THE POTENTIAL OF BROADLY NEUTRALIZING HIV ANTIBODIES TO FUNCTION AS MICROBICIDES

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ABSTRACT

Microbicides are products designed for vaginal or rectal use to prevent transmission of the human immunodeficiency virus (HIV). The first generation non-antiretroviral (non-ARV) microbicide candidates were intended to be a low-cost, female-controlled method of HIV prophylaxis because young women in the poorest regions of the world are disproportionately affected by HIV. However, these early microbicide candidates were not HIV specific and some disrupted the vaginal epithelium, increased immune activation in the female genital tract, or disturbed vaginal flora, while others simply did not work. Due to the poor clinical success of these first-generation candidates, there was a shift in focus to developing antiretroviral (ARV) compounds like tenofovir and dapivirine as microbicides. However, ARV-based microbicides may not prevent transmission of drug-resistant HIV. Moreover, not all persons may want to use an ARV-based product due to the potential of drug side-effects and the risk of developing drug-resistance if the product is used inappropriately. While there has been progress in developing a product for oral HIV pre-exposure prophylaxis (PrEP), there are still no commercially available topical microbicide products. Topical microbicides are desirable because they deliver active agents directly to the vaginal or rectal mucosa where HIV

transmission occurs while avoiding systemic drug exposure. Hence non-ARV based microbicides are of great public health significance as a user-controlled tool for reducing the sexual transmission of HIV toward achieving the 2030 Sustainable Development Goal of ending AIDS and ensuring good health and well-being for all. Consequently, several years after the failure of the first generation of non-ARV vaginal gel microbicides, the lessons learned from these early trials have given rise to more rigorous preclinical evaluation protocols and novel formulation and delivery technologies for microbicides. This has resulted in renewed interest and new approaches to developing non-ARV microbicides. The new generation of non-ARV microbicide candidates being developed includes active biologics like broadly neutralizing monoclonal antibodies. This dissertation presents a pre-clinical evaluation of the potential of unformulated monoclonal HIV neutralizing antibodies to function as topical HIV microbicides *in vitro* and using human *ex vivo* models of rectal and vaginal mucosal transmission.

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DEDICATION

I dedicate this body of work, this process, this journey to my family – both biological and those whom I was fortunate enough to have acquired. You have been supportive and present despite the distance. I thank you for your smiles, for sending your good wishes and for sharing your love.

PREFACE

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In addition, parts of the Introduction, and the Discussion and Conclusions have been submitted as a review article to the journal *AIDS Reviews* and will be published as the following: *Scott YM, Dezzutti CS. 2016. Non-antiretroviral microbicides for HIV prevention. AIDS Reviews. Vol 18.*

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INTRODUCTION

The women of sub-Saharan Africa bear the greatest burden of incident HIV infections in the world. This is due in part to social factors that restrict their ability to negotiate condom use in their sexual relationships; intravaginal hygiene practices (1, 2); lack of perceived risk (3-5); and biological factors that may make them more susceptible to HIV infection (6, 7). To address this disparity, research has been ongoing from the early days of the HIV epidemic toward the goal of producing microbicides for women (8). Microbicides are products designed to prevent the sexual transmission of HIV, and potentially other sexually transmitted diseases. The original paradigm was a gel product that could be self-administered vaginally. The first products were gels because vaginal gel and cream products such as spermicides and vaginal yeast medications already existed and the technology for their manufacture was readily accessible. However, since then, the various candidates being considered and the dosage forms used for microbicide products have expanded. This introduction provides an overview of the evolution of non-antiretroviral (non-ARV)-based microbicide development from the first generation candidates through the new generation of highly HIV-specific microbicide candidates. The progress in their evaluation and options for their potential application in reducing sexual transmission of HIV will also be discussed.

MUCOSAL HIV TRANSMISSION

The early events surrounding HIV transmission during receptive vaginal or anal intercourse have been widely studied using various models. Semen from an HIV-infected partner is deposited in the vaginal or rectal lumen. Cell-free virus in seminal plasma or cell-associated virus in the form of HIV infected leukocytes present in the ejaculate may cross the protective epithelial barrier of the vaginal or rectal lumen via multiple putative mechanisms. Viral transmission though the mucosal route is thought to impose a genetic bottleneck mediated by the selective pressure of the mucosal environment and mucosal immune response (9). These conditions select viruses with a unique genetic signature that is not observed in later stages of infection (9-11).

Cell-free virions are thought to enter the submucosal space through breaks in the vaginal or anorectal epithelium that may occur during intercourse. They may also diffuse through intercellular spaces between the cells of the stratified squamous epithelium (12) of the vagina, ectocervix and rectum, which lack tight junctions. Columnar epithelial cells, which characterize the endocervical and colorectal epithelium, can capture virions at their apical surfaces, carrying them through the cell body and releasing them at the basolateral surface into the submucosa via transcytosis (13). Alternatively, sub-epithelial dendritic cells or Langerhans cells resident in the mucosal epithelium can use long dendritic extensions to sample the surroundings as part of their immune surveillance function (14). In this way, these cells may capture virions in the lumen or intraepithelial space for transfer in endosomal compartments to local $CD4^+$ T cells or those in regional lymph nodes which may then become productively infected (15, 16).

Cell associated virus transmission has been less thoroughly studied but HIV-infected leukocytes derived from semen have been shown to infiltrate recipient mucosal tissues (17). They may also form intercellular synapses with host immune cells through which CD4-mediated viral transfer may occur (18). Infected seminal leukocytes may also form interactions with epithelial cells through which there is directional shedding of nascent virus. These virus particles are endocytosed by the epithelial cells and released into the submucosa (13, 19) where they may encounter immune target cells.

The mechanisms described demonstrate the need for HIV preventatives that are able to abrogate the numerous mechanisms of HIV entry into the vaginal and anorectal mucosa. Hence topical products that were applied shortly before intercourse became a popular approach to microbicide design.

Microbicide Mechanisms of Action

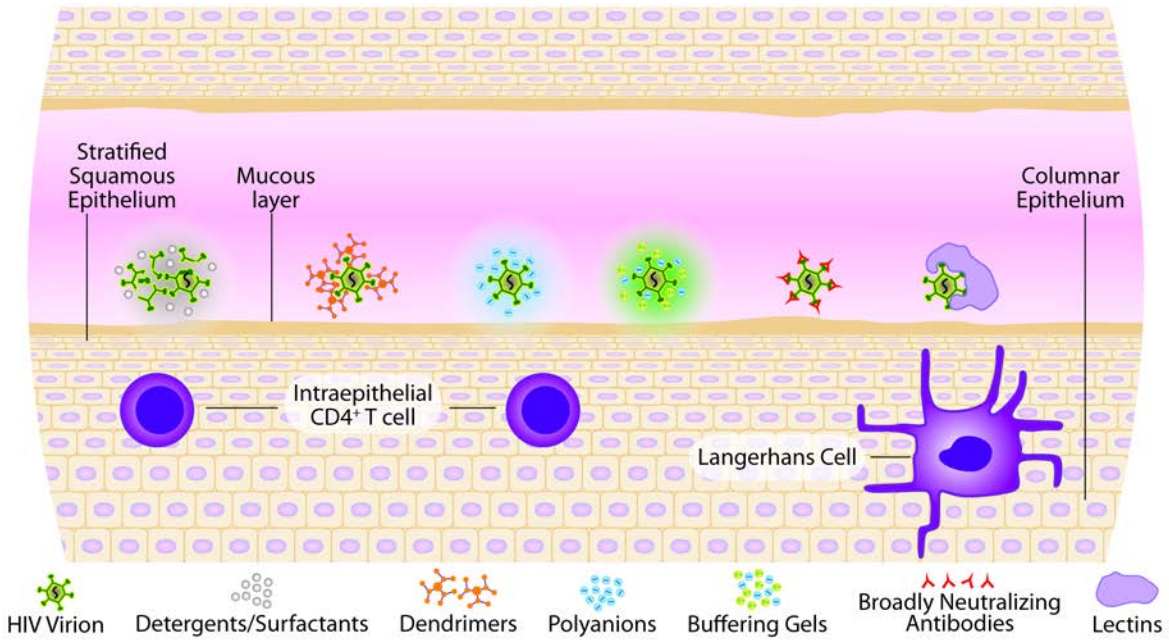


Figure 1. Non-antiretroviral (ARV) microbicide mechanisms of action in the vaginal lumen
Non-ARV microbicides were designed to prevent the establishment of HIV infection, mainly by preventing viral attachment and entry into target cells. In order to achieve this these products employed various mechanisms including lysing the virus to prevent infection (Detergents/Surfactants); inhibiting viral receptor or co-receptor engagement (Dendrimers, Broadly Neutralizing Antibodies, Lectins); bolstering natural defenses to infection (Buffering Gels); and disrupting viral envelope charge to prevent viral adsorption and fusion (Polyanions).

Topical microbicide products were originally intended to be applied inside the vagina where mucosal exposure to HIV-infected semen occurs. The first generation microbicides were designed to disrupt the HIV life cycle before infection was established. These microbicides were non-specific for HIV but exerted their anti-HIV activity by lysing the viral inoculum and preventing viral adsorption or entry into target cells (Figure 1). This was achieved by a variety of mechanisms including degrading the protective viral envelope; inhibiting successful viral interaction with entry receptors or co-receptors; restricting virion mobility to the epithelium by suspending viral particles in

high viscosity formulations; and disrupting the electrostatic interactions between virus and receptors that promote viral adsorption. The nature of some of these non-ARV-based products caused off-target effects which resulted in epithelial trauma and, in some cases, increased risk of HIV.

First Generation Non-Antiretroviral Microbicides

The first generation non-ARV microbicide candidates were a diverse array of compounds that included detergents, surfactants, polyanions, dendrimers, carrageenan derivatives and buffers, designed to inactivate the virus prior to infection of local immune cells and/or to bolster natural vaginal defenses to HIV infection. These early products had some degree of broad spectrum antimicrobial activity, partly due to their non-specific nature, which was desirable for simultaneous prevention of several sexually transmitted infections. An additional benefit of using non-ARV compounds was their inability to contribute to the development of drug resistance mutations in viral enzymes that could potentially arise with long-term use of ARV drugs. Thus non-ARV microbicides would be effective against wild-type and circulating drug resistant strains of HIV. Despite their promise, the first generation microbicide products were not successful in clinical evaluations due to lack of efficacy or poor safety indicators (Table 1).

Table 1. Outcomes of clinical trials evaluating first-generation non-ARV microbicides.

Microbicide Candidate	Clinical Trial Outcome	*References*
N-9 (Surfactant)	▪ No reduction in HIV incidence; higher incidence of genital ulcers	*(20)*
	▪ No reduced incidence of HIV, gonorrhea or chlamydia	*(21)*
	▪ Enhanced HIV acquisition	*(22)*
SAVVY (Surfactant)	▪ Did not prevent male-to-female HIV transmission	*(23)*
	▪ Higher incidence of reproductive tract adverse events	*(24)*
Carraguard (Carrageenan derivative)	▪ Did not prevent HIV transmission	*(25)*
BufferGel (Carbomer polyacrylic acid)	▪ Did not prevent HIV transmission	*(26)*
	▪ Did not prevent other sexually transmitted infections	*(27)*
PRO 2000 (Naphthalene sulfonate)	▪ Ineffective in reducing HIV acquisition	*(28)*
Cellulose Sulfate (Sulfated polymer)	▪ Increased HIV incidence with cellulose sulfate use, trial being halted early	*(29)*
VivaGel (Dendrimer)	▪ Mild to moderate perturbations of genital and urinary tract microflora	*(30, 31)*
	▪ Poor acceptability	*(32)*
ACIDFORM (Buffering gel)	▪ Mild to moderate genitourinary irritation	*(33)*
	▪ Inconclusive microbicidal efficacy	

The nonionic surfactant, nonoxynol-9 (N-9) has long been used in commercially available spermicidal products and was the first investigational microbicide product. N-9 showed potent activity against HIV-1 (34, 35) and herpes simplex virus type 2 (HSV-2) (35) *in vitro*, and consequently was evaluated as a microbicide for HIV prevention.

However, results from multiple clinical trials showed discrepancies in N-9 efficacy and safety. One prospective study enrolling HIV seronegative women to use an N-9 contraceptive sponge did not show reduced HIV incidence compared to placebo. In addition, participants in the N-9 arm had a higher incidence of genital ulcers than the placebo group (20). Another double-blind, placebo-controlled study evaluated N-9 efficacy in a cohort of HIV negative female sex-workers and showed no reduced incidence of HIV, gonorrhea or chlamydia (21). While these studies demonstrated no efficacy in reducing HIV incidence, one other study showed enhanced HIV acquisition in the N-9 treatment arm (22). Further *in vitro* studies showed that vaginal use of N-9 may cause tight-junction disruption in vaginal epithelial cells (36), and that N-9 was toxic to cells and tissues at doses suggested for therapeutic outcomes (37).

SAVVY vaginal gel, containing 1% C31G, was evaluated as a topical broad spectrum vaginal microbicide. C31G is a combination of two surfactants shown to have comparable spermicidal efficacy to N-9 (38). Similar to N-9, safety data for SAVVY was variable. In animal studies, SAVVY showed a favorable safety profile in pig tailed macaques (39) but increased inflammation and epithelial disruptions after a single use in a mouse model (40). In phase I safety studies to evaluate SAVVY as a contraceptive product, SAAVY was deemed less irritating than N-9 in women (41) and had favorable acceptability among their male partners (42). SAVVY also inhibited HIV-1 *in vitro* (43) and *Chlamydia trachomatis* infection (44) *in vitro* and in macaque studies (39). However this benefit did not translate to humans, as two phase III clinical efficacy studies were largely inconclusive. These trials indicated no benefit to using SAVVY to prevent male-

to-female HIV transmission (23, 24) and showed a higher incidence of reproductive tract adverse events with SAVVY use in one of the trial cohorts (24). Other products using various forms of surfactants did not reach human studies due to the concerns raised by these early trials. As a result, the microbicide field turned toward non-specific polyanionic gels, which were expected to be safer.

The polyanionic gels comprise a diverse group of large molecules, including sulfated and sulfonated compounds. These compounds interact non-specifically with the positively charged HIV-1 gp120, interfering with viral adhesion or fusion to target cell membranes. Additionally, some polyanionic compounds also demonstrated inhibitory activity against other viruses such as influenza, HSV type 1 and 2 (HSV-1, HSV-2), and sexually transmitted pathogens like *C. trachomatis* and *Neisseria gonorrhoeae (45)*, indicating their potential as broad spectrum microbicides.

Carraguard was one of multiple iterations of polysulfonated carrageenan derivatives evaluated for their antiviral properties. Carraguard gel, comprised of 3% κ- and λ-carrageenan, had inhibitory activity against HSV-2, human papilloma virus (HPV), and HIV transmission *in vitro* (46, 47). It also had favorable safety and acceptability profiles in phase I/II clinical studies (48, 49). However, more extensive pre-clinical testing of Carraguard showed that while it was safe, it was not efficacious against HIV, especially against clade A, C and CRF01_AE viruses (50) which are found in Africa and Asia. In a subsequent phase III clinical efficacy study, Carraguard failed to prevent HIV transmission (25).

PRO 2000 was a naphthalene sulfonate polymeric gel that demonstrated potent activity against HIV-1 (51) and HSV-2 (52), and reduced *N. gonorrhoeae* (53) infection *in vitro* and in animal studies. Despite these positive results, pre-clinical evaluation in human tissues *ex vivo* showed that although PRO 2000 gel was effective against HIV, use of the 4% PRO 2000 gel caused tissue toxicity (54), indicating a low therapeutic index. Lower doses were investigated and in a phase II/IIb clinical trial, use of 0.5% PRO 2000 gel did not affect the incidence of *N. gonorrhoeae*, *C. trachomatis* or *T. vaginalis* infections (27), and had a modest, but not significant 30% reduction in HIV acquisition (26). A subsequent phase III clinical trial of 9000 women ultimately showed that PRO 2000 was ineffective in reducing HIV acquisition (28).

