Microbicidal Approaches to Interdict Virus Infections in Human Epithelial Cell Cultures, Organ Tissue Cultures and Human Epithelial Xenografts

A Thesis

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Veronica M. Holmes

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Dedication

This dissertation is dedicated to my husband, Bill, for his unwavering love and support without which I would not have had the strength to endure and complete this enormous achievement.

I would also like to dedicate this dissertation to Dr. Mary K. Howett, a wonderful advisor, mentor and friend, who I will miss deeply. This dissertation would not have been possible without her constant guidance, teachings and support. Dr. Howett had wonderful perspective not only in science but also in life. She wanted everyone around her to live life to the fullest just as she had done and she would accept nothing less. According to Ralph Waldo Emerson's definition of success, Dr. Howett was the most successful person I have had the pleasure to have in my life and whether Emerson knew it or not he was decribing Mary K. Howett.

"To laugh often and much; to win the respect of intelligent people and the affection of children; to earn the appreciation of honest critics and endure the betrayal of false friends; to appreciate beauty; to find the best in others; to leave the world a bit better whether by a healthy child, a garden patch, or a redeemed social condition; to know even one life has breathed easier because you have lived. This is to have succeeded."

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Abstract

Microbicidal Approaches to Interdict Virus Infections on Human Epithelial Cell Cultures, Organ Tissue Cultures and Human Epithelial Xenografts

Veronica Holmes

Mary K. Howett, Ph.D.

Sexually transmitted infections (STIs) are causing a hidden epidemic. Everyday infected individuals and their families quietly suffer physically, psychologically and financially. Worldwide, STIs are a major economic burden with direct medical costs for treating these diseases in United States alone is $15.3 billion per year. As with many diseases and infections, prevention is the key and with our current technology the best hope for prevention is microbicides. The work set forth in this thesis examined three vastly different microbicides each with very different modes for preventing the initial stages of HIV-1 infection. The prototypic SDS Hydrogel is a broad-spectrum microbicide. We show that our prototypic SDS Hydrogel has potent antiviral activity against enveloped (HIV-1 and HSV-2) and non-enveloped (HPV-11) sexually transmitted viruses. The second microbicide is comprised of a liposomal delivered short interfering RNA (siRNA) pool targeting human chemokine receptor 5 (CCR5). The down-regulation of CCR5 along with other molecules (gp340, syndecans, a4ß7 integrin), which allow HIV-1 to remain infectious, may potentially inhibit binding of R5 forms of HIV-1 from binding to the co-receptor, therefore, preventing the subsequent infection. Lastly, the recombinant *L. plantarum* secreting cyanovirin is a pro-biotic microbicide that decreased HIV-1 infectivity *in vitro* and we were able to recolonize human vaginal epithelia *ex vivo* and *in vivo*.

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1. Introduction

1.1 The sexually transmitted disease epidemic, the crux of the problem.

The vastness of the sexually transmitted infection (STI) epidemic is astounding (for review see (Low, Broutet et al. 2006; Aral, Fenton et al. 2007; Da Ros and Schmitt Cda 2008)). The STI epidemic has been compared to an iceberg, what you see is just a minute fraction of a much larger problem, most of which is unseen. (Dymally 2008) As of December 2007, 34 million people worldwide are infected with human immunodeficiency virus type 1 (HIV-1) (UNAIDS 2007), approximately 20 million people are infected with human papillomavirus (HPV) (CDC 2007) and at least 45 million people, in the United States alone, are infected with herpes simplex virus type 2 (HSV-2).(CDC 2006) These infections may be life-long and there are no cures just pharmacological maintenance regimes or surgical treatments. If these infections are diagnosed in the early stages often the best a person can hope for is to manage the resulting recurrent or chronic disease. These diseases can cause major physical and psychological limitations in daily living for the people who are infected. Although these chronic viral diseases are among the most common and costly health problems, they are preventable. In tandem with prevention strategies there are direct cost benefits to society due to preventing infections versus the cost of treating these infections. According to the Center for Disease Control (CDC), for every $1 spent on the Safer Choice Program (a school-based HIV, other STI, and pregnancy prevention program), about $2.65 is saved on medical and social costs. Every $1 spent on preconception care programs for women with diabetes, can reduce healthcare costs by up to $5.19 by preventing costly complications in both mothers and babies. (CDC, 2008) A recent

government report estimated the numbers of newly acquired STIs in teenagers in California for 2005 was 1.12 million with a resulting combined cost of treatment of $1.11 billion. (Dymally 2008) This cost estimate included lifetime medical costs based on incidence rates, undiagnosed and untreated disease and treatment costs. This estimate does not include indirect costs, such as lost wages due to time off of work or productivity. (Dymally, 2008) In light of such reports combined with the major economic burden in the United States having nationwide direct medical costs for treating STIs as high as $15.3 billion per year, almost 60 congressman introduced a resolution to Congress asking the Federal Government, States and localities to provide additional funding for screening and increased programming to educate Americans of all ages on the risks and the prevention of STIs. (Williams 2008)

Several studies have shown that infection with one STI increases a person's susceptibility to another STI with women being the most susceptible population to STIs. (Madkan, Giancola et al. 2006) This is a special concern in regards to HIV-1 transmission. (Wald and Corey 2003; Freeman, Weiss et al. 2006) Many times it is not recognized that sexually transmitted diseases affect our society and the world population as a whole. These diseases cause some of the biggest problems in our society such as, infertility, cervical cancer and AIDS. The pandemic of HIV-1 infection emerged in the 1980's and has raised many new challenges to infectious disease paradigms that existed at that time. (Yang 2004) Continued lack of an effective anti-HIV-1 vaccine and the unabated spread of HIV-1 emphasize the

urgent need for the development of new preventative measures to stop transmission, such as topical microbicides (for review see (Balzarini and Van Damme 2007; van de Wijgert and Shattock 2007)). Currently there is uncertainty in the scientific community as to whether an efficacious vaccine can be developed with our current technology. One group, the AIDS Healthcare Foundation, has requested that National Institute of Allergy and Infectious Diseases (NIAID) suspend HIV-1 vaccine research. (IAVI 2008) They believe the wisest and most effective use of the limited public resources should be focused on preventing new infections. (IAVI 2008) The most prudent way to prevent new infections is the use of microbicides. A microbicide is defined as any compound that can be placed inside the vagina or rectum to prevent the transmission of STIs. As a complex retrovirus, HIV-1, the positive stranded RNA virus infects specific immune cell populations, which results in the infection eventually destroying the host's immune system leaving the infected individual in a severely immunocompromised state and vulnerable to many infectious pathogens and opportunistic infections. (Knipe and Howley 2001; Yang 2004) In developed countries the introduction of combination antiretroviral therapy has had a significant impact on the course of HIV-1 infection, with AIDS-related mortalities having decreased by more than 80% since the their introduction in the mid-1990s. (Egger, May et al. 2002; Porter, Babiker et al. 2003; Schneider, Gange et al. 2005) There are more than twenty different antiretroviral drugs in four different classes approved by the FDA for the treatment of HIV-1 infection (Knipe and Howley, 2007) The antiretroviral treatments each target different stages of the viral lifecycle. There are drugs that block fusion of virion to the host cells

(Lalezari, Henry et al. 2003; Fatkenheuer, Pozniak et al. 2005), drugs that block reverse transcription (Parker, White et al. 1991; Merrill, Moonis et al. 1996; Havlir, Tierney et al. 2000) (nucleoside, nucleotide and non-nucleoside, inhibitors) and drugs that block the processing of the gag-pol polyprotein by protease into their mature subunits.

The two primary methods of STI transmission are exposure of mucosal surfaces to infected semen or vaginal fluids. In contrast, genital ulcer and genital lesion diseases such as genital herpes and human papillomavirus, respectively, are primarily transmitted through contact with infected skin or mucosal surfaces. Latex condoms are designed to impede the contact of semen and/or vaginal secretions to mucosal surfaces thereby providing a greater level of protection against HIV-1. Condoms provide less protection against HSV-2 and HPV because these infections may be transmitted by exposure to infected areas that are not covered or protected by the condom. (CDC 2008) Vaccines against HIV-1 are in development but success is not expected until the distant future and while pharmaceutical treatments exist they are not always effective and many times increase the prevalence of multidrug resistant strains.(Wolkowicz and Nolan 2005) In addition, many of these therapeutics or preventatives are economically unfeasible and inaccessible in many parts of the world where the infection is the most prevalent.

1.2 HIV-1 pathogenesis and life cycle.

AIDS was first discovered in the early eighties after a spectrum of clinical disorders that included Kaposi's Sarcoma, *Pneumocytis jiroveci* pneumonia and *Candida esophagitis*, afflicting male homosexuals and intravenous drug users. (Knipe and Howley 2007) The causative virus, HIV-1 belongs to the family *Retroviridae* genus *lentiviridae*. The viral genome is a homodimer of linear, positive-sense, single stranded RNA with each monomer being approximately 9.7 kb in size.(Knipe and Howley 2001) The homodimer is maintained by interactions between the two 5' ends of the RNAs in a self-contain a string of poly (A) sequences, approximately 200 long at the 3' end. (Knipe and Howley 2001) The intrinsic mutation rate for HIV-1 is extremely high because of the non-proofreading ability of the viral reverse transcriptase that reverse transcribes the viral RNA genome into double stranded viral cDNA, which is later integrated into the host's genome. HIV-1 infects CD4 positive (CD4+) lymphocytes, with the M-Tropic form (R5) of HIV-1 using CCR5 as the co-receptor and the T-Tropic (X4) form using CXCR4 as the co-receptor. (Lederman 2006) When the virion binds to the target, cell it must bind the CD4 receptor as well as a co-receptor, CCR5 or CXCR4 before fusion can occur. This is mediated by binding of the viral envelope protein gp120 to two proteins on the surfaces of the target cells, namely CD4 and a co-receptor.(Agrawal, VanHorn-Ali et al. 2004) In the initial stages viral infection, trimeric gp120 binds the cell surface receptor, CD4, and undergoes a conformational change exposing a second binding site for the co-receptor to bind to

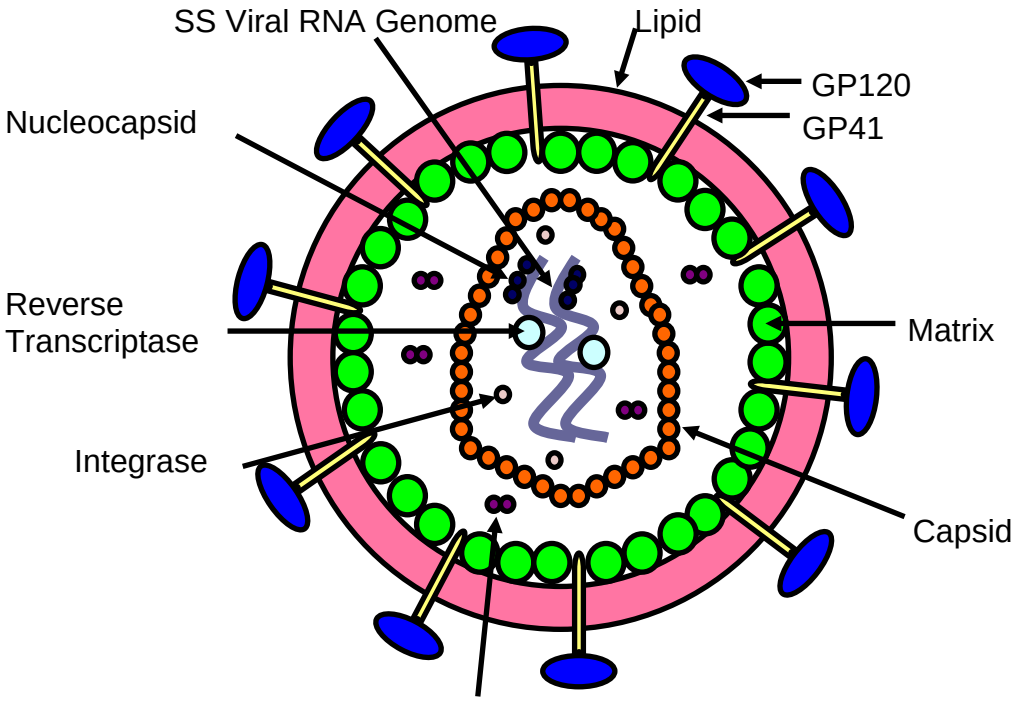


Figure 1: Diagram of mature the HIV-1 Structure

HIV-1 is an enveloped RNA retrovirus. The lipid bilayer is derived from the host and helps HIV-1 to evade the immune system. There are two viral glycoproteins, gp120, referred to as the surface unit that binds CD4 and co-receptor, and gp41, which is the transmembrane unit that is inserted into the host target cell drawing the virion and cell closer together allowing fusion to occur. The matrix protein (p17) interacts with the host cell membrane and plays an important role in infectivity. Capsid proteins (p24) form the core domains. Protease protein cleaves the polyprotein gp160 into gp120 and gp41. Integrase is a component of the pre-integration complex and is responsible for integrating the reverse transcribed double stranded viral cDNA into the host's cell genome. Reverse transcriptase is

responsible for reverse transcribing the viral RNA genome into viral cDNA. The nucleocapsid proteins encapsidates full length unspliced genomic RNA into virions.

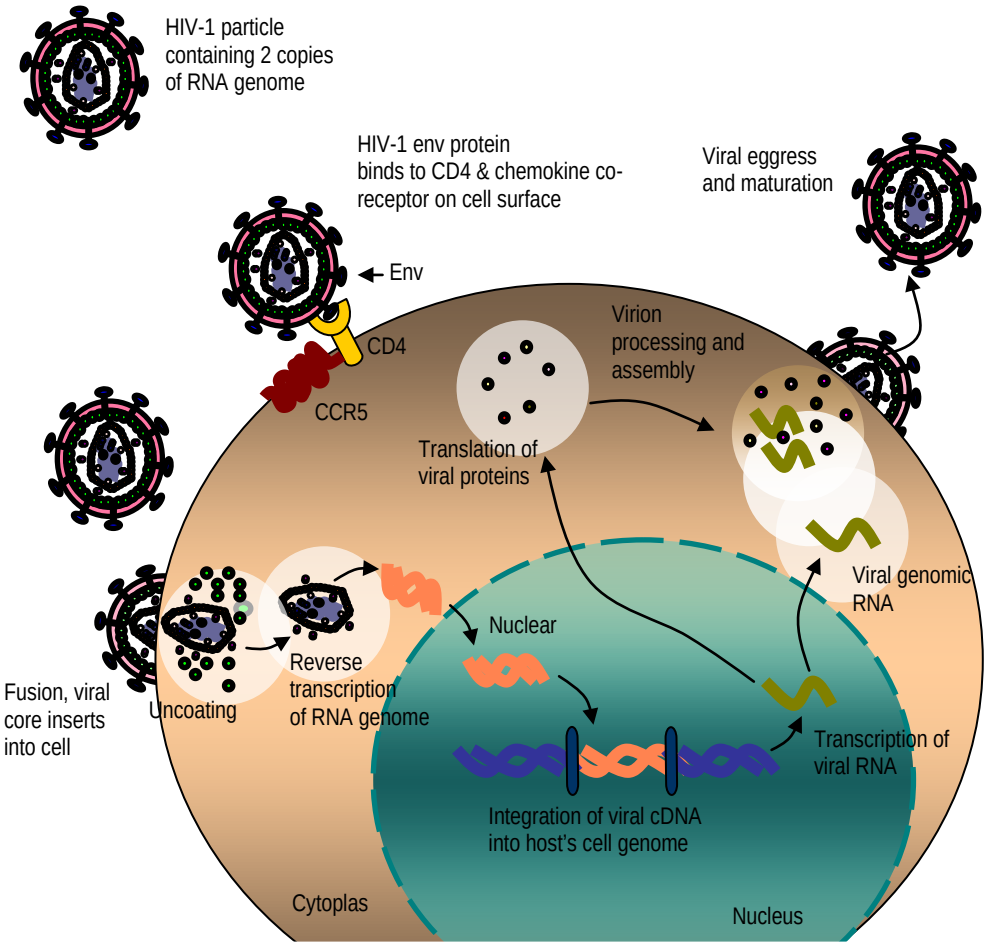


Figure 2: HIV-1 Lifecycle

HIV-1 glycoprotein 120 (gp120) binds CD4 and undergoes a conformational change exposing a binding site for a co-receptor, CCR5or CXCR4. There is another confirmational change in gp120 allowing gp41 to be inserted into the host membrane. After which, gp41 undergoes a confirmational change folding back on itself allowing fusion to occur. Following viral fusion, uncoating and reverse

transcription occurs with the resulting viral cDNA associating with viral proteins and translocating into the nucleus where integration occurs.

gp120. After gp120 has bound the co-receptor, CCR5 or CXCR4, gp120 undergoes a conformational change exposing the viral trimeric glycoprotein 41 (gp41) allowing gp41 to be inserted into the host cell membrane. (Lederman 2006) Once gp41 is inserted into the host cell membrane, it undergoes an additional conformational change forming a six helix bundle. This six helix bundle undergoes a conformational change, folding back on itself bringing the infectious virion and the CD4 positive lymphocyte closer together thereby allowing subsequent fusion to occur. Following fusion and internalization, uncoating of the capsid occurs and viral reverse transcriptase reverse transcribes the viral RNA genome into double standed cDNA in the cytoplasm of the host cell. The double stranded cDNA associates with several viral proteins, integrase, vpr, tat and matrix proteins, forming the pre-integration complex (PIC). The PIC is translocated into the nucleus with the help of integrase and vpr, which associates with the nucleoporin components and contains a nuclear localization signal. (Heinzinger, Bukinsky et al. 1994; Muthumani, Choo et al. 2005) Once inside the nucleus integration occurs in a two step process, processing and strand transfer. (Fujiwara and Mizuuchi 1988; Brown, Bowerman et al. 1989) In the processing step, the viral protein integrase endonucleolytically removes two or three nucleotides from the 3' termini of the blunt ended double stranded viral cDNA, creating staggered ends. The second step,

strand transfer, occurs when the viral integrase catalyzes a staggered cleavage cuts in the cellular target DNA creating 5’ overhangs and the 3’ recessed ends of the viral cDNA are joined to the 5’ overhanging termini of the cleaved cellular DNA.

