Candidate Microbicide PPCM Blocks HIV-1 Infection in Cell and Tissue Cultures
and Prevents Genital Herpes in a Murine Model

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ABSTRACT

A structurally novel candidate microbicide, PPCM, which is formed from the reaction of D,L-mandelic acid with sulfuric acid, provides activity against HIV and HSV and is not cytotoxic. The objectives of the current studies were to comprehensively evaluate its activity in cell and explant cultures, explore the possibility of combining PPCM with HIV-specific reverse transcriptase inhibitors and evaluate the efficacy of a formulated gel against genital herpes in a murine model. PPCM inhibited infection by laboratory and clinical R5 and X4 clade B and clade C HIV strains in cell culture. Ectocervical and endocervical tissue explants exposed to HIV-1\textsubscript{BaL} in the presence of PPCM were protected (IC\textsubscript{50} 3.9\(\mu\)g/ml for ectocervix; 3.1\(\mu\)g/ml for endocervix), and transfer of virus to target T-cells via migratory cells was significantly impaired (IC\textsubscript{50} 35.7\(\mu\)g/ml from ectocervix, IC\textsubscript{50} 54.6\(\mu\)g/ml from endocervix). The drug also blocked infection by cell-associated virus. Combinations of PPCM with UC-781 or PMPA \textit{in vitro} exhibited additive anti-HIV activity. PPCM was incorporated into a stable, low pH gel formulation at concentrations of 0.4\% and 4\%. Both gels prevented genital herpes infection in mice, even when virus was introduced in human seminal plasma. The ability of PPCM to inhibit primary HIV isolates, reduce infection by cell-associated virus and transfer of HIV from migratory to T cells, combined with the complete protection provided by formulated gel against genital herpes indicates that this drug is an excellent candidate for inclusion in a combination microbicide and would provide protection against both HIV and HSV.
INTRODUCTION

The human immunodeficiency virus (HIV) and herpes simplex virus (HSV) pandemics continue unabated and pose a major public health threat. HIV has emerged as primarily a sexually transmitted infection (STI) with women bearing a disproportionate burden of disease worldwide. Condoms are effective, but social barriers limit their use. Clinical trials demonstrate that male circumcision significantly reduces the risk for HIV infection among men, but may enhance transmission to their female partners in the immediate post-procedure time period. Moreover, social acceptance may limit its impact (20,45). While vaccines may hold great promise as a prevention strategy, obstacles to development of effective and safe vaccines persist, as highlighted by the lack of protection that HIV vaccines in phase III trials have afforded (5,38). Therefore, topical microbicides, a female-controlled strategy to prevent HIV and other STI, may be crucial in stemming the pandemic.

Development of a safe and effective vaginal microbicide has proven difficult. The premature closure of several first generation microbicide human clinical trials underscores the challenges in microbicide development. The increase in HIV infection among women using nonoxynol-9 (N-9), Savvy (35) or cellulose sulfate (CS) highlights the need for improved preclinical evaluation of both the safety and efficacy of candidate microbicides (43,15). Specifically, efficacy studies must include a more extensive evaluation of the activity of candidate drugs against HIV isolates representing multiple clades, in cell and explant culture systems reflective of the genital tract. The therapeutic success of combination systemic antiretroviral therapy suggests that a similar strategy may be necessary for prevention and supports prioritizing evaluation of candidate

3
microbicides in combination. Advantages of this approach include the ability to combine
drugs that target HIV at different stages of the viral life cycle thereby reducing the risk of
resistance. Additionally, combinations could provide activity against other sexually
transmitted pathogens that fuel the HIV epidemic, most notably, HSV (12,19). Recent
results suggest that HSV suppressive therapy fails to protect HSV-infected, HIV-
susceptible individuals from HIV acquisition, highlighting the important role that topical
microbicides may play in conferring protection against multiple pathogens (6).

The current studies were designed to further evaluate a structurally novel
candidate microbicide, PPCM, which is formed from the reaction of D,L-mandelic acid
with sulfuric acid. This compound was initially designated sulfuric acid modified
mandelic acid (SAMMA), and has since been named PPCM by Yaso Biotechnologies
Inc. based on further clarification of the chemical structure and synthesis (mspt in
preparation, DP Waller). Despite having no sulfations or sulfonations, earlier studies
indicated that this compound blocks the binding of HIV and HSV to cells by targeting the
envelope glycoproteins gp120 and gB-2, respectively and inhibits viral entry (22,49).

