernatants (100µl) were collected at the beginning of the culture (Day 0), and days 5, 7, 9 and 14 for p24 ELISA.

**Effect of continuous exposure to PRO 2000 on MDDC-target cell infection**

To determine the effect of continuous exposure to PRO 2000 on HIV infection in a single-cycle infection assay, MDDC were mixed with RD HIV$_{JRFL}$ in the presence or absence of
PRO 2000 and added to HeLa CD4⁺CCR5⁺ cells. After 2h incubation the medium was carefully changed and cells were cultured with or without PRO 2000. Luciferase activity of the lysates was measured as above after 72h of culture. In a multiple round infection assay MDDC were mixed with HIV_{BaL} in the presence or absence of PRO 2000 and then added to autologous PHA-activated CD4⁺ T cells. After 2h incubation MDDC and T cell mixtures were washed 4x and cultured with or without PRO 2000 for 14 days. Supernatants (100µl) for p24 ELISA were collected at the beginning of the culture (Day 0), and days 5, 7, 9 and 14.

**p24 ELISA**

p24 antigen was measured by ELISA (NCI, Frederick) according to the manufacturer’s protocol. The detection limit of the assay was 80 pg/ml of p24.

**Toxicity assay**

The toxicity of PRO 2000 was determined on CD4⁺ T cells and MDDC. The cells were exposed to PRO 2000 for 4 days. Cell viability was analysed using a tetrazolium compound assay (CellTiter96; Promega, Madison, WI).

**HIV-1 capture assay**

An HIV_{BaL} capture assay was performed as described elsewhere with modifications(21). MDDC were seeded at 2x10⁵ per well in 96-well flat-bottom plates. HIV_{BaL} alone (~420 TCID50/2x10⁵ MDDC) or in the presence of PRO 2000 was pre-incubated for 1h at 37°C. This higher dose of HIV was chosen based on the limits of detection in this system. To test the effect of PRO 2000 on virus binding, plated MDDC were incubated for 30min on ice, exposed to HIV for 2h on ice in the presence or absence of PRO 2000, washed 4x and lysed with 1% Triton X-100. To test the effect of PRO 2000 on binding/internalization,
cells were incubated with virus for 2h at 37°C, washed 4x and lysed. The amount of cell-associated virus was determined by quantitating HIV p24 by ELISA.

**HIV glycoprotein-mediated cell-cell fusion**

HeLa cells expressing Tat proteins (HeLa-Tat; from ARRRP) were transfected with the HIV$_{\text{JRFL}}$ envelope plasmid (gift of D. Littman, New York University) for 48h. Transfected HeLa-Tat cells and HL2/3 cells containing stably integrated copies of the HIV-1 molecular clone HXB2/3gpt (from ARRRP) expressing HIV glycoproteins were incubated with PRO 2000 or T20 at 37°C for 1h before addition to TZM-bl indicator cells expressing CD4, CXCR4 and CCR5 co-receptors, at 5x10$^5$ per well grown in a 48-well plate. TZM-bl cells contain HIV long terminal repeat (LTR)-driven beta galactosidase and luciferase reporter genes which are activated by Tat proteins when cell-cell fusion occurs. After incubation for 8-24h at 37°C, TZM-bl cells were treated with lysis buffer (Promega Corp.) and luciferase activity was measured using a Perkin-Elmer 1420 luminometer.

**Effect of PRO 2000 on MDDC cytokine production**

A series of experiments were performed to determine the effects of short- and long-term exposure of PRO 2000 on MDDC baseline cytokine production and response to LPS. LPS stimulation was done either in the presence of PRO 2000 or following washout. MDDC were exposed to PRO 2000 for 1h or 48h, washed 4x and re-cultured without the compound for 48h. LPS (100ng/ml) was added to the cells to test if prior exposure to PRO 2000 changes the cytokine response induced by LPS stimulation. To determine cytokine production in response to LPS stimulation in the presence of PRO 2000, both the compound and LPS were added to MDDC simultaneously for 48h. Supernatants were collected and stored at −20°C until use.
1 Luminex fluorescent bead assay

2 Cytokine measurement was done using Beadlyte human multi-cytokine detection system 2

3 (IL-1β, IL-6, IL-8, IL-10, IL-12p70, TNF-α) (Upstate Biotechnology, Lake Placid, NY).