Figure 3: HIV-1 provirus

(Fujiwara and Mizuuchi 1988; Brown, Bowerman et al. 1989; Roth, Schwartzberg et al. 1989) The host's cellular machinery ligates the integrated viral cDNA. The integrated viral cDNA is now referred to as the provirus. At this point in the viral life cycle the provirus can be actively transcribed into viral RNA genome, viral mRNA, which may or may not be spliced depending on the protein to be translated or alternatively the provirus can remain latent and transcriptionally inactive until the host target cell is activated. Once the provirus is stably integrated into a resting memory CD4+ lymphocyte's DNA that cell continually harbors replication competent provirus, creating a "latent reservoir" of infection regardless of treatment. (Chun, Carruth et al. 1997; Finzi, Hermankova et al. 1997) Medical treatment and the immune system are ineffective against these proviruses or the cells they infect because they are transcriptionally inactive and not producing any viral proteins. Due to the half-life of infected cells, current estimates to achieve viral elimination by slowly activating infected memory CD4+ lymphocytes is more than 70 years. (Zhou, Zhang et al. 2005) Thus, there is no way to eliminate the stably integrated provirus.

The exact mechanisms, of viral spread in the female genital tract are still unknown, including which cells and tissues are first infected. However, there are three proposed mechanisms of viral spread in women that have been described for

HIV-1 during vaginal intercourse. The first is the classical route of entry, which is characterized by the binding of cell-free virions in semen to a permissive host cell (vagina or cervix) via receptor interactions (binding of CD4 and co-receptor), followed by entry into the cytoplasm by fusion and subsequent steps in the replicative process. (Kilby and Eron 2003; Pierson and Doms 2003; Piguet and Quentin 2004) Second, resident immune cells, such as dendritic cells can capture virus via viral binding to the C-type lectins or other cell surface receptors, without necessarily becoming infected and re-present infectious virions to a permissive target lymphocyte (a process known as infection in *trans*). (Jameson, Baridaud et al. 2002; Piguet and Quentin 2004) Third, an HIV-1 infected cell (in semen or from a primary infected site) can infect a second cell without the requirement for release of cell-free virions into the surrounding extracellular environment, which represents viral transmission/propagation through direct cell-to-cell contact (Piguet and Quentin 2004) To understand the pathogenesis of HIV-1 and halt its spread heterosexually and perinatally, it is important to examine the virus in the genital tract of women. (Philpott, Burger et al. 2005) A recent study has demonstrated that the genital tract and blood harbor genetically distinct populations of replicating HIV-1 and provide evidence that recombination between strains from the two different compartments may contribute to the rapid evolution of viral sequence variation in infected individuals. (Philpott, Burger, et al. 2005) Another study reported that in both treated and untreated women, the plasma viral load may not be a reliable marker of transmission since one forth of the non-viremic patients in the study were still shedding virus in the genital tract, which indicates that caution is

required in judging the infectivity of women based on the plasma loads only in both sexual and mother to child transmission. (Fiore, 2004) Information about HIV-1 replication in the genital tract of infected women suggests that most of the cell-free viral quasispecies is shed in vaginal and cervical secretions are the products of local virus infection. (Sullivan, Mandava et al. 2005) Therefore, a successful topical microbicide applied vaginally should and would prevent the sexual transmission of HIV-1 from an infected person to a naïve or uninfected person. This latter property is in addition to the previously stated requirement of being able to inactivate incoming virus and virus infected cells.

1.3 The Human papillomavirus lifecyle, pathogenesis and association with cervical cancer.

Human papillomavirus (HPV) infection is the most common sexually transmitted disease in the United States (CDC 2007; Dunne, Unger et al. 2007; Forhan 2008), HPV DNA has been found in 90% of cervical cancers (zur Hausen 2002) and HPV-16 represents about 50% of the viral types identified in these cancers (Bosch,

Manos et al. 1995). Every year 450,000 women are diagnosed with cervical cancer and 250,000 women worldwide and 5,000 women in the United States die needlessly due to cervical cancer. In 2006, the vaccine Gardasil® developed by Merck Laboratories was approved by the FDA and made commercially available. This vaccine is comprised of coat proteins from HPV 6, 11, 16 and 18. Another vaccine, Cervarix™ developed by Glaxo SmithKline (GSK) is currently approved only in Europe but GSK is currently seeking approval by the FDA for use in the

United States. Both of these vaccines are designed for pre-sexually active females, ages 9-26, to prevent the initial HPV infection and the subsequent possibility of developing cervical cancer. These vaccines do not cure existing infections nor do not protect against other high-risk types. They only convey prophylactic protection

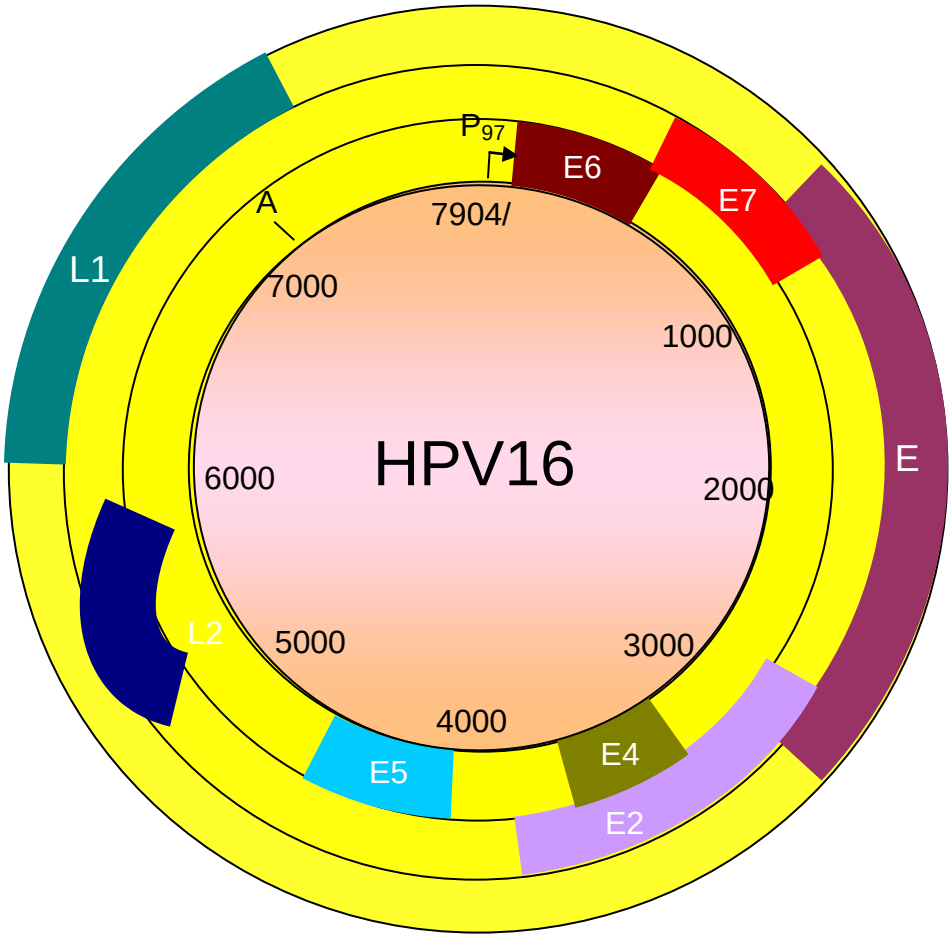


Figure 4: HPV16 genome structure

Note the overlapping open reading frames. The early 1 (E1) gene product controls viral replication and maintains the episome. The early 2 (E2) gene product regulates transcription and is a co-factor for viral replication. The early 4 (E4) gene product disrupts cytokeratins. The early 5 (E5) gene product interacts with growth factor receptors. The early 6 (E6) and early 7 (E7) produce an oncogenic gene products that associated with p53 and Retinoblastoma tumor suppressor genes, respectively. The late 1 (L1) gene product is the major capsid protein and late 2 (L2) is the minor capsid protein.

against the most prevalent types of HPV associated with cervical cancer. This leaves a significant number of the women, in the U.S. population and worldwide, unprotected and all women, vaccinated or not, vulnerable to infection by other types of human papillomaviruses. Thus, there is an urgent need for a broad spectrum microbicide that has been shown to have anti-HPV activity. HPVs are non-enveloped DNA viruses that contain supercoiled, ~8000 base pair circular, double stranded DNA genomes with a tightly packed and highly geometric proteinaceous capsid, consisting of major (L1) and minor (L2) capsid proteins. (Favre 1975; Gissmann, Pfister et al. 1977) Only one strand of the double stranded DNA genome is transcribed into mRNA, (for review see (Psyrri and DiMaio 2008)). Figure 4 illustrates how the HPV-16 genome is divided into three portions: a portion that encodes proteins primarily involved in viral DNA replication and cell transformation; ~3,000 base pair region that encodes the structural proteins (capsid) of the virus particle; and ~1,000 base pair noncoding region that contains the origin

of viral DNA replication and transcriptional regulatory elements. (Psyrri and DiMaio 2008; Munger, Baldwin et al. 2004)

HPV infects human epithelia with strict host and tissue target specificity and induces benign human neoplasms called “warts”, “papillomas” or “intraepithelial neoplasias”. Out of more than 100 HPV types, only a subset infects genital epithelia in men and women and a subset of these includes “high risk” viruses that may lead to cervical cancer. As shown in Figure 5, the natural history of HPV infection, it can take up to nine to fifteen years before cervical carcinoma develops and is diagnosed. (Goldie, Grima et al. 2003) The early gene 7 (E7) produces an oncogenic product that binds tumor suppressor protein, Retinoblastoma. A small subset of women demonstrate rapid onset of the disease. In the absence of external or frank genital lesions and because of a women’s anatomical structure many women are unknowingly infected. This is especially true of cervical infection. In male dominated, resource poor countries many women never receive the proper medical screening for early diagnoses and treatment, so unfortunately many do not find out until it is too late, when they are diagnosed with cervical carcinoma. One prominent example of this is cervical carcinoma associated with infection by HPV types 16, 18, 31, 35 and others (Ledwaba, Dlamini et al. 2004). In the U.S., fifty percent of cervical cancers are associated with HPV 16. Interestingly, not all women infected with high risk HPV develop cervical cancer and as shown in Figure 5 many cervical intraepithelial neoplasias (CIN I and II) can be cleared. It is accepted by the scientific community that another co-factor is required after HPV infection for progression to cervical intraepithelial neoplasia III (CIN III),

carcinoma *in situ* and frank cancer. For example, smoking increases the risk for cervical cancer by 4.5-7.0 fold relative to non-smokers. However, the mechanism remains unknown. For review, see (Harris, Kulasingam et al. 2004). Infection with a high-risk type human papillomavirus (HPV) constitutes the major and apparently required risk factor for developing cervical cancer in more than 90% of cases (zur Hausen 1994). For many years scientists were not able to grow HPV in the laboratory because of its fastidious nature. HPV requires the differentiation of the epidermis for its complete lifecycle. As illustrated in Figure 6, during the initial stages of the infection, HPV gains access through a cut or laceration in the squamous epithelia and infects the basal layer, the main mitotic layer, establishing its genome in low copy numbers as an episome in the nucleus of the cells. Very recently, one publication reported the presence of suprabasal stem cells in the epidermis. If correct, it remains possible that such cells could also be targets. Replication of the HPV genome is dependent on the host-cell DNA replication machinery and HPV-DNA replicates to high copy number only in terminally differentiated cells near the epithelial surface. The late viral genes, which encode the capsid proteins (L1 and L2), are expressed only in the highly differentiated cells, where infectious progeny virus may self assemble and are released in infected squames that are normally shed. (Psyrri and DiMao 2008) In many lesions, however, especially cervical lesions, L1 and L2 amounts are low and not much “infectious virus” appears to be present. It is not clear how this can be true for a virus group that successfully infects large numbers of women. This limitation in

virus particle production may not be representative of selected periods of the infection where virus assembly and shedding may be more active. In addition to the presence of HPV DNA in cervical neoplasias, active transcription of HPV DNA within dysplastic cervical lesions further establishes a strong molecular association of HPV with cervical neoplasia (Stoler 2000). The expression of early genes E6 and E7 predominates in high-grade CIN and invasive cancers, and E6 and E7 are also actively transcribed in cervical carcinoma cell lines containing HPV-16 or HPV-18 (Stoler 2000). Experimental studies on the viral oncoproteins E6 and E7 and their interactions with host cellular proteins provide a mechanistic basis for the

Figure 5: HPV Natural History of Infection
TO BE ADDED

link between HPV infection and cervical cancer. Several *in vitro* transformation assays have demonstrated that high-risk types of HPV E6 and E7 oncoproteins can immortalize primary human foreskin or cervical keratinocytes (McDougall 1994). However, the E6 and E7 genes can transform primary cells to cancers only in the presence of a second oncogene (Storey, Massimi et al. 1995), and continued expression of E6 and E7 is required to maintain the transformed phenotype. (DiPaolo, Popescu et al. 1993) The E6 proteins of the high-risk types inactivate the tumor suppressor protein p53 by inducing its degradation through the ubiquitin-mediated proteolysis pathway (Scheffner 1998). The E7 proteins of high-risk HPV bind to the tumor suppressor retinoblastoma protein (pRb). The binding of HPV E7 to pRb destabilizes pRb and results in the release of E2F (Berezutskaya and Bagchi 1997), which is capable of activating transcription of a variety of host genes, many

of which are involved in DNA synthesis and cell cycle progression (zur Hausen 2000). The activity of these oncoproteins results in genomic instability, and this may further contribute to the deregulation of HPV gene expression during carcinogenic progression and to mutation of host DNA. Cells with a growth advantage may be selected and give rise to clonal expansion and tumorigenic progression, which will ultimately result in full malignant transformation. Carcinomatous cells most often exhibit integration of the virus genome, which further increases the transcription of E6 and E7. Originally, scientists believed that HSV-2 was the causative agent of cervical cancer but it was later found to be HPV,

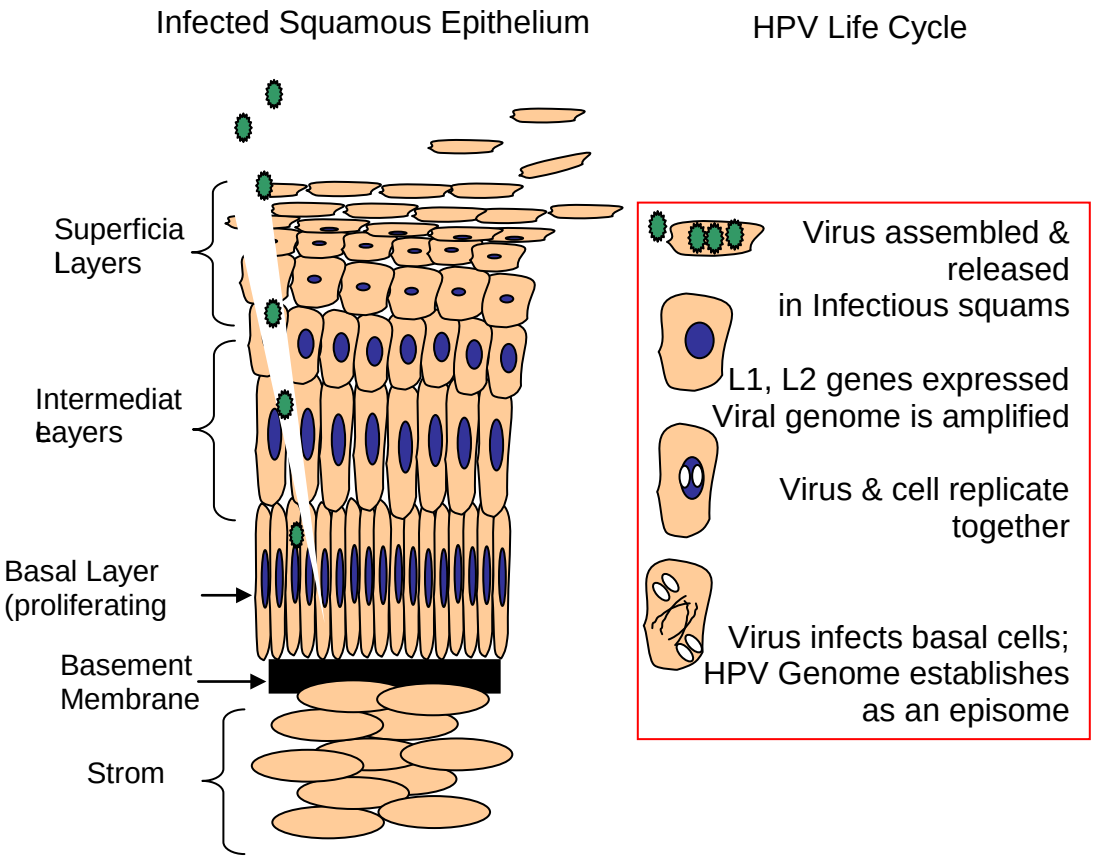


Figure 6: HPV Lifecycle

HPV infects the basal layer of the epithelia through a cut or wound and establishes its genome as an episome. Using the cell's machinery the cells and virus replicate together producing viral genomes and early gene products (E1, E2, E4, E5, E6 and E7). In the upper intermediate layers where the cells are beginning to change their morphology to a more flatten shape, the late gene products (L1 and L2) which are required for capsid assembly are produced. In the flattened most upper superficial layers viral assembly is completed and infected squames containing infectious HPV are released.

however, HSV-2 may still be a co-factor for development of cervical carcinoma in certain cases. (Orth, Jablonska et al. 1978; Boshart, Gissmann et al. 1984)