The current studies were undertaken to further define the extent of anti-HIV activity
using cell and explant culture models and to evaluate its anti-HIV activity when
combined in vitro with the candidate microbicide antiretroviral agents tenofovir (PMPA)
(1) and UC-781 (2). Formulated PPCM gels (0.4% and 4%) were also evaluated for
ability to prevent genital herpes in a murine model.
MATERIALS AND METHODS

Microbicides: Nonoxynol-9 (N-9) was purchased from Sigma-Aldrich Ltd. (Poole, UK), as were all reagents used, unless stated otherwise. PPCM was synthesized by researchers at the Program for the Topical Prevention and Conception of Disease (TOPCAD) at Rush University (Chicago, IL). The 0.4% and 4% PPCM gels (and matched placebo gel) were provided by Yaso Biotechnologies, Inc. (Coppell, TX). 9-[R-2-(Phosphonylmethoxy)propyl] adenine monohydrate (PMPA or Tenofovir) was obtained from Gilead Sciences, Inc. (Foster City, CA) and UC-781 was obtained from Biosyn, Inc. (Philadelphia, PA).

Cell and virus cultures: Cells and viruses were obtained from the AIDS reagent project, National Institute for Biological Standards and Control, Potters Bar, UK unless stated otherwise. ME-180 cells, a cervical epithelial cell line (obtained from American Type Culture Collection, Manassas, VA) and TZM-bl cells, a HeLa cell line stably expressing CD4 and CCR5 and used for quantitative analysis of HIV-1 with luciferase as a reporter, were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (complete DMEM) (36). Vero cells and the T cell lines PM-1 and H9 were cultured in RPMI 1640 supplemented as DMEM (complete RPMI). Jurkat-Tat-CCR5 cells, a T cell line which has been transfected with the HIV-1 tat gene and the CCR5 co-receptor, rendering it permissive to X4 and R5 viruses, were provided by Dr. Quentin Sattentau (Sir William Dunn School of Pathology, University of Oxford, Oxford, UK) and cultured in complete RPMI supplemented with 250µg/ml Hygromycin B (for Tat selection) and 500µg/ml Geneticin (for CCR5 selection). Raji cells stably expressing DC-SIGN (Raji/DC-SIGN cells) and negative control cells were provided by Dr. V. N. Kewal Ramani (National Cancer Institute, Frederick, MD) and
were cultured in complete RPMI (supplemented with 500µg/ml Geneticin for the transfected cells) (46). All cells were passaged every 3 to 4 days and cultured in a humidified incubator containing 5% CO₂.

The primary HIV-1 isolates belonging to clades B and C, a gift from Dr. John P. Moore (Weill Medical College, Cornell University, New York, NY), were grown in peripheral blood mononuclear cells (PBMCs) as described (30). The laboratory-adapted HIV-1 strains HIV-1$_r$RF (X4-utilizing strain) and HIV-1$_b$al (R5-utilizing strain) were grown in PM-1 cells and stored at -180°C after filtration through 0.2 µm filters (Millipore, MA). HSV-2 (G), a well-characterized laboratory strain, was grown and titered on Vero cells.

**Culture of mucosal tissue and infection with HIV-1 and HSV-2:** Cervical mucosal tissue was collected from pre-menopausal women undergoing therapeutic hysterectomies at St. George’s, St. Helier’s and Kingston Hospitals (London, UK) and male genital mucosal tissue was collected from patients undergoing gender reassignment surgery at Charing Cross Hospital, London, UK, all with informed consent. Tissue was cut into explants of approximately 3x3x2mm prior to culture and infection with 10$^5$ 50% tissue culture infective dose (TCID$_{50}$) HIV-1 or 10$^7$ PFU/explant HSV-2 as previously described for cervical tissue and more recently established for male genital tissue (17,21,30). Explants used for infection included the epithelial layer and underlying stromal tissue and were from the penile glans or the ectocervical or endocervical areas of the cervix. In brief, explants were exposed to cell-free or cell-associated HIV-1$_b$al for 2 hours in the presence or absence of different concentrations of PPCM in 200µl complete RPMI. Cell-associated virus consisted of chronically infected PM-1 cells (1x10$^6$
cells/explant), which had been pre-treated with 200μg/ml mitomycin C for 1 hour. After removal of the viral inoculum and compound by extensive washing with phosphate buffered saline (PBS), explants were incubated overnight at 37°C. Migratory cells (MCs) present in the overnight cultures were collected and co-cultured with 4x10^4 PM-1 cells. Both tissue explants and co-cultures were transferred to new 96-well plates and incubated at 37°C. Ten days post-infection, culture supernatants were collected and viral replication assessed using a p24 enzyme-linked immunosorbent assay (ELISA; p24 Antigen Capture Assay Kit, NCI-Frederick Cancer Research and Development Centre, AIDS Vaccine Program).

For HSV infection, explants were exposed to HSV-2 (G) (10^7 PFU/explant) in the presence of different amounts of PPCM in a total volume of 200 μl complete RPMI for 2 hours. After removal of unbound virus and compound by extensive washing, explants were cultured for 7 days in complete RPMI and fed with fresh medium every 2-3 days. Culture supernatants were harvested 7 days post-infection and assessed for infectious HSV-2 by plaque assay on ME-180 cells.