Cellulose sulfate gel, another polyanionic gel product, was evaluated for use as a contraceptive and HIV microbicide. It showed inhibition of HIV (51, 55) and HSV-2 (55, 56) transmission in *in vitro* studies; but in human efficacy trials cellulose sulfate increased HIV incidence, resulting in the trial being halted early (29). The increased transmission could be attributed to disruption of epithelial tight-junctions, which allowed HIV virions to gain better access to the underlying immune cells in the lamina propria (36). In addition, subsequent work in a murine model showed seminal fluid reduced the potency of polyanionic microbicide gels (14), suggesting that seminal proteins may neutralize the charge of the polyanionic compounds. The lack of efficacy demonstrated by the polyanionic gels in clinical trials decreased enthusiasm for further work with these compounds.

Dendrimers are synthetic three dimensional nanoparticles, consisting of several branching units that radiate from a central core (57) (pictured in Figure 1). While these macromolecules were originally used as tools for drug delivery, the use of the polyanionic $_{L}$-lysine-based SPL7013 dendrimer in an HIV candidate microbicide gel was among the first uses of dendrimers as bioactive agents. A carbopol gel formulation of SPL7013, VivaGel, showed efficacy in reducing HSV-2 infection in animal studies using mouse and guinea pig models (58). Also, vaginal application of SPL7013 gel protected pigtailed macaques from vaginal SHIV challenge (59). In human studies, although VivaGel was found to be safe; it caused mild to moderate shifts in genital and urinary tract microflora to Gram negative bacteria (31, 32) and was poorly accepted by study participants (30). Hence, VivaGel is no longer being investigated as an HIV microbicide product.

Buffering gels were another group of non-ARV products considered as microbicide candidates. These gels were acidic with modest buffering capacity and could maintain the vaginal pH of 4.5–5 after ejaculation in the vaginal lumen. Semen has been shown to raise the pH of the vaginal lumen to pH 7 or higher as quickly as 30s after ejaculation (60). The resulting higher pH can last for up to 2h and is thought to help stabilize HIV virions in the vagina, thus increasing opportunities for infection (61). One such gel, BufferGel, is a carbomer polyacrylic acid gel and demonstrated a favorable safety profile (62) and activity against *C. trachomatis* and *N. gonorrhoeae* infection *in vitro* and in animal models (53, 63). BufferGel also demonstrated contraceptive properties in

animal models (64), and inhibited sperm motility *in vitro* (46). However, in clinical trials, BufferGel was ineffective in reducing HIV (26) and other sexually transmitted infections (27); and women using BufferGel had no reductions in pregnancy compared to the placebo gel users (28). Another buffering gel, ACIDFORM, was also investigated due to its ability to maintain acidic pH and acidify semen in the vagina. ACIDFORM delivers $_{L}$-lactic acid to the vaginal lumen – the lactic acid isomer produced by commensal *Lactobacilli* that colonize the vagina. It also showed some efficacy as a possible treatment for bacterial vaginosis (BV) (33), had contraceptive properties, and demonstrated protection from *N. gonorrhoea* (65) and HSV-2 (66) transmission in animal studies. However, in phase I clinical safety studies ACIDFORM use was associated with mild to moderate genitourinary irritation (33).

Antiretroviral-Based Microbicides

Safety concerns and the lack of efficacy demonstrated by the first-generation microbicide candidates caused a general shift in the field of microbicides to explore HIV-specific compounds; hence ARV-based microbicides were investigated. The first ARV to be evaluated as a microbicide was the nucleotide reverse transcriptase inhibitor (NRTI), tenofovir (TFV). This drug was evaluated as a 1% TFV topical gel formulation and demonstrated mixed results in several phase IIb/III clinical studies. In CAPRISA 004, a 1% TFV vaginal gel was evaluated for efficacy in sexually active women using the product before and after sex (twice in a 24h period; BAT24) and showed a 39% reduction in HIV risk overall and 54% reduction in risk in those who achieved high levels of adherence (67). Two other studies of the efficacy of 1% TFV gel in cohorts of young

women unfortunately showed no benefit amidst poor adherence by the participants (68, 69). While these studies may indicate the need to explore other options to reduce HIV transmission in high-risk young women, they also provide proof-of-principle that topical PrEP could work if there is adherence to the regimen (70).

The NNRTI, dapivirine, is another ARV being evaluated for use as a microbicide. Dapivirine was licensed for development as a microbicide after the compound showed poor oral bioavailability that precluded its use as an HIV therapeutic. Dapivirine microbicides were first formulated as vaginal gels (71-73), which showed good efficacy against HIV in animal studies (74). Additionally, vaginal application of dapivirine gel formulations resulted in low systemic absorption (71, 72), while high sustained concentrations in cervicovaginal tissues were maintained – a favorable pharmacokinetic profile for an ARV-based microbicide. One of the caveats to using ARVs as microbicides is the increased risk of developing drug resistance with inappropriate use. Strategies to decrease this risk include reducing systemic exposure to the ARV and improving user adherence by using long-acting formulations that provide sustained delivery of the drug to susceptible tissues instead of on-demand preparations such as gels. Various formulation strategies have been attempted to optimize sustained dapivirine delivery. Among them are polymeric films (75-77) and vaginal rings (78, 79). The most developed dapivirine product is a vaginal ring that has shown favorable safety and acceptability data in phase I clinical trials (78), and a single entity vaginal ring formulation of dapivirine is being evaluated in two concurrent phase III trials (80, 81).

New Non-Antiretroviral Microbicides

Although ARV microbicide candidates have had some success in clinical trials, efforts have recently been refocused on developing non-ARV alternatives. This is amid concerns of increased transmitted HIV resistance with larger scale use of ARVs for prevention of mother-to-child transmission and increased access to combined antiretroviral therapy (cART). It has also been widely acknowledged that there is not a "one-size-fits-all" microbicide product to meet the needs of every susceptible niche population. Hence, non-ARV microbicides may increase the options available for HIV prevention. The new non-ARV microbicide candidates are highly HIV-specific, in comparison to the first generation microbicides, and include active biologics like algal and bacterial lectins, as well as broadly neutralizing monoclonal antibodies (nAbs) specific for HIV envelope epitopes.

Cyanovarin-N, a mannose-binding lectin isolated from the cyanobacterium *Nostoc ellipsosporum*, binds to HIV gp120 in a manner that is independent of CD4 receptor or coreceptor interactions (82). Its activity inhibits HIV binding and entry, thus reducing HIV transmission. Cyanovarin-N has been shown to prevent vaginal acquisition of SHIV in nonhuman primate models (83) and in human *ex vivo* tissue culture (84). Another lectin, Griffithsin is a mannose-specific lectin isolated from the marine red alga *Griffithsia sp*. This protein binds to HIV virions, preventing viral adsorption to target cells and causing an irreversible inhibition of HIV infectivity. Griffithsin has shown potent cross-clade anti-HIV activity (85) and broad spectrum antiviral activity against HSV-2 (86), hepatitis C (87), and coronaviruses (88). The lectins have generally had favorable safety profiles *in*

vitro (89, 90); and in macaque studies (83); although cyanovarin-N was shown to have mitogenic effects and cause increased expression of inflammatory markers in PBMC cultures (91). Development of a cyanovarin-N microbicide is ongoing and work is in progress to define clinical safety and efficacy metrics for a rectal-specific griffithsin gel product.

Broadly neutralizing antibodies to HIV were isolated from chronically HIV infected individuals and have demonstrated cross-reactivity to a variety of HIV strains *in vitro*. These antibodies develop broad neutralizing capacity over time through a process of somatic hypermutation (92). HIV neutralizing antibodies have been the focus of vaccine researchers. However, their potential as HIV microbicides is now being explored. These antibodies bind to key regions on the HIV gp120 envelope protein, preventing interactions with entry receptors and co-receptors on host target cells, thereby reducing viral entry. Neutralizing antibodies were shown to inhibit HIV infection *in vitro* (93-95) as well as in mouse (96) and macaque models (97-99) of infection. Importantly, animal studies involving topical vaginal application of neutralizing antibodies with subsequent vaginal viral challenge have provided proof-of-concept that antibodies can retain their function in mucosal secretions and are a viable microbicide (96, 100).

Although the first phase of microbicide research did not result in a successful non-ARV microbicide product, advances were made in HIV prevention science, and more comprehensive pre-clinical evaluation models were developed that could better predict the efficacy and safety of potential microbicide candidates. These include efficacy and

toxicity evaluations in human *ex vivo* tissue models of mucosal HIV transmission (54, 101); and the identification of safety biomarkers that may predict microbicide failure (102, 103).

In this dissertation, nAbs are assessed for their potential to function as HIV microbicides. This evaluation interrogates nAb efficacy in preventing attachment and entry of HIV into target cells in human ectocervical or colonic tissues *ex vivo,* incorporating models of cell-free and cell-associated HIV transmission. Most evaluations of HIV therapeutics and preventatives are undertaken using models of cell-free HIV transmission. However cell-associated transmission of HIV is believed to be more efficient than cell-free transmission at mucosal surfaces and has been shown to occur during sexual transmission of HIV (17). In addition, increases in circulating drug-resistance resulting from more widespread use of cART makes it necessary to ensure microbicide efficacy against drug resistant strains of HIV. Hence, the efficacy of nAbs to prevent transmission of cell-associated and drug-resistant cell-free HIV are investigated.

STATEMENT OF HYPOTHESES AND EXPERIMENTAL AIMS

Hypotheses

1) nAbs will reduce transmission of cell-free HIV but will show reduced potency in the presence of semen caused by factors in semen that enhance HIV infection and affect protein interactions.

2) nAbs will be effective against cell-associated virus but will demonstrate reduced potency due to reduced access to target viral epitopes during cell-associated transmission via intercellular synapses.

3) nAbs will retain efficacy against viruses that have resistance to HIV enzyme inhibitors.

Experimental Aims

Aim 1: Evaluate the efficacy and safety of nAbs against cell-free HIV using human tissue *ex vivo*.

The IC_{90} values for nAbs against cell-free HIV-1_{JR-CSF} will be established *in vitro* to determine the comparative efficacy of the nAbs used in this study. The functional efficacy of nAbs in preventing HIV transmission in the presence or absence of semen will then be evaluated *ex vivo*, using ectocervical and colonic tissue obtained from non-infected donors. Additionally, the safety of topically applied nAbs will be assessed by comparisons of tissue viability and architecture after nAb exposure, as well as quantitative cytokine and chemokine expression associated with the use of nAbs *ex vivo*. These data will establish the functional capacity of nAbs to prevent mucosal transmission of HIV in a biologically relevant human model.

Aim 2: Evaluate nAbs against drug-resistant and cell-associated HIV using human tissue *ex vivo*.

The IC_{90} of nAbs against a panel of WT and drug resistant strains of HIV will be used to determine their comparative susceptibility *in vitro*. nAb efficacy in preventing cell-cell transmission will also be evaluated in mucosal tissues *ex vivo* using a novel model of cell-associated HIV derived from the PM1 cell line. These studies will probe the limits of protection conferred by a putative nAb microbicide using inocula that are relevant but often overlooked in microbicide evaluations.

CHAPTER 1: EFFICACY OF BROADLY NEUTRALIZING ANTIBODIES IN PREVENTING MUCOSAL TRANSMISSION OF CELL-FREE HIV-1

1.1 CHAPTER OVERVIEW

Broadly neutralizing monoclonal antibodies (nAbs) specific for HIV are being investigated for use in HIV prevention. Due to their ability to inhibit HIV attachment and entry into target cells, nAbs may be suitable for use as topical HIV microbicides. As such, they would present an alternative intervention for individuals who may not benefit from using antiretroviral-based products for HIV prevention. We theorize that nAbs can inhibit viral transmission through mucosal tissue, thus reducing the incidence of HIV infection. The efficacy of PG9, PG16, VRC01, and 4E10 antibodies was evaluated in an *ex vivo* human model of mucosal HIV transmission. nAbs reduced HIV transmission; causing 1.5 – 2 $\log_{10}$ reduced HIV replication in ectocervical tissues and ≈ 3 $\log_{10}$ reduction in colonic tissues over 21 days. These antibodies demonstrated greater potency in colonic tissues, with a 50-fold greater dose being required to reduce transmission in ectocervical tissues. Importantly, nAbs retained their potency and reduced viral transmission in the presence of whole semen. No changes in tissue viability or immune activation were observed in colonic or ectocervical tissue after nAb exposure. Our data suggest that topically applied nAbs are safe and effective against

HIV infection of mucosal tissue and support further development of nAbs as a topical microbicide that could be used for anal as well as vaginal protection.

1.2 INTRODUCTION

With circulating drug-resistant virus on the rise in many communities where pre-exposure prophylactics (PrEP) will be used (104), the risk of transmitting virus with reduced susceptibility to ARVs is possible. To circumvent this risk, several non-ARV microbicide candidates are being considered. Previous investigations of non-ARV microbicides used non-HIV specific formulations of compounds like surfactants, polyanions, and buffering agents; yet all were ineffective in preventing HIV acquisition. Among them, the surfactant nonoxynol-9 (N-9), which is commercially available as a spermicide, was shown to have anti-HIV activity *in vitro* (35). However, clinical evaluation of N-9 was stopped due to increased HIV incidence in women using an N-9 vaginal gel (22). Additionally, N-9 was shown to cause tight junction disruptions in epithelial cells *in vitro* (36) and to be harmful to beneficial vaginal flora (105). Other candidates such as BufferGel (26); the carrageenan derivative, Carraguard (25); and the polyanionic gels (PRO 2000 (26) and cellulose sulfate (36)); were all unsuccessful as HIV microbicides. Unlike the previous candidates, the new non-ARV microbicide candidates being investigated are HIV-specific agents and include broadly neutralizing monoclonal antibodies (nAbs).

While nAbs have been investigated extensively in the development of HIV vaccines, increased focus has been placed on their development for HIV prevention. Originally isolated from chronically HIV infected individuals, nAbs were shown to retain potent neutralizing activity across a broad range of HIV clades (95). These highly cross-reactive antibodies are only found in a small subset of HIV-infected individuals (92, 106), and develop their broad cross-reactivity through a process of somatic hypermutation over 2-4 years (92, 107). They bind epitopes on key regions of the HIV envelope and directly inhibit the ability of the virions to engage entry receptors on target cells, thereby reducing viral infection. nAbs like VRC01, b12 and NIH 45-46 exert their HIV inhibitory activity by bind to the CD4 binding site (108); while 4E10, 10E8 and 2F5 bind to the membrane proximal external region (MPER) at the base of the viral envelope spike (108); and others, like PG9 and PG16, recognize quaternary glycosylation motifs on the exposed variable loops of gp120 (108). nAbs have shown efficacy in reducing HIV transmission *in vitro* (93, 95) and *in vivo* using animal models of HIV transmission (96, 100). This ability to inhibit viral transmission is particularly important in the context of HIV prevention, as it is better to prevent HIV acquisition, than to overcome the complications inherent to treating an established HIV infection. nAbs also bridge the gap between non-HIV specific compounds and ARV drugs in the spectrum of HIV microbicide candidates. The antibodies are specific for HIV but their activity has not been shown to affect viral sensitivity to HIV enzyme inhibitors. Conversely, viral neutralization by nAbs is not expected to be hampered by drug resistance mutations in traditional ARV drug targets – HIV reverse transcriptase, protease and integrase. This is because HIV envelope proteins are not under the selective pressure of HIV enzyme

inhibitors. Hence, nAbs are expected to retain efficacy against those viruses that are ARV-resistant.

In this chapter, the efficacy of nAbs – PG9, PG16, VRC01 and 4E10 – was evaluated in human mucosal tissue *ex vivo.* While studies of nAb efficacy have been conducted using animal models, this study is the first to evaluate the efficacy of topically applied nAbs using a relevant human mucosal model of HIV transmission. Individual nAb potency was assessed in the absence or presence of semen, in addition to their safety in mucosal tissue. The results of these assessments support the use of nAbs as a viable alternative to ARV-based HIV preventatives for dual compartment use.

1.3 MATERIALS AND METHODS

Reagents: Unless otherwise noted, culture media were purchased from Mediatech, Inc. (Manassas, VA); serum and media supplements were purchased from Gemini BioProducts (West Sacramento, CA). Whole human semen from pooled donors was purchased from Lee BioSolutions (St. Louis, MO).