1.4 Herpes simplex virus type 2, compounding the problem

Herpes simplex virus type 2 (HSV-2) are large double stranded enveloped DNA viruses in the *Herpesviridae* family. (Knipe and Howley 2007) The genome consists of two unique regions, U_L (long) and U_S (short), flanked by repeated sequences. (Wadsworth, Jacob et al. 1975; Latchman 1991) HSV-2 infection is acquired through close contact with an infected person who is shedding virus from their epithelia or genital secretions. (Gupta, Warren et al. 2007) In the initial stages of the primary infection the HSV-2 particle attaches to cell surface receptors in mucosal tissues followed rapidly by viral fusion with the host cell plasma membrane. HSV-2 attaches and fusions to cells in three stages: (1) attachment of virion to muscoal cell

surface (WuDunn and Spear 1989; Herold, WuDunn et al. 1991); (2) interactions of glycoproteins with cellular receptors (Spear and Longnecker 2003); and (3) fusion of viral envelope with host cell plasma membrane resulting the viral capsid and tegument entry into the cell. (Campadelli-Fiume, Cocchi et al. 2000) HSV-2 has the ability to bind multiple receptor targets giving the virus the ability to infect almost all cell types *in vitro*, however *in vivo* HSV-2 infects epithelia in part because of availability of target sites in genital skin and mucosal surfaces. During attachment viral glycoprotein D (gD) interacts with one of several cell surface receptors, herparin sulphates or other glycoaminoglycans (for example, chondroitin sulfate) and a co-receptor. The co-receptors HSV-2 can utilize and bind are proteins belonging to the

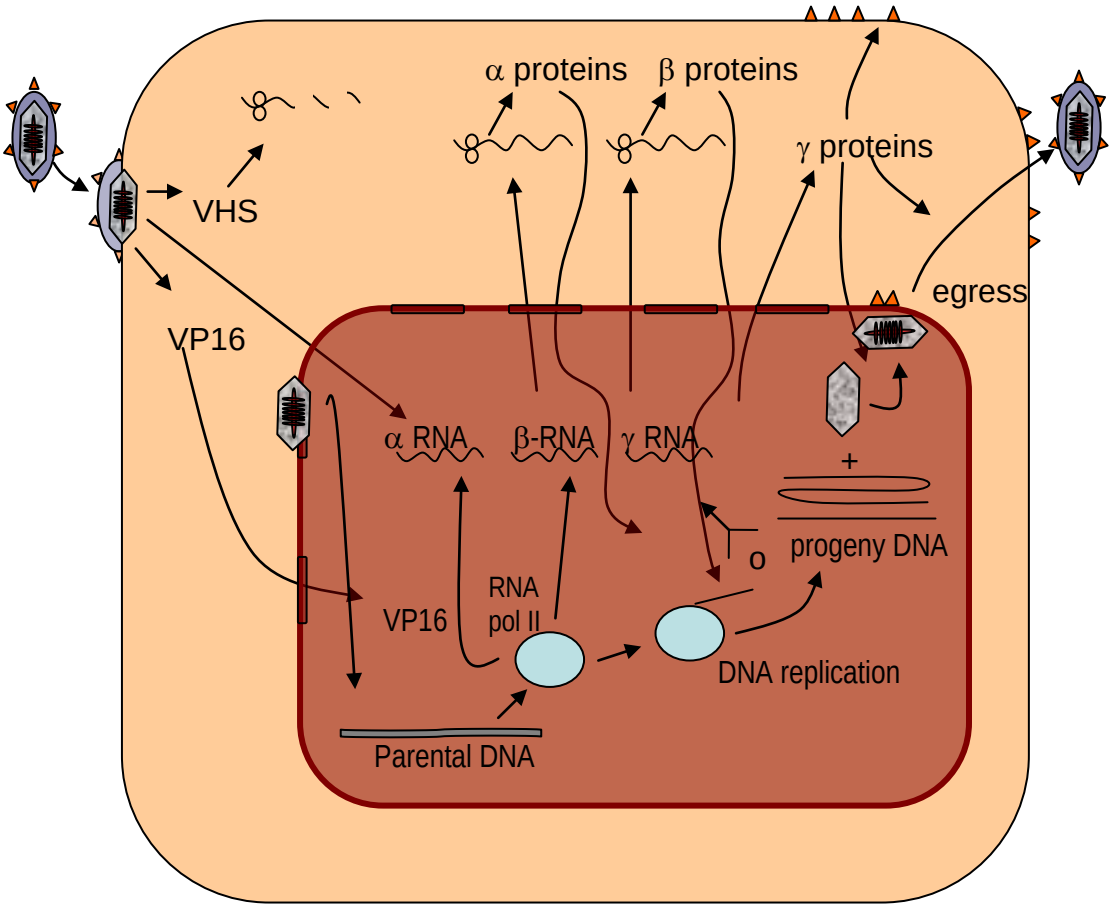


Figure 7: HSV-2 Lifecycle

HSV-2 particle attaches to mucosal cell surfaces and through interaction of viral glycoproteins with several different types of cells surface receptors, the virion fuses with the cell with viral capsid and tegument entry. Viral host shutoff protein (VHS) shuts off host functions and VP16 is translocated into the nucleus. Through interaction with the cell microtubule network, the capsid is transported to the nucleus where it releases the double stranded viral DNA into the nucleus. Viral DNA undergoes circularization and transcription begins. Transcription of the immediate early genes occurs first with the transcripts being transported into the cytoplasm for translation. Some of the immediate early proteins are transported back into the nucleus to shut off immediate early transcription and turn on genes for early gene transcription. The early gene products are translated in the cytoplasm and control cellular functions and some are translocated back into the nucleus to start viral DNA replication and late gene expression. The late gene products are structural capsid and tegument proteins required for assembly.

nectin family of proteins (Cocchi, Menotti et al. 2000), herpesvirus entry mediator proteins that belong to the tumor necrosis factor receptor family (Montgomery, Warner et al. 1996) or 3-*O*-sulfated heparan sulfate. (Shukla, Liu et al. 1999) Following viral envelope and host cell plasma membrane fusion some tegument proteins remain in the cytoplasm and start to effect cellular metabolism in several ways. Some proteins (viral host shut off factor) shut off host functions, while others (VP16) are translocated into the nucleus. The capsid interacts with the host cell microtuble network and is transported to the nucleus where it associates with a

nuclear pore releasing the viral double stranded DNA genome into the nucleus. (Kristensson, Lycke et al. 1986; Sodeik, Ebersold et al. 1997) After the viral double stranded DNA genome enters the nucleus it rapidly circularizes and transcription of immediate early genes commences. (McNamee, Taylor et al. 2000)

The host's RNA polymerase II and viral protein, VP16, facilitate this highly regulated process. The resulting immediate early mRNAs produced undergo splicing events and are transported to ribosomes in the cytoplasm for translation. Some of the resulting viral proteins are translocated back into the nucleus to shut off immediate early gene production and turn on early gene production. A portion of the subsequent proteins produced by early gene transcription and translation, remain in the cytoplasm to control cellular function and a portion translocates into the nucleus to start viral DNA replication via a rolling circular mechanism. (Strang and Stow 2005) This produces concatameric viral DNA, which serves as a template for late gene expression and viral genome replication. The proteins produced from late gene expression are mainly structural capsid proteins required for assembly, tegument and envelope proteins. Once the envelope proteins have been glycosylated in the Golgi they are transported to the host cell membrane for assembly. However, the capsid and tegument proteins are translocated into the nucleus where packing enzymes package the viral genome into the nucleocapsid. The completed resulting nucleocapsid combined with the tegument proteins line up at the nuclear membrane and bud into the endoplasmic reticulum (ER). After passing through the ER the partially assembled virus is processed in the Golgi, transported to the host cellular membrane where the fully packaged virus is released by exocytosis. However, when latency is established the virus enters sensory neurons and is transported via retrograde transport from the initial site of infection in the mucosal tissue to the sensory ganalion. (Gupta, Warren et al. 2007) The HSV-2 genome is maintained in a unintegrated circular latent state, with low level

expression of latently active transcript (LAT) gene products but without active replication of cytotoxicity. Studies suggest that cytotoxic T cells play a major role in maintaining viral latency by active surveillence. (van Lint, Ayers et al. 2004; Lang and Nikolich-Zugich 2005) When the immune system is stressed for some reason (i.e., fever, immunodeficiency, illness, stress, UV light) HSV-2 is reactivated in the neurons and is transported back to epithelia cells by anterograde transport and subsequent recurrent infection ensues. (Bloom 2004; Decman, Freeman et al. 2005)

HPV, HSV-2 and increasing HSV-1 infections (Xu, Sternberg et al. 2006) are all major causative agents of genital lesions or ulcers, respectively, causing lifelong infections with intermittent viral reactivation and vaginal shedding.(Gupta, Warren et al. 2007) When an infected individual has viral activation and an outbreak with genital lesions, CD4 positive T-lymphocytes infiltrate the tissue providing more target cells for HIV-1 infection (Rebbapragada, Wachihi et al. 2007; Kaul, Pettengell et al. 2008), thereby possibly increasing susceptibility to HIV-1 infection.

1.5 A modern approach to microbicides at a time when urgently needed.

The original concept of a microbicide arose from the over the counter (OTC) availability of spermicides. These spermicides, which are topical alternatives to birth control systems, are available in foams, creams, films and condom coatings, gained great popularity in the 1960s and 1970s with the onset of the sexual revolution.(Howett Kuhl 2005) Most of these spermicides contain N-9,

an non-ionic detergent that *in vivo* exhibits rapid killing of sperm at concentrations above 0.1%. By the mid 1970s scientists realized that N-9 worked by dissolving the sperm cell membrane and could possibly be used to dissolve the membranes of enveloped viruses. Several early reports (Rapp and Wrzos 1985; Hermonat, Daniel et al. 1992) showed efficacy *in vitro* against HSV-2 but not against non-enveloped viruses, such as bovine papillomavirus. A recent report presented at the National STD Conference in March 2008 by the CDC reported that one in four female adolescents (26%) in the United States has at least one of the most common sexually transmitted diseases (HPV, HSV-2, Chlamydia or trichomoniasis).

The impact of HIV-1 and consequent acquired immunodeficiency syndrome (AIDS) on women is severe, particularly in areas of the world where heterosexual transmission is the dominant mode of HIV-1 transmission.(UNAIDS 2004) According to the World Health Organization there were 2.5 million newly reported cases in 2007(UNAIDS 2007) and over 50% of the new infections are women.(UNAIDS 2003) Since 2003, women have increasingly become the face of the HIV-1 pandemic (UNAIDS 2003) and represent a source of infection for their uninfected partners and their children. The main HIV-1 prevention tools, condom use, reducing the number of sexual partners and treating other reproductive tract infections are not always feasible for many women living in resource poor settings and in male dominated societies. Women often have limited ability to enforce cooperation by their male partners to use condoms due to social, cultural and economic gender inequalities.(Van De Wijgert and Coggins 2002) Throughout the world, many women are submissive to their husbands or partners wishes due to

cultural, social or financial arrangements. In many instances, women are unable to refuse the sexual advances of their partners, thereby eliminating the opportunity to use contraceptive foams or creams. This issue is compounded by the ignorance of their partner's sexual activity or use of intravenous drugs, increasing the women's risk of acquiring STIs in both consensual and non-consenual relationships. Availability of an effective inexpensive and safe, female-controlled microbicide would provide women the tools to protect themselves against STI and impact the health of their uninfected partners and children.

Condoms, diaphragms and cervical caps all are coated with microbicides specifically designed for use with these devices. Since 2000 we have known that Nonoxynol 9 (N-9), the most broadly distributed spermicide in over-the-counter (OTC) products including lubricants in male latex condoms (FDA 2007), increases the risk of HIV-1 transmission to women frequently exposed to N-9.(Van Damme, Ramjee et al. 2002; van de Wijgert and Coggins 2002; Davis and Doms 2004) N-9 has not been banned by the US Food and Drug Administration (FDA), but in December 2007 the FDA emitted a final ruling requiring all OTC products containing N-9 to be prominently labeled indicating its risks of HIV-1 transmission to women.(FDA 2007) This ruling however, however, does not apply to condoms, which are subject to FDA's medical device regulation instead of under the jurisdiction of OTC products.(FDA 2007)

As previously described, HIV-1 is a sexually transmitted virus; it is found in two different infectious forms, cell-free and cell-associated. Since both forms are

found in semen and vaginal secretions an ideal microbicide must be efficacious against both forms. The complexity and multi-faceted aspects of HIV-1 infection have caused researchers to recognize the value of combining microbicides to interfere with infection using multiple modes of action or blocking multiple entry points. The most basic definition of a microbicide is a chemical or physical agent that can kill a microbe.(Howett Kuhl) But in the context of the STI epidemic, the term microbicide is defined by the World Health Organization (WHO) as "any compound that can be applied inside the vagina or rectum to protect against sexually transmitted diseases". Potential microbicides can be placed into one of three broad categories: (1) compounds that inactivate or disrupt viruses; (2) compounds that inhibit specific stages of virus infection and replication such as, fusion, uncoating, reverse transcription, etc.; and (3) compounds that specifically or non-specifically block infection by physically binding to the cell or virus surfaces (Davis and Doms 2004). The three different microbicides studied in this thesis each fall into one of these three broad categories and may be used accordingly based the person's circumstance. The SDS Hydrogels have broad spectrum activity and inactivate HIV-1, HSV-2 and HPV-11. The microbicide containing short interfering RNA (siRNA) targeting human chemokine receptor 5 (CCR5) may potentially inhibit binding of M-tropic HIV-1 forms from binding to the co-receptor, thereby, preventing subsequent infection. Lastly, is a pro-biotic approach by using recombinant lactobacilli secreting cyanovirin as a microbicide by specifically blocking infection by binding the HIV-1 glycoprotein 120 (gp120). Some microbicides have broad-spectrum activity against bacteria and viruses while

other microbicides are only specific for HIV-1, for example, attacking specific stages of the HIV-1 lifecycle. An ideal microbicide must have the following traits: broad spectrum activity against bacterial and viral sexually transmitted infections, efficacy against cell-free and cell-associated HIV-1, no or little effect on the structural integrity of vaginal and cervical mucosal epithelium, stability at tropical temperatures and resistant to acidic pH. To make it attractive and available for use, it must be odorless, colorless, tasteless, of low cost and readily accessible.(Balzarini and Van Damme 2007)

1.6 The prototypic SDS Hydrogel a versatile potential answer for the problem

While searching for compounds that would inactivate HPV, Dr. Howett and colleagues identified a prototype alkyl sulfate, sodium dodecyl sulfate (SDS) CAS #151-21-3, as a potential microbicide. Non-ionic surfactants, such as N-9, were found not to inactivate HPV because of the tightly packed protein coat of the virus. Sodium dodecyl sulfate (SDS) and other alkyl sulfates are anionic surfactants that also possess chaotropic properties. At concentrations below critical micellar concentration, (CMC), SDS enters into membranes and alters membrane fluidity. At and above the CMC concentrations, SDS will form micelles, solubilize cell envelopes and form mixed micelles with the solubilized lipids. When added to cells in culture, SDS will dissociate membranes into their lipid and protein components. In addition to previously being shown to disrupt enveloped viruses.(Becker, Helenius et al. 1975; Howett, Neely et al. 1999) Long term (24 hour) patching of SDS on skin can induce biochemical changes, namely

mobilization of dendritic cells and recruitment of lymphocytes, however, exposures up to three hours were not irritating.(Effendy and Maibach 1995; Aramaki, Loffler et al. 2001) Importantly, the combined surfactant and chaotropic properties of alkyl sulfates give SDS and other family members very effective anti-microbial and anti-viral activity. As far back as 1952, SDS was combined with sodium perborate for use as a douche to treat vaginal infections. (Karnaky 1952; O'Brien and Thoms 1955) Although SDS clearly can disrupt cells in culture, in intact mucosal tissues the surface keratinization, mucus and cellular exudates form a protective layer that is not easily disrupted by SDS. Sodium dodecyl sulfate and other alkyl sulfates are in the Generally Recognized As Safe (GRAS) list of the FDA (FDA 2006), are routinely used as a common ingredient in consumer products applied topically to the skin and mucosa (e.g. body lotions, baby wipes, toothpaste and shampoo) and as food additives (e.g. marshmallows and gelatin). SDS is metabolized and has been classified by the United Nations Environmental Program as readily biodegradable.(Howett Kuhl)

Our laboratory has shown that at concentrations ranging from 0.01% to 1% SDS kills HIV-1, HPV, herpes simplex virus (HSV) types 1 and 2 (Howett, Neely et al. 1999; Krebs, Miller et al. 1999; Krebs, Miller et al. 2000; Krebs, Miller et al. 2002; Howett and Kuhl 2005; Urdaneta, Wigdahl et al. 2005; Hartmann, Wigdahl et al. 2006), *Chlamydia trachomatis* (Achilles, Shete et al. 2002) *Neisseria gonorrhea* as well as Gram positive and Gram negative bacteria (Hartmann and Howett, unpublished results). Viral plaque assays proved that 0.1% and higher concentrations of SDS in aqueous solution inactivated HSV-2 and MAGI assays

proved that at these concentrations SDS also inactivated HIV-1. At the same concentrations, SDS prevented bovine papillomaviruses from inducing foci in epithelial cell monolayers, prevented Shope cottontail rabbit papillomavirus from forming papillomas in vivo, prevented HPV-11 from inducing experimental human papillomas. (Howett 1999) Control epithelia exposed to 1% SDS in vitro were not killed and the tissue yielded histologically normal epithelia 3 months after implantation in immunocomprised mice. 0.1% SDS in aqueous solution reduces HPV-11 virus titer by three fold, can directly affect dissolution of virions and release of virus DNA (Fang & Howett, Kish & Howett, unpublished observations). Because of its chaotropic properties SDS is also routinely used in laboratories for protein separation in polyacrylaminde gel electrophoresis. Our laboratory and others have shown that other alkyl sulfates including potassium laureth sulfate, lauric acid, lauryl alcohol, monolaurin and the related monocaprin also have antiviral activity. Yet, SDS remains the most efficacious.

