CCR5 ANTAGONISTS USED AS TOPICAL MICROBICIDES MODULATE CCR5 EXPRESSION AND MAY POTENTIALLY COMPOUND HIV INFECTION

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ABSTRACT

CCR5 antagonists, such as Maraviroc (MVC) and 5P12-RANTES, are being evaluated as HIV preventatives. The advantage of these drugs is that they restrict HIV entry via CCR5 receptor. However, sub-clinical concentrations could paradoxically increase HIV risk by increasing CCR5 expression.

Peripheral blood mononuclear cells (PBMCs) and cervical tissues were cultured with one HIV antiretrovirals (ARVs), Maraviroc (MVC), 5P12-RANTES, dapivirine (DPV), or griffithsin (GRFT). Immune cells isolated from PBMCs/cervical tissue were stained with live/dead stain and monoclonal antibodies. Samples were analyzed using a BD LSRII flow cytometer and FlowJo v. 10.1 software. To determine if suboptimal concentrations of antiretrovirals (ARVs) influence HIV susceptibility, PBMCs/cervical tissue were treated with MVC, 5P12-RANTES, DPV, or GRFT and challenged with HIV-1$_{BaL}$. Supernatant was collected on days 3, 7, and 11 and then tested for viral p24 by the AlphaLISA.

In PBMCs, 10 nM and 1nM MVC induced significant enhancement of CCR5+ expression levels when compared to DPV or GRFT treated cells, respectively. There were no significant changes in CCR5 MFI or percent positive cells found in the cervical tissue. HIV replication, via p24 production, did not increase for any drug concentration in PBMCs, but 975nM MVC significantly increased p24 expression in cervical tissue.

MVC is an effective therapeutic for HIV-infected persons, but may be problematic for HIV prevention due to its dose and time dependent qualities. Likewise, 5P12-RANTES showed similar trends in CCR5 expression. Topical application of CCR5 antagonists may be effective for only a short period in the genital tract. Conversely, DPV and GRFT did not affect CCR5 expression. The use of CCR5 antagonists for HIV prevention should be reconsidered as other options become available. The public health impact of improving topical HIV drugs could potentially reduce global HIV prevalence and shift toward an HIV-free world.

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PREFACE

Thank you to my family, friends, and colleagues for their constant love and support through this

journey.

1.0 INTRODUCTION

1.1 HUMAN IMMUNODEFICIENCY VIRUS

1.1.1 Epidemiology

In 2015, Human immunodeficiency virus (HIV) type 1 infected 36.7 million people globally, with 2.1 million new cases and 1.1 million deaths (1). The HIV virus was originally transmitted to humans via a primate species in Africa, such as western lowland gorillas and mangabeys, through a mutation in simian immunodeficiency virus (SIV) (2, p574). HIV was brought to the United States in the 1980's where it caused wide-spread concern due to its unknown infection route and spread (3). This disease predominately affected homosexual men that later in the disease progression developed rare, opportunistic infections with increased mortality from the general population (3). In 1983, the first isolate was discovered in France and Bethesda, Maryland, which spearheaded the pursuit to classify the newly identified virus (4). More than three decades following the discovery of HIV, rapid HIV detection techniques and preventative measures have been developed, but a successful vaccine is still absent (4). Due to increasingly successful treatments, HIV rates have gradually declined since the 1997 peak of 3.7 million new cases that year, with currently more than 9.7 million people accessing antiretroviral therapy (ART) in low- or middle-income countries (5).

While HIV infects all corners of the globe, the most HIV-impacted region of the world is sub-Saharan Africa. In 2000, 1.4 million people died from HIV-related illnesses, but by 2012 that number dropped to 1.2 million deaths and the HIV incidence was reduced by half (5). This dramatic drop in HIV deaths and incidence is related to an increase in widespread ART access, even in remote locations (5). The most common transmission route for HIV in Africa is unprotected, heterosexual intercourse, with an increased risk associated with multiple partners and other sexually transmitted infections, such as herpes simplex virus (5). A majority of new HIV infections can be attributed to discordant couples, which make up two-thirds of the heterosexual couples in Africa, where females are more likely to be HIV+ than their male counterparts (5). Mother-to-child HIV transmission is another substantial risk factor in Africa, but access to ART has reduced mother-to-child transmission to less than 1% of live births and 860,000 infant HIV acquisitions avoided in 2012 (5). To further reduce HIV acquisitions in Africa, innovative HIV prevention strategies to further control disease transmission and acquisition are needed.

1.1.2 Virology

HIV has several routes of transmission: sexual contact, intravenous drug use, and perinatal transmission. The virus is spread through contact with specific bodily fluids, such as blood, semen, vaginal/rectal secretions, and breast milk (6). HIV causes a gradual decline CD4$^+$ T cells and alterations in other immune cell levels such as macrophages, microglia, and dendritic cells, therefore reducing overall immunity of the individual. Normal CD4$^+$ counts range between 500 to 1,500 cells/mm^3, so when the virus depletes T cells to less than 200 CD4$^+$ T cells/mm^3 in circulation, HIV progresses to acquired immune deficiency syndrome (AIDS) (2, p573). Low CD4$^+$ T cells can make the body susceptible to opportunistic infections, such as Karposi

sarcoma, and eventually lead to death (2, p573). HIV infects and replicates within activated immune cells such as CD4$^+$ T cells, macrophages, and dendritic cells. For CD4$^+$ cells, the virus requires specific cellular surface proteins – CD4 in conjunction with CCR5 or CXCR4 – to gain entry (2, p576). Once the virus enters the cell and establishes infection, it replicates using host cell machinery and newly formed virus spreads throughout the body (2, p.576-77). HIV uses viral reverse transcriptase to convert viral RNA to complementary DNA (cDNA), which is ushered into the nucleus and the viral integrase incorporates the cDNA into the host DNA (2, p.576). After integration, productive viral infection is established, where viral particles begin to assemble (2, p.576). Viral proteins, including gp120 Env, are assembled at the cell membrane and bud from the infected cells to continue the spread of infection (2, p.576). Upon budding from the infected cell, new viral particles can infect CD4$^+$ T cells in the surrounding area and continue the HIV replication cycle.

The gp120 binds to the CCR5 or CXCR4 receptor to facilitate HIV entry. CCR5 (R5) tropic HIV viruses predominate early stages of infection, where CXCR4 tropic (X4) viruses arise over time (7). Persons with a CCR5 delta32 mutation, a 32-base pair deletion in the CCR5 gene, can render the CCR5 binding site unusable to R5 tropic viruses (8). The CCR5 gene deletion produces an abbreviated gene that does not produce a functioning CCR5 surface receptor (9). Delta32 homozygous individuals are resistant to R5 tropic HIV infection and delta32 heterozygous individuals are categorized as 'long-term progressors' (9). The primary mode of transmission worldwide is sexual contact through vaginal/penile or rectal/penile intercourse (10). While better access to treatment has reduced overall transmission, the lack of an effective vaccine still warrants research into other prevention options, such as blocking the sexual transmission route (10).

1.2   DRUGS AND TREATMENT

1.2.1   Drug Classification

There are several targets within the HIV replication cycle that are utilized as points for therapeutic interventions. The HIV drug arsenal is comprised of: entry inhibitors, nucleoside/non-nucleoside reverse transcriptase inhibitors (NRTI/NNRTI), integrase inhibitors, and protease inhibitors. These critical points have been exploited to create a combination treatment strategy comprised of three antiretroviral drugs, known as highly active antiretroviral therapy (HAART) which affects multiple points of the viral replication cycle (10). Entry inhibitors block the virus's ability to bind to surface receptors of the target cell (10). The virus enters the cell by, using its glycoprotein 120 (gp120) trimer to bind with the CD4 receptor and a co-receptor, which consists of either CCR5 or CXCR4 (11). Entry inhibitors target CCR5 thereby preventing the binding viral gp120 or other molecules/residues such as carbohydrate moieties to block entry (11).