Antibodies: VRC01-N, 4E10-N and HSV-8-N monoclonal antibodies were generously provided by Dr. Kevin Whaley (Mapp Biopharmaceuticals Inc., San Diego, California). These antibodies were produced in genetically engineered *Nicotiana benthamiana* plants, a species closely related to tobacco (109); hence in this paper they have been given a '–N' designation. PG9 and PG16 monoclonal antibodies produced in Chinese

hamster ovary (CHO) cells, were provided by the International AIDS Vaccine Initiative (La Jolla, CA). All antibodies used are IgG_1 isotype. The HSV-8-N antibody has specificity for an HSV-1/2 envelope epitope and was used as a non-HIV-specific isotype control.

Virus: HIV-1_{JR-CSF} was chosen as the inoculum for the current studies because it is a CCR5-using virus which preferentially infects T cells over macrophages (110) and retains transmitter/founder virus qualities (111, 112). The pYK-JR-CSF molecular clone was purchased from ATCC (Manassas, VA). 293T cells were a gift from Christina Ochsenbauer-Jambor and John C. Kappes from the University of Alabama, Birmingham. 293T cells and were cultured in complete DMEM (cDMEM; 1× DMEM supplemented with v/v 10% heat-inactivated fetal bovine serum (FBS) and 1% Pen-Strep-L-Glut). 293T cells were transfected with proviral DNA using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). After 48h, mature HIV virions were harvested from the culture medium by filtration through a 0.45 µM pore syringe filter. The tissue culture infectious dose ($TCID_{50}$) was determined in activated human peripheral blood mononuclear cells (Central Blood Bank, Pittsburgh, PA) using the Reed-Muench method (113).

Assessment of nAb neutralization activity *in vitro*: TZM-bl cells (114) were obtained through the NIH Research Reagent Program, Division of AIDS, NIAID, NIH: Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc., and were cultured in cDMEM. Individual wells of a 96-well plate were seeded with 1×10^4 cells which were allowed to adhere

overnight. Culture medium in each well was replaced with 100 µL of 2× nAb dilutions or with cDMEM in untreated control wells, and cells were incubated for 1h at 37°C. Treated cells and untreated controls were inoculated with 3,000 $TCID_{50}$ $HIV\text{-}1_{JR\text{-}CSF}$ in 100 µL and cultured for 48h. Tat-activated luciferase expression was detected using the Bright-Glo Luciferase Assay reagent (Promega, Madison, WI) and luminescence was measured using the SpectraMax M3 plate reader (Molecular Devices, LLC; Sunnyvale, CA). The 90% inhibitory concentrations (IC_{90}) were determined using SigmaPlot software version 11.0 (Systat Software, Inc., San Jose, CA).

***Ex vivo* tissue culture:** Normal human ectocervical tissues were obtained from pre-menopausal women undergoing routine hysterectomy after informed consent (IRB #PRO09110431) or purchased from the National Disease Research Interchange (NDRI; http://ndriresource.org/) and transported overnight on wet ice. Polarized explants were prepared from the specimens as described elsewhere (54). Briefly, the ectocervical epithelium was trimmed of the muscularis and 5 mm diameter explants were mounted with the epithelium upward in 12 mm permeable trans-well supports and sealed in position with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA). Ectocervical explants were activated with 1 µg/mL phytohemmaglutinin-P (PHA-P) and cultured in DMEM supplemented with v/v 10% human A/B serum, 1% Pen-Strep-L-Glut, 100 U/mL human interleukin-2 (hIL-2) (Roche Diagnostics, Indianapolis, IN) and 1% non-essential amino acids (Lonza, Walkersville, MD).

Human colonic tissue was obtained after informed consent (IRB #PRO09110431) from individuals undergoing scheduled colon resection for non-inflammatory conditions (101). The epithelium and lamina propria were trimmed of excess adipose tissue and muscle, and 5mm diameter explants were mounted with the epithelium upward on gel foam inserts in 12mm permeable trans-well supports and sealed with Matrigel. Colonic explants were activated with 1 mg/mL PHA-P and cultured in RPMI-1640 supplemented with v/v 5% human A/B serum, 1% Pen-Strep-L-Glut, 0.5 mg/mL Zosyn (Wyeth, Collegeville, PA), 100 U/mL hIL-2 (Roche Diagnostics, Indianapolis, IN) and 2.5 mM Hepes (Hyclone, Logan, UT).

Efficacy evaluations in human tissue *ex vivo*: Paired polarized explants were treated with nAbs, 24h (colon) or 48h (ectocervix) after set-up. nAbs were applied at 2× the final concentration to the apical surface of the appropriate explants in duplicate and cultured for 1h at 37°C. Controls were treated similarly with either medium only or with the HSV-8-N antibody. After 1h, each explant was inoculated apically with 50,000 $TCID_{50}$ (ectocervix) or 10,000 $TCID_{50}$ (colon) HIV-1_{JR-CSF}, suspended in RPMI-1640 or 50% v/v pooled, whole human semen (Lee BioSolutions, St. Louis, MO). Tissues were incubated for 24h after which the basolateral medium was collected and the explants were washed with 1× DPBS (Mediatech, Inc., Manassas, VA). Basolateral media was replenished with fresh media supplemented with 100 U/mL hIL-2, and subsequently collected every 3 to 4 days, up to 21 days post infection and stored at -80°C. Viral replication was monitored by measuring HIVp24 in the basolateral media using the Alliance HIV-1 p24 Antigen ELISA kit (PerkinElmer, Waltham, MA). Individual ectocervical explants were

fixed in formalin at 21 days post-infection and processed for immunohistochemical (IHC) analysis of intracellular HIVp24 antigen (54).

Evaluation of immune activation and tissue viability *ex vivo*: Non-activated, polarized ectocervical and colonic explants were treated on the apical surface with 1.5 µM or 0.03 µM nAbs, respectively. For controls, tissues were treated with medium only (negative control) or with 1 µg/mL PHA-P and 100 U/mL hIL-2 in the basolateral medium (positive control). All treatments were performed in duplicate and tissues were cultured for 24h at 37°C and 5% CO_2. Basolateral media was sampled 24h after treatment and used for quantitative comparison of inflammatory cytokines (IFN-γ, IP-10, IL-1β, IL-6, IL-8, TNF-α, GM-CSF, IL-12, MIP-1β, IL-18 and IL-15) using a Milliplex™ Human Cytokine/Chemokine Magnetic Bead panel (EMD Millipore Corp., Billerica, MA). For viability assays, non-activated, polarized explants were treated with nAbs as described above for 24h. Control tissues were treated apically with medium only or a 1:5 dilution of Options Gynol II® Extra Strength gel (Caldwell Consumer Health LLC, Madison, NJ), containing 3% N-9. After 24h treatment, explants were processed for histology and viability determination by MTT assay.

Statistical analysis: All variables were inspected using descriptive statistics and graphical methods. For analyses, raw HIVp24 values were log-transformed because their distribution was heavily skewed. Linear mixed models were used to investigate the effects of semen, nAb treatment and different nAb doses on the trajectory of HIVp24 during the 21 day culture period. For all models, random effects for explants and tissue

donors were included to adjust for any clustering effect of repeated measures within each explant and tissue donor. Separate models were used for ectocervix and colon data.

The model used to evaluate the effect of different nAbs on HIVp24 included dummy variables for nAbs, day of culture, and the interaction between day of culture and individual nAbs as fixed effects. To evaluate the effect of semen on nAb potency, the model included the presence or absence of semen, day of culture, and the interaction between these factors as fixed effects. The effect of semen on HIVp24 was also investigated while adjusting for the effect of nAbs using a model that included the presence or absence of semen, dummy variables for each nAb, day of culture, the interactions between day of culture and semen or nAb treatment as fixed effects. The effect of individual nAbs at each dose was investigated with dose, day of culture, dummy variables for each nAb, and interactions between day of culture and dose, or each nAb as fixed effects. A model using dose, day of culture, and their interaction term as fixed effects was used to interrogate the effect of nAb dose on HIVp24. Finally, the effect of semen and nAb dose was investigated separately for each antibody, using a model that included the effects of semen, nAb dose, and their interaction separately for each antibody. All analyses were performed in R (R Foundation for Statistical Computing, Vienna, Austria).

Comparison was made between treatment and viability outcomes from MTT viability experiments using one-way ANOVA. The effect of nAb treatment on cytokine

concentrations were compared using two-way ANOVA and the Holm-Sidak correction for multiple comparisons. Colon and ectocervix data were analyzed separately and analysis was performed using GraphPad Prism version 6.05 (GraphPad Software, La Jolla, CA).

1.4 RESULTS

1.4.1 Assessment of nAb efficacy *in vitro*

Using TZM-bl cells, the IC_{90} of all nAbs against HIV-1_{JR-CSF} infection *in vitro* was derived. The potency of nAbs, VRC01-N and 4E10-N, produced in the transgenic *N. benthamiana* expression system were also compared to the same nAbs produced in the traditional CHO cell system (VRC01 and 4E10) and were shown to have equivalent inhibitory activity ($p>0.05$) (Table 2). The IC_{90} values indicated that nAb potency followed the order of PG16 > PG9 > VRC01/VRC01-N >> 4E10/4E10-N. Due to their equivalence to CHO cell-produced nAbs and greater availability, the *Nicotiana*-produced antibodies were used in the subsequent work.

Table 2. Comparative *in vitro* efficacy of nAbs against HIV_{JR-CSF} infection of TZM-bl cells.

nAb	IC_{90} (μM)	SDev	*p-value*
PG16	0.000435	0.000268	
PG9	0.00410	0.003981	
VRC01	0.0734	0.059379	*0.165*
VRC01-N	0.0298	0.019791	
‡**4E10**	0.727	n/a	*0.350*
4E10-N	1.19	0.375102	

Results are the mean of 4 or more experiments.

‡Result of 1 experiment

1.4.2 nAbs reduce HIV transmission in human ectocervical tissue *ex vivo*

In experiments to define nAb potency in mucosal tissue, equivalent molar concentrations of IgG for all nAbs was used. As the IC_{90} value of VRC01-N was intermediate in the range of the other nAbs used in this study (Table 2), the doses of all nAbs were standardized to the effective concentrations of VRC01-N. For ectocervical tissue, 1.5 μM and 0.3 μM IgG were used, which was the dose of IgG equivalent to 50× and 10× the IC_{90} of VRC01-N, respectively. Similarly, for colonic tissue, 0.03 μM and 0.003 μM were used, which was equivalent to 1× and 0.1× the VRC01-N IC_{90}. The HSV-8-N isotype control antibody was also used at a concentration of 0.3 μM in ectocervical tissues or 0.003 μM in colonic tissues.

nAbs were applied to the apical surface of tissues for 1h before inoculation with virus to simulate peri-coital application of a topical microbicide preparation. Using this strategy, treatment of ectocervical tissues with 1.5 µM and 0.3 µM doses of VRC01-N, PG9 and PG16 significant reductions in HIVp24 over the 21 day culture period ($p<0.0001$) compared to the untreated control. Treatment with 1.5 µM VRC01-N, PG9 or PG16 caused median reductions of 1.5, 1.9 and 1.8 $\log_{10}$ pg/mL HIVp24 at day 21 of culture, respectively, as compared to the untreated control (Figure 2a, Table 3). Conversely, treatment with 4E10-N did not significantly reduce HIVp24 ($p=0.5797$) (Figure 2a, Table 3), as the concentrations used were below the *in vitro* IC_{90} of this nAb (Table 2). However, tissues pre-treated with doses of 59.5 µM and 11.9 µM 4E10-N in ectocervical tissues and 1.19 µM and 0.119 µM in colonic tissues showed reduction in HIV transmission (Appendix B). These doses correspond to the 50×, 10×, 1× and 0.1× IC_{90} of 4E10-N (Table 2), respectively; and also reflect an 8 to 40-fold greater concentration than the effective doses of nAbs used in this study. Treatment with the isotype control antibody, HSV-8-N, also did not affect HIV replication ($p=0.2830$) (Table 3). There was also a greater reduction in HIVp24 with the use of 1.5 µM PG9 ($p=0.0171$) and PG16 ($p=0.0302$) at day 21 compared to 0.3 µM of these nAbs (Table 3). While there was a trend, the high dose of VRC01-N was not significantly different from the low dose ($p=0.0810$). This suggests that the neutralizing capacity of 0.3 and 1.5 µM doses of VRC01 may have been saturated by the inoculum used in the ectocervical model. The dose effect observed with PG9 and PG16 antibodies in the same model supports that these nAbs are more potent, and is concordant with the nAb hierarchy of potency observed *in vitro* (Table 2).

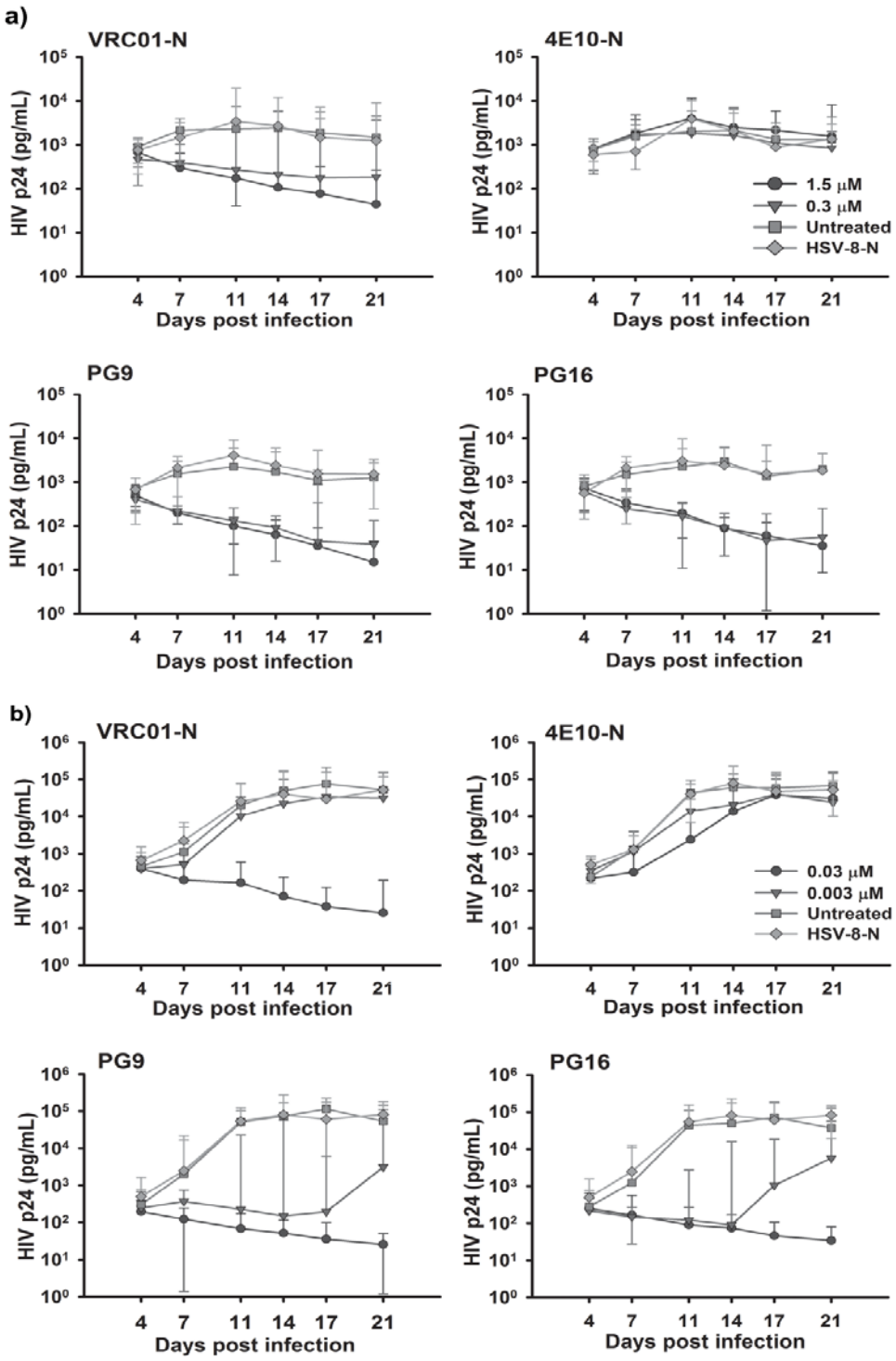


Figure 2. nAb efficacy in human mucosal tissue *ex vivo*
Tissues were treated for 1h before inoculation with HIV_{JR-CSF}. Viral replication was monitored in a) ectocervical and b) colonic tissues by p24 antigen ELISA on basolateral culture supernatants collected 4, 7, 11, 14, 17 and 21 days post infection. Data points represent the median and interquartile range of ≥5 tissues from individual donors.