4 Statistical analysis

5 Two-tailed Student’s t-test with significance set at the p<0.05.
Results:

**PRO 2000 present during RD HIV\textsubscript{JRFL} capture by MDDC inhibits virus transfer**

We first determined whether PRO 2000 interferes with MDDC-mediated transfer of RD HIV\textsubscript{JRFL} reporter virus in a synchronized system using a single-cycle infection assay. MDDC were exposed to RD HIV\textsubscript{JRFL} in the presence of PRO 2000 for 2h and washed before addition to HeLa cells expressing CD4 and CCR5, which provided a better luciferase read-out in comparison to primary CD4\textsuperscript{+} T cells. As expected, MDDC efficiently transferred RD HIV\textsubscript{JRFL} to target cells in the absence of the compound (Fig. 1A). Consistent with a published report (29) there was no infection in MDDC cultured in the absence of target cells (not shown). PRO 2000 present during exposure of MDDC to RD HIV\textsubscript{JRFL} inhibited subsequent infection of HeLa CD4\textsuperscript{+}CCR5\textsuperscript{+} cells in a dose-dependent manner (Fig. 1B). Exposure to PRO 2000 at 100µg/ml, a concentration similar to the one detected post-application of a 0.5% formulation into a human vagina (23), resulted in ~55% inhibition of virus transfer (Fig. 1A, B). This inhibitory effect present even when MDDC were extensively washed to eliminate cell-free virus and residual compound prior to co-culture with HeLa CD4\textsuperscript{+}CCR5\textsuperscript{+} cells likely reflects decreased amount of virus sequestered by MDDC in the presence of PRO 2000, although we cannot exclude inhibition of transfer by residual compound.

We also examined the effect of PRO 2000 on mature MDDC (mMDDC)-mediated HIV transfer as encounter with sexually transmitted pathogens in the setting of co-existing STI can induce DC maturation (15, 39). Similar to the effect observed on MDDC-mediated HIV transfer, PRO 2000 inhibited mMDDC-mediated HIV transfer (Fig. 1B).
Continuous exposure to PRO 2000 inhibits RD HIV\textsubscript{JRFL} infection in MDDC-target cell co-cultures

A microbicide could be present during both DC HIV uptake and subsequent transfer to T cells via DC-T cell conjugates in the mucosa and sub-mucosa. To test the efficacy of PRO 2000 in this setting, MDDC and HeLa CD4\textsuperscript{+}CCR5\textsuperscript{+} mixtures were infected with RD HIV\textsubscript{JRFL} in the presence of PRO 2000 and cultured in the presence of the compound. Continuous exposure to PRO 2000 at 10 and 100\,µg/ml inhibited HeLa CD4\textsuperscript{+}CCR5\textsuperscript{+} infection more effectively than when compound was present only during capture (Fig. 1C). However, this experimental approach does not discriminate which step(s) during RD HIV\textsubscript{JRFL} uptake or transfer is(are) affected. For comparison, infection of HeLa CD4\textsuperscript{+}CCR5\textsuperscript{+} cells with cell-free virus was effectively inhibited in the presence of PRO 2000 (Fig.1C).

PRO 2000 present during HIV\textsubscript{BaL} capture by MDDC inhibits virus transfer

To assess the extent of PRO 2000 inhibition on replication competent virus transfer, MDDC were exposed to HIV\textsubscript{BaL} in the presence of PRO 2000 for 2h and washed before co-culture with autologous CD4\textsuperscript{+} T cells. PRO 2000 at 100\,µg/ml inhibited MDDC-mediated HIV\textsubscript{BaL} infection of T cells up to 87-100\% (Days 5-14 of culture) (Fig. 2A, B). However, in 1 out of a total of 7 experiments, PRO 2000 at 100\,µg/ml did not have a significant inhibitory effect (not shown) suggesting that donor cell variability influences efficacy of the compound. Exposure to the compound at 10\,µg/ml inhibited CD4\textsuperscript{+} T cell infection up to Day 9 with breakthrough virus replication at Day 14 of the culture (Fig. 2B). Exposure of MDDC to higher dose of HIV\textsubscript{BaL} (∼1900 TCID50) resulted in less inhibition of infection in the transfer experiments (not shown). MDDC were not infected
with HIV$_{Bal}$ (Fig. 2A). PRO 2000 at 100µg/ml effectively (up to 100%) inhibited direct cell-free virus infection of T cells (Fig. 2A).

**Continuous exposure to PRO 2000 inhibits HIV$_{Bal}$ infection in MDDC-target cell co-cultures**

PRO 2000 efficiently inhibited HIV$_{Bal}$ replication in MDDC-T cell co-cultures when DC-T cell mixtures were exposed to the virus in the presence of PRO 2000, washed and then cultured in the presence of the compound (Fig. 2C).