**Luciferase assay for detection of HIV-1 infection:** TZM-bl cells were exposed to 10^3 TCID_{50} HIV-1 in the presence of varying concentrations of PPCM alone or in combination with the reverse transcriptase inhibitors UC-781 and PMPA. Virus and drugs were left in culture for 48 hours at 37°C and then removed by washing once with 200μl PBS. Following cell lysis with luciferase cell culture lysis reagent (Promega, Southampton, UK), luciferase activity in lysates was determined as previously described (30).
Anti-HIV activity of PPCM in cell models: Cell-free HIV-1 (BaL or RF) was captured to 96-well plates coated with a monoclonal antibody against human HLA-DR as previously described (18,30). Unbound virus was removed by washing and immobilized virus was treated with 100 µl of serial dilutions of PPCM for 1 hour at 37°C. To assess direct virucidal activity, the compound was removed and the plates washed four times with 200 µl PBS before addition of target cells (4x10^4 Jurkat-Tat-CCR5 cells per well). Alternatively, cells were added without removal of compound or, to assess cell protection, Jurkat-Tat-CCR5 cells (4x10^4 cells/well) were exposed to the same concentrations of compound in U-bottom 96-well plates and washed in the same way before transfer to plates with immobilized virus. Viral replication was assessed by measuring reverse transcriptase (RT) levels in culture supernatants 7 days post-infection as described previously (36).

To evaluate the activity of PPCM against cell-associated HIV-1, PM-1 or H9 cells chronically infected with B clade isolates HIV-1RF, HIV-1IIIB, or HIV-1BaL or the clade C clinical isolate Za003/97, were treated with 200µg/ml mitomycin C in complete medium for 1h at 37°C. Infected cells were washed twice with 50ml PBS, added to 96-well plates (500 cells/well) and incubated with varying concentrations of PPCM for 1h before addition of 4x10^4 Jurkat-Tat-CCR5 cells per well. Co-cultures were incubated at 37°C for 5 days and culture supernatants collected and stored at -20°C prior to measurement of RT activity as before.

Blockade of HIV-1 binding to DC-SIGN-expressing cells and subsequent transfer to HIV targets. To assess potential blockade of HIV-1 transmission to target cells via DC-SIGN, Raji cells (1x10^4 cells/well) expressing DC-SIGN (and negative control cells) were
exposed to different concentrations of PPCM or 200µg/ml mannan for 1h. HIV-1\textsubscript{BaL} or
HIV-1\textsubscript{RF} (2ng p24) was added and allowed to bind to the cells for 2h. Non-bound virus
and PPCM were washed from the cells three times with 200µl PBS and cells were co-
cultured with 8x10\textsuperscript{4} Jurkat-Tat-CCR5 cells per well for 7 days. Culture supernatants
were collected and stored at -20°C prior to measurement of RT activity. Alternatively,
and to assess the ability of compounds to prevent virus binding to DC-SIGN, Raji/DC-
SIGN cells (1x10\textsuperscript{5} cells/well) were exposed to different concentrations of compound or
mannan for 1h. Virus was added and allowed to bind as before but this time, the amount
of HIV-1\textsubscript{RF} was increased 10-fold. After 2h incubation, cells were washed as described
previously and 100µl PBS 1% TritonX was added to each well to lyse the cells and any
bound virus. Virus present in lysates was quantified by p24 ELISA.

**Combination studies:** PPCM and RTIs were combined at 1:1 ratios in terms of 50%
Inhibitory Concentrations (IC\textsubscript{50}) and treated as a single drug that was used at a range of
dilutions in the viral inhibition assay (luciferase assay), in conformity with the model of
Chou and Talalay (10). To determine the ratios of compound that should be used in these
studies, IC\textsubscript{50} values were determined following exposure of TZM-bl cells to each viral
isolate in the presence of single drugs for 48 h. PPCM:PMPA ratios were 1:13.213 and
1:0.015 for HIV-1\textsubscript{RF} and HIV-1\textsubscript{BaL}, respectively, whereas PPCM:UC-781 ratios were
1:0.055 and 1:0.015 for the X4 and R5 virus, respectively. In parallel, single drugs were
tested at the same concentrations. The resulting inhibitory effects were calculated and
introduced in CalcuSyn software for determination of the Combination Index (CI) for
each combination at different inhibitory concentrations (IC\textsubscript{75} and IC\textsubscript{90}) (8).
**HSV plaque assays:** ME-180 cells seeded in 24-well plates were exposed in duplicate to serial dilutions of culture supernatants from ectocervical explants infected with HSV-2 in the absence or presence of PPCM. After incubation for 1 h, the inoculum was removed and the cells were washed thrice and overlaid with fresh medium. Viral titers were determined by counting plaques 48 hours post infection (23). Only wells in which the number of plaques ranged from 20 to 100 were used to calculate the viral titer.