Table 3. Effect of nAbs on HIVp24 at day 21 post-infection in human tissues *ex vivo*.

	Cervix			Colon		
Treatment	**Dose (μM)**	**Log_{10} Δ HIVp24**	***p-value***	**Dose (μM)**	**Log_{10} Δ HIVp24**	***p-value***
VRC01-N	1.5	-1.523	*<0.0001*	0.03	-3.315	*<0.0001*
	0.3	-0.898		0.003	-0.221	
4E10-N	1.5	+0.067	*0.5797*	0.03	-0.342	*0.1994*
	0.3	-0.192		0.003	-0.458	
PG9	1.5	-1.924	*<0.0001*	0.03	-3.319	*<0.0001*
	0.3	-1.516		0.003	-1.233	
PG16	1.5	-1.752	*<0.0001*	0.03	-3.037	*<0.0001*
	0.3	-1.547		0.003	-0.813	
HSV-8-N	0.3	-0.005	*0.2830*	0.003	+0.102	*0.3117*

p-values represent comparisons of the change in p24 over time in tissues treated with each nAb, regardless of dose, relative to untreated tissue.

1.4.3 Lower concentrations of nAbs are required to protect human colonic tissues *ex vivo*

Using 0.03 or 0.003 μM nAbs (Figure 2b), treatment with PG16 ($p<0.0001$), PG9 ($p<0.0001$), VRC01-N ($p<0.0001$) caused significant reductions in HIVp24 over the treatment period. Pretreatment of colonic tissues with 0.03 μM PG16, PG9 and VRC01-N yielded reductions of 3.0, 3.3 and 3.3 log_{10}, respectively, in median HIVp24 by day 21 of culture, compared to untreated controls (Figure 2b). A dose effect was observed in colonic tissues as tissues treated with the low doses (0.003 μM) of VRC01, PG9 or PG16 had decreases in median HIVp24 of 1.2 log_{10} or less at day 21 ($p<0.0001$)

compared to tissues treated with 0.03 µM nAbs (Table 3). However, treatment with 4E10-N had no effect on HIVp24 at any of the concentrations used (p=0.1994). Median reductions of <0.5 $\log_{10}$ observed in median HIVp24 at day 21 in tissues treated with 4E10-N did not reach statistical significance and support the lack of potency observed with 4E10-N in this study. Infection curves for those tissues treated with 0.003 µM PG9 or PG16 showed a delay in infection, not observed with VRC01-N, with virus production expanding 14-17 days post infection (Figure 2b). These results support that PG9 and PG16 demonstrated superior potency in colonic tissue as compared to ectocervical tissue (Figure 2a), and are also concordant with the order of nAb potency described in our *in vitro* assays (Table 2). Higher doses of nAbs showed greater response than lower doses of the same nAb in colonic tissues, with the exception of 4E10-N.

1.4.4 nAbs retain potency in the presence of semen

During *in vitro* evaluations of some non-ARV microbicides like the polyanionic gel, PRO 2000, semen was shown to abrogate its anti-HIV activity (115). The counteractive effect of semen on microbicides has been attributed to an activity of seminal proteins on microbicide moieties (115); the formation of amyloid fibrils that enhance HIV attachment to target cells (116); and the neutralizing effect of semen that lowers the vaginal pH, prolonging the survival of HIV virions (61, 117-119). Hence, the potency of nAbs was compared in the presence and absence of whole human semen in ectocervical (Figure 3a) and colonic (Figure 3b) tissues. Semen had no effect on HIVp24 concentrations (p=0.2382) or on nAb potency in ectocervical tissue (p = 0.0670) as compared to no-semen controls. In colonic tissues (Figure 3b), the presence of semen had no effect on

nAb potency during the 21 day culture period (p=0.0940), and comparison of HIVp24 also showed similar levels of HIV replication in the presence or absence of semen (p=0.1177). These data show no enhancement of HIV infection or effect on nAb activity in the presence of semen, and collectively support that nAbs applied topically would be effective in preventing sexual transmission of HIV.

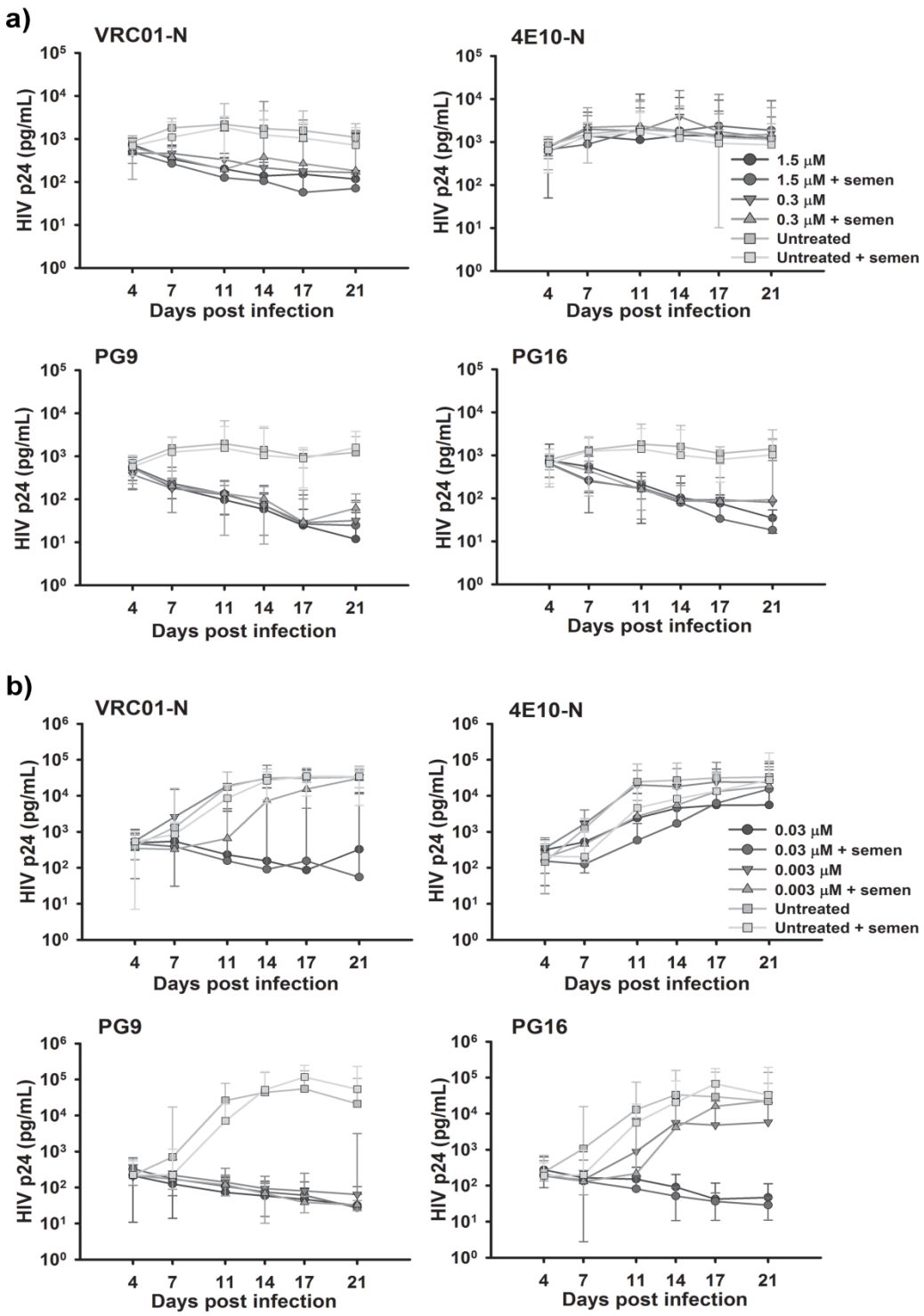


Figure 3. Potency of nAbs in the presence of semen in human tissues ex vivo
Tissues were treated for 1h before inoculation with HIV_{JR-CSF} in the presence or absence of 50% human semen. Viral replication was monitored in a) ectocervical and b) colonic tissues by p24 antigen ELISA on basolateral culture supernatants collected 4, 7, 11, 14, 17 and 21 days post infection. Data points represent the median and interquartile range of ≥5 tissues from individual donors.

1.4.5 Safety of nAbs

The safety of nAb treatment was assessed in ectocervical and colonic tissue explants using the MTT assay and histology. Tissue viability after 24h treatment with nAbs was compared to untreated tissues and tissues treated with N-9 (Figure 4a, b). There was no loss of viability with nAb treatment, compared to untreated tissues. However, treatment with N-9 decreased tissue viability to a mean of 13% percent ($p = 0.0007$) relative to untreated tissues in ectocervical tissue (Figure 4a) and 17% percent ($p = 0.0002$) in colonic tissue (Figure 4b). Colonic and ectocervical tissues treated with nAbs were histologically comparable to untreated tissues after 24h exposure to treatment. However, N-9-treated tissues showed considerable sloughing of the epithelium and focal necrosis (Figure 4a, b). Inflammatory cytokine levels were measured in the culture supernatants of tissues treated for 24h with 1.5 μM nAbs in ectocervix or 0.3 μM nAbs in colon. In ectocervical tissues, concentrations of GM-CSF, IFN-γ, IL-1β, MIP-1β and TNF-α were significantly elevated ($p<0.01$) by PHA + hIL-2 as compared to the untreated ectocervical tissues (Figure 4c). Cytokine concentrations between nAb-treated and untreated ectocervical tissues were not significantly different, with the exception of IP-10, which had significantly lower concentrations in tissues treated with VRC01-N compared to untreated tissues ($p<0.01$). In colonic tissues, 24h treatment with

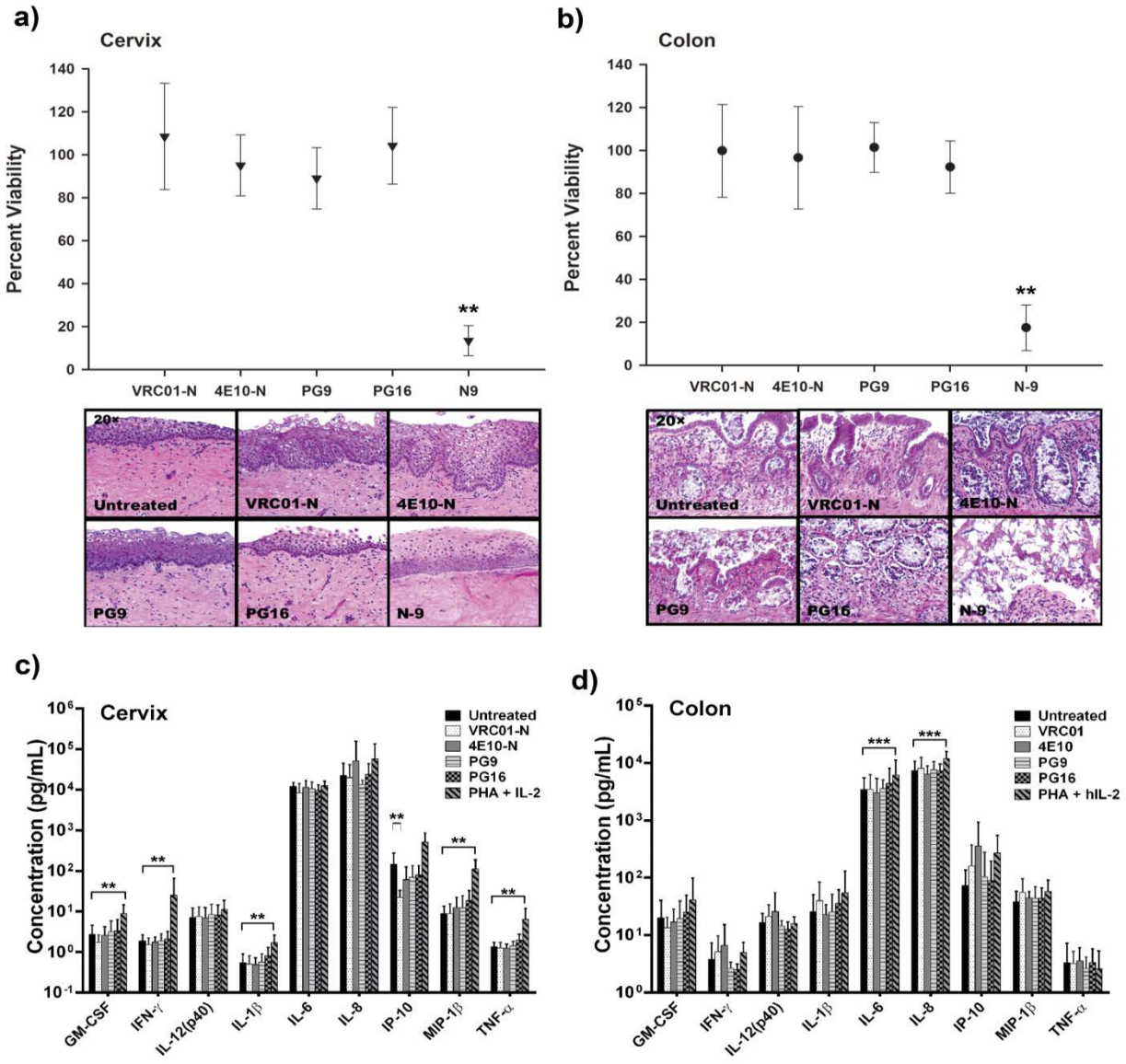


Figure 4. nAb safety in mucosal tissue

a) Ectocervical or b) colonic explants were treated for 24h with 1.5 µM (ectocervix) or 0.03 µM (colon) nAbs or N-9 for 24h. Viability was measured using the MTT viability assay and represented as the mean percent viability relative to untreated tissues and standard deviation; n = 5. Histologic panels show images of comparative hemotoxylin and eosin staining of representative tissues after 24h treatment. Images were captured at 20× magnification. Quantitative Luminex analysis was performed on c) ectocervical or d) colonic tissue culture supernatants after 24h treatment with nAbs or an immune activation cocktail containing PHA-P + hIL-2. The histogram represents the mean cytokine concentration (pg/mL) and standard deviation for each treatment, n = 3-5 tissues. Statistical comparisons were made using One- or Two-Way ANOVA with the Holm-Sidak correction for multiple comparisons, where $\alpha = 0.05$; ** $p<0.01$, *** $p<0.0001$.

nAbs did not cause significant differences in inflammatory cytokine concentrations compared to untreated tissues (Figure 4d). However, there was a significant increase in concentrations of IL-6 and IL-8 between untreated tissues and tissues treated with PHA + hIL-2 ($p<0.0001$). These data suggest that 24h treatment with nAbs does not adversely affect viability or expression of inflammatory cytokines in ectocervical or colonic tissues *ex vivo*.

1.5 DISCUSSION OF RESULTS

Early microbicide development was focused on vaginal products. However, recent efforts have expanded to optimize products to safely prevent HIV transmission during receptive anal intercourse as well. While these new products are directed at reducing HIV incidence among MSM, it has also been acknowledged that heterosexual anal intercourse may be under-reported due to social taboos and other factors (120-122). This issue has shifted the paradigm of microbicide development toward a new generation of dual-compartment microbicides that are safe for both vaginal and rectal use (123, 124). Hence, in this study we used models of rectal and vaginal mucosal transmission to evaluate the potential of topically applied nAbs. This study presents the first comprehensive pre-clinical evaluation of nAbs as a topical microbicide using a human *ex vivo* model of sexual HIV transmission. These data show that nAbs are potent and effective in reducing mucosal HIV transmission in human ectocervical and colonic tissues *ex vivo.* These results are concordant with animal studies where nAb preparations applied to the vaginal lumen before vaginal inoculation with SHIV were

protective (100, 125). Importantly, nAb potency was not affected by the presence of whole semen. In addition, this study shows that nAbs have a good safety profile, with no loss of tissue viability or immune activation that could preclude their use as an effective topical HIV microbicide.