Another potential use for SDS as a microbicide is the treatment of HIV-1 infected human breast milk. At low treatment concentrations (0.1-1%), SDS has been shown to inactivate high concentrations of purified HIV-1 added to expressed human breast milk. (Urdaneta). In addition, SDS was successfully removed, >90%, with a commercial resin (SDS Detergent Out) without substantially altering the nutritional or immunologic properties of the treated milk. The presence of the microbicide was sufficient to reduce infectivity of both cell-free and cell-associated virus and to allow rapid degradation of virus RNA in the treated milk. Thus, we are

proposing that prototypic SDS Hydrogel can be used as a vaginal microbicide but the compound SDS can also be utilized as a microbicidal treatment for breast milk.(Urdaneta)

1.7 Using RNAi to outsmart HIV-1

In 1996 a Hemophilia Growth and Multicenter AIDS Cohort study (Dean, Carrington et al. 1996) found that mutations in the Chemokine Receptor 5 (CCR5) co-receptor, confers some resistance in humans to infection by HIV-1 M-tropic forms. This mutation was found to be present at a frequency of ~0.10 in the Caucasian population in the United States.

The rate of HIV-1 disease progression varies considerably among infected individuals. A small but rare subset of chronically infected, therapy naïve individuals appear to maintain high and stable CD4+ and CD8+ T cell counts with low to undetectable plasma viral loads for an prolonged number of years.(Dean, Carrington et al. 1996; Mikhail, Wang et al. 2005) The importance of CCR5 in HIV-1 infection and pathogenesis was revealed by the discovery of the CCR5Δ32 allele, a 32 base pair deletion in the portion of the human CCR5 open reading frame that encodes the second extracellular protein loop between transmembrane domains four and five of the seven transmembrane architecture.(Dean, Carrington et al. 1996; Agarwal, Zainab Van Horn et al. 2004; Oppermann 2004) CCR5Δ32 encodes a truncated protein that is retained in the endoplasmic reticulum, which is not detectable on the cell surface.(Benkirane, Jin et al. 1997; Agrawal, VanHorn-Ali et al. 2004) People who are homozygous for the CCR5Δ32 mutation are resistant to HIV-1 infection from M-Tropic strains, which is the form most commonly sexually

transmitted, and heterozygous individuals expressing CCR5Δ32 progress more slowly to AIDS.(Dean, Carrington et al. 1996) Also, people that are homozygous for the CCR5Δ32 mutation appear to be phenotypically normal and to date the only immunodeficiency found was an increased susceptibility to a fatal West Nile Virus infection. For these reasons CCR5 has become a very attractive target of investigation to many researchers.

CCR5 is a G-coupled protein receptor, which has seven transmembrane domains with an extracellular N-terminal portion and a cytoplasmic C-terminal portion. It is found on CD4 positive T cells with memory/effector phenotype, monocytes, macrophages and Langerhan cells.(for review see Oppermann, 2004)

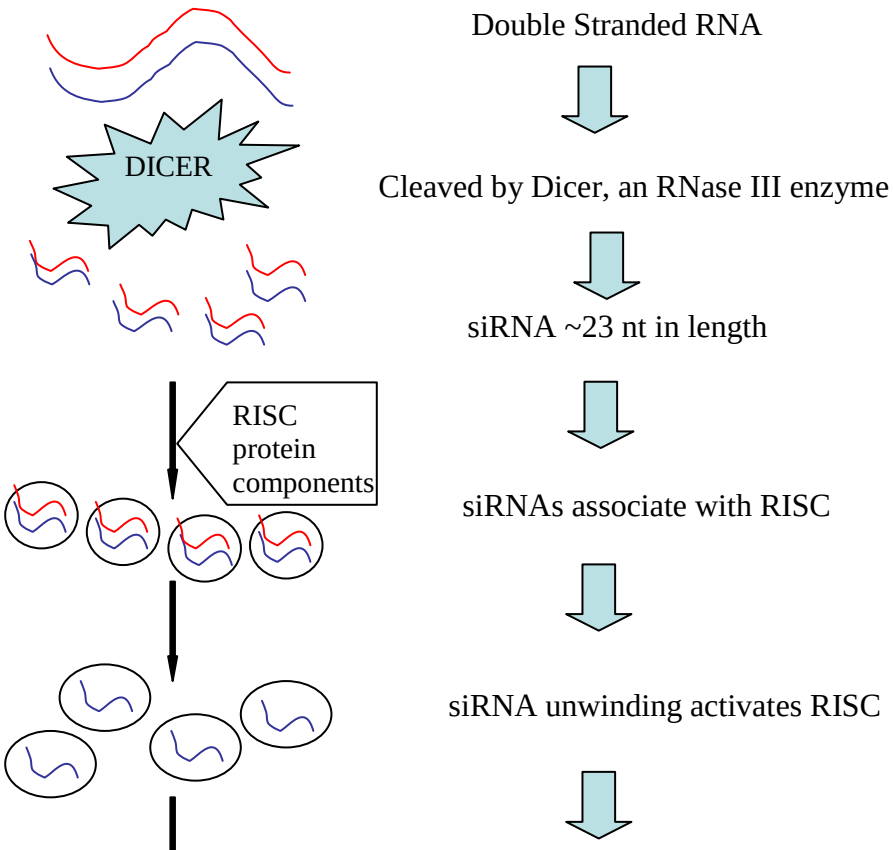


Figure 8: RNAi Pathway

This pathway is activated by the introduction of double stranded RNA into the cytoplasm of a cell. An RNase III type enzyme, Dicer, cleaves the double stranded RNA into short 20-23 nucleotide segments, which associate with the RISC complex. The RISC is comprised of Argonant proteins that are activated by the unwinding siRNA. The RISC helps to guide the siRNA to its complementary mRNA, which in turn is degraded.

CCR5 binds MIP-1α, MIP-1β and RANTES ligands. Initially, chemokines were identified as chemoattractant cytokines involved in leukocyte trafficking to sites of inflammation however, their biological functions do not end with chemotaxis. Chemokines have been shown to be involved in many different biological functions, such as angiogenesis, haematopoiesis, embryonic development and metastasis.(Oppermannm 2004; Yoshie, 2001; Gerard 2001) CCR5 is an important co-receptor for HIV-1 and is preferentially used by HIV-1 during the primary infection macrophages and lymphocytes.(Shea & Deng Nature 1997)

One promising approach scientists have taken in creating microbicides is creating synthetic chemokines molecules that compete with HIV-1 for the CCR5 co-receptor. CCR5 is a human chemokine that normally binds MIP-1ą, MIP-1β and RANTES chemokines. The most successful of the synthetic chemokines to inhibit binding of HIV-1 is PSC-RANTES, which is a RANTES analog chemically identical to native RANTES except for the substitution of a nonanoyl group, thioproline and cyclohexylglycine for the first three N-terminal amino acids of the native protein.(Lederman, Veazey et al. 2004) This synthetic PSC-RANTES was vaginally applied to rhesus macaques and upon challenging with SHIV, a simian immunodeficiency virus and human immunodeficiency virus chimera, it was shown that 12 out of the 15 monkeys treated were protected by 100 ųM treatment.(Lederman, Veazey et al. 2004)

Much of the human genome consists of remnants of previous transposon/virus invasions and elements that are still active to date, therefore one would expect that organisms need to fight off such invasions to prevent the genome from being completely taken over by molecular invaders.(Plasterk 2002) A novel approach in developing a microbicide is to use RNA interference (RNAi) (for review see Shrivastava, 2008; de Fougerolles, 2007) targeted towards the CCR5 chemokine receptor. This RNA-based silencing mechanism has emerged as an ancient mechanism that is conserved among species from different kingdoms (fungi, animals and plants) and very likely acts as the “immune system” of the genome.(Plasterk 2002) RNA interference was originally identified in *Caenorhabditis elegans*, while

studying a gene encoding a nonessential myofilament protein, *unc-22*.(Fire) Fire and colleagues demonstrated that the injection of a mixture of sense and antisense RNA molecules homologous to a 742 nucleotide segment of the *unc-22* produced more potent gene inhibition as compared to using sense or antisense molecules injected alone. As illustrated in Figure 9, RNAi is a sequence specific posttranslational gene silencing, which is triggered by double stranded RNA. After double stranded RNA introduction or formation, the long double stranded RNAs are cleaved by Dicer, a member of the RNase III family, into 21 – 23 nt small interfering RNA (siRNA) with two nucleotide 3' overhangs and 5' phosphate termini.(McManus, Haines et al. 2002; Milhavet, Gary et al. 2003) Then the processed siRNAs are incorporated into a multi-component nuclease complex, known as RNA induced silencing complex (RISC), which is responsible for the association and degradation of the target mRNA.(Martinez, Patkaniowska et al. 2002; Yu, DeRuiter et al. 2002) It has been shown that siRNA has been successful in down-regulating CCR5. In U87-CCR5 cells, which are engineered to express CCR5, CCR5 was down-regulated by 48% decreasing viral entry by 55% resulting in inhibition of HIV-1 replication.(Martinez, Gutierrez et al. 2002) siRNA targeted against CCR5 and HIV-1 p24 antigen together in infected macrophages also down-regulated CCR5 and inhibited viral entry for fifteen days.(Song, Lee et al. 2003) Another study showed down-regulation of CCR5 in MAGI-CCR5 cells that decreased CCR5 expression by ten fold, which in turn decreased viral replication by three to seven fold.(Qin, An et al. 2003)

1.8 Can recombinant probiotics be used to secrete a microbicide?

Probiotic is from the Greek meaning “for life” and is used to describe a specific type of live, beneficial bacteria that may be used to replace or replenish resident, commensal bacterial populations. The United Nations’ Food and Agriculture Organization (FAO) and the World Health Organization (WHO) formally define probiotics as live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host. (Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, Amerian Córdoba Park Hotel, Córdoba, Argentina. 1-4 October 2001). Therefore, probiotics are a class of beneficial bacteria that help to restore and maintain a healthy balance of “good” versus “bad” bacteria.

The bacterial species that colonize the human body are both numerous and diverse. These bacteria or normal flora inhabit the skin, the entire digestive system and urogenital tract in women. Many of these normal flora contribute to the healthy development of the immune system, prevent infection from pathogenic and opportunistic microbes as well as maintain the intestinal barrier function. Illness, poor nutrition, use of antibiotics and other factors can affect this delicate balance and cause emergence of harmful bacterial. In 1907 the Russian scientist, Elie Metchnikoff, hypothesized that replacing or diminishing the number of putrefactive bacteria in the gut with lactic acid bacteria could normalize bowel health and prolong life.(Anukam, 2007) For decades gynecologists have recommended women

include yogurt in their normal diet because of the beneficial nutritional and probiotic properties. Yogurt helps to maintain the normal flora in the vaginal environment. Daily ingestion of yogurt containing live lactobacilli has been shown to decrease vulvovaginal *Candida* infections by three fold.(Hilton, 1992)

The predominate normal flora found in the vagina is lactobacilli. These bacteria are gram-positive rods, primarily facultative anaerobes that generally have a fastidious growth environment. They prefer an acidic environment and help create one by producing lactic and other acids.(Reid 2001) Byproducts produced from lactobacilli metabolism have antagonistic effects against urinary and vaginal pathogens. One approach for delivering microbicides is to use probiotics, specifically recombinant normal flora in the vaginal environment.

Recent studies have shown that exogenously applied lactobacilli or indigenous lactobacilli can reduce the risk of urinary tract infections, where lactobacilli depletion is associated with bacterial vaginosis and yeast vaginosis.(Reid, 2001; Reid, 1995; Raz, 1993; Gupta, 1998) Ingested lactobacilli, specifically *L. rhamnosus* GR-1 and *L. reuteri* RC-14, have been shown to influence the vaginal environment by being delivered to the vagina following oral ingestion via the natural passage through the intestine and along the perineal and vulval skin.(Reid and Bruce, 2001; Anukam, 2007) An imbalance in or a depletion of normal flora in the vaginal environment has been associated with an increased risk of acquiring opportunistic infections, including HIV-1. (Chang, 2003; Taha, 1998) Recently, recombinant lactobacilli as a delivery method for microbicidal molecules and compounds has attracted a lot of attention and may provide a unique opportunity

to prevent the transmission of HIV-1. The first recombinant lactobacilli tested was a recombinant *L. jensenii* Xna, which secreted CVN and a two domain soluble CD4 (2D CD4) molecule.(Chang, 2003) *In vitro* testing of this lactobacilli showed a small decrease in HIV-1 binding.(Chang, 2003) Our collaborator has designed a recombinant lactobacilli that secretes CVN and optimized the CVN secretion in the lactobacilli *L. plantarum*.(Pusch, 2006). The studies presented in this thesis are the first steps in testing our recombinant L. plantarum for reconstituting human vaginal tissue ex vivo and in vivo as well as for anti-HIV-1 activity.

In 1997 a novel protein, Cyanovirin-N (CVN), was discovered to have potent anti-HIV-1, anti-HIV-2 and anti-SIV activity.(Boyd, 1997) CVN is an 11-kD protein, which is produced by the cyanobacterium, *Nostoc ellipsosporum*. This protein has been shown to be resistant to physico-chemical degradation, multiple freeze-thaw cycles, heat and can withstand treatment with denaturants, detergents and some organic solvents. (Bewly, 1998; Boyd, 1997) E*x vivo* experiments utilizing human cervical tissue and *in vivo* experiments utilizing adult female cynomolgus macaques (*Macaca fasciularis*) showed treatment with low doses of CVN in a gel formulation prevented infection from HIV-1 and SIV, respectively, when treatments occurred up to an hour before inoculation. (Tsai, 2004) Another recent study has shown that combining CVN with the peptide 12p1, which was isolated from a phage display library, has a synergistic effect increasing the anti-HIV-1 activity of both compounds allowing lower dosages to be used without decreasing efficacy.(McFadden, 2007) The antiviral activity of CVN is due to the irreversible

multivalent interactions between CVN and high mannose residues on the HIV-1 envelope glycoprotein, gp120.(TSal, 2004; Botos, 2002; Shenoy, 2002) It is these molecular interactions that prevent fusion of the virus particle with the target cells and make CVN an ideal candidate for investigation as a potential microbicide.

1.9 ex vivo *and* in vivo *experimental systems utilized to test microbicides.*

When testing microbicides it is imperative to test the compounds in the exact tissues that will be exposed to the compound in the vaginal environment. A very important aspect of the work set forth in this thesis involves utilizing human vaginal, cervical and foreskin tissue in both *ex vivo* and *in vivo* model systems. Before beginning experiments utilizing human tissues the compounds were tested *in vitro* for toxicity and efficacy using several different cell lines. The different cell lines and the experimental procedures used will be discussed in detail in each individual chapter for each microbicide. However, a brief overall description of the *ex vivo* and *in vivo* model systems are discussed below.

The organ tissue culture (OTC) system, originally developed by Gupta and colleagues, was the *ex vivo* model system selected to test the compounds before going into the immunocompromised mouse human epithelial model system. The

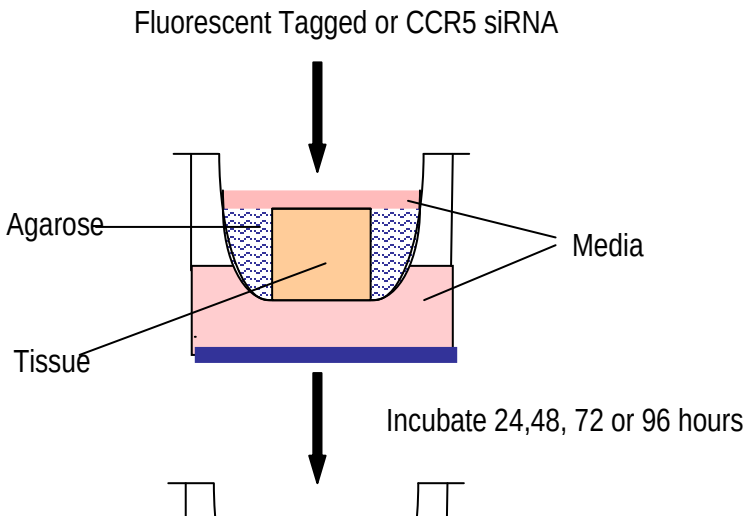


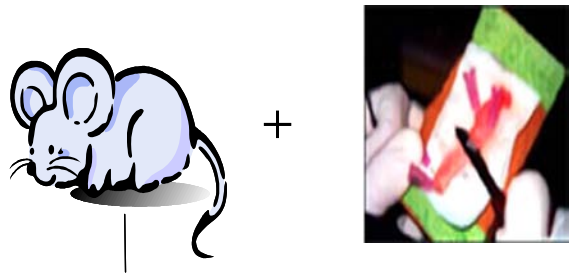
Figure 9: Organ Tissue Culture System

Originally developed by Gupta and colleagues, to study microbicides to prevent the transmission of HIV-1. Human tissue is placed epithelia surface upwards in a transwell dish, surrounded by agarose and then placed in a 12 well dish that has 1 ml of media in each well. The OTC was used to test FITC-tagged siRNA absorption into human vaginal tissue. After treatment with non-specific FITC-tagged siRNA the tissue was snap frozen, cryosectioned and examined by immunofluorescent microscopy (IF). Also, the bottom chamber was examined by IF to check for leakage. This system was also used to determine CCR5-siRNA down-regulation in human vaginal tissue. After treatment the tissue was homogenized and Western Blot analysis was performed, as described in Chapter 3.