**Cytotoxicity studies:** To assess potential toxicity of PPCM, human cervical cells (ME180), TZM-bl cells, Jurkat-Tat-CCR5 cells, TZM-bl cells, cervical stroma tissue and penile glans tissue were exposed to different concentrations of compound for varying durations that mimicked exposure during antiviral assays. The surfactant agent nonoxynol-9 (N-9) was used as a toxic control. Cell and tissue viability was determined by measuring tetrazolium salt (MTT) cleavage into a blue coloured product (formazan) by viable cells (41). Cells or explants were incubated with 100 or 200 µl complete RPMI 0.5 mg/ml MTT at 37°C for 3 hours, respectively. The formazan salts were solubilised by addition of 100 µl 20% SDS in 1:1 H2O:N,N-Dimethylformamide (DMF) (cells) or 1 ml methanol (explants) and viability was determined by measuring the optical density at 570 nm (690 nm reference) in a Synergy-HT plate reader. For tissue studies, this value was corrected for explant dry weight.

**Murine studies:** Five days prior to infection, mice were treated with 2 mg/mL Depo-Provera. On day 0 the mice were treated with 30 µl of 0.4% or 4% PPCM or a matched placebo gel 15 min prior to challenge with 20 µl of HSV-2(G) (1x10^5 PFU) diluted in PBS or in pooled human seminal plasma obtained from males at low risk for STI (34). Mice were monitored for signs of disease for 14 days post-infection on a 0-4 point scale:
0, no apparent infection; 1, slight redness of the vagina; 2, moderate redness and swelling of the vagina and surrounding tissue; 3, severe redness, swelling, and hair loss of the genital and surrounding tissue; 4, genital ulceration with severe redness, swelling, and hair loss of the genital and surrounding tissue. Mice reaching Stage 4 genital disease or exhibiting neurologic signs (hindlimb paralysis) were euthanized.
RESULTS

PPCM inhibits HIV-1 infection in cell culture. PPCM prevents infection of TZM-bl indicator cells by laboratory-adapted and primary clade C and B HIV isolates (Figure 1). At a concentration of 100µg/ml, a concentration that should be readily found in genital tract secretions following application of either a 0.4% or 4% PPCM formulation (26), PPCM completely blocked infection by all isolates tested. No cytotoxicity was observed at any concentration tested.

Additional in vitro studies were conducted to further define the mechanism of anti-HIV activity. Treatment of immobilized HIV-1RF (X4) with 15µg/ml PPCM for one hour prior to addition of target cells was sufficient to completely inactivate viral particles before contact with target cells (Pre-treat virus, IC₅₀=1.82±0.5µg/ml), while when the compound was present for the duration of the assay, less than 8µg/ml was needed to achieve complete inhibition (Leave in, IC₅₀=0.63±0.3µg/ml) (Fig. 2A). In contrast, pre-treatment of HIV-1BaL (R5) with 1mg/ml PPCM did not completely inactivate virions (Pre-treat virus, IC₅₀>1mg/ml) (Fig. 2B). Nevertheless, if the compound was left in culture (Leave in), a mean IC₅₀ of 2.02±0.5µg/ml was obtained, indicating that although direct inactivation of the R5 virus is not readily achieved, the compound is still very effective at blocking attachment and/or fusion to target T cells. Little anti-viral activity was observed if the target T cells were treated with PPCM and then washed to remove drug prior to viral exposure, suggesting that the drug needs to be present at the time of viral exposure. These data are consistent with earlier studies indicating that the drug primarily targets the virus and has greater antiviral activity against X4 viruses compared to R5 (22).
PPCM blocks infection of target cells by cell-associated HIV-1. Both cell-free and cell-associated virus may contribute to sexual transmission. To evaluate the activity of PPCM against cell-associated HIV-1, PM-1 or H9 cells chronically infected with B clade isolates HIV-1_RF, HIV-1_IIIB or HIV-1_BaL, or the clade C clinical isolate Za003/97 were incubated with varying concentrations of PPCM for 1h and then co-cultured with Jurkat-Tat-CCR5 cells. PPCM completely blocked infection by cell-associated virus, although greater activity was observed for X4 compared to R5 strains (IC\textsubscript{50} values observed for BaL, Za008/93, RF and IIIB were 7.16, 6.73, 2.32 and 1.90µg/ml, respectively) (Fig. 3A). Furthermore, PPCM potently inhibited infection when ectocervical explants were challenged with cell-associated virus (mitomycin-treated PM-1 cells chronically-infected with HIV-1_BaL) in the presence of drug (Fig. 3B).

PPCM inhibits HIV-1 infection of human ectocervical, endocervical and penile tissue at concentrations that showed no tissue toxicity. Explant cultures may provide a more rigorous evaluation of the anti-viral activity of microbicides and offer the opportunity to examine the effects of the drugs on multiple cell types. As men will be exposed to microbicides and these products have the potential to be used by both women and men, it is important to also evaluate efficacy and safety in penile tissue. Ectocervical, endocervical and penile explants were cultured in a non-polarized manner and exposed to the R5 virus, HIV-1_BaL, for 2h in the presence or absence of a range of PPCM concentrations. An R5 isolate was selected for these studies because R5 isolates predominate following sexual transmission (50). The compound inhibited infection of all three types of tissue in a dose-dependent manner (Fig. 4), with very similar IC\textsubscript{50} values (3.9µg/ml for ectocervix; 3.1µg/ml for endocervix and 3.1µg/ml for penile glans).
Notably, complete protection was not achieved in the penile model. No toxicity was observed on any of the tissues.