The effective nAb dose for ectocervical tissue (1.5 µM) was 50 times more than the effective dose for colonic tissue (0.03 µM) (Figure 2). It is currently unclear why there is a different effect of nAbs between colonic and ectocervical tissues. Pharmacokinetic studies of intravenous, intramuscular and orally administered ARVs have shown differential deposition of drugs in female genital and rectal compartments. Higher drug levels are typically found in the rectal tissue compared to the female genital tract (17, 126); and topically applied drugs are metabolized differently in the vaginal and rectal mucosae (127). However, in the context of a topically applied preparation of nAbs, where pharmacokinetic coverage is limited to the lumen, those previous observations do not explain why higher concentrations of topically applied nAbs are required to prevent viral transmission in cervical tissue compared to colonic tissue. The greater potency of nAbs observed in this model of rectal mucosal transmission is not easily explained by differences in the size of the inoculum used and merits further exploration. nAb potency in the order, PG16>PG9>VRC01-N>>4E10-N was resolved *in vitro*. However, in the *ex vivo* model, the distinction between the potency of PG9 and PG16 was not well defined. Lower doses of both of these nAbs caused a delay in infection (Figure 2), further attesting to the greater potency of these nAbs in comparison to VRC01-N or 4E10-N. This may suggest that further dilutions of PG9 and PG16 may

have been needed in both tissue models to better define the comparative potency of these antibodies.

The delay in infection observed with PG9 and PG16 treatment (Figure 2) is likely the result of a small portion of the inoculum that was not neutralized by nAb treatment. This low level infection would have taken some time to expand enough to generate p24 concentrations that were above the ELISA limit of detection. Hence, the observed outgrowth is likely due to insufficiency of the antibody dose used. It is unlikely that this outgrowth of virus was due to the emergence of escape mutants as exposure to a single dose of nAbs would not have provided sufficient selective pressure in this model. However, it must be acknowledged that viruses with natural polymorphisms in envelope glycoproteins that make them resistant to neutralization by a single antibody may be more effectively prevented by using combinations of anti-HIV antibodies that simultaneously target multiple key envelope epitopes.

The relative lack of potency demonstrated by 4E10-N was not surprising as this antibody, although broadly neutralizing, has been characterized as being only moderately potent (128). 4E10 exerts its main HIV inhibitory activity by MPER-binding; however this antibody is also polyreactive, engaging in relatively short-lived, low avidity interactions with other hydrophobic moieties (129). These may contribute to the decreased potency of 4E10 in HIV neutralization. The observed lack of potency may also be attributed to slower neutralization kinetics seen in MPER antibodies like 4E10 and 2F5. These antibodies require an epitope conformation that is thought to only be

realized post-receptor engagement in more neutralization-resistant HIV-1 isolates like HIV-1_{JR-CSF} (130).

Seminal plasma contains factors that enhance HIV transmission (116, 131), and others that may interfere with microbicide activity (115). Previous microbicide evaluations showed that PRO 2000 gel protected mice from HSV-2 transmission. However in the presence of semen, the product showed decreased efficacy *in vitro* (115). It was postulated that this effect may have been due to seminal protein interactions with the polyanion that blocked binding to HIV. This effect of semen essentially precludes the use of PRO 2000 for prevention of mucosal HIV transmission, where the putative inoculum is HIV-infected semen. In comparison, evaluation of nAb potency in the presence of semen, shows that nAb activity in this model was not affected.

Preliminary safety data on the use of topically applied nAbs (Figure 4) suggest that they are suitable for use as a topical on-demand prophylactic. In experiments to determine if there was immune activation due to nAb treatment, nAbs generally caused no changes in concentrations of inflammatory mediators, suggesting their suitability as topical microbicides. Additional safety evaluations may be necessary to determine the safety of other nAb applications. Previous non-antiretroviral microbicide candidates were shown to cause immune activation. Increased expression of IP-10 in the female genital tract has been associated with increased HIV acquisition (132) and is indicative of an inflammatory milieu. Treatment of ectocervical tissue with VRC01-N was associated

with decreased expression of IP-10 (Figure 3c), suggesting that these nAbs do not increase production of soluble pro-inflammatory mediators.

Regarding a nAb microbicide, antibodies are highly specific for their cognate HIV epitopes and are native to the human body, hence nAbs applied luminally are expected to have negligible adverse effects that would preclude a therapeutic benefit. Traditional pharmaceutic antibody production uses transgenic mouse or human cell systems – both of which can be cost prohibitive to producing an affordable antibody-based topical microbicide. The cost of producing a pharmaceutic grade supply of antibodies using mammalian cell or animal systems was estimated at $5-6 million over 18 months. In comparison, the use of transgenic plant production systems has made large scale production of antibodies feasible and relatively cheap. Production of a pharmaceutic grade supply of antibodies in transgenic plant production systems was estimated to cost $0.5–0.8 million over 12 months (109). These time and cost savings are important factors for making a nAb microbicide accessible for use in resource-limited settings.

These data show that nAbs are effective in models of both rectal and vaginal transmission, and may be considered for formulation as a dual-compartment microbicide product. Hence, an antibody-based microbicide could possibly expand microbicide application beyond vaginal use in high-risk women to rectal application and use in other vulnerable populations. A nAb microbicide may also be recommended for use by heterosexual couples engaging in both vaginal and anal sex. nAb efficacy is not expected to be affected by HIV mutations selected as a result of suboptimal ARV use,

hence a nAb microbicide could be used by HIV serodiscordant couples. In addition, nAbs may provide a non-ARV microbicide option for individuals who choose to avoid the side-effects of using an HIV drug. Our findings using unformulated nAbs suggest that these antibodies can prevent mucosal transmission of HIV when applied pericoitally, and would likely be safe and effective as topical vaginal or rectal microbicides.

CHAPTER 2: POTENCY OF BROADLY NEUTRALIZING ANTIBODIES AGAINST ALTERNATIVE INOCULA

2.1 CHAPTER OVERVIEW

Due to the increased use of cART, there is an increased risk of developing ARV drug resistance and transmitting drug resistant HIV. Hence non-ARV-based microbicides like nAbs present a promising alternative where ARV-based products may fail. Additionally, most evaluations of HIV microbicides use models of cell-free HIV infection; however in the context of sexual transmission, cell-associated HIV transmission is believed to be more efficient than cell-free viral transmission. Semen from HIV positive men has been found to contain HIV-infected seminal leukocytes that can usurp normal intercellular interactions with epithelial cells and target cells to efficiently transfer virus without releasing free virions into the extracellular space. Hence, cell-associated virus and drug-resistant HIV are both important targets for HIV microbicides but are often overlooked in pre-clinical evaluations.

In this chapter, the efficacy of topically applied nAbs in preventing cell-associated HIV transmission in human tissues *ex vivo* and transmission of cell-free drug-resistant strains *in vitro* was assessed. Viral strains used also included transmitted/founder

viruses. Neutralization by nAbs, PG9 and VRC01-N, was not affected in viruses that had drug resistance mutations for NRTIs or NNRTIs. However, variations in envelope genotype and glycosylation greatly affected antibody neutralization. The transmitted/founder strain, THRO, was found to be highly resistant to neutralization by PG9 and VRC01. CXCR4-tropic strains were also more neutralization-resistant than CCR5-tropic strains. In comparisons of nAb efficacy against cell-associated HIV transmission *ex vivo*, PG9 and PG16 were more potent than the CD4-binding site antibody, VRC01-N, and reduced HIV transmission as effectively as dapivirine (DPV) in polarized ectocervical explants. These studies suggest that using combinations of nAbs with complementary neutralization breadth may improve the functional efficacy of a topical antibody-based microbicide product.

2.2 INTRODUCTION

With the increased use of cART in recent years, and the amended World Health Organization guidelines that advocate initiation of cART for anyone living with HIV regardless of $CD4^+$ cell count (133), there is an increased risk of transmitting drug-resistant strains of HIV. These viral strains may have mutations that can confer reduced sensitivity to multiple classes of drug (134), hence transmission of these strains may not be effectively prevented using ARV-based microbicides. This underscores the utility of non-ARV-based microbicides for HIV prevention in the current treatment landscape, and highlights the need for pre-clinical evaluation of microbicides for their efficacy in preventing transmission of drug resistant strains of HIV.

Historically, most models used for evaluating candidate HIV therapeutics and preventatives have used cell-free HIV inocula. However, sexual HIV transmission can occur from an infected cell to a target cell without free virus being released into the extracellular medium. Cell-to-cell transmission occurs via intercellular virological synapses that allow directed transfer of nascent virus to the target cell within a protected space (17). Viral transfer from an infected cell may also occur via transcytosis of nascent virus by epithelial cells and subsequent transfer to subepithelial or intraepithelial immune cells (13, 135). Additionally, infected leukocytes have the inherent ability to infiltrate the mucosal barriers of the rectal and vaginal compartments where they can directly engage target cells in the submucosa (17, 18). These processes improve the kinetics of virus-receptor engagement or occur in restricted spaces that may protect the virus from some host restriction factors and innate immune factors that may have inhibited cell-free virus. Hence, cell-to-cell HIV transmission is thought to be more efficient than transmission of cell-free virus.

In the context of sexual transmission of HIV, free viral particles can be found in seminal plasma as well as in infected leukocytes present in the ejaculate. Virus has been successfully propagated from these HIV infected seminal leukocytes (17, 136), indicating that HIV-infected cells in semen are a potential source of infectious virus. Hence evaluation of microbicide efficacy against cell-associated HIV transmission is warranted.

In this study, the efficacy of nAbs in preventing transmission of cell-free drug resistant strains of HIV and cell-associated virus are investigated *in vitro*. The panel of nAbs used includes VRC01-N, 4E10-N, PG9 and PG16, which had been evaluated previously for their efficacy in preventing transmission of cell-free wild-type (WT) HIV (Chapter 1, Figure 2). These antibodies each bind different viral epitopes – VRC01-N binds to the CD4 binding site; 4E10-N binds to the MPER at the base of HIV gp41; and PG9 and PG16 recognize glycosylation motifs on variable regions 1 and 2 (V1/V2) of HIV gp120 (108). To interrogate the ability of nAbs to inhibit cell-to-cell transmission of HIV in human cervical and colonic tissues *ex vivo,* a model of cell-associated HIV transmission was developed using the PM1 $CD4^+$ T cell line. This study presents a pre-clinical evaluation of the efficacy of topically applied nAbs in preventing transmission of inocula that are not commonly used, but could be transmitted if there are gaps in microbicide protection. Hence nAbs are evaluated for efficacy against a panel of cell-free drug-resistant HIV inocula *in vitro* and cell-associated virus in a human model of mucosal transmission.

2.3 MATERIALS AND METHODS

Culture Media: All culture media were purchased from Mediatech, Inc. (Manassas, VA); serum and media supplements were purchased from Gemini BioProducts (West Sacramento, CA).

Antibodies: Plant-produced VRC01-N and 4E10-N monoclonal antibodies were generously provided by Dr. Kevin Whaley (Mapp Biopharmaceuticals Inc., San Diego, California). PG9 and PG16 monoclonal antibodies were provided by the International AIDS Vaccine Initiative (La Jolla, CA). All antibodies used are IgG_1 isotype.

Virus strains: The pTHRO.c/2626 and pCH077.t/2627 subtype B infectious molecular clones of transmitted/founder viruses (137) were obtained from the NIH AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH: Dr. John Kappes and Dr. Christina Ochsenbauer. pTHRO is known to have a wild-type (WT) reverse transcriptase (RT) phenotype and CH077 has N/NRTI drug resistance mutations K65R and Y181C. The pYK-JR-CSF molecular clone was purchased from ATCC (Manassas, VA), and the xxLAI-162 and xxLAI-182 molecular clones were a gift from Dr. Urvi Parikh, University of Pittsburgh. The xxLAI strains were constructed by inserting full length subtype C RT sequences derived from study participants into the xxHIV-LAI backbone. Hence these strains have subtype B CXCR4-tropic envelopes, but subtype C RT. xxLAI-162 has a WT RT genotype and xxLAI-182 has the L100M, K101E, V106M, E138K and F227L mutations in RT that contribute to DPV resistance.

Cell-free virus stocks: 293T cells were a gift from Christina Ochsenbauer-Jambor and John C. Kappes from the University of Alabama, Birmingham. 293T cells and were cultured in complete DMEM (cDMEM; 1× DMEM supplemented with v/v 10% heat-inactivated fetal bovine serum (FBS) and 1% Pen-Strep-L-Glut). 293T cells were transfected with proviral DNA using the Lipofectamine 2000 reagent (Invitrogen,

Carlsbad, CA). After 48h, mature HIV JR-CSF WT/K65R, CH077 and THRO virions were harvested from the culture medium by filtration through a 0.45 µM pore syringe filter. xxLAI-162 and xxLAI-182 were harvested similarly after 24h. The tissue culture infectious dose ($TCID_{50}$) was determined in activated human peripheral blood mononuclear cells (Central Blood Bank, Pittsburgh, PA) using the Reed-Muench method (113).

Viral envelope sequence alignments: Full-length protein sequences for gp160 of JR-CSF, THRO, CH077 and the subtybe B reference strain HXB2 were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) – accession numbers U45960.1, JN944930, JN944909 AND K03455.1, respectively. Sequences were aligned using the N-GlycoSite tool available from the online HIV sequence database (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html), which predicts putative sites of N-linked glycosylation (138). Sequence output was annotated manually.

Cell-associated virus stocks: PM1 cells (139) were obtained through the NIH AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH: Dr. Marvin Reitz, and cultured in complete RPMI (cRPMI; RPMI supplemented with v/v 10% FBS and 1% Pen-Strep-L-Glut). TZM-bl cells (114) were also obtained through the NIH AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH: Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc., and were cultured in cDMEM.

PM1 cells were inoculated with HIV-1_{JR-CSF} at an m.o.i. of 0.5 in the presence of 2.5 µg/mL diethylaminoethyl-dextran (DEAE-dextran) for 24h. Cells were washed, cultured for 3 days, and then harvested and stored in liquid nitrogen. Comparative infectivity of $PM1_{JR-CSF}$ cells and cell-free HIV-1_{JR-CSF} was determined by titration in TZM-bl cells. Briefly, PM1 cells were thawed and washed before being treated with 200 mg/mL mitomycin C (Sigma-Aldrich, St. Louis, MO) for 1h at 37°C. Treatment with mitomycin C inhibits PM1 cell division, preventing expansion of the $PM1_{JR-CSF}$ inoculum. Cells were washed 3 times, and titrations of mitomycin C-treated PM1 cells were used to inoculate monolayers of TZM-bl cells. HIV-1 tat-activated luciferase induction was measured after 48h using the Bright-Glo Luciferase Assay reagent (Promega, Madison, WI). Luminescence was measured using the SpectraMax M3 plate reader (Molecular Devices, LLC; Sunnyvale, CA). Controls were uninfected TZM-bl cells and TZM-bl cells inoculated with 3,000 $TCID_{50}$ cell-free HIV-1_{JR-CSF}.

Flow cytometry: Surface staining of $PM1_{JR-CSF}$ cells for CD4 antigen was performed using mouse anti-human CD4-FITC (BD Biosciences, San Jose, CA). Viability was assessed using the LIVE/DEAD™ Fixable Aqua Dead Cell stain (Invitrogen, Carlsbad, CA), and quantitative assessment of intracellular HIV core antigen was performed in viable $PM1_{JR-CSF}$ cells using the mouse monoclonal antibody, KC57-PE (Beckman Coulter, Inc. Indianapolis, IN). Uninfected PM1 cells were used as staining controls and used to set the sorting gates. Multicolor flow cytometry was performed using the BD LSRFortessa X20 cell analyzer instrument (BD Biosciences). Analysis was performed using FlowJo Data Analysis Software V.10.0.8 (FlowJo LLC., Ashland, OR).