**PRO 2000 is not cytotoxic to MDDC and CD4$^+$ T cells**

To ensure that the inhibitory effect of PRO 2000 on MDDC-mediated HIV transfer and infection in the co-cultures was not due to cytotoxicity, we examined viability of MDDC and CD4$^+$ T cells in the presence of PRO 2000 by MTS assay. PRO 2000 at 10 or 100µg/ml did not decrease viability of T cells (0-7%, 2 experiments) and MDDC (0-3%, 2 experiments) (Fig. 3).

**PRO 2000 inhibits HIV$_{Bal}$ capture**

To test the effect of PRO 2000 on HIV binding and internalization by MDDC, cells were incubated with HIV$_{Bal}$ in the presence or absence of PRO 2000 at 4°C or 37°C, carefully washed and lysed. We observed an inhibitory effect of PRO 2000 (100µg/ml) on HIV$_{Bal}$ binding (4°C) and binding/internalization (37°C) by MDDC (Fig.4A, B). The inhibition of binding and binding/internalization in the presence of PRO 2000 relative to no treatment control was 51-73% (2 experiments) and 63-72% (3 experiments), respectively. Of note is that in one out of a total of 4 experiments exposure to PRO 2000 increased virus binding (42%) (not shown). This suggests that compound efficacy can be influenced by donor cell variability.
Inhibition of HIV glycoprotein-mediated cell-cell fusion

The effective inhibition of HIV activity in MDDC - T cell co-cultures in the presence of the compound and the fact that virus transfer via DC-T synapse is inhibited by entry and fusion inhibitors (24) led us to test the effect of PRO 2000 on HIV glycoprotein-mediated cell-cell fusion. PRO 2000 effectively inhibited HIV_{JRF} (R5) envelope-mediated cell-cell fusion (Fig. 5). PRO 2000 also inhibited envelope-mediated cell-cell fusion when donor cells expressed HIV_{HXB2} (X4) envelope (not shown).

MDDC cytokine profile after short- and long-term exposure to PRO 2000

Exposure to microbicides might have indirect effects on virus replication in vivo through changes in local cytokine milieu (9). We tested the effect of in vitro short- and long-term exposure to PRO 2000 on cytokine production.

In the first series of experiments cells were exposed to PRO 2000 for 1h (Fig. 6A) or 48h (Fig. 6B) followed by washout and then stimulated with LPS for 48h. The latter stimulation mimics mucosal exposure to bacterial pathogens including sexually transmitted pathogens such as C. trachomatis and N. gonorrhoeae. Transient or prolonged exposure to PRO 2000 did not significantly alter baseline or LPS-induced MDDC cytokine profile, however, there were trends toward increased IL-8 and IL-12p70 after 1h exposure to PRO 2000 as well as trends toward increased IL-1\(\beta\) and TNF-\(\alpha\) in response to LPS stimulation after 1h and 48h exposure to PRO 2000 (Fig. 6A, B). In modification of the protocol, cells were stimulated with LPS in the presence of PRO 2000 (Fig. 6C). In this case, although the non-LPS cytokine profile was not significantly altered by PRO 2000, LPS-induced changes in IL-1\(\beta\), -6, -8 and -10 were significantly decreased.

Discussion
PRO 2000, at a concentration achieved in vivo after a single vaginal administration, inhibited MDDC-mediated HIV infection of HeLa CD4+CCR5+ and CD4+ T cells when the compound was present only during HIV capture by MDDC. PRO 2000 also inhibited target cell infection when present throughout the MDDC-target cell coculture period. These observations suggest that the compound may alter DC-mediated HIV transfer and amplification of infection whether this occurs locally or at a distance.

PRO 2000 most likely interferes with a number of steps during DC capture and transfer of HIV. Exposure to the compound led to partial inhibition of HIV\textsubscript{BaL} capture by MDDC as indicated by the binding and internalization studies. This is consistent with previous reports that PRO 2000 interferes with DC-SIGN-mediated HIV capture (11) and inhibits HIV binding to epithelial cells (37). The increased binding seen in one of four experiments utilizing different donor sources for cells has been an important variable and warrants further investigation. The compound also inhibited HIV envelope-mediated cell-cell fusion, which may contribute to the inhibitory effect on cell-mediated HIV transfer when PRO 2000 was present in the co-culture. Although the exact mechanism of this interference is uncertain, the process of virus transfer via DC-T synapse is inhibited by entry and fusion inhibitors (24).