NRTIs/NNRTIs interrupt the conversion of viral RNA to cDNA via HIV reverse transcriptase disruptions. NRTIs prevent the addition of nucleotides to the growing DNA chain during transcription, so HIV DNA synthesis is halted (10). NNRTIs bind directly to the reverse transcriptase enzyme, inhibiting the conformational changes needed to elongate the DNA chain (10). Integrase inhibitors prevent HIV cDNA from integrating into the target cell DNA by blocking the function of the HIV integrase enzyme (12). The HIV integrase enzyme relocates viral cDNA into the target cell chromosome (13). For retroviruses to replicate, the integrase enzyme ligates viral cDNA with host DNA (13). Integrase inhibitors prevent the integration between host DNA and viral cDNA, so the replication cycle cannot be completed (13). Finally,

protease inhibitors prevent viral assembly by interfering with HIV protease enzyme function (12). HIV proteases cleave non-functional, structural polyproteins into functional proteins that the virus needs to mature the capsid (14). In the presence of protease inhibitors, the virion particle cannot form a complete capsid, therefore halting viral replication (14). These effective drugs have provided opportunities for use in HIV treatment and prevention.

1.2.2 Maraviroc

HIV CCR5-facilitated entry occurs at the initial stage of infection and more frequently than CXCR4-facilitated entry making the CCR5 receptor a prime target for drug development (15). The oral formation of CCR5 entry inhibitor, Selzentry (Maraviroc) tablets, is FDA approved and recommended to be used in conjunction with other ARV drugs as second-line combination therapy. Maraviroc (MVC) is well tolerated, safe, and effective at treating HIV with adjustable dosing to accommodate the needs of the patient (16). MVC's optimal effectiveness window for oral and topical MVC application varies by species and application time in relation to viral exposure. The oral treatment is effective up to 4 hours post-administration while topical application is most effective 0.5 hours after application but loses 50% of its efficacy after 4 hours (15,17). Timing the administration and application of MVC is crucial in preventing HIV infection; if the optimal effectiveness window is missed, HIV infection may occur. MVC is effective via allosteric modification, which disrupts the interaction between the CCR5 receptor and gp120 surface HIV protein by the insertion of small MVC molecules (7). While MVC does not bind to the same structural component of CCR5 as HIV does, but it does prevent HIV entry by modifying the shape of the receptor and preventing proper HIV binding

(18).   In patients infected with X4 and duel tropic viruses, MVC presented little to no virologic advantage (7).

Blocking the CCR5 receptor modifies the cytokines signaling by allosterically inhibiting agonist activation, indicating that MVC stabilizes CCR5 in an inactive conformation (19, 20). CCR5 is a co-activation molecule on the CD4$^+$ T cell and can enhance T cell activation when accumulation occurs at the immunological synapse, but when CCR5 is blocked by entry inhibitors, like MVC, there is a decrease in CD4$^+$ proliferation (21).   MVC has been shown to diminish *in vitro* chemotactic activity of T cells towards an assortment of cytokines, such as CCL3 and CCL5 (21). The CD4$^+$ T cells associated with CCR5 antagonists could inadvertently prevent T cells trafficking between circulation and the lymphatics through decreased cytokine activity (21). High concentrations of MVC can reduce TNF-α, IFN-γ, and IL-4 in the supernatant and alter levels of chemoattractants CCL2 and CCL5 leading to suppression of T cell activation (19). These two factors could reduce overall CD4$^+$ T cell recruitment and chronic inflammation. Due to its anti-inflammatory properties, MVC can be utilized to mitigate persistent inflammatory illness, such as certain cancers and autoimmune diseases (22).

In the early stages of oral MVC efficacy and safety evaluations, MOTIVATE-1 and 2 trials were conducted with R5 tropic infected individuals to determine the efficacy of one or two doses of oral MVC (7). MOTIVATE-1 and 2 were randomized, double blind control trials, where oral MVC demonstrated a significant decrease in viral load and significant increase in CD4$^+$ T cells up to 48 days after the initial MVC administration with two, 300mg doses of MVC daily (7). The MOTIVATE trials demonstrated that long-term efficacy can be achieved with oral MVC by blocking the host protein with a small molecule antagonist, rather than only blocking the viral protein receptor (7). While the oral formulation has proven effective, topical gel

formulations have been created for HIV prevention to halt infection at the mucosal membrane within the vaginal/colorectal tract.

Topical application of MVC has demonstrated to be effective at preventing HIV transmission in mouse and macaque models, but has advantages and disadvantages with its formulation and effectiveness. The topical CCR5 microbicides are geared toward prophylactic use at the mucosal site of infection, where a topical product is inserted vaginally or rectally directly preceding intercourse (17). In a humanized mouse model, of the seven mice treated with topical MVC and exposed to HIV, none of the MVC-treated mice became infected compared to the placebo mice group after a 16-week treatment period (23). HIV DNA and RNA viral loads remained at undetectable levels (less than 10^2 copies/mL in plasma) over the 16-week period compared to all the placebo mice which had much higher plasma viral loads (more than 10^6 copies/mL of plasma) (23). Finally, the $CD4^+$ counts remained constant in the MVC treatment mice post-HIV exposure, which was not seen in the placebo gel treated (23). Rhesus macaque models were utilized to test if the gel formulation of MVC is equally as efficacious in a species that are comparable to humans. The results mirrored the humanize mice results with similar concentrations of the drug; yielding complete protection at higher concentrations of MVC and an effective time frame between 0.5 to 4 hours post-application (18).

1.2.3   5P12-RANTES

In addition to MVC, 5P12-RANTES is a CCR5 entry inhibitor that prevents HIV infection and is being developed into a topical microbicide. RANTES (CCL5) is a naturally occurring cytokine that attracts immune cells to the site of inflammation and regulates the immune response (24). RANTES, along with MIP-1α and MIP-2β, can suppress HIV infection through binding the

CCR5 receptor with chemokines and blocking HIV gp120 binding (24). The CCR5-blocking attributes along with its antigen independent T-cell activation makes RANTES an ideal candidate for an HIV entry inhibitor drug because immune cells will be recruited, but not susceptible to HIV infection (24). RANTES binds to the CCR5 receptor leading to internalization of the receptor:ligand complex and removes the HIV entry target from the cells surface (25). Hours after RANTES exposure, receptor cycling occurs, which allows more CCR5 receptors to appear on the surface of the CD4 cell, and the cell is vulnerable to infection once again (25). Receptor cycling ceases after a few days, so an analogous long-acting RANTES drug was developed to mimic CCR5 blockage without the inflammatory effects over several days (25). 5P12-RANTES is a large molecule entry inhibitor derived from the RANTES cytokine (24). This HIV drug does not activate CCR5 and will therefore reduce unwanted inflammatory effects (24). 5P12-RANTES acts similarly to MVC; it binds to the CCR5 receptor and does not allow viral entry through CCR5 binding and internalization (25).

1.2.4 Dapivirine and Griffithsin

To compare the effects of MVC and 5P12-RANTES, Dapivirine (DPV) and Griffithsin (GRFT) were used. DPV is a small molecule NNRTI that demonstrated reduced HIV acquisition in women using an intravaginal ring containing the drug (26). GRFT is a novel, 121 amino-acid carbohydrate-binding, protein entry inhibitor that is under consideration as treatment options for multiple diseases, such as Hepatitis C or Human Papillomavirus (27). GRFT mechanism of action blocks infection by binding to the high mannose N-linked glycan portion of the viruses rendering them unable to enter the target cell (28). This binding allows GRFT to be effective against CCR5 and CXCR4-tropic HIV viruses (27). GRFT is also stable at high temperatures and low pH,

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making it an ideal compound for topical application in resource poor settings (27). Both DPV and GRFT can be used as pre-exposure prophylaxis drugs (PrEP) and represent small molecule and protein controls for MVC and 5P12-RANTES respectively (26, 27).

1.3   PUBLIC HEALTH SIGNIFICANCE

HIV infects millions of people globally, costs billions of dollars in the healthcare industry, and leaves people with a life-long illness that eventually leads to death (1). Making topical HIV drugs more effective and efficient for the most vulnerable populations would greatly improve HIV-infected areas and reduce community viral loads. The cost to formulate some of the drugs into pill or ring form can be inexpensive for people who truly need it. For example, one 300mg MVC tablet, which costs about $15 in the US, can be made into a gel formulation that was shown to protect several macaques against SHIV infection (17), suggesting that this could be a feasible option for humans. With rising HIV resistance, there is a need for innovative HIV drug development to combat this growing concern. The more common second-line and PrEP drug usage becomes, the risk for drug resistance within the viral genome increases. MVC drug resistance can occur when the gp120 mutates allowing for HIV binding despite the presence of the drug altering the receptor binding site (29). Novel drug development, such as GRFT, should be a priority in the pharmaceutical industry to stay ahead of HIV mutations. In certain parts of the world with a high HIV prevalence, taking pills or condom use to prevent HIV infection are stigmatized (30). Therefore, other strategies, such as topical products, that are user controlled may increase uptake and use.