Assessment of nAb inhibition of cell-associated virus transmission *in vitro*: Individual wells of a 96-well plate were seeded with 1×10^4 TZM-bl cells which were allowed to adhere overnight. Culture medium in each well was replaced with 100 µL of 2× nAb or drug dilutions, or with cDMEM in untreated control wells. Cells were incubated for 1h at 37°C. Treated cells and untreated controls were inoculated with 3,000 $TCID_{50}$ cell-free HIV-$1_{JR\text{-}CSF}$ or an equivalent inoculum of $PM1_{JR\text{-}CSF}$ cells in 100 µL and cultured for 48h. Tat-activated luciferase expression was detected using the Bright-Glo Luciferase Assay reagent (Promega, Madison, WI) and luminescence was measured using the SpectraMax M3 plate reader (Molecular Devices, LLC; Sunnyvale, CA). The 90% inhibitory concentrations (IC_{90}) were determined using SigmaPlot software version 11.0 (Systat Software, Inc., San Jose, CA).

***Ex vivo* tissue culture:** Normal human ectocervical tissues were obtained from pre-menopausal women undergoing routine hysterectomy after informed consent (IRB #PRO09110431) or purchased from the National Disease Research Interchange (NDRI; http://ndriresource.org/) and transported overnight on wet ice. Polarized explants were prepared from the specimens as described elsewhere (54). Briefly, the ectocervical epithelium was trimmed of the muscularis and 5 mm diameter explants were mounted with the epithelium upward in 12 mm permeable trans-well supports and sealed in position with Matrigel basement membrane matrix (BD Biosciences, San Jose, CA). Ectocervical explants were activated with 1 µg/mL phytohemmaglutinin-P (PHA-P) and cultured in DMEM supplemented with v/v 10% human A/B serum, 1% Pen-Strep-L-Glut,

100 U/mL human interleukin-2 (hIL-2) (Roche Diagnostics, Indianapolis, IN) and 1% non-essential amino acids (Lonza, Walkersville, MD).

Efficacy evaluations in human ectocervical tissue *ex vivo*: Paired polarized explants were treated with nAbs 48h after setup. nAbs were applied at 2× the final concentration to the apical surface of the appropriate explants in duplicate and cultured for 1h at 37°C. Controls were treated similarly with either medium only or with DPV. After 1h, each explant was inoculated apically with the equivalent of 50,000 $TCID_{50}$ $PM1_{JR-CSF}$, suspended in RPMI-1640. Tissues were incubated for 24h, after which the basolateral medium was collected and the explants were washed with 1× DPBS (Mediatech, Inc., Manassas, VA). Basolateral media was replenished with fresh media supplemented with 100 U/mL hIL-2, and subsequently collected every 3 to 4 days, up to 21 days post infection and stored at -80°C. Viral replication was monitored by measuring HIVp24 in the basolateral media using the Alliance HIV-1 p24 Antigen ELISA kit (PerkinElmer, Waltham, MA).

2.4 RESULTS

2.4.1 nAbs reduce transmission of cell-free transmitted/founder and drug resistant HIV *in vitro*

In vitro evaluations of nAb efficacy were conducted against a panel of cell-free HIV strains using TZM-bl cells that had been pre-treated with titrations of nAbs, TFV or DPV

for 1h before inoculation. The IC_{90} of each treatment was determined for each strain used. These experiments generally showed that viruses remained susceptible to nAb neutralization regardless of decreased susceptibility to TFV or DPV (Table 4). Drug resistant strains like JR-CSF K65R and CH077 shared similar neutralization sensitivity with the wild-type reference strain, JR-CSF WT. They showed 0.7 – 1.6 fold change in susceptibility to nAb neutralization despite the presence of mutations in RT. A similar observation was made for the xxLAI strains that included wild-type LAI-162 and NNRTI-resistant variant LAI-182, which had a 2.8-fold increased IC_{90} compared to the wild-type. These data show that virus neutralization is not affected by the RT genotype. It may also be inferred that a similar outcome would be observed with viruses that have developed drug resistance to other HIV enzyme inhibitors like integrase and protease inhibitors.

Table 4. Comparative susceptibility of a panel of HIV viruses to nAb neutralization and ARV inhibition.

	HIV Strain					
	JR-CSF WT	**JR-CSF K65R**	**CH077**	**THRO**	**LAI-162**	**LAI-182**
Treatment	**IC_{90} *(Fold change relative to reference strain)*[‡]**					
TFV	16.46 µM *(1)*	125.75 µM *(7.6)*	63.21 µM *(3.8)*	17 µM *(1.03)*	27.46 µM *(1)*	46.43 µM *(1.7)*
DPV	2.31×10^{-3} µM *(1)*	5.77×10^{-3} µM *(2.5)*	8.77 µM *(3797)*	1.45×10^{-3} µM *(0.6)*	6.17×10^{-3} µM *(1)*	9.96×10^{-1} µM *(161)*
PG9	4.16×10^{-4} µM *(1)*	6.69×10^{-4} µM *(1.6)*	6.60×10^{-4} µM *(1.6)*	> 5 µM *(>12,000)*	>1.5 µM *(1)*	>1.5 µM *(1)*
VRC01-N	7.51×10^{-2} µM *(1)*	5.56×10^{-2} µM *(0.7)*	5.39×10^{-2} µM *(0.7)*	1.12 µM *(15)*	2.48×10^{-2} µM *(1)*	6.91×10^{-2} µM *(2.8)*

Values represent the mean of 3 independent experiments.
[‡] JR-CSF WT is the reference strain for JR-CSF K65R, CH077 and THRO, and LAI-162 is the reference strain for LAI-182.
CH077 RT mutations: K65R, Y181C
LAI-182 RT mutations: L100M, K101E, V106M, E138K and F227L
Drug resistance was defined as a fold change ≥ 3 relative to the reference strain.

Interestingly, the THRO transmitted/founder virus, which has a wild-type RT genotype, showed >12,000 fold reduced susceptibility to PG9 compared to the reference strain, JR-CSF WT. THRO also showed reduced susceptibility to VRC01, requiring 15× the IC_{90} of JR-CSF WT. Both the WT and drug-resistant variants of the CXCR4-tropic xxLAI virus showed similar susceptibility to PG9 neutralization, while the IC_{90} of the xxLAI viruses was considerably greater than that of JR-CSF WT (>3000-fold). These results suggest that nAbs that recognize glycan-dependent epitopes may be less efficient at neutralizing CXCR4-tropic viruses. Collectively, these data confirm that nAbs are not affected by mutations in HIV RT; but nAb efficacy is affected by envelope polymorphisms and variations in envelope glycosylation patterns which may be harder to predict.

To address this hypothesis, full length gp160 envelope sequences for JR-CSF, THRO and the neutralization-sensitive CH077 were compared against a sequence for the HXB2 strain to identify specific genotypic differences in THRO that could mediate PG9-neutralization resistance. Previous studies had identified mutations in residues V127, N156, S/F158-159, K/N160, T/S162, Y173, F176 and V/I181, in the V1/V2 loops of gp120 that contributed to PG9 neutralization resistance (140). Putative N-linked glycosylation sites N136, N141, N156, N160, N186 and N197, were also identified in the loops (140). The positions of N-linked glycosylation sites and mutations in the envelope sequences were noted, and differences between THRO and JR-CSF that were not shared with CH077 were denoted as THRO-specific PG9-resistance mutations. These include substitutions N156K, N160E, Y173T, F176R, and V/I181R (Figure 5), which had been previously identified as affecting PG9 neutralization (140). Interestingly, non-K residues at positions 168-170 had been associated with PG9 resistance (141) and THRO was the only strain with no consensus to the reference sequence at these residues (Figure 5). THRO also contained the unique R166N and Q170N mutations that resulted in putative N-linked glycosylation sites that were not present in the CH077 or JR-CSF WT sequences. THRO, JR-CSF and CH077 each had 6 putative N-linked glycosylation sites within the V1/V2 sequence; although the position of those residues varied (Figure 5). CH077 also had some deviations from the JR-CSF V1/V2 loop sequence and differed in the position of putative N-glycosylation sites (Figure 5); but those mutations did not seem to affect PG9 neutralization in this strain (Table 4). These findings support a role for differences in both sequence and N-linked glycosylation patterns in the PG9-resistance observed in the THRO transmitted/founder virus. As

THRO was also resistant to VRC01 (Table 4), sequence homology in the V5 loop, loop D and CD4-binding loop sequences of gp120 was also compared as these regions have been identified as key interfaces for VRC01 binding (142, 143). There was substantial sequence variation at the V5 loop, loop D and CD4-binding loop in both THRO and CH077. However, CH077 remained susceptible to VRC01 neutralization, whereas THRO showed 15-fold VRC01 resistance. This suggests that loss of sensitivity to VRC01 may be mediated by multiple mutations within the various key contact sites and warrants further investigation to identify the determinants of VRC01 resistance.

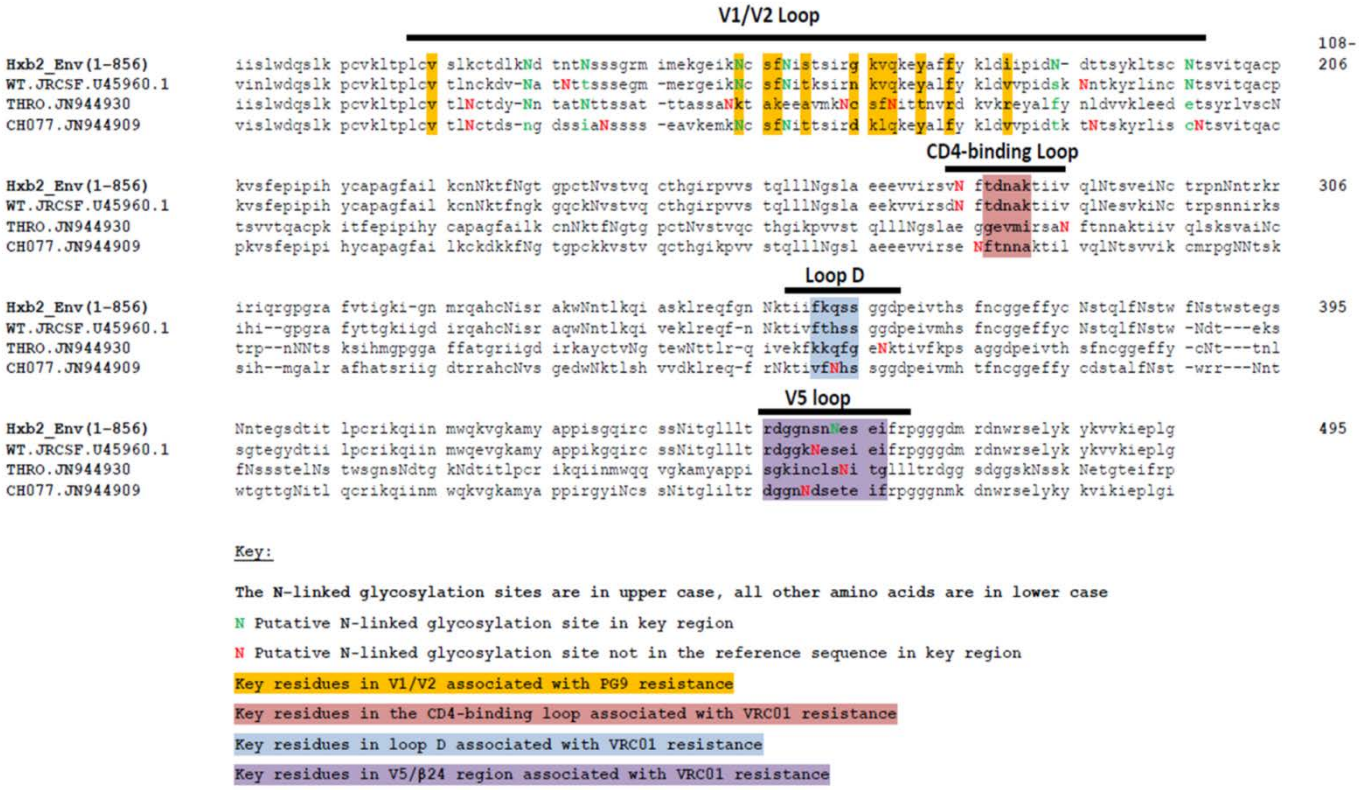


Figure 5. Comparison of viral envelope sequences and glycosylation
Multiple envelope sequence alignment compares JR-CSF WT, THRO and CH077 to the HXB2 subtype B reference sequence. Alignments show putative sites of N-linked glycosylation that are in concordance with the reference sequence (green text) and N-linked glycosylation sites resulting from sequence deviations (red text), that may interfere with nAb neutralization. The V1/V2 loop forms the PG9 epitope and the VRC01 epitope spans contacts in the CD4-binding loop, loop D and the V5 loop. Yellow residues were previously identified as being important for PG9 neutralization, while regions highlighted in pink, blue and purple were identified as having a role in VRC01 neutralization sensitivity. THRO demonstrated resistance to PG9 and VRC01 neutralization *in vitro* (Table 4), while CH077 remained sensitive to both nAbs.

2.4.2 Developing a cell-associated inoculum

HIV-infected $CD4^+$ T cells have been found in semen and their number contributes to the cell-associated inoculum in those secretions. To evaluate the efficacy of nAbs in preventing cell-associated HIV transmission, a model was developed using the PM1 $CD4^+$ T cell line and $HIV_{JR\text{-}CSF}$, and is denoted as $PM1_{JR\text{-}CSF}$. PM1 cells were inoculated with $HIV_{JR\text{-}CSF}$ for 24h before being washed. Cells were cultured for another 48h before

being frozen and stored in liquid nitrogen. The percentage of HIV-infected cells in the $PM1_{JR\text{-}CSF}$ culture was determined by flow cytometric detection of surface CD4 antigen expression and intracellular HIVp24 on thawed cells. 3.6% of viable PM1 cells were found to be infected ($CD4^+$, $p24^+$) (Figure 6a).

The proportion of $PM1_{JR\text{-}CSF}$ infection that was attributable to shedding of cell-free virus particles that were either loosely attached to $PM1_{JR\text{-}CSF}$ cells or newly released into the supernatant was examined. This was assessed by comparing the magnitude of HIV infection generated in TZM-bl cells that were seeded in the basolateral chamber of plates containing 0.4 µM-pore transwell supports. Cell-free $HIV_{JR\text{-}CSF}$ or $PM1_{JR\text{-}CSF}$ inocula were introduced in the apical chamber. As cells were too large to pass through the 0.4 µM pore, any infection in the basolateral layer of TZM-bl cells was attributed to shedding of cell-free virus. The magnitude of this infection was compared to that in TZM-bl monolayers that were directly inoculated with cell-free $HIV_{JR\text{-}CSF}$ or $PM1_{JR\text{-}CSF}$ cells in the basolateral chambers. After 48h incubation, inoculation with $PM1_{JR\text{-}CSF}$ cells in the apical chamber of the transwell support caused 2% of the magnitude of infection caused by the infection control – an equivalent inoculum of cell-free $HIV_{JR\text{-}CSF}$ deposited directly on the TZM-bl monolayer (Figure 6b). In comparison cell-free $HIV_{JR\text{-}CSF}$ in the apical chamber caused 45% the magnitude of the cell-free infection control. Hence minimal cell-free virus was shed from $PM1_{JR\text{-}CSF}$ cells (Figure 6b).