OTC model system permits the compound or substance to naturally traverse the tissue from epithelial to stromal surface, just as it would in the normal vaginal environment. As shown in Figure 9, the OTC system uses human tissue placed epithelia surface upwards in a 0.3 μm membrane transwell dish, surrounded by 5% sterile agarose. The sterile agarose forms an intact seal around the top of the

epithelial tissue, simulating a fully intact epithelia layer as found in normal vaginal walls. Once the agarose solidifies around the epithelia the transwell dish is placed in a twelve well plate, which has one milliliter of cell culture media in each well. This allows the tissue to be fed from underneath with the appropriate nutrients from the cell culture media. The application of the specified compound is then added to the top of the tissue in the top chamber of the OTC and following incubation in a humidified chamber the tissue is processed for either immunofluorescent microscopy, standard hematoxylin and eosin staining or Western blot analysis. This *in vitro/ex vivo* system was selected because it most closely emulates the natural process that the microbicide and the virus must traverse to infect the tissue in the vaginal environment.

The human vaginal epithelia system was selected as the *in vivo* model system to test the efficacy of the compounds. This system was developed by Howett and Krieder to grow a fastidious organism, human papillomavirus, that could not be grown in cell culture.(Kreider, Howett et al. 1985) This model system overcomes many of the problems that limit biological experimentation with HIV-1; it is a human system with human tissue.(Kish, Budgeon et al. 2001) This system



Vaginal epithelial tissue cut, rolled and implanted in mice with estrogen pellet. Allowed to heal

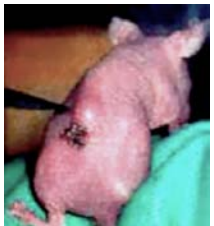


Figure 10: Human Epithelial Xenograft System

Cervicaovaginal tissue is prepared by slicing away the epithelia from the stroma, taking minimal amounts of stroma and implanting the epithelia with an estrogen pellet in the subcutaneous space in the back of NOD/SCID mice. The epithelia is allowed to heal for three weeks forming a tube similar to the vaginal tube in women, after which the lumen of the graft is treated with specified compound and challenged with virus. It is important to note in the diagram how the vaginal tissue takes on a tube conformation, modeling the vaginal tube in women and reproducing how the heterosexual transmission of STI occurs.

uses non-obese diabetic severely compromised immunodeficient (NOD/SCID) mice, which do not have any humoral or cell mediated immunity, lack natural killer cells and circulating complement system.(Janeway, Travers et al. 2001; Kish, Budgeon et al. 2001) By using the NOD/SCID mice graft rejection is eliminated

and decreases other variables, such as, mouse immune cells infiltrating the xenograft and affecting the analyses. Previous studies have shown that this *in vivo* model system can sustain active HIV-1 transmission

and replication in human vaginal tissue, vagina or cervix, its normal environment during transmission.(Kish, Budgeon et al. 2001) In the NOD/SCID animals complete restoration of the epithelial layer across the entire xenograft occurred within three weeks and the human CD45+ cell populations and distribution were present at baseline levels (equivalent to freshly excised tissue) up to two months after engraftment.(Kish, Budgeon et al. 2001) Figure 10, outlines the basic procedure for the xenograft system. Briefly, human vaginal or cervical tissue is first prepared by separating the epithelia from the stroma (leaving a minimal amount of stroma) then the tissue is rolled with the epithelium on the inside and subcutaneously implanted, along with an estrogen pellet, on the back (in between the skin and muscle) of the mouse. After healing for approximately three weeks, the tissue has re-epithelialized with resulting tissue morphology and architecture comparable to normal vaginal epithelial tissue. The healed xenograft forms a tube, similar to the vaginal tube in women, and since the treatment and/or virus is introduced directly into the lumen of the xenograft it mimics the natural heterosexual transmission of HIV-1.

Specific Aims:

Chapter 2: SDS Hydrogel

To determine if formulated SDS Hydrogels have broad spectrum anti-viral activity (HIV-1, HSV-2 & HPV). We hypothesize that our formulated SDS Hydrogels will

have potent anti-HIV-1, anti-HSV-2 and anti-HPV activity at very low concentrations.

Chapter 3: Liposomal delivered CCR5 siRNA in human vaginal epithelial xenografts.

To determine whether liposomal delivered siRNA can mediate knockdown *in vivo* of CCR5 on immune cells found in vaginal epithelium and if so ascertain the effects on transmission. We hypothesize that down-regulating human CCR5 will decrease HIV-1 binding and fusion *in vitro*, *ex vivo* and *in vivo*, thereby, providing proof-of-principle that liposomal delivered siRNA targeting human genes can successfully be used as a microbicide.

Chapter 4: Cyanovirin secreting L.plantarum

To determine whether lactobacilli secreting Cyanovirion can effect HIV-1 transmission *in vitro* and *in vivo.* We hypothesize that utilizing recombinant forms of normal vaginal flora that secretes cyanovirin, a anti-HIV-1 compound, decreasing HIV-1 transmission *in vivo* providing proof-of-principle for using recombinant *L.plantarum* as a means of re-colonizing the vaginal environment as a microbicide.

2. A Hydrogel Formulation Containing Alkyl Sulfate Inactivates a Broad Spectrum of Sexually Transmitted Viruses

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The following chapter is written as an independent manuscript to allow for an easy transition from thesis format to submission format.

2. 1 Introduction

The breath and depth of the sexually transmitted infection (STI) epidemic is staggering (for review see (Low, Broutet et al. 2006; Aral, Fenton et al. 2007; Da

Ros and Schmitt Cda 2008)). As of December 2007, 34 million people worldwide are infected with human immunodeficiency virus type 1 (HIV-1) (UNAIDS 2007), approximately 20 million people are infected with human papillomavirus (HPV) (CDC 2007) and at least 45 million people, in the United States alone, are infected with herpes simplex virus type 2 (HSV-2).(CDC 2006). In especial concern to HIV-1 susceptibility and transmission, several studies have shown that infection with one STI increases a person's susceptibility to another STI with women being the most susceptible population to STI.(Wald and Corey 2003; Freeman, Weiss et al. 2006; Madkan, Giancola et al. 2006) The pandemic of HIV-1 infection emerged in the 1980's and has raised many new challenges to infectious disease paradigms that existed at that time.(Yang 2004) Continued lack of an effective anti-HIV-1 vaccine and the unabated spread of HIV-1 emphasizes the urgent need for the development of new preventative measures to stop transmission, such as topical microbicides (for review see (Balzarini and Van Damme 2007; van de Wijgert and Shattock 2007)).

When HIV-1 is sexually transmitted it is found in two different forms, cell-free and cell-associated. Since both forms are found in semen and vaginal secretions an ideal microbicide must be efficacious against both forms. Topical microbicides are defined as any compound that can be applied inside the vagina or rectum to protect against sexually transmitted diseases. Potential microbicides can be placed into one of three broad categories: (1) compounds that inactivate or disrupt viruses; (2) compounds that specifically or non-specifically block infection

by physically binding to the cell or virus surfaces; and (3) compounds that inhibit specific stages of virus infection and replication such as, fusion, uncoating, reverse transcription, etc.(Davis and Doms 2004). An ideal microbicide must have the following traits: broad spectrum activity against bacterial and viral sexually transmitted infections, efficacy against cell-free and cell-associated HIV-1, no effect on the structural integrity of vaginal and cervical mucosal epithelium, stability at tropical temperatures and resistant to acidic pH. To make it attractive for use, it must be odorless, colorless, tasteless, low cost and readily accessible.(Balzarini and Van Damme 2007)

HPV infection is the most common sexually transmitted disease in the United States (CDC 2007; Dunne, Unger et al. 2007; Forhan 2008) and HPV DNA has been found in 90% of cervical cancers.(zur Hausen 2002) Every year 250,000 women worldwide and 5,000 women in the United States die needlessly due to cervical cancer. HPV, HSV-2 and increasing HSV-1 infections (Xu, Sternberg et al. 2006) are all major causative agents of genital lesions or ulcers, respectively, causing lifelong infections with intermittent viral reactivation and vaginal shedding.(Gupta, Warren et al. 2007) When an infected individual has viral activation and an outbreak with genital lesions, CD4 positive T-lymphocytes infiltrate the tissue providing more target cells for HIV-1 infection (Rebbapragada, Wachihi et al. 2007; Kaul, Pettengell et al. 2008), thereby increasing susceptibility to HIV-1 infection.

The impact of HIV-1 and consequent acquired immunodeficiency syndrome (AIDS) on women is severe, particularly in areas of the world where heterosexual

transmission is the dominant mode of HIV-1 transmission.(UNAIDS 2004) According to the World Health Organization there were 2.5 million newly reported cases in 2007(UNAIDS 2007) and over 50% of the new infections are women.(UNAIDS 2003) Since 2003, women have increasingly become the face of the HIV-1 pandemic (UNAIDS 2003) and represent a source of infection for men and their children. The main HIV-1 prevention tools, condom use, reducing the number of sexual partners and treating other reproductive tract infections are not always feasible for many women living in resource poor settings and in male dominated societies. Women often have limited ability to enforce cooperation by their male partners to use condoms due to social, cultural and economic gender inequalities.(Van De Wijgert and Coggins 2002) In addition, since 2000 we have known that Nonoxynol 9 (N-9), the most broadly distributed spermicide in over-the-counter (OTC) products including lubricants in male latex condoms (FDA 2007), increases the risk of HIV-1 transmission to women frequently exposed to N-9.(Van Damme, Ramjee et al. 2002; van de Wijgert and Coggins 2002; Davis and Doms 2004) N-9 has not been banned by the US Food and Drug Administration (FDA), but in December 2007 the FDA emitted a final ruling requiring all OTC products containing N-9 to be prominently labeled indicating its risks of HIV-1 transmission to women.(FDA 2007) This ruling however, however, does not apply to condoms, which are subject to FDA's medical device regulation instead of under the jurisdiction of OTC products.(FDA 2007) Availability of an effective

inexpensive and safe female-controlled microbicide would provide women the tools to protect themselves against STI and impact the health of the men and children.

Sodium dodecyl sulfate (SDS) and other alkyl sulfates are anionic surfactants that possess chaotropic properties. SDS will dissociate membranes of cells in culture into their lipid and protein components and has previously been shown to disrupt enveloped viruses.(Becker, Helenius et al. 1975; Howett, Neely et al. 1999) Long term (24 hour) patching of SDS on skin can induce biochemical changes, namely mobilization of dendritic cells and recruitment of lymphocytes, however, exposures up to three hours were not irritating.(Effendy and Maibach 1995; Aramaki, Loffler et al. 2001) Importantly, the surfactant and chaotropic properties of alkyl sulfates give SDS very effective anti-microbial and anti-viral activity. As far back as 1952, SDS was combined with sodium perborate for use as a douche to treat vaginal infections. (Karnaky 1952; O'Brien and Thoms 1955) In intact mucosal tissues, surface keratinization, cellular exudate and mucus form a protective layer that is not easily disrupted by 1% or lower SDS. Sodium dodecyl sulfate and other alkyl sulfates are in the Generally Recognized As Safe (GRAS) list of the FDA (FDA 2006), are routinely used as a common ingredient in consumer products applied topically to the skin and mucosa (e.g. body lotions, baby wipes, toothpaste and shampoo) and as food additives (e.g. marshmallows and gelatin). Our laboratory has shown that at concentrations ranging from 0.01% to 1% SDS kills HIV-1, HPV, herpes simplex virus (HSV) types 1 and 2 (Howett, Neely et al. 1999; Krebs, Miller et al. 1999; Krebs, Miller et al. 2000; Krebs, Miller et al. 2002; Howett and Kuhl 2005; Urdaneta, Wigdahl et al. 2005; Hartmann,

Wigdahl et al. 2006), *Chlamydia trachomatis* (Achilles, Shete et al. 2002) *Neisseria gonorrhea* as well as Gram positive and Gram negative bacteria (Hartmann and Howett, unpublished results). Based on these results we concluded that the three different concentrations of SDS in the formulated gel would be 0.1%, 0.5% and 1%, which would provide a range of anti-viral activity with the lowest possibility of adverse effects. Because of its chaotropic properties SDS is also routinely used in laboratories for protein separation in polyacrylaminde gel electrophoresis.

Our laboratory has previously shown that low concentrations of sodium dodecyl sulfate (SDS) in cell culture media is relatively non-toxic compared to Nonoxynol-9 (N-9) and C31G (Krebs, Miller et al. 2000) and has broad-spectrum activity against enveloped and non-enveloped viruses. (Howett, Neely et al. 1999) Our collaboration with Hydromer, Inc. (Branchburg, NJ), has produced a formulated SDS Hydrogel for use as a potential vaginal microbicide. Hydromer, Inc., has combined SDS with their patented Aquadapt® hydrophilic polymer medical technology, which incorporates chitosan derivatives complexed with polyvinylpyrrolidone.(Hydromer 2008) Aquadapt® gels also exhibit lubricious properties when in the vaginal environment. These highly biocompatible polymer systems naturally bind one another to form gels, providing excellent physical strength and stability. Currently, the Aquadapt® gels are being utilized to coat commercially available medical devices, such as catheters, without exhibiting and any signs of tissue toxicity. It is also used as a vehicle of pharmaceutical formulations to mucosal surfaces, such as paranasal sinuses In this study we

demonstrate that our prototypic formulated SDS Aquadapt® gel (SDS Hydrogel) has potent anti-HIV-1, anti-HSV-2 and anti-HPV-11 activity *in vitro* and *ex vivo* without being toxic to human vaginal, cervical or newborn foreskin tissue.

2.2 Materials and Methods

2.2.1 Hydrogels.

SDS has been combined with Hydromer's patented Aquadapt® hydrophilic polymer medical technology, which incorporates chitosan derivatives complexed with polyvinylpyrrolidone.(Hydromer 2008) These highly biocompatible polymer systems naturally bind one another to form gels, providing excellent physical strength and stability

2.2.2 Ex vivo toxicity assay

Human vaginal tissue was obtained from patients undergoing elective reconstructive surgeries for vaginal prolapse. All experiments utilizing vaginal tissue were performed within three hours of excision from the patient and in accordance with Drexel University and Temple University Hospital Internal Review Board rules and regulations. Human vaginal tissue was cut into 2 mm x 2 mm x 0.5 cm pieces and placed with the epithelia oriented up on filter paper (Whatman, Philadelphia, PA) saturated with DMEM (Mediatech Inc., Herdon, VA) supplemented with 10% fetal bovine serum. Tissue was then treated with either placebo, 0.1%, 0.5% or 1% SDS Hydrogel and incubated for 3, 5, 16 or 24 hours. Following the incubation periods the tissue was harvested, immediately fixed in 10% neutral-buffered formalin and processed by standard histological techniques for staining with hematoxylin and eosin.

2.2.3. HSV-2 Inactivation Assay

Experiments were performed as previously described.(Howett, Neely et al. 1999) For inactivation of HSV-2, 100 μl of virus stock (initially diluted 1000-fold in medium, an amount equal to approximately $8.7x10^4$ plaque forming units (pfu/ml)) was combined with 100 μl of each of the SDS Hydrogel formulations to achieve final concentrations of 0.05%-0.5% and incubated for 10 min at 37°C. Following the incubation, each virus/Hydrogel mixture was further diluted in cell culture medium (1:50), and 200 μl of each sample was adsorbed onto CV-1 monolayers for 1 h at 37°C. The monolayers were then re-fed with medium and incubated at 37°C in 5% CO_2. At 48 h post-infection, the

cells were fixed and stained with crystal violet and plaques were counted with a dissecting microscope. Each datum in Table 1 represents an average for duplicate plates. A two-tailed unpaired Student t-test was performed using GraphPad Software to determine statistical significance.

2.2.4 HIV-1 Cell Free Inactivation Assay.

Experiments were performed as previously described.(Urdaneta, Wigdahl et al. 2005) Briefly, one day prior to the assay, P4-R5 HeLa cells (Charneau, Mirambeau et al. 1994) were seeded into 12-well culture dishes at a concentration of $8x10^4$ cells per well and incubated overnight at 37°C with 5% CO_2. The cells used in this assay, P4-R5, are HeLa cells that have been engineered to express HIV-1 receptors (CD4, CCR5 and CXCR4) and have an integrated HIV-1 promoter fused to a B-Galactosidase (β-gal) reporter gene.(Charneau, Mirambeau et al. 1994) This allows

β-gal expression to be measured when the HIV-1 promoter has been activated. On the day of the assay 5 μl of a high titer stock ($10^{7.17}$ tissue culture infective dose 50/ml) ($TCID_{50}$) of HIV-1 (strain IIIB; Advanced Biotechnologies, Inc., Columbia, MD) was treated with an equal volume (5 μl) of placebo, 0.1%, 0.5% or 1% SDS Hydrogel and incubated for ten minutes at 37°C. To stop the reaction and to dilute the SDS, 990 μl of RPMI media (supplemented with 10% FBS) was added to the virus/SDS Hydrogel mixture and 300 μl of this dilution was applied to the P4-R5 cells in triplicate. Following a two-hour incubation at 37°C with 5% CO_2, 2 ml of fresh DMEM media (supplemented with 10% FBS and with half the concentration of puromycin 250 ug/ml) was added to each well. After incubation for 46 hours at 37°C with 5% CO_2, cells were washed twice with phosphate buffered saline, lysed and incubated for one hour at room temperature with reaction buffer supplied in Galacto-Star™ β-Galactosidse Reporter Gene Assay System (Applied Biosystems, Foster City, CA), per the manufacturer's instructions. Following the incubation β-gal expression was quantified utilizing Fluoroskan Ascent FL Luminometer (Thermo Scientific, Waltham, MA). β-gal expression was measured in relative luminescence units per second (RLU/s). A one-way ANOVA followed by Dunnett's (compare all versus control) post-test was performed using GraphPad Software to determine statistical significance.