Topical HIV treatments can be used more discretely and have less stigma attached to them than a pill. In Africa, disclosing a person's HIV status has negative consequences, such as violence and infidelity accusations, and can lead to severe stigmatization within their community (30). The potential for such stigma and the economic and social repercussions may cause an HIV-positive individual to forgo medication and treatment (30). The use of a topical gel or vaginal ring is more discrete than traveling to a clinic to get daily HIV medication or requiring partners to wear condoms. The topical gels can be applied without medical personnel and vaginal rings can last up to a month before needing replaced. The independence associated with gels and rings can empower women to manage their lives and sexual health. The continuing improvement of topical HIV products will further benefit public health globally by reducing the transmission and spread of HIV.

2.0   SPECIFIC AIMS

2.1   PROJECT STATEMENT

While CCR5 antagonists appear to be effective for treatment, their use for HIV prevention may raise some public health concerns. CCR5 antagonists, such as MVC, prevent natural binding of CCR5 receptor:ligand complex reducing internalization (31). The lack of the complex internalization leads to an increase in surface CCR5 and circulating CCR5 ligand (31). Because MVC has such a short half-life in the genital tract, the effects of sub-clinical concentrations of MVC on the immune cell population within the genital mucosa should be explored for alterations in CCR5 expression (17). For this study, we hypothesize that sub-clinical concentrations of MVC and/or 5P12-RANTES could paradoxically increase HIV risk by increasing CCR5 expression on the surface of target cells. More CCR5 receptors would make target cells more susceptible to HIV infection and exacerbate the spread of HIV in already high risk areas.

2.2   PROJECT SPECIFIC AIMS

Specific Aim 1

Examine the effect of sub-clinical concentrations of MVC, 5P12-RANTES, DPV, or GRFT will have on CCR5 surface receptor expression. PBMCs and immune cells isolated from cervical tissue will be analyzed through flow cytometry to quantify surface CCR5 expression on individual

cells and the number of CCR5 positive cells within a lymphocyte population, through mean fluorescence intensity (MFI) and percent positive metrics. An increase in CCR5 positive cells and number of CCR5 receptors could increase the likelihood of HIV infection within vulnerable populations.

Specific Aim 2

Examine the effect of sub-clinical concentrations of MVC, 5P12-RANTES, DPV, and GRFT will have on HIV infectivity and replication. PBMCs and immune cells isolated from cervical tissue will be infected with HIV and treated with an HIV drug, then a structural p24 protein analysis, p24 AlphaLISA, will be performed to quantify HIV replication within CD4$^+$ cells. An increase in p24 expression is a result of increasing HIV infection in target cells that could cause HIV to spread.

3.0   METHODS

3.1   REAGENTS

The HIV strain used was the laboratory strain HIV-1$_{BaL}$. The reagents used for flow cytometry included: near-IR live-dead (Molecular Probes, Eugene, OR), CCR5 (BV421, clone 3A9), CD69 (PE, clone FN50), CD45 (PerCP-Cy5.5, clone HI30), CD4 (BB515, clone RPA-T4), CD8 (BV510, clone SK1), and CD3 (APC, clone UCHT1) monoclonal antibodies (BD Biosciences, San Diego, CA). Optimal antibody concentration was determined through antibody titrations to ensure the brightest signal with the lowest background noise.

3.2   PBMC FLOW CYTOMETRY ASSAY

Buffy coats were obtained from healthy donors at the Central Blood Bank. PBMCs were isolated from buffy coats using Ficoll-sodium metrizoate separation gradient. The PBMCs were treated with the indicated concentrations of MVC, DPV, GRFT, or 5P12-RANTES for 48 hours in 5% CO_2 /37°C. After treatment, PBMCs were washed and adjusted to 1×10^6 cells per mL in PBS and stained with near-IR live-dead viability dye for 25 minutes protected from light at room temperature. The cells were then washed and stained with the following antibodies in order: CCR5 (BV421, clone 3A9), CD69 (PE, clone FN50), CD45 (PerCP-Cy5.5, clone HI30), CD3 (APC, clone UCHT1), CD4 (BB515, clone RPA-T4), and CD8 (BV510, clone SK1). The cells were incubated for 25 minutes protected from light at room temperature. Cells were washed and

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fixed with 1× red blood cell lysis buffer (Roche, Basel, Switzerland). The fixed cells were

suspended in FACS buffer (BD Bioscience) and analyzed using BD LSR II Flow Cytometer (BD

Bioscience) (32). Fluorescent minus one (FMO) and isotype controls were utilized to determine

proper gate placement and spectral overlap was accounted for by single color compensation

controls. Cells were gated on singlet, live lymphocytes (Figure 1). Live, CD45$^+$ cells were gated

from the single cells to further analyze the CD4+ cell population expressing CD69 and CCR5.

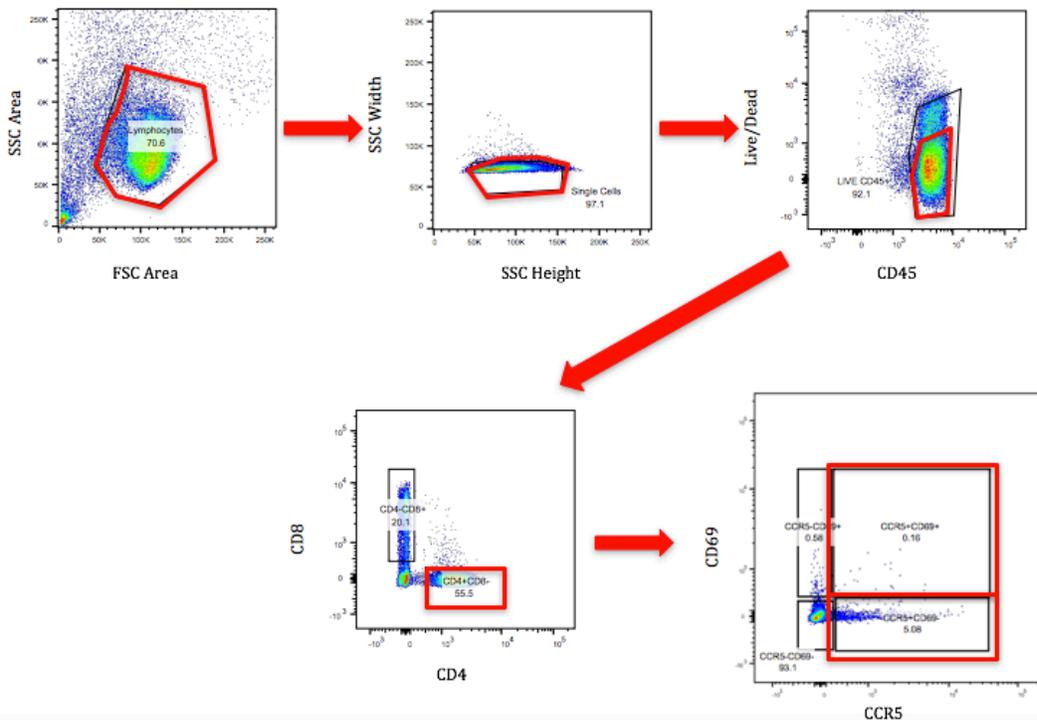MFI and percent positive metrics were collected.

Figure 1. Flow cytometry gating strategy.

The analysis template was used for PBMCs and immune cells isolated from cervical tissue. The flow cytometry
data was analyzed using FACS Diva v6.2 (BD Bioscience) and FlowJo v.10 software (Tree Star Inc., Ashland, OR).