**PPCM prevents transfer of HIV from migratory cells to T cells.** Migratory cells, which include immature dendritic cells (DCs) located in the epithelium and subepithelium have been previously described and play a critical role in the transmission of HIV-1 to target T cells in lymphoid tissue (24). Therefore, we examined the ability of PPCM to block the transmission of HIV-1\textsubscript{Bal} to permissive PM-1 cells, via migratory cells emigrating from cervical explants that had been infected with HIV in the presence of PPCM (Fig. 5A). Transmission via cells migrating from ectocervix was greatly reduced with an IC\textsubscript{50} of 35.7µg/ml; however, slightly higher concentrations of drug were needed to block transfer of virus from migratory cells emanating from endocervical explants, with an IC\textsubscript{50} of 54.6µg/ml.

These observations could reflect the ability of PPCM to directly block binding of HIV to DCs or the ability to prevent the transfer of hijacked virus to target cells. To further evaluate this, we took advantage of a cell culture system with the B-cell transfected cell line (Raji/DC-SIGN). PPCM greatly reduced the transmission of HIV-1\textsubscript{RF}, but not HIV-1\textsubscript{Bal}, to Jurkat-Tat-CCR5 cells (Fig. 5B). Notably, PPCM had only modest inhibitory effects on capture of HIV-1\textsubscript{RF} by the Raji/DC-SIGN cells, suggesting that the compound rendered the bound virus incapable of being transferred, rather than competitively inhibiting binding of HIV to DC-SIGN (Fig 5C). These data are consistent with a recent study demonstrating that transmission of HIV-1\textsubscript{Bal} via DCs was not completely blocked unless PPCM was present at the time of transfer to target cells (7).
Anti-HIV activity of PPCM in combination with UC-781 or PMPA. The therapeutic success of combination antiretroviral therapy systemically suggests that a similar approach may be necessary for effective microbicide development. Combining vaginal microbicides could inhibit HIV infection at multiple steps and reduce the risk of selecting for resistant isolates. The optimal in vitro assays for evaluating combinations are not known. The multiple-drug effect analysis of Chou, based on the median effect principle and the isobologram technique, has been used to analyze combined-drug effects, but for this model to be applicable, the drug ratio must be fixed (8,10). This method plots dose-effect curves for each drug and for multiply diluted fixed-ratio combinations of drug by using the median-effect equation. Using this approach, we exposed TZM-bl cells to HIV-1\textsubscript{BaL} or HIV-1\textsubscript{RF} in the presence of PPCM alone or combined with UC-781 and PMPA at fixed ratios as described above. Synergy was obtained when PPCM and PMPA were combined at concentrations that inhibited 75% and 90% of both R5 and X4 viruses in culture. However, when PPCM was combined with UC-781, we observed additive activity, and there was a suggestion of antagonism when the two drugs were combined at concentrations that inhibited 75% of the R5 isolate (Fig. 6 and Table 1).

PPCM gel protects mice from genital herpes both in the presence and absence of semen. Prior studies have demonstrated that PPCM inhibits HSV infection primarily by binding to viral envelope and inhibiting viral binding and entry (22). We have extended these in vitro observations and demonstrated that PPCM also blocks HSV infection of cervical explant cultures (Fig. 7) and in a murine model of genital herpes infection (Fig. 8). A stable, acid-buffered (pH = 4.5 - 4.8) gel containing PPCM at two different concentrations, 0.4% and 4% was produced and evaluated for efficacy in the murine
model. Both active gels provided significant protection against HSV-2 with 94% survival and no signs of disease in 33/35 of the mice treated with a single dose of 4% gel. Control gel, which consisted of the GRAS excipients used in the active gel protected only 9/35 (26%) of mice (Fig. 8). Moreover, the 4% gel retained activity when virus was introduced diluted in human semen and protected 16/20 (80%) mice. These findings differentiate PPCM from PRO 2000 or cellulose sulfate, as both of these drugs showed a significant reduction in anti-HSV activity when virus was introduced in semen (34).
DISCUSSION