2.2.5 HIV-1 Cell Associated Inactivation Assay.

Experiments were performed as previously described.(Urdaneta, Wigdahl et al. 2005) Briefly, three days prior to the assay, Sup-T1 (ATCC, Manassas, VA) cells were seeded at a density of $3x10^6$ in 30 ml per T75 flask, infected with a high titer

stock ($10^{7.17}$ tissue culture infective dose 50/ml) ($TCID_{50}$) of HIV-1 (strain IIIB; Advanced Biotechnologies, Inc., Columbia, MD) diluted 1:10 (30 μl) in serum free antibiotic free media and incubated for 72 hours at 37°C with 5% CO_2. At 48 hours post-infection, P4-R5 HeLa cells were seeded into 12-well culture dishes at a concentration of $8x10^4$ cells per well. At 72 hours post-infection, the infected Sup-T1 cells, $1x10^6$ cells in 95 μl for each group, were treated with 5 μl of placebo, 0.1%, 0.5% or 1% SDS Hydrogel and incubated for ten minutes at 37°C. To stop the reaction and to dilute the SDS, 900 μl of RPMI media (supplemented with 10% FBS) was added to the infected Sup-T1/SDS Hydrogel mixture and 300 μl of this dilution was subsequently applied to the P4R5 cells in triplicate. Following a two-hour incubation at 37°C with 5% CO_2, the mixture was aspirated from each well, the P4-R5 cells were washed twice with PBS and 2 ml of fresh DMEM media (supplemented with 10% FBS and with half the concentration of puromycin 250 ug/ml) was added to each well. Following an incubation period of 46 hours at 37°C with 5% CO_2 β-gal expression was quantified and analyzed as described in the previous section for the HIV-1 cell free inactivation assay. β-gal expression was again measured in relative luminescence units per second (RLU/s).

2.2.6 HPV-11 Inactivation Assay and RT-PCR.

Experiments were performed as previously described.(Fang, Budgeon et al. 2006) One day prior to the assay A431 cells (established from human epidermoid carcinoma(Giard, Aaronson et al. 1973)) were seeded in six-well plates at a density of $5x10^5$ cells per well and incubated overnight at 37°C with 5% CO_2. On the day

of the assay 30 μl of HPV-11 ($2x10^6$ per ml) was mixed with equal volumes of either placebo, 0.1%, 0.5% or 1% SDS Hydrogel and incubated for ten minutes at 37°C. Immediately following the incubation, 450 μl of media was added to the HPV-11/SDS Hydrogel mixture followed by serial dilutions with a total of 1 ml being added to each well. Cells were incubated overnight at 37°C with 5% CO_2 then re-fed with an additional 2 ml media and allowed to incubate for an additional 2 days. Total cellular RNA was extracted 72 hours postinfection using a QIAshredder and RNeasy mini kit (Qiagen, Valencia, CA). Reverse transcription polymerase chain reaction (RT-PCR) analysis of the HPV-11 E1^E4 transcript was used to determine the end-point dilution of virus that still initiated infectivity in 50% of the samples as previously described. (Smith, Foster et al. 1995; Ludmerer, McClements et al. 2000; Fang, Budgeon et al. 2006) The final expected size for the E1^E4 amplified product was 294 bp, and the amplified product for β-actin was 429 bp.

2.3 Results:

2.3.1 Concentrations of SDS Hydrogel formulations are non-toxic to intact vaginal and cervical tissue at the histologic level.

To determine toxicity to the tissues that would be exposed to an SDS-based microbicide, human vaginal, cervical and newborn foreskin tissues were exposed *ex vivo* to either placebo, 0.1%, 0.5% or 1% SDS Hydrogel formulations and incubated for 3, 5, 16 or 24 hours. In total we tested three concentrations (0.1%, 0.5% and 1%) for four different treatment times (3, 5, 16 and 24 hours) with Figure 11 and Figure 12 containing representative pictures showing foreskin, cervical and vaginal tissues after treatment for three hours (Figure 12) and cervical tissue after

16 hour treatment and vaginal tissue after 24 hour treatment (Figure 13). Results were similar after exposure to either 0.1% or 0.5% SDS Hydrogel formulations (data not shown). In all tissues there was no histologically visible squamous epithelium disruption after treatment with all of the different concentrations and time points.

2.3.2 SDS Hydrogel formulations inactivate HSV-2.

Our laboratory has previously shown effective HSV-2 inactivation by 0.1% SDS diluted in cell culture media.(Howett, Neely et al. 1999) We performed HSV-2 plaque assays with SDS Hydrogels to determine the potency of the formulated SDS Hydrogels against this enveloped virus. In two separate experiments with duplicate samples all of the SDS Hydrogel treatment concentrations showed strong anti-HSV-2 activity. As shown in Table 1, treatment concentrations as low as 0.05% to 0.5% were effective in eliminating the ability of the virus to induce plaques in a monolayer of monkey kidney cells by completely inactivating HSV-2 *in vitro*. Since there was an increased number of plaques counted in the placebo group a two tailed unpaired Student t-test was performed comparing the HSV-2 control to the placebo group. No statistically significant difference was found between the HSV-2 control and placebo group in inducing plaque formation ($p=0.2625$). This data is consistent with previously published reports, which demonstrate that aqueous SDS solution completely inactivated HSV-2 by dissolving the lipid envelope and denaturing viral proteins.(Howett, Neely et al. 1999)

2.3.3 Inactivation of cell-free and cell associated HIV-1 (IIIB) by SDS Hydrogel formulations.

HIV-1 is found in two different infectious forms, cell-free and cell-associated, in semen and vaginal secretions. We tested our SDS Hydrogels against both forms.

Figure 11: H&E of Hydrogel treated tissue 3 hr

Staining shows treatment with 1% SDS Hydrogel did not disrupt squamous epithelium in human foreskin (A, D, G), cervical (B, E, H) and vaginal tissue (C, F, I) when compared to untreated controls. The untreated controls (A, B, C) show tissue integrity; placebo treated controls (D, E, F)) show the Hydrogel vehicle also did not disrupt the epithelium; and 1% SDS Hydrogel treated tissues (G, H, I) show no visible disruption of epithelium from a histological perspective. All tissues were treated for 3 hours. Arrows indicate basal layer. Above the basal layer is the squamous epithelium and below is the stroma, the connective tissue. All magnifications shown are 10X.

Our laboratory has previously shown that SDS diluted in cell culture media in semenand vaginal secretions. We tested our SDS Hydrogels against both forms. Unformulated SDS inactivated cell free HIV-1 within 1 minute.(Howett, Neely et al. 1999; Urdaneta, Wigdahl et al. 2005) To confirm that the SDS Hydrogel formulations have the same efficacy against HIV-1 as SDS in cell culture media, five separate experiments with triplicate samples was performed to determine inactivation of each of the HIV-1 forms, cell-free and cell-associated. In each experiment highly statistically significant anti-HIV-1 activity ($p<0.0001$) was observed in all concentrations of SDS Hydrogel against both infectious forms relative to untreated HIV-1 control, as shown in Figure 13, HIV-1 infectivity experiments were performed using P4R5 MAGI HeLa cells to test if the formulated SDS Hydrogels had anti-HIV-1 activity against both cell-free and cell-associated virus as demonstrated in Figure 13 the 0.5% and 1% SDS Hydrogels completely inactivated cell-associated HIV-1. Furthermore, Figure 13 shows that all concentrations of the formulated SDS gels were highly active against cell free HIV with a highly statistically significant p value of 0.0001. In the cell free HIV-1 infectivity assays it was observed in all experiments that the placebo slightly increased HIV-1 infectivity. However, this pro-infectivity effect was completely

ablated in the presence of even the lowest concentrations of SDS in the Hydrogel formulations. SDS has such potent anti-viral activity that at 1% and 0.5% SDS Hydrogel completely inactivated HIV-1 and at the lowest concentration (0.1%) of the SDS formulation viral infectivity decreased >95%.

Figure 12: H&E of Hydrogel treated tissue 16/24 hr

Staining shows treatment with 1% SDS Hydrogel did not disrupt squamous epithelium in human cervical (A, C, E) and vaginal tissue (B, D, F) when compared to untreated controls. The untreated controls (A, B) show tissue integrity; placebo treated controls (C, D) show the Hydrogel vehicle also did not disrupt the epithelium; and 1% SDS Hydrogel treated tissues (E, F) show no visible disruption of epithelium from a histological perspective. All cervical tissues were treated for 16 hours and all vaginal tissues were treated for 24 hours. Arrows indicate basal layer. Above the basal layer is the squamous epithelium and below is the stroma, the connective tissue. All magnifications shown are 10X.

2.3.4 SDS Hydrogel formulations decrease HPV-11 infectivity.

To determine if the SDS Hydrogel formulations had activity against non-enveloped virus, HPV-11 infectivity assays were performed followed by RT-PCR using nested primers targeted towards the viral mRNA transcript, E1^E4. This viral transcript is the most abundant transcript detected during the HPV-11 life cycle and infection. Therefore, its detection by RT-PCR would be indicative of active viral transcription. As shown in Figure 14, treatment with 0.5% or 1% SDS Hydrogels resulted in at least a 10 fold decrease in the levels of viral transcript, E1^E4. The lowest concentration 0.1% (final concentration 0.05%) SDS Hydrogel did not decrease HPV-11 infectivity (data not shown). The RT-PCR results show that formulated SDS Hyrdogel decreases HPV-11 infectivity by 90% *in vitro*. This is evident by the loss of the E1^E4 band in the 10^{-5} columns in the 0.5% and 1% SDS Hydrogel experimental groups as compared to the 10^{-6} column in the Placebo and HPV experimental control group, which show viral infectivity.

2.4. Discussion:

Globally, there is an urgent need to prevent the transmission of sexually transmitted diseases, such as HIV-1, HSV-2 and HPV. Previous studies have shown that infection with HSV-2 and/or HPV increases susceptibility to infection by HIV-1. Several strategies must be employed to stop the transmission of these incurable

diseases. These include the accepted ABCs (A-abstinence, B-be faithful and C-condom use), education, socioecomonic equality between genders and microbicide development and use. A recent report presented at the National STD Conference in March 2008 by the CDC reported that one in four female adolescents (26%) in the United States has at least one of the most common sexually transmitted diseases (HPV, HSV-2, chlamydia and trichomoniasis). (Forhan 2008) Several studies (Balzarini and Van Damme 2007; van de Wijgert and Shattock 2007) have indicated that an ideal microbicide has to be non-toxic to the vaginal

and cervical tissue to prevent host immune cells from being recruited into these tissues, thereby introducing more target cells for HIV-1 to infect.

Worldwide, a large portion of HIV-1 infections have been transmitted through exposure to the virus or virus infected cells in the female genital tract. The exact mechanisms of viral spread remain unknown. However, there are three proposed mechanisms of viral spread that have been described for HIV-1. The first is the classical route of entry, which is characterized by the binding of cell-free virions to a permissive host cell via receptor-ligand interactions (binding of GP120 and GP41 to CD4 and co-receptor, respectively), followed by entry into the cytoplasm via virion fusion and initiation of subsequent steps in the replicative process.(Kilby and Eron 2003; Pierson and Doms 2003; Piguet and Quentin 2004) Second, cells such as dendritic cells can capture virus via viral binding to the C-type lectins or other cell surface receptors, without necessarily becoming infected and re-present infectious virions to a permissive target cell (a process known as infection in *trans*).(Jameson, Baridaud et al. 2002; Piguet and Quentin 2004) Third, an HIV-1

infected cell can infect a second cell without the requirement for release of cell-free virions into the surrounding extracellular environment, which represents viral propagation through direct cell-to- cell transmission.(Piguet and Quentin 2004)

Table 1: Inactivation of HSV-2 Infectivity by SDS Hydrogel Formulations

Prior to infection of CV-1 cells, HSV-2 was pre-treated with either 0.1%, 0.5% or 1% SDS Hydrogel, placebo or untreated medium control in equal volumes at 37° C for 10 minutes. Final concentrations of SDS Hydrogels were 0.05%, 0.25% and 0.5%, respectively. Results are respresentative of 2 independent experiments with n=2/group with similar outcomes.

Treatment	Average No. of plaques/plate at 48 hours post-infection
Medium	99, 114
Placebo	119, 121
1% SDS Hydrogel	0, 0
0.5% SDS Hydrogel	0, 0
0.1% SDS Hydrogel	0, 0

The first candidate microbicide for HIV-1 to reach phase III trials was the spermicidal detergent nonoxynol-9 (N-9), which inactivated HIV-1 *in vitro* by disrupting the outer membrane but failed to prevent sexual transmission *in vivo*.(Van Damme, Ramjee et al. 2002; Davis and Doms 2004) Women using N-9 were shown to have higher rates of infection by HIV-1, most likely because the detergent disrupted the membranes of the epithelial cells in the genital tract which normally serve as a protective barrier against viral and bacterial infection.(Van Damme, Ramjee et al. 2002; Van De Wijgert and Coggins 2002; Davis and Doms 2004) In murine studies that assessed the toxicity of N-9, as early as 2 hours post-application epithelial disruption of the cervix occurs accompanied by an intense infiltration of immune cells just below the basal layers, primarily in the regions without epithelial disruption.(Catalone, Kish et al. 2004) These findings indicate that N-9 greatly enhances inflammation and that the recruited lymphocytes may serve as targets for HIV-1.(Catalone, Kish et al. 2004) Another candidate microbicide to reach phase III trials was C31G, which is also a surfactant but is longer being developed as a vaginal microbicide.(van de Wijgert and Shattock 2007) The trial was underway for 23 months and after a recommendation from an independent monitoring board the trial was halted because too few women were becoming infected with HIV-1 to

provide the statistical power needed to show a difference between C31G and the placebo. (Mascolini 2007; Peterson, Nanda et al. 2007) The phase III trials of the Ushercell microbicide, a cellulose sulfate

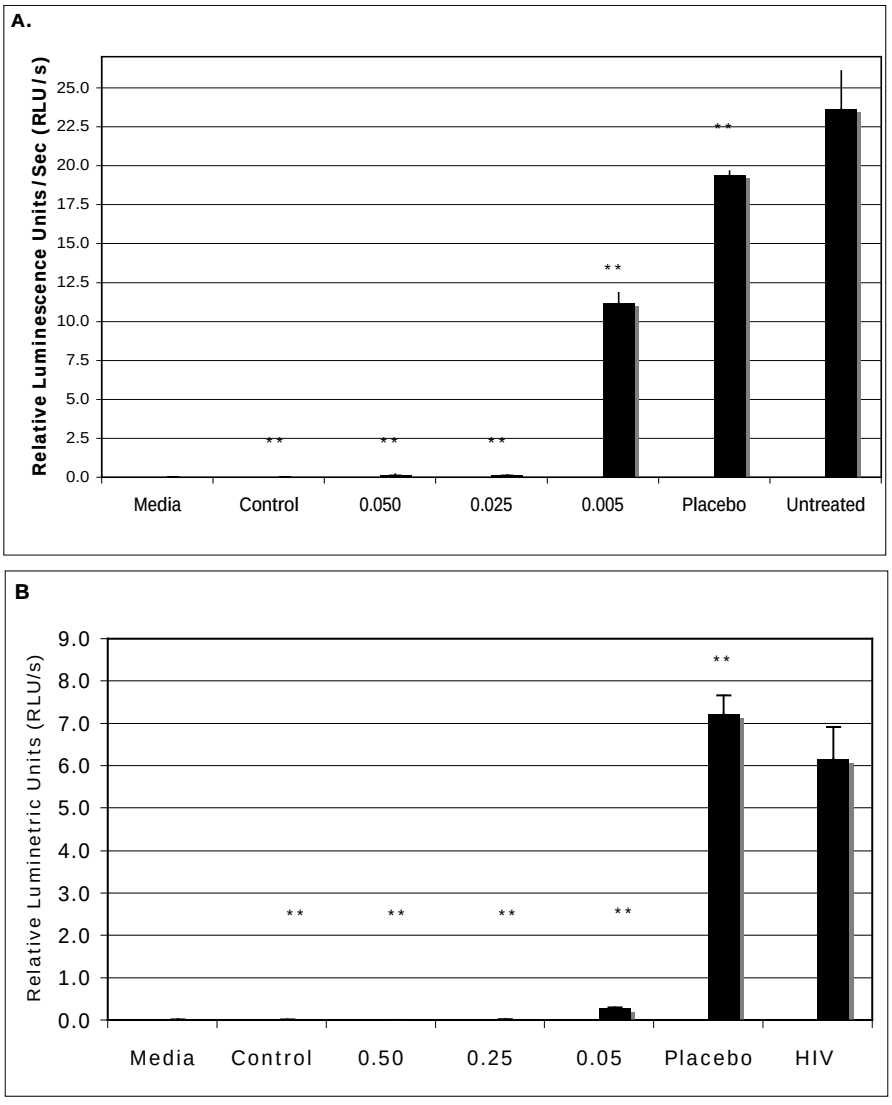

Figure 13: SDS Hydrogels inactivate HIV-1

SDS Hydrogels inactivate cell-free and cell-associated HIV-1 *in vitro*. Cell-associated (A) or cell-free (B) HIV-1 were pre-treated with either 0.1%, 0.5% or 1% SDS Hydrogel, placebo or left untreated, prior to infection of P4-R5 indicator cells which express β-gal if infected. Viral inactivation with 1% SDS in media was included as an intra-assay control (Control). Media only group show baseline expression of β-gal. X-axis labeled with final concentrations of SDS in Hydrogel. Results are representative of five independent experiments with n=3/group with similar outcomes. **=$p<0.0001$.

compound that blocked viral entry into cells and that was developed by CONRAD, was also halted because interim analysis indicated that the rate of HIV-1 infection was higher in groups using the Ushercell as compared to the placebo group.(Honey 2007) The first candidate microbicide to complete phase III trials, Carraguard (developed by the Population Counsel) contains carrageenan and blocks viral entry into cells. Carraguard failed the phase III trials again because the microbicide did not prevent HIV infections to a statistically significant level after comparing Carraguard to placebo.(2008) In stark contrast, our prototypic SDS Hydrogel differs because it inactivates and disrupts sexually transmitted viruses (HIV-1, HPV, HSV-1 and HSV-2) instead of blocking infection by physically binding to virus surfaces as compared to other microbicides that have recently completed phase III clinical trials.