3.3   CERVICAL TISSUE FLOW CYTOMETRY ASSAY

The cervical and colorectal tissues were obtained through the University of Pittsburgh Health Sciences Tissue Bank (Pittsburgh, PA) under IRB-approved protocols (IRB PRO09110431), or procured from tissue donated to the National Disease Research Interchange (NRDI, Philadelphia, PA). Tissue pieces were divided into four treatment groups: negative control, positive control, MVC versus DPV or 5P12-RANTES versus GRFT. Each group contained 4-6 pieces of tissue which was approximately 500mg. After treatment, tissues were minced using sterile scissors and digested in 20mL RPMI containing 1% Collagenase B (Roche) and 0.5% DNAse (ThermoFisher, Waltham, MA). After digestion, samples were filtered through a 40μm cell sieve to generate a single-cell suspension (Becton Dickenson, Franklin Lakes, NJ). The isolated mucosal immune cells were stained for flow cytometry following the same procedure used for PBMCs.

3.4   HIV INFECTION OF PBMCS AND CERVICAL TISSUE ASSAY

Isolated PBMCs were treated with MVC, DPV, 5P12-RANTES, or GRFT for 48 hours in a 96-well plate at 5% CO_2 and 37°C. After 48 hours, the samples were washed with media to remove the drug then 5×10^3 or 5×10^2 tissue culture infectious dose for 50% infectivity ($TCID_{50}$) of HIV-1$_{BaL}$ was added to the appropriate wells. After 3 hours, the virus was washed with media and incubated for 7 days. On days 3 and 7, 100μL of supernatant was removed and frozen at -80°C until a high sensitivity AlphaLISA p24 ELISA (Perkin Elmer, Bridgeville, PA) was performed. Previously collected and analyzed AlphaLISA p24 data for HIV-infected cervical

tissue was used for statistical analysis in determining HIV replication in MVC, DPV, GRFT, and 5P12-RANTES treated cervical tissue (33, unpublished data).

3.5   STATISTICAL ANALYSIS

One-way ANOVA was used to compare relationship between drug concentrations and $CCR5^+CD4^+$ percent positive and MFI. Dunnett's post hoc analysis was used to compare the negative control to drug treatment groups. Both tests were measured at a level of $\alpha=0.05$. Graphpad Prism 7 software (Graphpad Software Inc., La Jolla, CA) was used to statistically analyze the data and generate graphical representations of the data. For the HIV-infected PBMC analysis, a one-way ANOVA was used for compare the combined day 3 and 7 p24 levels of the various treatment groups using Prism 7. Previously collected AlphaLISA p24 cervical tissue data was statistically analyzed comparing MVC, DPV, GRFT, and 5P12-RANTES drug dilutions to an HIV control (33, unpublished data). For the HIV-infected tissue, the two p24 replicates from day10/11 of the AlphaLISA were averaged and entered into Stata14 software (StataCorp LP, College Station, TX). A linear or quadratic correlation between the data was established. If there was a linear correlation, the data were analyzed with a generalized estimating equation (GEE) linear model. If there was a quadratic correlation, the data were analyzed with a GEE non-correlated model.

4.0   RESULTS

4.1   PBMC FLOW CYTOMETRY DEMONSTRATE INCREASE CCR5 EXPRESSION WITH MVC

To determine whether CCR5 antagonists affect cellular expression of CCR5, two CCR5 antagonists, one a small molecule (MVC) and other a large protein (5P12-RANTES), were compared to other HIV prevention compounds, DPV and GRFT, which reflects a small molecule and large protein as controls. In PBMCs, there were significant differences in the mean fluorescence intensity (MFI) between the untreated controls and those treated with MVC, the 1µM and 10nM dose ($p = 0.006$ and $p=0.0231$, respectively) (Figure 3A). There were statistically significant differences between the DPV, 5P12 RANTES, or GRFT treatments, but 5P12 RANTES MFI expression mirrored that of MVC. For DPV, 5P12 RANTES, and GRFT no concentrations exhibited a significant percent positive cells or MFI difference from the negative controls (Figure 2A-2D or 3A-3D). The changes in percent positive cells were not significant for 1µM or 10nM MVC, but there were several replicates with higher concentrations of CCR5$^+$ cells. 5P12-RANTES showed a similar trend in that both concentrations (10nM and 1nM) had higher expression of CCR5 (percent positive cells and MFI; Figure 2C and 3C) and a dose response was noted, but these differences did not reach statistical significance.   While the change in CCR5 was not statistically significant, there was a 2 to 3.6-fold increase in CCR5 expression after culture with 5P12-RANTES and MVC, respectively (Table 1).
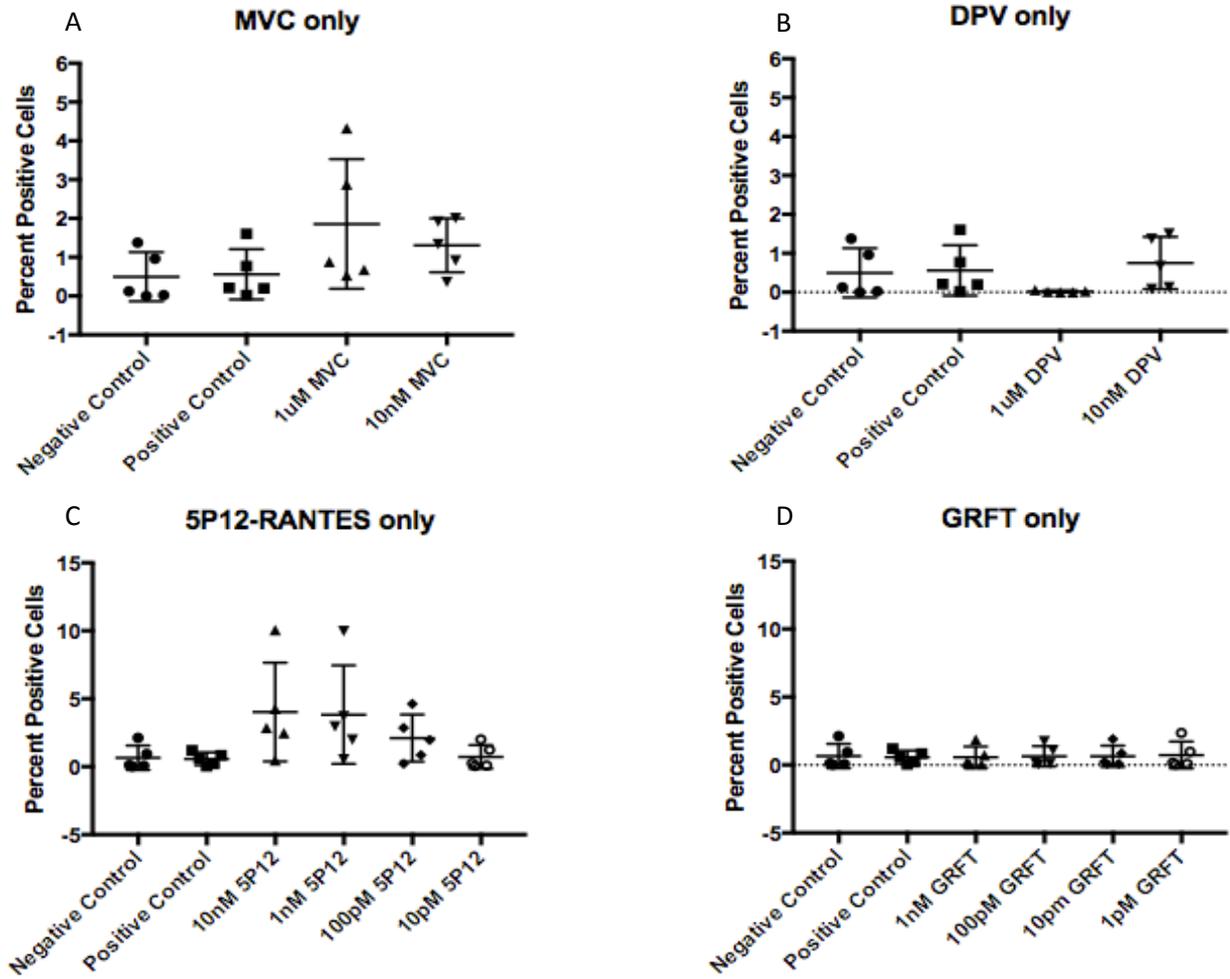
Figure 2. Percent positive cells from flow cytometry analysis of drug-treated PBMCs.