SAMMA, a mandelic acid condensation polyanion that is being developed as a topical microbicide, has recently been reported to have similar inhibitory effects on DC-mediated T cell infection (5).

Langerhans cells, Langerin-negative lamina propria DC, macrophages and T cells are the first cells to be infected in the vaginal epithelium following atraumatic intravaginal challenge of macaques with SIV (20). In our study, MDDC were chosen as a model based
on their phenotypic similarity with lamina propria DC. The latter have been shown to mediate HIV transfer to susceptible target cells in the human cervical explant tissue model (21). Lamina propria DC may have a central role in early HIV infection in the setting of an inflamed or traumatized squamous epithelium (reviewed in (45) (41).

Our data are in agreement with a recent report describing >90% inhibition of HIV transmission by cervical tissue derived dendritic cells upon tissue exposure to PRO 2000 at 100µg/ml, with an IC_{50} of 29.1µg/ml (11). A similar effect of PRO 2000 (EC_{50} = 32µg/ml) on MDDC-mediated transmission was reported recently as well, however, it was less pronounced compared to other carbohydrate-binding agents tested in the study (3).

A critical function of DC is triggering a potent immune response through the production of cytokines. Pro-inflammatory cytokines may play opposing roles in HIV as they may induce protective immune response as well as directly enhance viral replication (9). It has been shown that genital mucosal fluids from women with bacterial vaginosis have high levels of TNF-α and IL-1β, cytokines known to up-regulate HIV replication through activation of the LTR promoter (33). Bacterial vaginosis and STIs have been associated with increased acquisition of HIV (44) (27). Changes in pro-inflammatory cytokines (increased IL-1, IL-6, IL-8) are also considered surrogate markers of microbicide toxicity (10). A single application of 0.5% PRO 2000 gel was not associated with any significant change in the levels of pro-inflammatory cytokines in CVL from women given the compound compared to women given a placebo-gel (23). PRO 2000 at 1mg/ml has been shown to enhance production of IL-1β and RANTES in cervical tissue in vitro (11); a concentration higher than the level of PRO 2000 detected following the in vivo application of the 0.5% gel (23).
We investigated if exposure to PRO 2000 influenced MDDC cytokine production. Although PRO 2000 did not induce significant pro-inflammatory cytokines production, it blocked the cytokine response induced by LPS when the compound was present during LPS exposure. This could lead to less of an inflammatory response during HIV exposure and/or co-existing STIs and could have a protective effect. On the other hand, an inadequate cytokine response may lead to an impaired immune response against HIV and other mucosal pathogens. It is difficult to predict overall whether these effects of PRO 2000 on DC function would have a beneficial or deleterious outcome relative to HIV transmission. MDDC cytokine response to LPS stimulation after long-term exposure to PRO 2000 (followed by wash-out) was comparable to non-exposed cells indicating that PRO 2000 did not induce any persistent changes in DC function at the level of cytokine production.

In summary, PRO 2000 effectively blocked HIV transfer and infection in DC-T co-cultures suggesting that this compound may be effective at inhibiting viral infection and spread within the mucosa as well as spread to local lymph nodes via DC. However, it is important to consider that PRO 2000 interferes with a host immune response and may alter the balance between virus dissemination and a local antimicrobial immune response.
Acknowledgments

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References


**Fig.1-** PRO 2000 inhibits MDDC-mediated RD HIV\textsubscript{JRFL} transfer to HeLa CD4$^+$CCR5$^+$ cells and infection in MDDC - HeLa CD4$^+$CCR5$^+$ cell co-cultures.

In transfer experiments (A) and (B), MDDC or LPS-matured MDDC (mMDDC) were mixed with RD HIV\textsubscript{JRFL} pre-incubated with PRO 2000 or medium for 1h at 37°C. After 2h incubation MDDC were washed and plated with HeLa CD4$^+$CCR5$^+$ cells. A representative experiment (mean ± s.d. of duplicate wells) (A) and the summary of 3-8 experiments (mean ± s.d.) (B) are shown. Percentage inhibition of HeLa CD4$^+$CCR5$^+$ cell infection by PRO 2000 in transfer experiments and significant differences are shown relative to infection in the absence of the compound which represents 0\% inhibition. In co-culture experiments (C), MDDC were mixed with RD HIV\textsubscript{JRFL} pre-incubated with PRO 2000, immediately added to target cells, and then incubated in the presence or absence of the compound for 2h. Then medium containing the virus and the compound was aspirated and cells were co-cultured in fresh medium with or without PRO 2000 for 72h. HeLa CD4$^+$CCR5$^+$ cells were infected with cell-free RD HIV\textsubscript{JRFL} in the presence or absence of PRO 2000, washed and incubated with or without the compound. Percentage inhibition of infection by PRO 2000 and significant differences are shown relative to infection in the absence of the compound which represents 0\% inhibition. Summary of 3-8 experiments (mean ± s.d.) is shown. * - p<0.05; ** - p<0.01; *** - p<0.001; **** - p<0.0001.