It is known that SDS in an aqueous solution can cause skin irritation when introduced at high concentrations and for long exposure times. This irritation has been proven to be dose and time dependent. (Aramaki, Loffler et al. 2001) Albeit brief, multiple daily exposure of the oral mucosa to toothpaste, which contains up to 8% of SDS, proves that SDS is innocuous to some mucosal tissues. This result

is supported by billions of exposures. Previous studies from our laboratory have shown that at low concentrations, (1% or less) SDS in an aqueous solution has broad spectrum antiviral activity and is relatively non-toxic to vaginal keratinocytes and newborn foreskin in

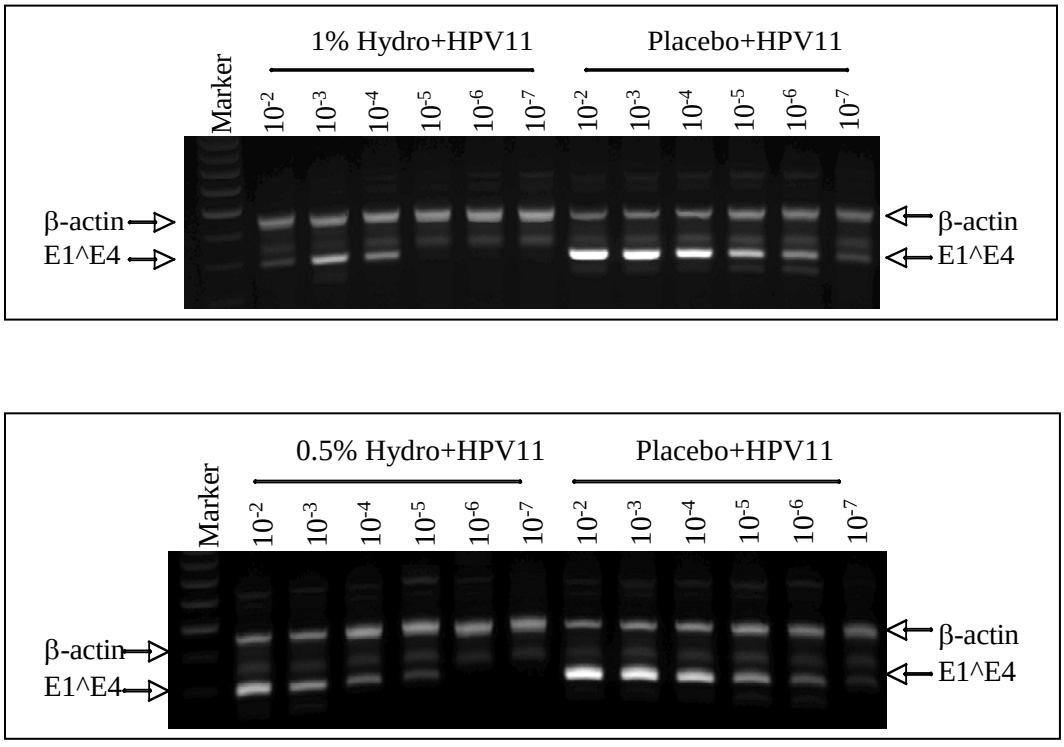

Figure 14: SDS Hydrogel decrease HPV11 Infectivity

RT-PCR shows treatment of HPV-11 with SDS Hydrogels decreased viral transcript, E1^E4, by at least a ten fold reduction relative to placebo. Beta actin and the viral E1^E4 transcript is indicated by arrows. The placebo hydrogel controls for viral infectivity.

comparison to N-9 and C31G.(Howett, Neely et al. 1999; Krebs, Miller et al. 2000; Krebs, Miller et al. 2002) To ensure that our SDS Hydrogels were not toxic, we treated human vaginal, cervical and newborn foreskin tissue with 0.1%, 0.5% and 1% SDS Hyrdogels and incubated the treated tissues continuously for up to 24 hours. As seen in Figure 11 and 12, the Hematoxylin and Eosin staining shows that even the highest concentration of 1% SDS Hydrogel did not cause apparent disruption in the squamous epithelium of the vaginal tissue at the histologic level even after 24 hours of continued exposure. Furthermore, there was no decrease in the thickness of the squamous epithelia in the SDS Hydrogel treated groups as compared to the no treatment groups. A recent study by Ghosh and Blankschtein has shown that SDS in glyercol reduces pore number density and radius, decreasing SDS micelle penetration into the stratum cornuem and therefore decreasing the perturbation of the skin barrier as compared to SDS in an aqueous solution. (Ghosh and Blankschtein 2007) Viscous gels containing SDS may also decrease micelle penetration into the stratrum corneum, thereby, further decreasing toxicity. Testing is currently being conducted to evaluate the differences between aqueous and gelatinous SDS toxicities as well as

repeated exposure testing to determine the implications for efficacious microbicide development.

In 2006, a vaccine (Gardasil®, Merck Laboratories) containing the coat proteins of HPV (6, 11, 16 and 18) was approved by the FDA and made commercially available. Cervarix™ (Glaxo SmithKline) is another preventive vaccine and currently approved only in Europe. Both of these vaccines are currently for pre-sexually active females (ages 9-26), to prevent the initial HPV infection and the future possibility of the development of cervical cancer. These vaccines do not cure existing infections. These two vaccines do not protect against other high-risk types they only offer prophylactic protection against the most prevalent types of HPV associated with cervical cancer. This leaves a significant number of the women in the population unprotected and all women, vaccinated or not, vulnerable to infection by other types of human papillomaviruses. Thus, there is an urgent need for a broad spectrum microbicide that has been shown to have anti-HPV activity. Our *in vitro* results presented here support our previous findings of the microbicidal activity of SDS against non-enveloped virus.(Howett, Neely et al. 1999; Krebs, Miller et al. 2000; Krebs, Miller et al. 2002; Kish, Ward et al. 2003; Urdaneta, Wigdahl et al. 2005; Hartmann, Wigdahl et al. 2006) Our studies show that $\leq$1% SDS disrupts HPV (Kish and Howett, unpublished results). This is a unique feature of SDS that sets it apart from other candidate microbicides with a more limited scope of action. Currently, experiments are underway to determine if the formulated SDS Hydrogel has the same anti-HPV activity *in vivo* utilizing our laboratory's mouse xenograft

model system. This system emulates the full life cycle of HPV-11 and produces infectious virions. Howett and Kreider pioneered this human tissue xenograft model to grow a fastidious organism, human papillomavirus, that could not be grown in cell culture (Kreider, Howett et al. 1985) and overcomes some of the problems that limit biological experimentation with HPV, for example, it is a human system with human tissue.(Kish, Budgeon et al. 2001) This model system has been adapted to grow many different organisms, such as, bovine papillomavirus, cottontail rabbit papillomavirus, poxviruses, HSV-2, varicella zoster virus and most recently HIV-1.(Howett, Christensen et al. 1997)

Many times it is overlooked that sexually transmitted diseases affect not only women, they also affect men, children, families and society as a whole. These diseases cause some of the biggest problems in our society such as, infertility, cervical cancer and AIDS. Several strategies must be employed simultaneously at both the population and individual levels to combat and overcome these diseases. Microbicides, vaccines, condom use, education and raising the socio-economic status of women and the poor all need to be addressed before we can defeat these diseases.

Based on the *in vitro* and *ex vivo* work set forth in this paper and our laboratory's previous reports, our prototypic SDS Hydrogel meets the criteria for an ideal microbicide. Experiments are currently under way to determine if the formulated SDS Hydrogels will have the same activity *in vivo* as *in vitro*. Together the data presented in this article along with the ongoing *in vivo* work currently being conducted in our laboratory provide the basis for moving the SDS Hydrogels into Phase I Clinical Trials. The topical, non-toxic, broad activity of the formulated SDS

Hydrogels has the potential to prevent many of the health problems associated with sexually transmitted diseases, such as, AIDS, cervical cancer and genital ulcers. The versatility of the SDS Hydrogels will have a significant impact on women's health regardless of where they live and irrespective of their personal circumstances.

Acknowledgements:

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3. siRNA Microbicide to Impede HIV-1 Binding and Fusion by Down-regulating CCR5 in Human Vaginal Tissue and Human Epithelial Xenografts

Veronica Holmes, Yana Thaker, Li Ming, Bharat Ramratnam and Mary K. Howett

The following chapter is written as an independent manuscript to allow for an easy transition from thesis format to submission format.

3.1 Introduction

Chemokine receptors are G protein coupled receptors (GPCR) that have seven transmembrane domains with three extracellular loops and four intracellular loops.(Lederman 2006) Chemokine receptor 5 (CCR5) is a human receptor, which R5 forms of HIV-1 utilize as a co-receptor for binding. In 1996 a Hemophilia Growth and Multicenter AIDS Cohort study (Dean, Carrington et al. 1996) found that mutations in the CCR5 co-receptor, confers some resistance in humans to infection by R5 forms of HIV-1. This mutation was found to be present in the Caucasian population worldwide at a frequency of approximately 10% while the frequency in the United States was 1%.

The rate of HIV-1 disease progression varies considerably among infected individuals. A small but rare subset of chronically infected, therapy naïve individuals appear to maintain high and stable CD4+ and CD8+ T cell counts with low to undetectable plasma viral loads for an prolonged number of years.(Dean, Carrington et al. 1996; Mikhail, Wang et al. 2005) The CCR5Δ32 mutation is a 32 base pair deletion in the portion of the human CCR5 open reading frame that encodes the

second extracellular protein loop between transmembrane domains four and five of the seven transmembrane architecture.(Dean, Carrington et al. 1996; Agarwal, Zainab Van Horn et al. 2004; Oppermann 2004) CCR5Δ32 encodes a truncated protein that is retained in the endoplasmic reticulum, which is not detectable on the cell surface.(Benkirane, Jin et al. 1997; Agrawal, VanHorn-Ali et al. 2004) People who are homozygous for the CCR5Δ32 mutation are resistant to HIV-1 infection from R5 strains, which is the form most commonly sexually transmitted, and heterozygous individuals expressing CCR5Δ32 progress more slowly to AIDS.(Dean, Carrington et al. 1996) People that are homozygous for the CCR5Δ32 mutation appear to be phenotypically normal except for an increased susceptibility to contracting a fatal form of West Nile Virus infection. For these reasons CCR5 has become a very attractive target of investigation to many researchers.

Many scientists have tried to develop microbicides with anti-HIV activity but microbicides are multi-faceted because the exact mechanisms by which HIV-1 gains entry is still largely unknown. Several neutralizing monoclonal antibodies targeted towards gp120 have been developed that conferred immunity to rhesus macaques that were vaginally challenged with virus, as long as the antibodies were present within a few hours of the virus challenge.(Shibata, Igarashi et al. 1999; Nishimura, Igarashi et al. 2003; Veazey, Shattock et al. 2003) However, even though monoclonal antibodies seem to be effective they are also very expensive. When an individual is infected there is a genetic diversity of HIV-1 within that individual, which is a collection of closely related but genetically distinct viral variants.(Sullivan, Mandava et al. 2005)

Taken together it has not been definitely proven that these monoclonal antibodies will be effective at conferring immunity in humans.

One encouraging method scientists have taken in developing microbicides is creating synthetic chemokines molecules that compete with HIV-1 for the CCR5 co-receptor. CCR5 is a human chemokine that normally binds MIP-1ą, MIP-1β and RANTES chemokines. The most successful of the synthetic chemokines to inhibit binding of HIV-1 is PSC-RANTES, which is a RANTES analog chemically identical to native RANTES except for the substitution of a nonanoyl group, thioproline and cyclohexylglycine for the first three N-terminal amino acids of the native protein.(Lederman, Veazey et al. 2004) This synthetic PSC-RANTES was vaginally applied to rhesus macaques and upon challenging with SHIV, a Simian Immunodeficiency Virus and Human Immunodeficiency Virus chimera, it was shown that 12 out of the 15 monkeys treated were protected by 100 ųM treatment.(Lederman, Veazey et al. 2004)

Much of the human genome consists of remnants of previous transposon/virus invasions and elements that are still active, therefore one would expect that organisms need to fight off such invasions to prevent the genome from being completely overrun by molecular invaders.(Plasterk 2002) A novel approach in developing a microbicide is to use RNA interference (RNAi) targeted towards the CCR5 chemokine receptor. This RNA-based silencing mechanism has emerged as an ancient mechanism that is conserved among species from different kingdoms (fungi, animals and plants) and very likely acts as the immune system of the

genome.(Plasterk 2002) RNAi is a sequence specific posttrancriptional gene silencing, which is triggered by double stranded RNA. It has been shown that siRNA has been successful in down-regulating CCR5. In U87-CCR5 cells, which are engineered to express CCR5, CCR5 was down-regulated by 48% decreasing viral entry by 55% resulting in inhibition of HIV-1 replication.(Martinez, Gutierrez et al. 2002) siRNA targeted against CCR5 and HIV-1 p24 antigen together in infected macrophages also down-regulated CCR5 and inhibited viral entry for fifteen days.(Song, Lee et al. 2003) Another study showed down-regulation of CCR5 in MAGI-CCR5 cells that decreased CCR5 expression by ten fold, which in turn decreased viral replication by three to seven fold.(Qin, An et al. 2003)

It has recently been shown that siRNA based microbicides can protect mice from a lethal form of Herpes Simplex Virus 2 (HSV2) infection when treated with siRNA liposomal treatment two to four hours prior to viral challenge.(Palliser, Chowdhury et al. 2005) Previous studies have shown that siRNA can decrease CCR5 expression in cells but it needs to be further validated in human vaginal epithelial tissue. We used human vaginal epithelial xenograft model to determine if CCR5 could be down regulated in human vaginal tissue using a cationic liposome delivery system, Lipofectamine 2000 (Invitrogen). Worldwide, a large portion of HIV-1 infections have been transmitted through exposure of the virus in the female genital tract. The exact mechanisms of viral spread are still unknown. The human epithelial xenograft model was first developed to grow a fastidious organism, human papillomavirus, that could not be grown in cell culture.(Kreider, Howett et al. 1985) This model system has been adapted to grow many different organisms, such as, bovine papillomavirus

(BPV), cottontail rabbit papillomavirusCRPV), poxviruses, herpes simplex virus type 2 (HSV-2), varicella zoster virus and HIV-1.(Howett, Christensen et al. 1997) This model system uses the natural human tissue which HIV-1 normally infects.(Kish, Budgeon et al. 2001) We used NOD/SCID mice, which do not have any humoral or cell mediated immunity, they lack natural killer cells and a complement system.(Janeway, Travers et al. 2001; Kish, Budgeon et al. 2001) Previous studies have shown that this *in vivo* model system can sustain active HIV-1 transmission and replication in human vaginal tissue, vagina or cervix, its normal environment during transmission.(Kish, Budgeon et al. 2001)

***3.2 Materials and Methods*:**

3.2.1 Validation of Pooled CCR5-siRNA in P4-R5 MAGI HeLa Cell Lines and Testing of Different Transfection Reagents:

The pooled CCR5-siRNA needed to be validated to prove that it could down-regulate CCR5 *in vitro* before attempting treatment *in vivo*. The pooled siRNA (Dharmacon) was resuspended per the manufacturers' instructions. On the day prior to the experiment P4-R5 cells were seeded in twelve well plates at a density of $8x10^4$ cells per well and incubated overnight at 37° with 5% CO_2. On the day of the assay when the P4-R5 cells were approximately 50% confluent, cells were washed serum free antibiotic free (SFAF) DMEM. The appropriate concentration of Lipofectamine 2000 at a 1:5 ratio or pooled CCR5-siRNA (2nmol, 4nmol or 10nmol) was added to SFAF DMEM bringing the total volume to 525 μl, mixed and incubated at room temperature for 5 minutes. Following the incubation equal volumes of the CCR5-siRNA and Lipofectamine 2000 was mixed and incubated for 20 minutes at room

temperature. The subsequent mixture was added dropwise (300 µl per well) to triplicate samples of P4-R5 cell monolayers and incubated at 37° with 5% CO_2 for four hours with gentle rocking every thirty minutes. At the end of the four hour incubation 650 µl of P4-R5 cell culture media was added to each well and incubated for 48, 72 or 96 hours at 37° with 5% CO_2. At the end of the incubation, the cells were washed with ice cold PBS, and cell lysate was collected by adding 120 µl of RIPA buffer (150mM Sodium Chloride,1% NP40, 0.5% DOC, 1% Sodium Dodecyl Sulfate, 50mM Tris, 1mM PMSF, 5µg/ml Aprotinin, 1µg/ml Pepstatin-A, 2 µg/ml Leupeptin and 200 µM Sodium Orthovandate) to each well and to ensure all of the cells were collected the bottom of the cell culture plate was scraped with a cell scraper. The resulting cell lysate underwent three freeze/thaw cycles, was sonicated three times for 10 seconds each with cooling on ice in between each sonication and centrifuged. A modified Lowry Protein assay was performed to determine total protein in each cell lysate and OD was read on a Beckman Coulter DU800 spectrophotometer. Also, following the protocol described above we tested other transfection reagents to determine if other reagents would increase transfection efficiency and decrease cell toxicity, as shown in the summary in Table 3.