Expression of CCR5 on CD4+ cells treated with Maraviroc (MVC; A), Dapivirine (DPV; B), 5P12-RANTES (C), and Griffithsin (GRFT; D) for 48 hours. Total lymphocytes were gated on the live CD4+CD8- cells (n=5 buffy coats. Horizontal bars represent mean ± standard deviation (SD).

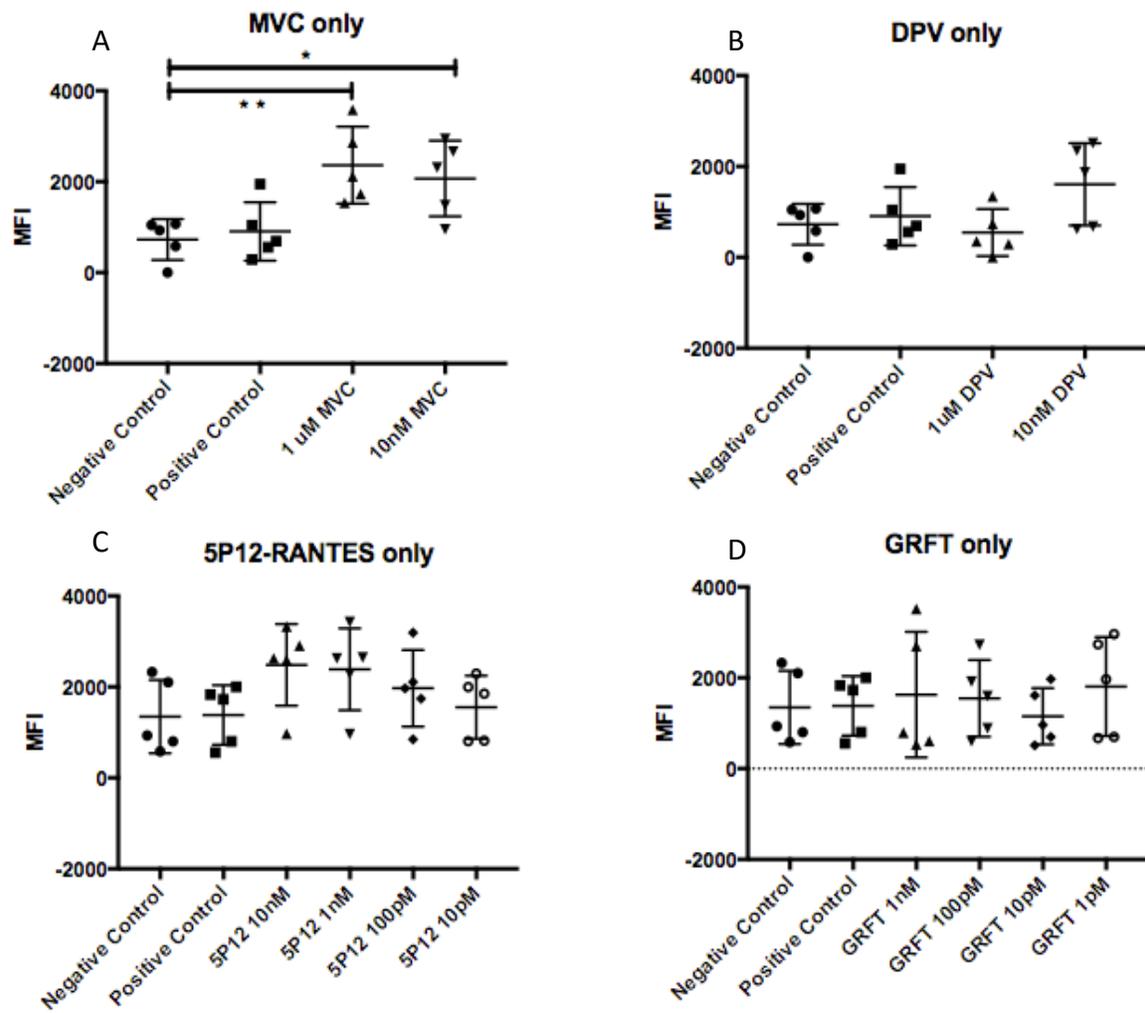**Figure 3**. Mean fluorescence intensity from flow cytometry analysis of drug-treated PBMCs.

Expression of CCR5 on CD4+ cells treated with Maraviroc (MVC; A), Dapivirine (DPV; B), 5P12-RANTES (C), and Griffithsin (GRFT; D) for 48 hours. Total lymphocytes were gated on the live CD4+ CD8- cells (n=5 buffy coats). ** p=0.006; * p=0.0231. Horizontal bars represent mean ± standard deviation (SD).

Table 1. MFI characteristics of drug-treated PBMCs

Treatment	CCR5 MFI change	Fold change from negative control
DPV 1uM	457.52	0.745
DPV 10nM	1498.76	2.442
Maraviroc 1uM	2253.7	3.673
Maraviroc 10nM	2029.58	3.307
Negative control	613.64	-
GRFT 1nM	1511.2	1.238
GRFT 100pM	1440.2	1.180
GRFT 10pM	1037.56	0.850
GRFT 1pM	1702.96	1.395
5P12-RANTES 10nM	2357.38	1.931
5P12-RANTES 1nM	2272.88	1.862
5P12-RANTES 100pM	1849	1.515
5P12-RANTES 10pM	1430.54	1.172
Negative control	1220.6	-

CCR5 measurements in PBMCs for MVC, DPV, GRFT, or 5P12-RANTES compared to the negative control (n=5).

4.2   CERVICAL FLOW CYTOMETRY DEMONSTRATED NO SIGNIFICANT CHANGE IN CCR5 EXPRESSION

Isolated immune cells from cervical tissue were treated with MVC or DPV. The treated tissue did not show a significant increase in either $CD4^+CCR5$ percent positive cells or MFI (Figure 4A & 4C). For 5P12-RANTES or GRFT, there was no significant increase of CCR5 expression, percent positive cells or MFI, on $CD4^+CCR5$ cells (Figure 4B & 4D). At the concentrations tested, MVC was the only drug that demonstrated any increase in CCR5 expression compared to the negative control (Table 2).

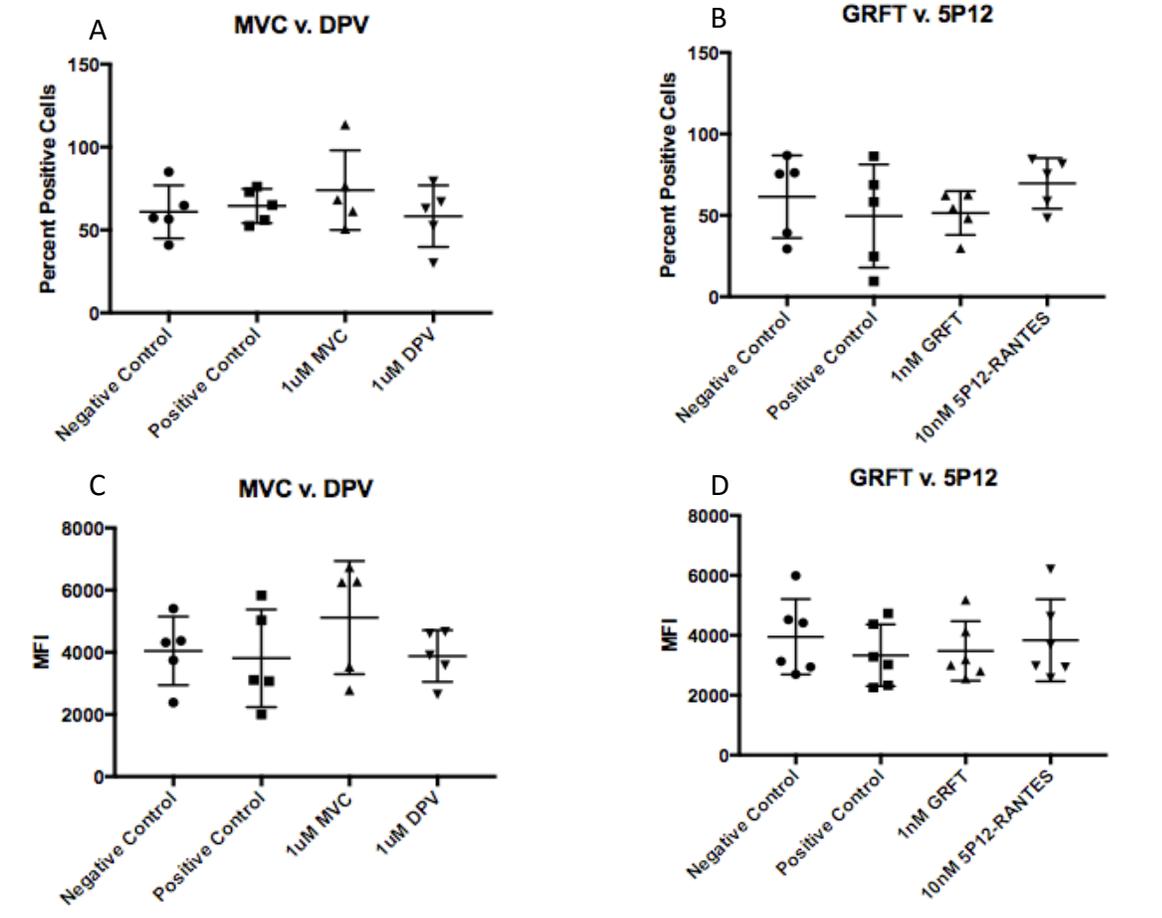**Figure 4.** Flow cytometry analysis of isolated immune cells from cervical tissue.