Development of topical microbicide combinations that target both HIV and HSV may prove a powerful strategy to reduce HIV as epidemiological studies consistently demonstrate synergy between these two pathogens. This is particularly important in light of recent data indicating that acyclovir suppressive therapy does not protect against HIV acquisition in HSV-positive individuals at high risk of acquiring HIV (6). PPCM is a structurally unique compound active against both viruses. PPCM inhibits primary clade B and clade C isolates at concentrations that would be predicted to be present in the genital tract following application of either a 0.4 or 4% gel. For example, in a recent study, we found that the concentration of PRO 2000 found in cervicovaginal lavage (CVL) fluid one hour post application of a 0.5% gel ranged from ~100-300 µg/ml (26). Moreover, in a different study, 25µg/ml PRO 2000 was detected in CVL samples collected 12 hours following application of 4% gel (26,28). The in vitro activity against clade C isolates is significant as clade C remains the most prevalent HIV strain worldwide and in Central and South Africa. Few published studies have documented the antiviral activity of microbicides against non-clade B isolates prior to the initiation of clinical trials and in vitro studies have shown that Carraguard displays little or no activity against clade C isolates (29,30,14,16). This lack of activity in vitro may have contributed to the lack of efficacy in the recently completed phase III Carraguard trial (25). These disappointing results highlight the importance of extensive pre-clinical evaluation of candidate microbicides against HIV isolates from multiple clades.

Consistent with other anionic inhibitors of viral entry, PPCM is more active against X4 compared with R5 isolates under each experimental system evaluated. This
presumably reflects the ability of the anionic drug to bind irreversibly with the greater
aptic to the CD4-induced co-receptor
binding site. This may explain why compounds belonging to this class inhibit R5
viruses more effectively when present at the time of attachment to the target cell. This
differential activity suggests that X4 isolates could be inactivated by anionic polymers
within the vaginal lumen, while R5 virus would require compound to reach target cells
within the mucosa with equal efficiency as the virus itself. However, despite the higher
concentrations required, PPCM provided substantial protection against R5 viruses,
particularly when the drug was present throughout the experiment and most importantly,
in the human explant model.

Early algorithms for pre-clinical assessment of candidate microbicides focused
primarily on \textit{in vitro} cell culture assays with cell-free virus. However, infection also may
be initiated by cell-associated virus as well as by infected cells (27,37). Similarly,
successful sexual transmission of HIV-1 requires that the virus gain access to immune
cells permissive for infection. Migratory cells, which include immature dendritic cells
located in the genital tract epithelium and subepithelium, play a critical role in the
transmission by sequestering the virus and then transferring HIV-1 to target T cells in
lymphoid tissue (33). The current work demonstrates that PPCM inhibits infection by
cell-associated virus and significantly reduces dissemination by migratory cells in explant
culture systems. This reduction in MC-mediated transmission of HIV-1$_{\text{BaL}}$ was greater
than that observed in a cell culture system where little protection was observed using DC-
SIGN-expressing Raji cells, suggesting that the former results from inhibition of both \textit{cis-}
and trans-infection. Additionally, ongoing studies from our lab demonstrate that PPCM also blocks binding of HIV to human cervical epithelial cells and prevents subsequent transfer of the bound virus to T cells (Mesquita and Herold, work in progress). Several studies have demonstrated that epithelial cells are capable of sequestering large amounts of HIV particles, possibly through engagement of syndecan proteoglycans, and that the sequestered virions remain infectious for days and can then be effectively transferred to immune target cells (3,13,39,47). Together these findings suggest that PPCM should be active in reducing sexual transmission of HIV.

A truly safe and effective microbicide is likely to require a combination of drugs that target different steps in the HIV life cycle and provide protection against other sexually transmitted infections (STI) known to facilitate HIV infection. The advantages of combinations have led to a change in oral pre-exposure prophylaxis trials to focus on Truvada (a fixed dose combination of tenofovir disoproxil fumarate (TDF) and emtricitabine), rather than monotherapy with TDF alone. Whether resistance will be as great a problem with topically delivered microbicides as it is with oral therapy is not yet known, but there is a clear scientific rationale to support a combination strategy. First, there is the potential for transmission of resistant HIV strains that may overcome a microbicide consisting of a single antiretroviral drug (ARV). Approximately 8-20% of all new HIV infections in North America and Western Europe are attributable to viruses that contain at least one drug resistance-associated mutation and it is likely that this will increase over time both in developed and developing countries (42). An additional risk of the use of a single drug microbicide is the potential for selection of drug resistance by a woman who uses an ARV-based microbicide without knowing that she is infected, or by
an HIV-infected woman who uses a microbicide in an effort to protect her partner. Another distinct advantage of combination microbicides is the inclusion of a second drug that also targets other STI, particularly HSV. Epidemiological studies consistently demonstrate a strong link between HSV-2 infection and the risk for HIV acquisition and transmission (19). The prevalence of HSV-2 infection among Africans with HIV ranges from 50-90% (32). Asymptomatic shedding is common and is associated with both a higher frequency and a higher amount of HIV-1 in genital secretions (32). In addition to the effect of HSV on HIV replication, genital herpes may facilitate HIV acquisition by disrupting the epithelial barrier, thereby increasing exposure of target cells to virus. Both clinical and sub-clinical shedding of HSV-2 is associated with the influx of activated CD4+ T cells into genital mucosa (11).