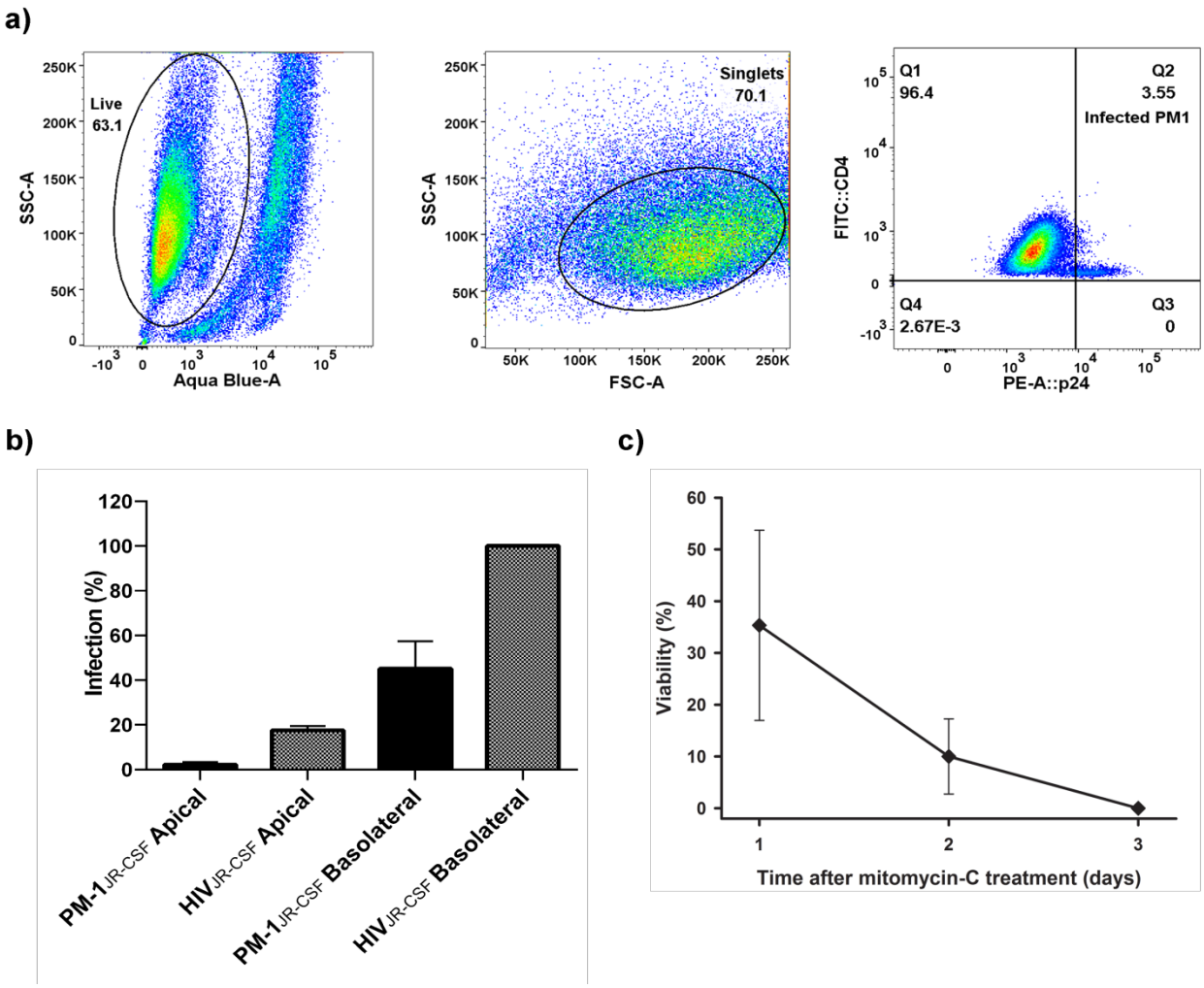


Figure 6. Developing the PM1$_{JR-CSF}$ cell-associated inoculum
a) HIV infection was assessed by flow cytometry in thawed PM1$_{JR-CSF}$ cells. b) The proportion of infection that was due to cell-free virus shedding from PM1$_{JR-CSF}$ cells across a 0.4µM membrane was assessed in TZM-bl cells, and c) PM1$_{JR-CSF}$ cell viability was monitored after mitomycin C treatment. Cell-free transmission b), and viability of mitomycin C-treated PM1$_{JR-CSF}$ cells c), are represented as the mean and standard deviation of 3 independent experiments.

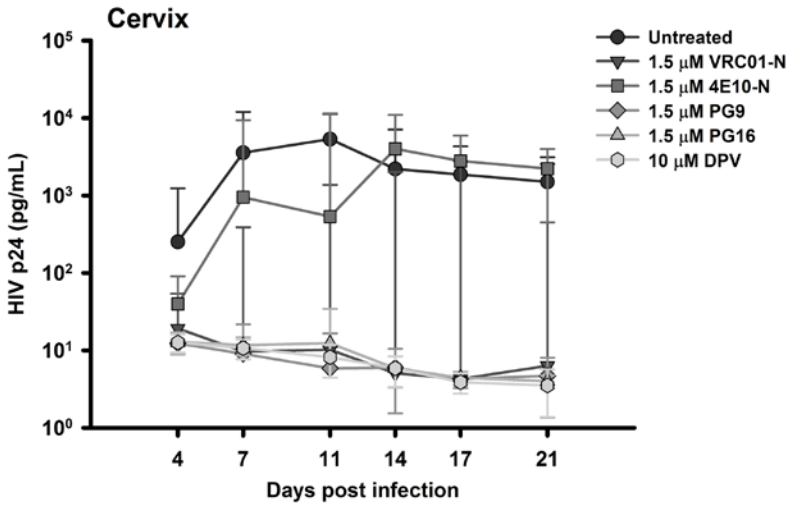
To confirm that mitomycin C-treated PM1$_{JR-CSF}$ cells did not replicate in culture, effectively expanding the inoculum, their viability was monitored over time. Viable mitomycin C-treated PM1$_{JR-CSF}$ cells were identified by trypan blue exclusion and counted daily. Cell viability decreased to 0% 72h after mitomycin C-treatment (Figure

6c). These experiments suggest that the $PM1_{JR-CSF}$ cells generated in this study are a suitable model inoculum for cell-associated HIV transmission.

2.4.3 nAbs show differential efficacy in reducing cell-associated HIV transmission

Ectocervical tissues were pre-treated in duplicate with 1.5 µM nAbs or 10 µM DPV for 1h before inoculation with mitomycin C-treated $PM1_{JR-CSF}$ cells. The 1.5 µM dose of nAbs used represents the highest concentration of nAbs that was previously shown to provide protection from cell-free HIV transmission in ectocervical tissues *ex vivo* (Chapter 1). Similarly, 10 µM DPV added to the culture medium was found to be protective in *ex vivo* cervical and colonic tissues using cell-free $HIV\text{-}1_{BaL}$ (144). Tissues were washed after 24h to remove the inoculum and treatments, and HIVp24 was measured in culture supernatants collected at 4, 7, 11, 14 and 21 days after inoculation. The protective index of each treatment was calculated as the proportion of explants that were protected out of all explants treated with an individual treatment. In comparisons of median HIVp24, nAbs PG9 and PG16 consistently reduced cell-associated HIV transmission as effectively as the ARV, dapivirine (Figure 7). In contrast, 4E10-N provided poor protection from transmission of $PM1_{JR-CSF}$ cell-associated virus in ectocervical tissues *ex vivo*, with a protective index of 0.17; and 4E10-N-treated tissues had similar median levels of viral replication to untreated control tissues. VRC01-N generally appeared protective, with median HIVp24 levels similar to dapivirine-treated tissues. However, this protection was not consistent across tissues, as shown by the large interquartile range of HIVp24 in VRC01-N-treated tissues and the marginal

protective index of 0.67 (Figure 7). Additionally VRC01-N demonstrated approximately 10-fold decreased potency against the cell-associated $PM1_{JR-CSF}$ inoculum *in vitro* compared to cell-free HIV_{JR-CSF} (Appendix C). Collectively these data suggest that nAbs, PG9 and PG16 are more effective than VRC01-N in reducing cell-associated HIV transmission in human ectocervical tissues *ex vivo*, and that PG9 and PG16 may be more potent against this inoculum.



Comparison of protection conferred by nAb treatments		
Treatment	**# of explants protected**	**Protective index**
1.5 μM VRC01-N	4/6	0.67
1.5 μM 4E10-N	1/6	0.17
1.5 μM PG9	6/6	1.00
1.5 μM PG16	6/6	1.00
10 μM DPV	6/6	1.00

Figure 7. nAb efficacy against cell-associated HIV transmission in ectocervical tissues *ex vivo*
Tissues were treated in duplicate with nabs for 1h before inoculation with $PM1_{JR-CSF}$. Viral replication was monitored by HIVp24 antigen ELISA on supernatants collected 4, 7, 11, 14, 17 and 21 days post infection. Data points represent the median and interquartile range of 3 tissues from individual donors. The protective index was calculated as the proportion of explants that were protected out of all explants treated with an individual treatment.

2.5 DISCUSSION OF RESULTS

Pre-clinical evaluations of nAbs applied as topical microbicides have shown that they can reduce mucosal transmission of HIV. While most models used cell-free HIV or SHIV, this study presents a comprehensive evaluation of topically applied nAbs against inocula not commonly included in pre-clinical evaluations. These include strains of HIV with drug resistance mutations in RT, and cell-associated HIV. nAbs retain efficacy against viruses that are resistant to ARVs like TFV and DPV. NRTIs and NNRTIs like TFV and DPV have been used in cART regimens in resource-limited settings and can select viruses with cross resistance that reduces their susceptibility to entire classes of RT inhibitory molecules (134). Additionally, DPV and TFV products are undergoing clinical evaluation for their suitability as HIV preventatives, hence comparisons of their efficacy against alternative inocula is warranted.

The viral strains used in this study include the CCR5-tropic JR-CSF wild-type and K65R mutant strains; subtype B transmitted/founder strains CH077 and THRO; and two CXCR4-tropic xxLAI strains, one with a wild type RT genotype (LAI-162) and the other with multiple NNRTI drug resistance mutations (LAI-182). THRO was highly resistant to neutralization by the CD4-binding site antibody, VRC01-N, compared to JR-CSF and other subtype B viruses used in this study (Table 4). VRC01 interaction with the HIV CD4-binding site has been shown to be mainly inhibited by antigenic variation in variable region 5 (V5) and loop D sequences of gp120, but these changes did not affect binding of soluble CD4 (sCD4) (142). However, THRO was previously shown to be more susceptible to inhibition by sCD4 than JR-CSF (137), suggesting that the sCD4

binding site on THRO is intact. This indicates that the observed THRO resistance to VRC01-N neutralization may be due in part to genotypic differences in the variable region 5 and loop D sequences of gp120, and are confirmed by discovery of envelope polymorphisms in these regions (Figure 5). THRO was also highly resistant to PG9 neutralization (Table 4). Sequence comparisons of CH077, THRO and the reference strain JR-CSF identified envelope polymorphisms in the V1/V2 loops of THRO gp120 that resulted in changes in key residues and altered positioning of putative N-linked glycosylation sites. As PG9 recognizes a quaternary glycosylation motif, its binding is greatly affected by changes in protein sequence, glycosylation and electrostatic interactions (145). Hence, it may be inferred that the polymorphisms present in the THRO envelope sequence translated to sufficient differences in the quaternary glycan structures to disrupt key contacts for PG9 binding, rendering THRO resistant to that nAb. These data also highlight how vulnerable the neutralization efficacy of nAbs with glycan-based epitopes can be to the plasticity of HIV envelope glycosylation. HIV envelope glycosylation can be highly heterogeneous, may vary across subtypes (146, 147), the stage of infection at viral isolation (11), co-receptor usage (148, 149), and even according to the cell-type used to produce virus (150). Therefore, nAbs like PG9 that bind gp120 glycan epitopes, despite their potency, may not provide sufficient microbicidal efficacy if used singly due the inherent heterogeneity of envelope glycan structures in various HIV strains. In this study, all viruses were produced using the same cell types; hence there would be reduced heterogeneity in glycan processing for the viral envelope across strains used in this study.

CXCR4-tropic xxLAI viral envelopes showed similar responsiveness to VRC01-N neutralization compared to CCR5-tropic JR-CSF, but have dramatically decreased susceptibility to PG9 neutralization (Table 4). CXCR4-tropic viruses evolve longer variable loops and increased envelope glycosylation with the switch from preferential CCR5 to CXCR4 co-receptor usage (148, 149), and this may account for the greatly reduced sensitivity to PG9 neutralization. It has been postulated that establishment of HIV infection by mucosal transmission of HIV is mediated by CCR5-tropic virus strains that have been selected by the mucosal transmission bottleneck (10). Although the relative proportion of CXCR4-tropic HIV transmission is low, it has been reported at up to 20% in various cohorts (151-153); consequently CXCR4-tropic HIV may still be transmitted. Most entry inhibitors target CD4-CCR5-mediated HIV entry, but the CXCR4 mechanism is neglected. Hence, the evaluation of nAb efficacy against CXCR4-tropic HIV is necessary.

These data demonstrate the potential envelope diversity of HIV inocula and highlight the importance of using combinations of nAbs in any antibody-based microbicide product. nAbs neutralize 70-90% of HIV strains (95, 154), hence if a single nAb is used there may be gaps in coverage that could result in HIV transmission even if the product is used correctly. Hence it is advisable to formulate nAb microbicides with combinations of antibodies that have complementary neutralization profiles to provide the broadest neutralization coverage and prevention efficacy possible.

Not surprisingly, the presence of drug resistance mutations in HIV RT did not affect nAb potency as the viral envelope is not expected to be under the selective pressure of HIV enzyme inhibitors. This was demonstrated in comparisons of nAb neutralization in the JR-CSF and xxLAI strains, where both wild-type and drug resistant variants show similar susceptibility to nAb neutralization, despite having much greater differences in ARV susceptibility. The demonstrated efficacy of nAbs in reducing transmission of viruses with drug resistance to NRTIs and NNRTIs supports that nAbs are suitable as a non-ARV microbicide candidate. These data also underscore the utility of nAbs in HIV prevention in the current landscape of increased ARV use and the resulting increased risk of transmitted drug resistance. Hence, nAbs would be a useful tool for preventing transmission in sero-discordant couples, especially where pregnancy is desired.

In evaluations of nAbs against cell-associated HIV in ectocervical tissues *ex vivo*, DPV was used as the ARV control. Due to its poor solubility and cellular uptake and because it does not require cellular metabolism to be active, most of the DPV activity would occur extracellularly, providing the basis for comparison to the extracellular viral inhibition demonstrated by nAbs. PG9 and PG16 consistently reduced HIV transmission to levels that were comparable to DPV-treated tissues, lending further support to the superior neutralization potency of PG9 and PG16 (155) demonstrated in Chapter 1. However, VRC01-N was less potent against cell-associated virus, suggesting that the dose of VRC01-N used was not sufficient to achieve similar neutralization coverage to that observed against cell-free HIV (155) (Chapter 1). This may also be due to reduced efficacy of CD4-binding site antibodies like VRC01 against cell-associated virus (156).

Due to the use of a T cell-associated inoculum, it may have been inferred that the main mechanism of cell-cell HIV transmission in the ectocervical explant model is via the virological synapse and is CD4-mediated (157). However, the reduced efficacy of VRC01-N against cell-associated HIV transmission in the *ex vivo* explant model may suggest that cell-to-cell transmission in this model may rely on a CD4-independent mechanism. Hence, cell-cell transmission of HIV in the cervicovaginal mucosa may be characterized more by uptake of virions by epithelial cells and their subsequent transcytosis and transfer to submucosal immune cells, than by transfer using the virological synapse, which is CD4-mediated. Although these mechanisms cannot be credibly defined in this study, these data do suggest a role for cell-to-cell virus transfer in the natural history of HIV transmission and predict that higher doses of nAbs may be required to reduce cell-to-cell compared to cell-free HIV transmission.

Taken together, data from pre-clinical evaluations of nAbs as topical microbicides show that they can reduce mucosal transmission of various HIV inocula. These include transmitted/founder strains with reduced ARV susceptibility, and cell-associated HIV. nAbs also reduced transmission of tenofovir- and dapivirine-resistant strains of HIV. It was noted that CD4-binding site nAbs like VRC01 may be less potent against cell-associated HIV, an observation that could advise dosing of a microbicide containing CD4-binding site antibodies. Additionally, the variable potency of nAbs against different strains of recommends the use of nAb combinations over single nAbs for effective

prevention of HIV. These data suggest that nAbs would be an effective non-ARV microbicide option to reduce transmission of HIV.

DISCUSSION AND CONCLUSION

ADVANCES IN MICROBICIDE DEVELOPMENT

The focus of microbicide design has expanded from producing vaginal gels to generating a variety of formulation and delivery platforms to create many options for populations at high-risk of HIV acquisition, which includes the first products for rectal use. This is a departure from the previous efforts that sought a one-size-fits-all product. While gel formulations are still being considered, advances in vaginal drug delivery have moved toward solid dosage forms (e.g. quick dissolving films, tablets and suppositories). Those dosage forms can deliver active molecules with more diverse physical properties including hydrophobic molecules that have proven challenging to deliver in aqueous formulations (158). Use of these new dosage forms expands the repertoire of potential microbicide candidate compounds to include those that may have had unfavorable release profiles in an aqueous gel format. Solid dosage forms would also circumvent some user acceptability issues such as messiness, discharge or leakage that have been reported for vaginal gels (158). Another benefit to using these dosage forms is the ability to co-formulate active compounds with incompatible physicochemical properties into a single delivery platform. This technology could also lend itself well to developing multipurpose products that incorporate multiple active

compounds to prevent simultaneous infection by various pathogens or concomitantly provide contraception.