Isolated immune cells from 5 independent cervical tissues treated with MVC, DPV, GRFT, or 5P12-RANTES and analyzed by flow cytometry (n=5). No significant changes in CCR5 expression, percent positive cells or MFI, for any drug concentration of MVC/DPV (A and C) or GRFT/5P12-RANTES (B and D) was noted. Horizontal bars represent mean ± SD.

Table 2. MFI characteristics of isolated immune cells from cervical tissue.

Treatment	CCR5 MFI change	Fold change compared to negative control
DPV 1uM	3573.24	0.951
Maraviroc 1uM	4783.84	1.273
Negative control	3758.22	-
GRFT 1nM	2966.03	0.870
5P12-RANTES 10nM	3287.62	0.965
Negative control	3407.52	-

CCR5 in isolated immune cells from cervical tissue for MVC, DPV, GRFT, or 5P12-RANTES (n=5)

4.3   PBMC P24 ALPHALISA DEMOSTRATES HIV SUPPRESSION WITH GRFT

To determine if the increase in CCR5 resulted in enhanced HIV infection, PBMCs were treated with several concentrations of MVC, DPV, GRFT, or 5P12-RANTES, washed and then exposed to HIV. For HIV 5×10^3 and 5×10^2 $TCID_{50}$, DPV and MVC suppressed HIV infection at 10nM and 1nM, but suppression was not significant (Figure 5A & 5C). For 1nM GRFT and 10nM 5P12-RANTES, there was significant suppression at the 5×10^3 $TCID_{50}$ (p=0.0199 and p=0.0308, respectively) (Figure 5B). No significant increase or decrease in p24 expression at 100pM GRFT or 1nM 5P12-RANTES (Figure 5B). The 5×10^2 $TCID_{50}$ had poor viral replication for both MVC/DPV and GRFT/5P-12 (Figure 5C & 5D).
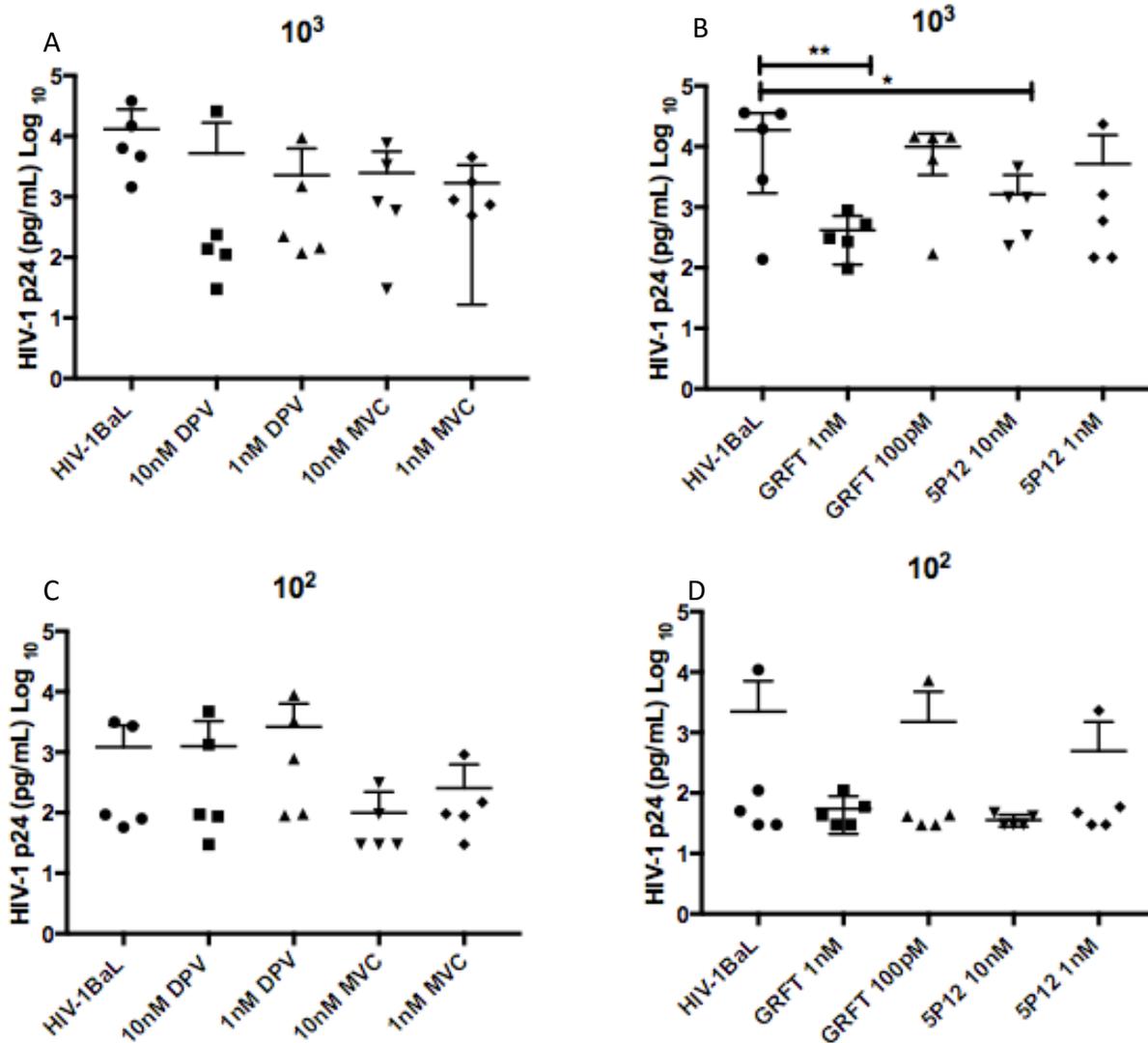
Figure 5. p24 analysis of drug-treated PBMCs.

Day 3 and day 7 cumulative, median p24 values for five PBMC donors. HIV-1$_{BaL}$ is the untreated control. MVC/DPV (A and C) showed no significant increase or suppression of p24 expression. GRFT (1nM) and 5P12 RANTES (10nM) significantly decreased p24 expression (B). ** p=0.0199; * p=0.0308. The data represents treatment of five independent blood donors and tested in duplicate. Horizontal bars represent mean ± SD.