Despite the clear scientific rationale for a combination approach to microbicides, development has been slow, in part because of the complexities of Intellectual Property (IP) conflicts and regulatory requirements. Recent efforts by the FDA to expedite the development of microbicide combinations include the requirement for limited bridging studies if one of the active agents of a combination is being clinically evaluated as a microbicide. This should significantly accelerate the advancement of candidate combinations.

In the current studies, we demonstrated synergistic activity if PPCM were combined with PMPA against R5 and X4 virus, and at least additive activity when PPCM was combined with UC781. It should be emphasized that the optimal in vitro assay for evaluating combinations is not established and in vitro synergy or additive activity is not a prerequisite for advancing a microbicide combination; combinations that are indifferent
in vitro may prove beneficial in the clinical setting where other factors such as drug resistant isolates play a role.

Experience with systemic antiretroviral therapy suggests that PMPA induces less NRTI resistance than other drugs in its class, although a recent study found that HIV-1 subtype C viruses rapidly develop K65R resistance to PMPA in cell culture (4). Whether repeated exposure to vaginal PMPA could select for resistant viruses in women who use the gel and are HIV-positive is not known. However, a combination of PMPA with PPCM could overcome this problem by blocking local HIV infection at distinct steps, thus decreasing the likelihood for selection of resistant viruses.

The anti-HSV and contraceptive activities of PPCM provide an additional benefit for a combination microbicides, as both UC-781 and PMPA are HIV-specific and provide no contraception (48). Importantly, formulated PPCM protected mice from genital herpes, even when virus was introduced diluted in seminal plasma. We recently found that human seminal plasma interferes with the activity of PRO 2000 and cellulose sulfate, increasing the concentration required by 100-fold to inhibit 90% of infection in cell culture. The interference translated in vivo into a loss in protection in a murine model. 2% PRO 2000 gel protected 100% of mice challenged intravaginally with HSV introduced in PBS, whereas only 55% of the mice were protected if virus were introduced in seminal plasma (34).

PPCM is not cytotoxic in vitro against cervical epithelial or immune cells (9,22) and shows no cytotoxicity using cervical or penile glans explant tissue. PPCM gel is not cytotoxic toward lactobacilli, is not mutagenic, has low acute oral toxicity, and is safe in the rabbit vaginal irritation assay (49). Together these studies support further
development of PPCM, preferably in combination with a reverse transcriptase inhibitor such as PMPA or other HIV-specific drugs as a topical microbicide.
Acknowledgments

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Table 1: Combination indices obtained for combination of PPCM with UC-781 and PMPA. TZM-bl cells were exposed to HIV-1 in the presence of PPCM alone or in combination with UC-781 or PMPA at a fixed ratio and a range of concentrations. % inhibition values were calculated relative to control for each concentration tested and were used for a computerized calculation of a combination index using Calcusyn software. The calculation was based on the slope of the median-effect plot, which signifies the shape of the dose-effect curve, and the x intercept of the plot, which signifies the potency of each compound and each combination. Combination indices of <0.9 indicate synergy (i.e., greater than the expected additive effect when two agents are combined), combination indices of 0.9 to 1.1 indicate nearly additive effects, and a combination index of >1.1 indicates antagonism (i.e., less than the expected additive effect).

<table>
<thead>
<tr>
<th>Combination</th>
<th>HIV-1RF</th>
<th>HIV-1Bal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC75</td>
<td>IC90</td>
</tr>
<tr>
<td>PPCM:UC-781</td>
<td>1.03</td>
<td>0.78</td>
</tr>
<tr>
<td>PPCM:PMPA</td>
<td>0.91</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. **PPCM inhibits HIV infection in cell culture.** TZM-bl cells were infected with $10^3$ TCID$_{50}$ of each of the indicated HIV-1 strains in the presence or absence of varying concentrations of PPCM. Virus and drug were left in culture for 48 hours at 37°C, and infectivity was monitored by luciferase assay. Cell viability was determined by MTS assay following exposure to drug for 48 hours. Results are means ± SEM obtained from 3 experiments conducted in triplicate.

Figure 2: **PPCM targets HIV to prevent infection.** The effects of PPCM on HIV-1$_{RF}$ (A) or HIV-1$_{BaL}$ (B) infectivity were compared under three different conditions: (i) drug present throughout the experiment (leave in); (ii) pre-treatment of virus followed by removal of the drug by washing (pre-treat virus); or (iii) if the target T cells were treated with PPCM and then washed to remove drug prior to viral exposure (pre-treat cells). Results are presented as mean reverse transcriptase (RT) activity as a % of untreated cells and are means ± SEM obtained from 4 experiments conducted in 6 replicates.