Though the first generation microbicides were optimized to preserve the low pH vaginal environment and the *Lactobacillus*-dominant vaginal microbiome (159), the normal colorectal compartment has a distinctly different homeostatic environment. The lower genital tract has a pH closer to neutral and is sensitive to low pH and hyperosmolar conditions (160-162). Previous studies showed that rectal application of hyperosmolar products resulted in reduced epithelial barrier function and caused epithelial disruptions in the rectal lumen that could enhance HIV infection (105). Hence, new rectal-specific gel formulations of non-ARV microbicides are being optimized to address the need for rectal microbicides. Non-ARV rectal products that were considered include a topically applied cyanovarin-N gel that was effective in preventing rectal SHIV transmission in macaques (163), and a Griffithsin gel that is currently being investigated as the first rectal-specific non-ARV microbicide (K. Palmer, personal communication). These rectal studies are important as they could help further define rectal microbicide formulation parameters. This would pave the way for new rectal delivery formats and development of dual-compartment microbicide products that may be used vaginally and rectally. A nAb-based rectal specific product is not currently in development, however the pre-clinical safety and efficacy data for nAbs in colonic tissues (Chapter 1) (155) strongly support further evaluation of nAbs for rectal use.

Multipurpose products had been conceived of early on in microbicides research, and microbicide candidates that also had purported contraceptive efficacy – N-9, SAVVY, Cellulose sulfate and BufferGel – were highly desirable, yet these early products were not safe or did not show antiviral or contraceptive activity. In this new phase of microbicide development multiple investigational products are being developed, but the most progress has been made with ARV-based multipurpose technologies, including intravaginal rings for sustained release of combinations of dapivirine or tenofovir, and the hormonal contraceptive levonorgestrel (164, 165). Combinations of TFV with the anti-herpetic, acyclovir (165) are also being developed to prevent HIV and HSV-2 infection. Similarly, non-ARV multipurpose options are being investigated. Engineered *Lactobacillus jensenii* which produce cyanovarin-N are being developed to prevent BV and HIV (166). These engineered bacteria are intended to colonize the vagina and promote a healthy vaginal microbiota, while producing sustained protective concentrations of cyanovarin-N. Also, a phase I safety study of the first nAb microbicide product, a vaginal film containing a combination of VRC01 and the anti-HSV-2 antibody HSV-8 is ongoing (Clinical Trial #NCT02579083).

The results of comparisons of nAb efficacy against the transmitted/founder virus strain, THRO (Chapter 2, Table 4) raise important questions about the success of this investigational VRC01/HSV-8 combination film microbicide. THRO demonstrated resistance to VRC01 neutralization *in vitro*, likely due to natural envelope polymorphisms in the THRO envelope gene (Chapter 2, Figure 5). That observation, along with the consideration that VRC01, despite its broad neutralization activity was

not 100% neutralizing against the panel of viruses used in its evaluation (154), collectively suggest that use of a single nAb intervention may not be effective as a microbicide. As microbicides are required to have great breadth of efficacy to accommodate the diversity of potential inocula, combinations of complementary HIV nAbs like PG9 or PG16 with VRC01, may provide better functional protection from HIV transmission.

Alternative delivery formats are also being explored for non-ARV microbicides and the expansion of PrEP products. These new formulations are generally long-acting or sustained release delivery formats that are desirable for improving adherence as they do not rely on the user to adhere to a daily dosing regimen or use with every sex act. An example of the potential benefit of a long-acting formulation could be found in the results of the VOICE and FACTS 001 trials. These trials evaluated the efficacy of pericoitally applied tenofovir 1% gel in high-risk young women. Poor efficacy in these trials was attributed to poor adherence (67, 167); hence reliance on coitally dependent topical formulations may ultimately jeopardize the success of a microbicide, especially when adherence to the product is not optimal. Preclinical data for neutralizing antibodies and lectins indicate that they may be successful as topical microbicides but their pharmacokinetic coverage has not been fully defined in that context. However their activity is expected to be limited to the tissues of the vaginal or rectal lumen and provide protection over just a few hours. Hence, long-acting formulations of non-ARV microbicides may be most beneficial as they would accommodate less rigid application

guidelines, allowing the user a wider window of protection. That leeway may result in non-ARV microbicides being more effective overall.

A long-acting injectable formulation containing the NNRTI, rilpivirine, is being developed as PrEP for monthly administration. Most of the drug candidates being developed for HIV prophylaxis to date have been either RT inhibitors or entry inhibitors; but a long-acting injectable product containing the integrase inhibitor, cabotegravir is currently undergoing clinical evaluation. The cabotegravir long-acting nanosuspension has a long half-life that favors a quarterly dosing window (168). The major concern with the use of long acting injectable formulations is that due to their long-acting pharmacokinetic properties, they persist long after their therapeutic dose has waned, resulting in suboptimal amounts of drug for up several months (169). This creates a scenario where drug resistant virus may be selected if HIV infection occurs during this period (170) – the same problem they were designed to prevent. The use of long-acting nAb formulations may circumvent this risk as the likelihood of developing drug resistance is remote to nAbs. Ongoing clinical trials of a long acting VRC01 injectable for HIV prevention will test this hypothesis (Clinical Trial #NCT02568215).

Alternative delivery formats are also being considered for non-ARV microbicide candidates. Studies are ongoing to determine the safety and pharmacokinetic metrics of a passive infusion of the broadly neutralizing HIV antibody, VRC01, in children, and cohorts of HIV-positive and negative adults (171-173). In addition, exploratory studies conducted to evaluate the sustained release of HIV neutralizing antibodies achieved

through the intramuscular administration of adenoviral vectors showed that humanized mice were protected from HIV infection (174). This novel delivery platform would provide longer lasting protection and represents a trend toward designing HIV preventatives with more prolonged protective effects than traditional on-demand microbicide products.

Hence, advances in microbicide development encompass new formulations for topical microbicide products and have expanded the scope of microbicides to include rectal products. Novel delivery formats like injectables further broaden the scope of microbicides beyond topical products and effectively expand PrEP options. Also the focus on multipurpose products incorporates contraceptives that can add value to microbicide use for the user and may help avoid the stigma of taking an HIV preventative if contraceptives are widely used and accepted. However the heavy reliance on ARVs for prevention may ultimately create a landscape of increasing circulating drug resistance and decreased treatment options. This highlights a role for non-ARVs in HIV prevention.

THE FUTURE OF NON-ARV MICROBICIDES

The new generation of active biologic non-ARV microbicides represents a departure from the non-HIV specific compounds that had been considered previously. They are highly specific for HIV and are expected to inhibit transmission of viral strains that are resistant to HIV reverse transcriptase, integrase, and protease inhibitors. These non-

ARVs hold promise for the future of HIV prevention, presenting an array of options to individuals who may not benefit from ARV-based microbicide products. Antibody- and lectin-based microbicides provide options for individuals in HIV serodiscordant partnerships who are trying to conceive, or where the infected partner is on ARV therapy. They may also be a more attractive non-chemotherapeutic option for individuals who want to avoid the potential side effects of taking a drug or the stigma of taking an ARV for HIV prevention. Preclinical safety and efficacy data on these compounds have been promising, and although clinical evaluations of non-ARV microbicide candidates are just beginning, they provide hope for a novel non-ARV microbicide product in the near future.

Despite the many experimental microbicides in the development pipeline, the only approved biomedical HIV preventative has been Truvada, an oral ARV-based PrEP product. Formulation and delivery options have expanded to include more long-acting products; although the reliance on ARV-based prevention still presents the risk of developing drug-resistance. Additionally, most of the products are designed to prevent HIV infection, but there are currently no broad spectrum ARV-based microbicides in development to decrease the risk of concomitant transmission of other sexually transmitted pathogens that may also increase the risk of HIV acquisition.

A vaginal ring containing a combination of TFV and acyclovir, and the VRC01/HSV-8 vaginal film discussed previously are both being developed to simultaneously prevent HIV and HSV-2 transmission. The non-ARV candidate Griffithsin has broad spectrum

inhibitory activity against HSV-2 (86), hepatitis C (87), and coronaviruses (88) *in vitro* and is being investigated to define its broad spectrum activity. However there still remains a gap in biomedical interventions that provide comprehensive protection from sexually transmitted infections caused by both bacterial and viral pathogens.

Combinations of nAbs could be used to prevent transmission of multiple sexually transmitted pathogens without the risk of drug interactions that may be a factor with combining active pharmaceutical compounds. While generalized multipathogen microbicides could be developed, it may be more beneficial to design microbicides that are tailored to the prevention needs of a specified geographic region or vulnerable population. Hence a nAb microbicide could be used to prevent HIV, chlamydia and HPV in one population, while another nAb product that prevents HIV, HSV-2 and gonorrhea may be used in another population. In this way nAb-based microbicides have the capacity to generate multiple iterations of tailored microbicide products and could generate more flexibility for their implementation. Additionally, nAbs could be combined with hormonal contraceptives as a multi-purpose product. Alternatively, antibodies to various contraceptive targets may become an option for non-hormonal contraception in the future. nAbs to seminal targets may be engineered using some of the strategies utilized by vaccine researchers to generate antibodies to novel epitope targets, although data on the antigenicity of spermatozoa is limited.

PUBLIC HEALTH SIGNIFICANCE

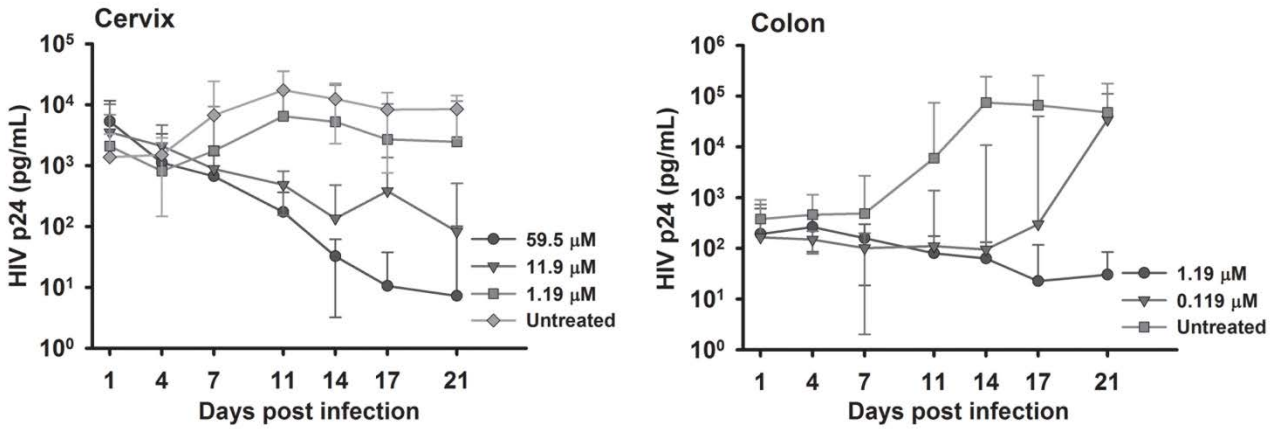
There are approximately 37 million people living with HIV globally, and current estimates of incidence anticipate 2 million new HIV infections. While the number of new infections has fallen over the past 14 years (175), HIV/AIDS remains one of the greatest threats to public health in this century. There are few biomedical interventions to prevent sexual transmission of HIV, which is the most common route of infection. Oral PrEP has been approved but is only available in a few populations, and an HIV vaccine remains elusive. Hence the potential of HIV microbicides looms large as a viable tool to reduce HIV incidence.

The data presented in this body of work suggest that neutralizing antibodies are safe and effective options for HIV prevention and present a much-needed alternative to ARV-based preventatives. Such heavy reliance on ARV products increases the risk of circulating drug resistance in the population. This could abolish the utility of current therapeutic ARV regimens and jeopardize the great progress made in controlling the spread of HIV, and improving the health of persons living with HIV. Use of nAb-based microbicides would avoid this risk because antibodies are not expected to contribute to ARV resistance and can reduce transmission of drug-resistant HIV. Hence nAb-based microbicides can be used to provide truly universal protection from sexually transmitted HIV infection and their development is a real opportunity to abolish new HIV infections toward the goal of ending AIDS.

APPENDIX A: ABBREVIATIONS USED

ARV	Antiretroviral
BV	Bacterial vaginosis
cART	Combination antiretroviral therapy
CHO	Chinese hamster ovary
DPV	Dapivirine
ELISA	Enzyme-linked immunosorbent assay
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
MPER	Membrane proximal external region
N-9	Nonoxynol-9
nAbs	Broadly neutralizing HIV antibodies
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitor
PrEP	Pre-exposure prophylaxis
RT	Reverse transcriptase
TFV	Tenofovir

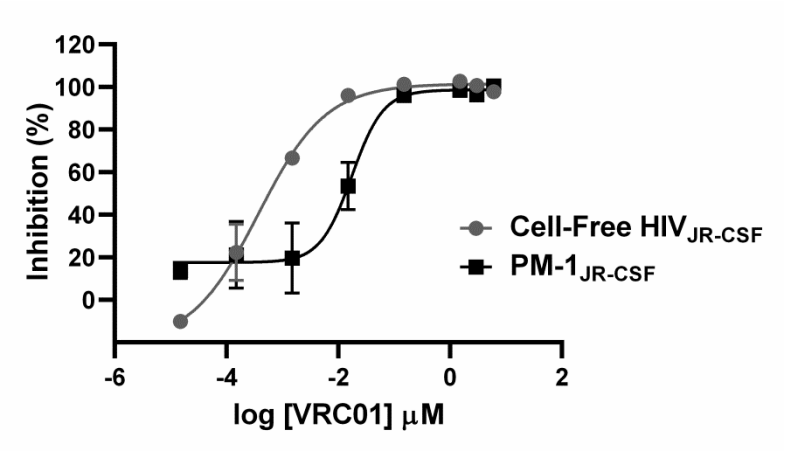
APPENDIX B: EFFICACY OF HIGHER CONCENTRATIONS OF 4E10-N AGAINST CELL-FREE HIV_{JR-CSF}



Efficacy of 4E10-N against cell-free HIV_{JR-CSF} at higher concentrations

Tissues were treated with 4E10-N at concentrations equivalent to 50× (59.5 µM), 10× (11.9 µM), 1× (1.19 µM) and 0.1× (0.119 µM) the *in vitro* IC_{90} before inoculation with HIV_{JR-CSF}. Viral replication was monitored by p24 antigen ELISA on basolateral culture supernatants collected 4, 7, 11, 14, 17 and 21 days post infection. Data points represent the median and interquartile range of ≥3 tissues from individual donors.

APPENDIX C: COMPARISON OF VRC01-N NEUTRALIZATION OF CELL-FREE HIV$_{JR-CSF}$ AND CELL-ASSOCIATED PM1$_{JR-CSF}$ INOCULA



Comparative VRC01-N IC_{90}	
Cell-Free HIV$_{JR-CSF}$	PM1$_{JR-CSF}$
7.9×10^{-3} μM	7.3×10^{-2} μM

Comparison of VRC01-N neutralization of cell-free HIV$_{JR-CSF}$ and cell-associated PM1$_{JR-CSF}$ inocula

TZM-bl cell monolayers were treated with titrations of VRC01-N for 1h before being inoculated with the equivalent of 3,000 $TCID_{50}$ of cell-free HIV$_{JR-CSF}$ or PM1$_{JR-CSF}$ and cultured for an additional 48h. Tat-activated luciferase expression was detected using the Bright-Glo Luciferase Assay reagent (Promega, Madison, WI) and luminescence was measured using the SpectraMax M3 plate reader (Molecular Devices, LLC; Sunnyvale, CA). The IC_{90} of VRC01-N was determined for each inoculum using GraphPad Prism software version 6.05 (GraphPad Software, La Jolla, CA). Data are from 3 experiments performed in triplicate.

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