4.4   CERVICAL P24 ALPHALISA DEMONSTRATES INCREASE HIV
REPLICATION WITH MVC

Cervical tissue treated p24 data with varying drug concentrations of DPV or MVC (33) and GRFT or 5P12-RANTES (unpublished data) were evaluated for efficacy against HIV infection. These data were used here to determine if the suboptimal dose of drug influence HIV infection as reflected by the viral p24 quantification. GEE analysis was used to evaluate the average p24 response at day 10/11 among the control and treatment groups and to determine if the p24 expression was dose dependent. The p24 expression was higher in the 97.5nM MVC-treated tissue as compared to the HIV control, but the increase was not significant (Figure 6A). The 975nM MVC-treated tissues showed significantly increased p24 expression above the HIV-1_{BaL} control p24 levels (p=0.025), while higher MVC concentrations significantly suppressed p24 expression at day 10/11 (Figure 6A). DPV at 79.5nM showed a significant increase in p24 expression above the HIV control levels (p = 0.007; Figure 6B); however, four tissues were used in this experiment which may have influenced the results. All concentrations of DPV greater than 759nM significantly suppressed p24 expression (Figure 6B). All concentrations of 5P12-RANTES and GRFT significantly suppressed HIV replication in cervical tissue (Figure 6C & 6D).
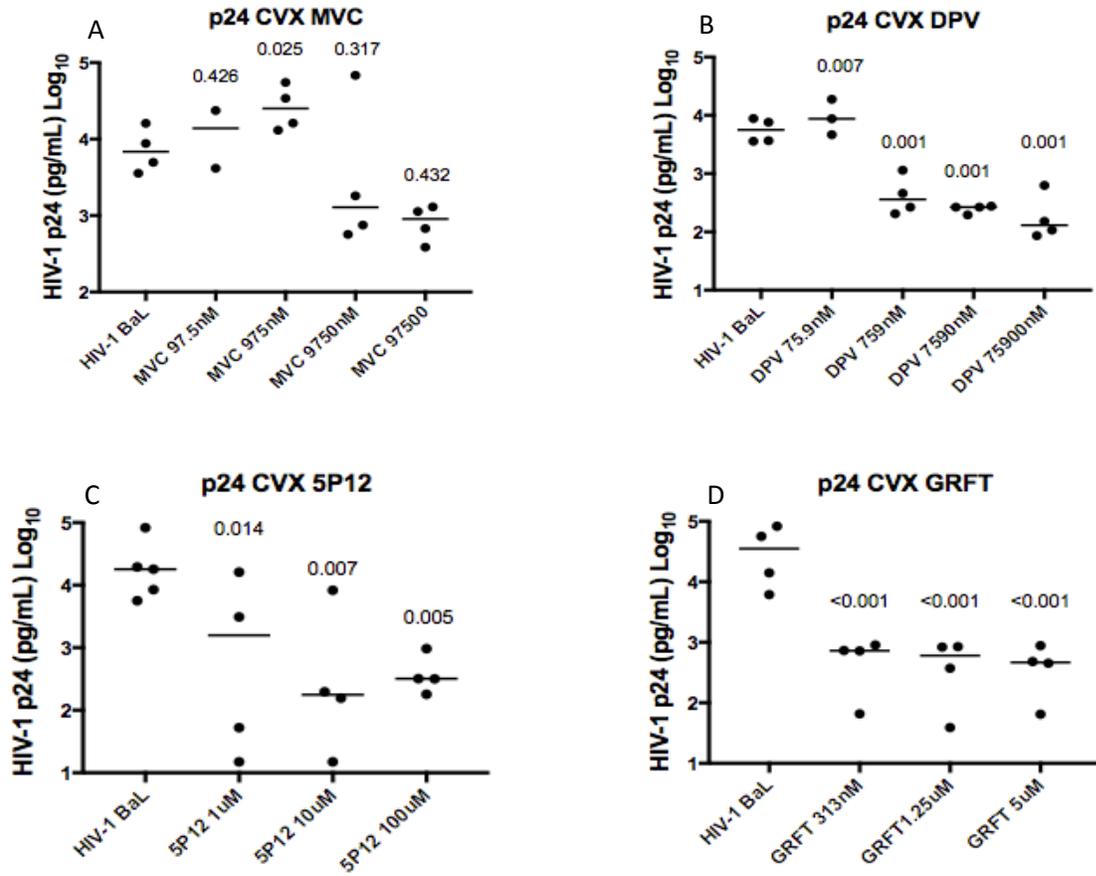
Figure 6. p24 analysis of isolated immune cells from cervical tissue.

MVC (A), DPV (B), GRFT (C), or 5P12-RANTES (D) treated cervical tissue was analyzed for HIV-1 p24 expression (n=5). MVC 975nM significantly increased p24 expression (5A, p=0.025). Horizontal bar represents the median. Five independent cervical tissues tested in duplicate.

5.0 DISCUSSION

Different categories of HIV drugs target multiple sites of the viral replication cycle to halt the virus replication at different points. The first line of defense in preventing infection is the entry inhibitor class of drugs, such as MVC, 5P12-RANTES or GRFT (10). These drugs block the viral gp120 surface protein from binding to its respective co-receptor, through either CCR5 or CXCR4 (10). The next drug categories are non-NRTIs/NNRTIs, such as DPV (10). These drugs prevent viral RNA from being converted to DNA, which would then be integrated into the host genome (10). Once in the nucleus of the host cell, integrase inhibitors are used to prevent integrating the newly converted viral cDNA into the host genome (13). If the virus manages to surpass all those antiretroviral road blocks, then protease inhibitors can prevent viral assembly at the very last stage of viral replication (12). These drugs prevent the virus from maturing and exiting the host cell to infect more cells (12).

CCR5 entry inhibitors were at the forefront of this study, examining whether sub-clinical concentrations of entry inhibitor drugs could alter CCR5 expression (31). The CCR5 entry inhibitors had some impact on CCR5 receptors in PBMCs and immune cells in cervical tissue. In the PBMCs, MVC increased the number of CCR5 receptors on the surface of CD4+ T cells as demonstrated with CCR5 MFI increases, but MVC did not have an impact on the number of cells expressing CCR5$^+$. While there were similar trends, the other entry inhibitor, 5P12-RANTES, did not significantly alter the number of percent positive cells or the number of surface CCR5 receptors. The other drugs used here, DPV and GRFT, did not have any significant impact on the number of percent positive or surface CCR5. Interestingly, little change in CCR5 expression or numbers of positive cells was found after culturing cervical tissue with sub-optimal

concentrations of the drugs tested. It is not clear why similar findings were not found between the PBMCs and the immune cells from cervical tissue. However, additional time points after treatment should be considered as 48 hours may have been too soon after treatment to quantify changes in CCR5 expression in intact tissue. Moreover, higher concentrations of the drugs could be considered for testing as we have shown more drug is needed in the tissue models compared to the *in vitro* models (34).

In HIV-infected PBMCS, none of the drugs caused a significant increase in HIV infection, even at sub-optimal concentrations of drug. The higher concentrations of 5P12-RANTES and GRFT, 10nM and 1nM respectively, had a significant protective effect on PBMCS. In the cervical mucosa, the number of CCR5$^+$ cells and the number of CCR5 receptors on the cell surface were consistent with the negative control amounts across all drug treatment groups. The HIV p24 expression in cervical tissue significantly increased for the lowest concentration of MVC (97.5nM) at day 11 of infection. The lowest concentration of DPV (75.9nM) also significantly increased in p24 expression, but that treatment had fewer cervical tissues tested, which could have impacted our findings. All other concentrations of DPV, GRFT, and 5P12-RANTES exhibited significant HIV protective effects in cervical tissue. The other concentrations had significantly lower p24 values than the HIV control, which demonstrates the drugs are preventing HIV infection in cervical mucosa.

Subclinical concentrations of MVC caused an increase of surface CCR5 receptors of PBMCs and increased p24 immune cells from cervical tissue. The concentrations evaluated of MVC affected peripheral and mucosal CD4$^+$ T cells differently leading to antiviral activity in PBMCs but not cervical tissue (12). For peripheral CD4 T cells, complete HIV protection was achieved at a lower concentration and shorter exposure time in the presence of MVC; however,

this result was not mimicked in mucosal immune cells (12). Immune cells isolated from cervical

tissue did not achieve complete HIV protection, despite the prolonged and constant drug

exposure (12). The CCR5 receptor is a dynamic modulated G-protein-coupled receptor, which

can be expressed differently at various stages of activation, so the gp120 binding to CCR5 is

affected by the co-receptor conformational state (12). Contrary to the tissue-specific CCR5

response, MVC has been found effective in PBMCs at concentrations as low as 47.2nM (35).