**Fig.2-** PRO 2000 inhibits MDDC-mediated transfer of HIV\textsubscript{BaL} to CD4$^+$ T cells and infection in MDDC-CD4$^+$ T cell co-cultures.

For transfer experiments (A), MDDC were mixed with HIV\textsubscript{BaL} pre-incubated with PRO 2000 (100μg/ml) or medium for 1h at 37°C. After 2h incubation MDDC were washed and
added to PHA activated CD4+ T cells. As a control, MDDC were cultured without T cells after exposure to the virus. Alternatively, T cells were infected directly with cell-free HIV\textsubscript{BaL} in the presence or absence of PRO 2000, washed and cultured with or without the compound. Supernatants were collected at the beginning of the culture (Day 0) and days 5, 7, 9 and 14. A representative experiment (mean ± s.d. of duplicates) is shown. (B)

Summary of 3-5 transfer experiments (mean ± s.d.) done as in (A) is shown. Percentage inhibition of CD4+ T cell infection by PRO 2000 in transfer experiments and significant differences are shown relative to the infection in the absence of the compound (control) which represents 0% inhibition. * = p<0.05; *** = p<0.001; **** = p<0.0001 (C) For co-culture experiments, MDDC were mixed with virus in the presence or absence of PRO 2000 (100µg/ml) and immediately added to PHA activated CD4+ T cells. After 2h incubation, MDDC and T cells were washed and plated with or without PRO 2000. Supernatants were collected as in (A). A representative of 4 experiments (mean ± s.d. of duplicates) is shown.

Fig.3– PRO 2000 does not cause cytotoxicity in MDDC and CD4+ T cell cultures. MDDC or PHA-activated CD4+ T cells were cultured in the presence of PRO 2000 for 4 days at 37°C. Cytotoxicity was determined by MTS assay (Promega). Data are means ± s.d. of triplicated samples and represent two experiments each with MDDC and T cells.

Fig.4 – HIV\textsubscript{BaL} capture by MDDC is inhibited in the presence of PRO 2000. HIV\textsubscript{BaL} binding at 4°C (A) and binding/internalization by MDDC at 37°C (B) in the presence of PRO 2000 (100µg/ml; mean ± s.d. of triplicates). The minimum levels of p24 detected in binding and binding/internalization experiments were 1.1ng/ml and 0.82ng/ml,
respectively. Mean background values were subtracted. A representative of 2 binding and
3 binding/internalization experiments is shown.

**Fig.5 – PRO 2000 inhibits R5 envelope fusion.**

Fusion assay with R5 envelope expressing cells was performed in the presence of PRO
2000 or T20. Data are means ± s.d of triplicates. Mean background value was subtracted
(one of 2-3 experiments is shown).

**Fig.6 - MDDC cytokine profile after short- and long-term exposure to PRO 2000.**

(A) MDDC were exposed to PRO 2000 (100µg/ml) for 1h, washed and re-plated in the
absence (PRO 2000 1h_Med 48h) or presence of LPS at 100ng/ml (PRO 2000 1h_LPS 48h) for 48h. Controls included MDDC cultured in the presence of LPS or medium alone.
Cell-free supernatants were collected in the end of the culture. (B) The experiments were
performed as in (A) except that MDDC were exposed to PRO 2000 for 48h prior to
culture in the absence (PRO 2000 48h_Med 48h) or presence of LPS (PRO 2000 48h_LPS 48h). In (C) LPS was added simultaneously with PRO 2000 for 48h. The indicated
cytokines were measured with Beadlyte human multi-cytokine detection system. Each
supernatant was tested in duplicates. Of note is that the difference in baseline cytokine
production in experiments in A, B and C derives from the fact that there are differences in
the experimental setups including washouts vs. no washout and total duration of MDDC
culture prior to the measurements. The mean level of each cytokine (mean pg/ml ± s.d.) is
shown for 3-4 experiments. * = p<0.05; *** = p<0.001.