Figure 3: **PPCM prevents infection by cell-associated HIV-1.** Cells chronically infected with different isolates of HIV-1 were treated with 200µg/ml mitomycin C for 1h at 37°C and with varying concentrations of PPCM 1h before addition of target Jurkat-Tat-CCR5 cells. Supernatants were collected 5 days post-infection and viral replication was assessed by determining RT activity. Results are expressed as % RT activity relative to virus control and are means ± SEM of 3 independent experiments where each condition
was tested in 6 replicates (A). Mitomycin-treated infected T cells (PM-1) were added to ectocervical explants in the presence of PPCM. Cells and drug were removed by extensive washing after 2h incubation and explants were cultured in complete medium for 10 days. 50% of the culture medium was replaced every 2-3 days and culture supernatants were analyzed for p24 content by ELISA. Results are means± SEM of 2 independent experiments from different donors where each condition was tested in triplicate (B).

**Figure 4:** PPCM inhibits infection of human mucosal tissue. Ectocervical (squares, continuous line), endocervical (circles, dashed line) and penile explants (triangles, dotted line) were exposed to HIV-1\textsubscript{BaL} in the presence or absence of varying concentrations of PPCM. Virus and PPCM were removed by extensive washing after 2 h. Following overnight incubation, explants were transferred to a new plate and cultured for 10 days. 50% of the culture medium was replaced every 2-3 days and culture supernatants were analyzed for p24 content by ELISA. Toxicity was assessed by MTT assay following 24 h exposure. Results are means ± SEM of 3 independent experiments (2 for penile tissue) from different donors. Each condition was tested in triplicate.

**Figure 5:** PPCM inhibits transfer of HIV-1 via DC-SIGN and cervical MCs. Ectocervical (squares, continuous line) and endocervical (circles, dashed line) were exposed to HIV-1\textsubscript{BaL} in the presence or absence of varying concentrations of PPCM. Virus and PPCM were removed by extensive washing after 2 h. Following overnight incubation, MCs were co-cultured with $4\times10^4$ target T cells (PM-1) for 10 days. Culture
supernatants were analyzed for p24 content by ELISA and results are means ± SEM of 3 independent experiments from different donors. Each condition was tested in triplicate (A). Raji/DC-SIGN cells were treated with varying concentrations of PPCM or mannan (200µg/ml) for 1 h before addition of HIV-1_{BaL} (open bars) or HIV-1_{RF} (solid bars). Cells were extensively washed after 2 h incubation and then either target Jurkat-Tat-CCR5 cells were added in order to amplify any transmission of virus, cultured for 7 days and viral replication assessed by determining RT activity (B) or the bound virus was lysed immediately and quantified by p24 ELISA (C). The amount of HIV-1_{RF} added for the binding assay was 10-fold that of HIV-1_{BaL} in order to achieve similar levels of bound virus in the absence of PPCM. Results are expressed as % RT activity (B) or p24 antigen (C) relative to the control where no compound was added and are means ± SEM of 3 independent experiments where each condition was tested in 6 replicates.

**Figure 6:** PPCM inhibits HIV-1 infection in combination with RTIs. TZM-bl indicator cells were exposed to HIV-1_{RF} (A and B) or HIV-1_{BaL} (C and D) in the presence or absence of varying concentrations of PPCM alone or combined with different amounts of the RT inhibitors PMPA (A and C) and UC-781 (B and D). Virus and drug were left in culture for 48 hours at 37°C, and infectivity was monitored by luciferase assay. The effective concentrations for inhibition of HIV-1 replication by a compound alone and in combination with another compound are plotted in two curves for each drug. The shift between the two curves represents the dose reduction in compound that can be used to achieve the effect of the single drug. Results are means ± SEM obtained from 3 independent experiments.
Figure 7: **PPCM prevents HSV infection of cervical explants.** Cervical explant cultures were exposed to $10^7$ PFU/explant HSV-2(G) or in the absence or presence of the indicated concentration of drug. Heat inactivated virus (HSV HI) was used as a control for input virus. Supernatants were collected 7 days post infection and assayed for HSV by plaque assays on ME180 cells. Results are presented as PFU formed in the presence of drug as a % of PFU formed in the presence of medium alone and are means ± SEM obtained from 3 experiments conducted in triplicate. Asterisks indicate statistical significance (p<0.05).

Figure 8: **PPCM protects mice challenged vaginally with HSV-2.** Female Balb/c mice were pretreated with 30ul of either 0.4% PPMC, 4% PPMC or matched placebo gel and then 15 minutes later inoculated with 20ul of 1x $10^5$ (LD90) dose of HSV-2G diluted in either PBS or semen. Results show survival pooled from 4 independent experiments (n=5-10 mice/group/experiment). 4% PPMC significantly protected the mice when virus was delivered in both PBS and semen (p<0.0001 and p<0.0004, respectively, log rank test). The 0.4% PPMC also afforded significant protection (p<0.002).


Fig. 1

IC₅₀ values (µg/ml)
- CC1/85: 3.81
- HC4: 0.05
- DJ259: 0.27
- ZB20: 0.003
- BaL: 0.05
- RF: 0.02

PPCM (µg/ml)

RLU (% of untreated)

Viability (% of untreated)
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8