The variance in drug efficacy between PBMCs and cervical immune cells could be attributed to

sample drug exposure. PBMC T cells are more readily exposed to MVC than resident immune

cells within the tissue. MVC needs to penetrate into the tissue to reach its target cells, which

could alter the effective concentration when compared to PBMCs. MVC is a small molecule

HIV drug and there is an inverse relationship between drug molecule size and drug penetrating

abilities (36). The larger molecules, 5P12-RANTES and GRFT, cannot diffuse across the

mucosal membrane to the target cells. In PBMCs, penetrating qualities are not necessary

because the target CD4 cells are in single-cell suspension making CCR5 binding readily

available. Molecular penetration could account for the disparity in the PBMC and cervical tissue

data.

In more recent findings, MVC has not demonstrated effective HIV prevention as was

once thought. The HIV Prevention Trials Network (HPTN) 069 study is a phase two study

comparing oral MVC alone with two different combination therapies, MVC + emtricitabine

(FTC) or MVC + tenofovir disoproxil fumarate (TDF), to be used as PrEP for men who have sex

with men (MSM) in the United States (37). The study yielded contrasting results from what has

been demonstrated in animal models. Over the course of the study, five participants acquired a

R5 tropic HIV infection, four of them where in the MVC only group (4.5% of MVC-only study

arm) (37). Two of those five participants had no detectable MVC in measured plasma levels and two more has suboptimal plasma concentrations at the time of seroconversion (37). Previous PrEP MVC efficacy studies have shown varying levels of success between animal models. The results from this study contradict results from Neff et al that demonstrated HIV prevention using oral MVC in humanized mouse models with HIV vaginal challenge (23). In macaque models, Massud et al found that one MVC dose 24 hours prior to rectal viral challenge resulted in five of the six macaques acquiring simian/human immunodeficiency virus (SHIV) (38). Sufficient drug concentration sustainability within the mucosal tissue should also be considered when determining PrEP formulations. Veazy et al demonstrated effective SIV prevention with topical MVC in macaques, but the sample size was small and the drug doses administered were above the recommended clinical dosing (17). While oral PrEP regimens may differ compared to topical regiments, the lack of efficacy demonstrated by Gulick et al should be considered when moving forward with MVC as a PrEP option (37).

There were several limitations associated with this project that will need additional research to confirm the findings. The tissues were obtained from women undergoing surgery and may have had hormone or chemotherapy treatments leading up to the surgery date. The resections are typically obtained from older women, 35 years of age and older, and may not be generalizable to younger women. Limited information was provided from the blood bank with the buffy coats. Gender and ages of blood donors were not identified unless that information was specifically requested from the blood bank.

To understand the effect of MVC and 5P12-RANTES may have on RANTES production from treated PBMCs and immune cells isolated from cervical tissue, multiplex ELISA analysis can be used to capture and determine how much RANTES (CCL5) is being expressed in HIV

infected and drug treated CD4+ cells. Combining the amount of RANTES produced with the quantified number of CCR5 receptors through flow cytometry, future studies may be able to determine CCR5 internalization and cycling on the target cell surface. A better understanding of this process could be used to determine which sub-optimal drug concentrations could result in increased risk of infection. Cell migration assays can also be used to determine the chemotactic effects of altering CCR5 surface expression. Higher drug concentrations should be explored with cervical tissue with a short dosing period to determine if these exposures lead to CCR5 modulation.

Further development of topical HIV prevention drugs may improve the quality of care for many people, especially in resource-poor areas. The data provided here suggest subtle alterations of CCR5 could occur with CCR5 antagonist drugs. Other entry inhibitor drug classes, such as GRFT, may be a better option for future topical microbicide development. Giving women safe, effective, and alternative options for HIV prevention will hopefully empower women and reduce stigmatization within their communities. Improving HIV drug treatment and management is leading us one step closer to creating an AIDS-free generation.
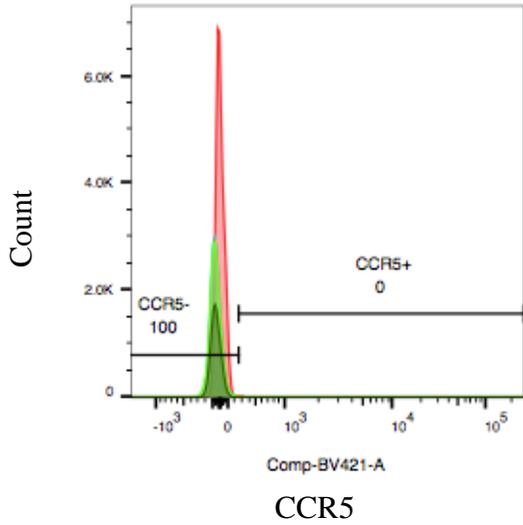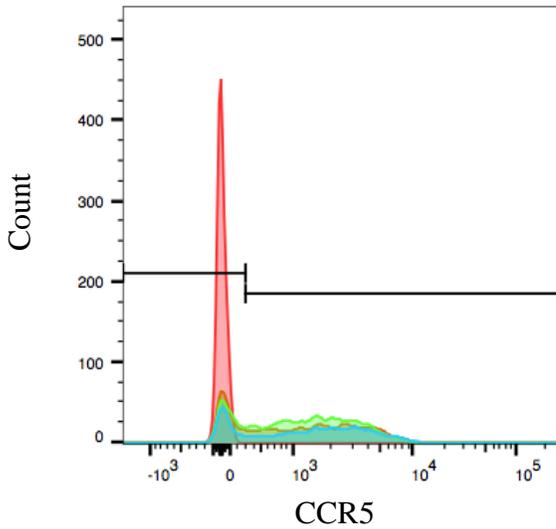
	Sample Name	Subset Name	Count
■	BC97 DPV dilution_1uM DPV_029.fcs	Single Cells	14116
■	BC97 DPV dilution_10nM DPV_030.fcs	Single Cells	24150
■	BC97 Maraviroc dilution_1uM Maraviroc_026.fcs	Single Cells	24829
■	BC97 Maraviroc dilution_10nM Maraviroc_027.fcs	Single Cells	25005
■	BC111_Untreated_001.fcs	Single Cells	43880

Ancestry Subset Statistic For	Lymphocytes/ Single Cells CCR5+ Freq. of Single Cells	Lymphocytes/ Single Cells CCR5- Freq. of Single Cells
BC97 DPV dilution_1uM DPV_029.fcs	0.17	99.8
BC97 DPV dilution_10nM DPV_030.fcs	0.56	99.4
BC97 Maraviroc dilution_Negative Control_025.fcs	0.60	99.4
BC97 Maraviroc dilution_1uM Maraviroc_026.fcs	3.19	96.8
BC97 Maraviroc dilution_10nM Maraviroc_027.fcs	2.36	97.6
BC111_Untreated_001.fcs	0	100
Mean	1.15	98.8
SD	1.31	1.31

Supplemental Figure 1. Example histogram of MVC or DPV treated PBMCs.

	Sample Name	Subset Name	Count
■	NRDI 213B_MVC 1uM NRDI 213B_003.fcs	Single Cells	1927
■	NRDI 213B_DPV 1uM NRDI 213B_004.fcs	Single Cells	3031
■	NRDI 213B_Negative Control NRDI 213B_001.fcs	Single Cells	2546
■	NDRI217B_Untreated_014.fcs	Single Cells	2853

Ancestry Subset Statistic For	Lymphocytes/ Single Cells Comp-BV421-A- Freq. of Single Cells	Lymphocytes/ Single Cells Comp-BV421-A+ Freq. of Single Cells
NDRI217B_Untreated_014.fcs	99.8	0.21
NRDI 213B_DPV 1uM NRDI 213B_004.fcs	22.3	77.7
NRDI 213B_MVC 1uM NRDI 213B_003.fcs	26.2	73.8
NRDI 213B_Negative Control NRDI 213B_001.fcs	29.7	70.3
Mean	44.5	55.5
SD	37.0	37.0

Supplemental Figure 2. Example histogram of MVC or DPV treated immune cells isolated from cervical tissue.

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