3.2.2 Absorption of fluorescent siRNA by vaginal tissue:

The organ tissue culture model system (Collins, 2000) using human vaginal tissue was utilized to see absorption of fluorescently tagged siRNA targeted against CCR5. Briefly, vaginal tissue obtained from women undergoing reconstructive surgeries for non-cancerous reasons were collected immediately prior to performing the experiment, and transported in serum free Minimum Essential Media (Fisher

Scientific) containing 0.05% sodium bicarbonate (Mediatech, Herndon, VA) and 10 mmol/L HEPES (Mediatech, Herndon, VA) as well as the following antibiotics: penicillin (100 U/ml; Mediatech, Herndon, VA); streptomycin (0.08 mg/ml; Mediatech, Herndon, VA); gentamicin (0.4 mg/ml; Mediatech, Herndon, VA); and fungizone (2.5 ųl/ml; Life Technologies). Only grossly normal samples released by surgeon were used. All samples were from tissues that would normally be discarded. Tissue from a single donor was divided into 6 contiguous 6.0 mm circular pieces by an Acu-punch® biopsy scalpel. The cut tissue sections were soaked in an antibiotic wash containing fungizone (250 ug/ml: Life Technologies) and nystatin (12ul/ml; Sigma), penicillin and streptomyocin (20,000 U each; Mediatech), for 5-8 minutes

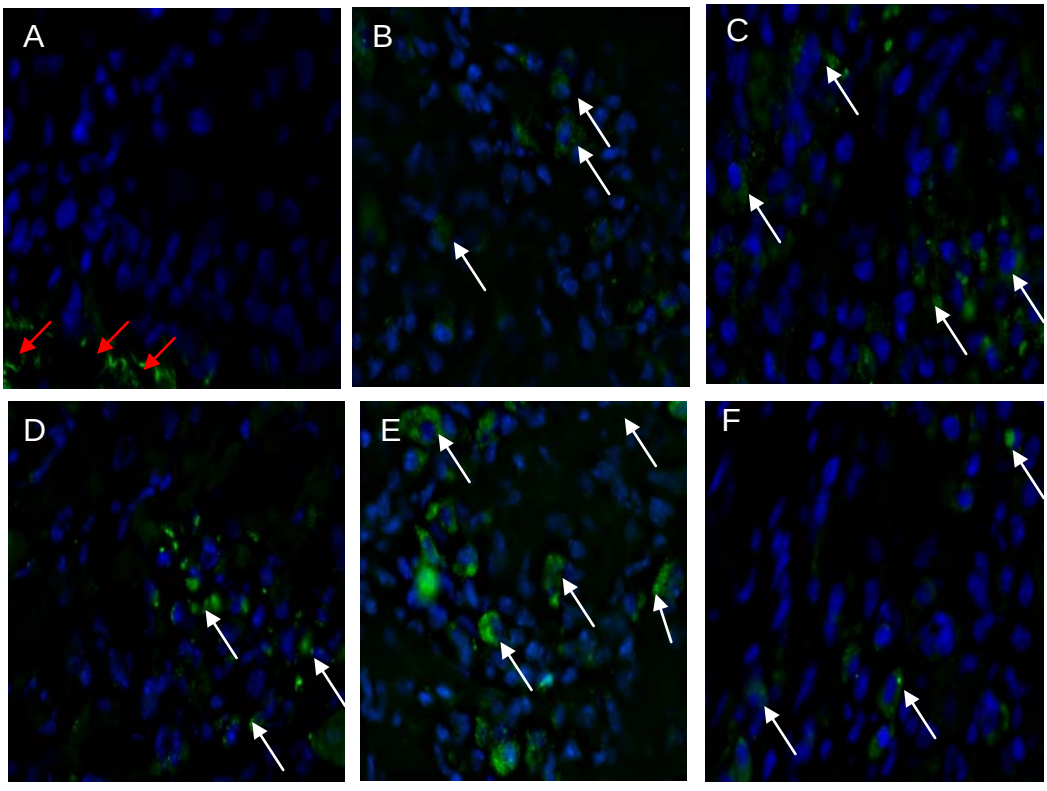


Figure 15: FITC-siRNA treated vaginal xenografts

Human vaginal epithelial xenografts treated for 2, 4, 12, 24 or 48 hours with non-specific fluorescein-tagged (FITC) siRNA nanoparticles to determine the tissue absorption of liposomoal delivered siRNA into the xenografts. By 2 hours (B) post-treatment a green positive FITC signal is clearly observed in the cytoplasm of the epithelial cells and at 4 hours (C), 12 hours (D) the signal continues to brighten. The signal is the brightest at 24 hours (E) and starts to fade by 48 hours (F) in the xenografts. The untreated control (A) xenografts do not show a positive signal. Red arrows indicate autofluorescence of connective tissue in the stroma, presumably type I collagen. White arrows indicate green positive signal from the FITC-siRNA. DAPI staining was performed coloring the cell nuclei blue and to characterize the architecture of tissue. All pictures shown are 60X magnification.

and washed three times in serum-free and antibiotic-free DMEM (Mediatech, Herndon, VA). The circular pieces of tissue with the epithelial layer oriented on top, were placed into the top chamber of 12-well Transwell dish. A sterile 5% solution of agarose in Hank's medium was added to the area surrounding the tissue and allowed to solidify creating a tight seal around the tissue. Serum free and antibiotic free DMEM (1 ml) is added to the bottom chamber to keep the Transwell membrane in contact with the medium. Also, 300 µl of serum-free and antibiotic-free DMEM containing different concentrations of FITC-tagged siRNA (Block-It™ Invitrogen) (either 10pM, 100pM, 2nM or 4nM siRNA) was added to the top chamber and incubated at 37º for 24 or 48 hours. At each time point the tissue was removed from the agarose, snap frozen and cryosectioned. Following the cryosectioning, the tissues

were fixed with 4% paraformaldehye and stained with DAPI (Santa Cruz Biotechnology), as per the manufacturer's instructions. The tissue sections were examined by immunofluorescent microscopy using an Olympus IX-81 Immunofluorescent microscope at a magnification of 10X. All of the resulting images were subjected to deconvolution to eliminate light emissions from other tissue planes. To test for fluorescent siRNA leakage from the top chamber of the organ tissue culture system, at 24 or 48 hours the Raft media from the bottom chamber was examined by immunofluorescent microscopy. During the initial validation experiments after surrounding vaginal tissue with agarose, 10% blue dextran in sterile phosphate buffer saline (PBS) was added to the top chamber and allowed to incbuate

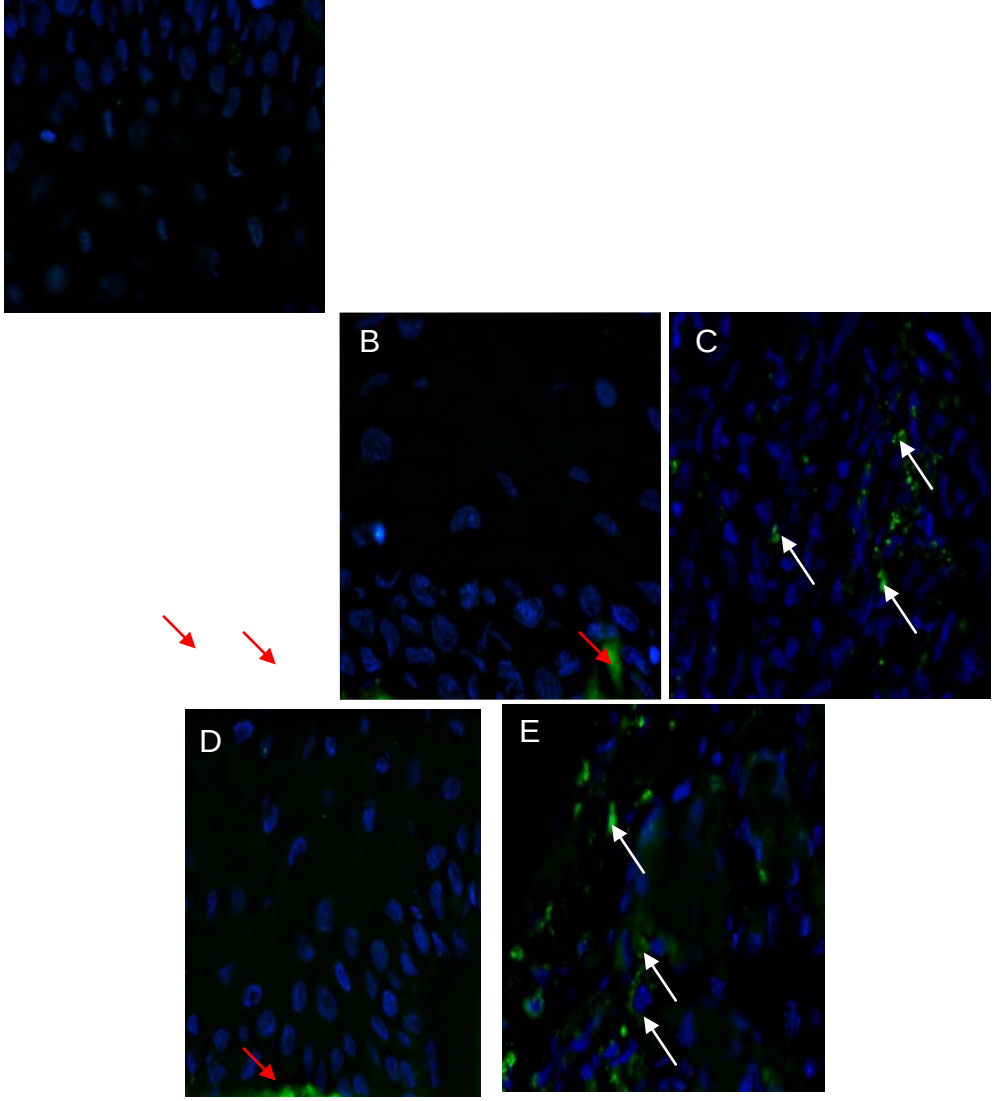


Figure 16: FITC-nanoparticle treated Xenografts

Human vaginal epithelial xenografts treated for 6 or 24 hours with fluorescein-tagged (FITC) polyethyleneglycol nanoparticles to assess the effectiveness of an alternate delivery method for the CCR5-siRNA. A green positive FITC signal is clearly observed in the cytoplasm of the epithelial cells in 6 hour (C) and 24 hour (E) FITC-nanoparticle treated xenografts. The untreated control (A), 6 hour (B) and 24 hour (D) sodium fluorescein nanoparticle treated xenografts do not show a positive signal. Red arrows indicate autofluorescence of connective tissue in the stroma, presumably type I collagen. White arrows indicate green positive signal from the FITC-nanoparticles. DAPI staining was performed coloring the cell nuclei blue and to characterize the architecture of tissue. All pictures shown are 60X magnification.

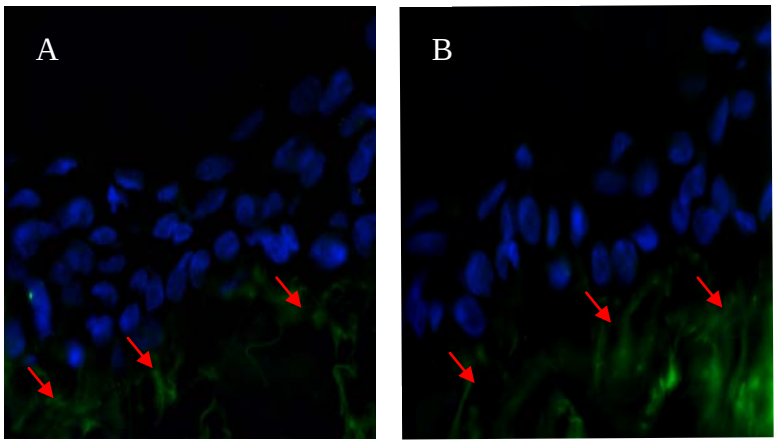


Figure 17: Human vaginal tissue treated with a lentiviral vector system expressing green fluorescent protein (GFP).

To assess the effectiveness of an alternate delivery method for an alternate molecule, CCR5-shRNA, human vaginal tissue was treated with a lentiviral vector expressing GFP for 24 hours. There is no difference between the untreated (A) and the lentiviral vector treated (B) tissue that does not show any positive signal. Red arrows indicate autofluorescence of connective tissue in the stroma, presumably type I collagen. DAPI staining was performed coloring the cell nuclei blue and to characterize the architecture of tissue. All pictures shown are 60X magnification.

for 48 hours. Following the incubation 500 ul of media was removed from the bottom chamber and subjected to spectrophotometry (Beckman Coulter, DU800) at 595 nm. The instrument was able to detect dilutions of blue dextran as high as $1x10^4$ in media.

3.2.3 Validation of Pooled CCR5-siRNA in Human Vaginal Tissue Using the Organ Tissue Culture System:

The OTC was set up as described in previous section. The vaginal tissue was treated with 300 µl of serum-free and antibiotic-free DMEM containing liposomal complex containing10nmol of CCR5-siRNA (Dharmacon) was added to the top chamber and incubated at 37º for 72 or 96 hours. At each time point the tissue was removed from the agarose, snap frozen and homogenized using FastPrep Machine and Bio101 RNApro kit, per the manufacturers' instructions.

3.2.4 Human vaginal xenograft model treatment with either different siRNAs or infection HIV-1:

Six to fourteen week old female NOD/LtSz-*scid/scid* mice (Jackson Laboratory, Bar Harbor, ME) were maintained under specific pathogen-free environmental conditions, in accordance with all Drexel University's Institutional Review Board and Institutional Animal Care and Use Committee's rules and bylaws. Vaginal tissue was obtained from patients undergoing reconstructive surgeries for non-cancerous reasons. The vaginal tissue samples were cut into split-thickness grafts, ~ 2cm x 2cm x 0.5 mm in size, consisting of the vaginal epithelial layer with minimal stroma. The tissues were rolled into a tubular conformation with the epithelial layer directed inward and placed within the subcutaneous space on the backs of the NOD/SCID mice. Estrogen pellets were also implanted so that the circulating peripheral blood estrogen levels

were 200 pg/ml, the average normal level in pre-menopausal women. This allowed sustained growth of the human vaginal xenografts. Grafts were allowed to heal for ~21 days after implantation, at which time they were infected with HIV-1 BaL or treated with liposomal complex (Lipofectamine 2000, Invitrogen) according to the manufacturer's instructions, containing 2nmol or 4nmol FITC-siRNA (Block-It™ Invitrogen), 4nmol or 10nmol CCR5-siRNA, FITC-nanoparticles or untreated controls. At specified time points the mice were euthanized, the xenografts were removed and processed. For the xenografts treated with fluorescein molecules (FITC-siRNA and nanoparticles), immediately after removal the xenografts were snap frozen and cryosectioned. Following the cryosectioning, the tissues were fixed with 4% paraformaldehye and stained with DAPI (Santa Cruz Biotechnology), as per the manufacturer's instructions. The tissue sections were examined by immunofluorescent microscopy using an Olympus IX-81 Immunofluorescent microscope at a magnification of 10X and 60X. All of the resulting images were subjected to deconvolution to eliminate light emissions from other tissue planes. For xenografts treated with CCR5-siRNA (Dharmacon), immediately after harvesting the xenografts were cut in half with one half being snap frozen for Western Blot analyzes and the other half being fixed in 5% formalin, paraffin embedded and subjected to immunohistochemical analyzes. For xenografts infected with HIV-1 (BaL) upon harvesting the xenografts homogenized, total tissue RNA was extracted and RT-PCR was performed for viral Gag/Pol or Tat/Rev transcripts, as previously described. To

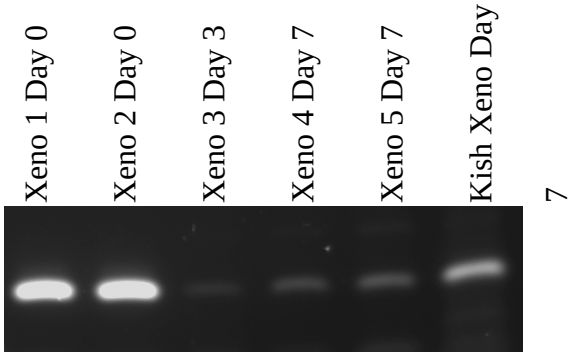


Figure 18: Successful HIV-1 infection in human vaginal epithelial xenografts.

RT-PCR was performed on 200 ng of total cellular RNA from HIV-1 BaL infected vaginal xenografts. Vaginal xenografts were infected and on days 0, 3 and 7 xenografts were harvested, homogenized and total RNA was extracted. Following RNA extraction RT-PCR was performed using primers specific for viral Gag/Pol transcripts to detect successful HIV-1 infection. On day 0 the positive signal observed in both xenograft 1 and 2 is a residual signal from the inoculum; the signal almost completely disappears in xenograft 3 on day 3; the positive signal in xenograft 4 and 5 on day 7 is indicative of active HIV-1 replication; and Kish xenograft day 7 is a positive control from our laboratories previous work for the RT-PCR.

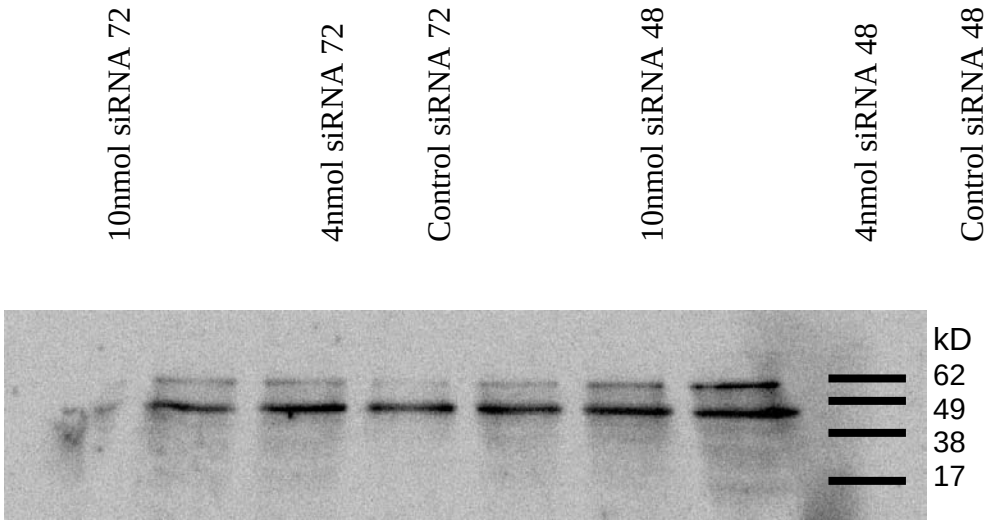


Figure 19: Western Blot of CCR5-siRNA treated cell lysates

Western Blot showing down-regulation of CCR5 in P4-R5 cell lysates. Cells were treated with CCR5-siRNA and incubated for either 48 or 72 hours after which lysates were collected as described in Materials and Methods. Unglycosolated (immature) forms of CCR5 are ~49 kD and glycosolated (mature) forms are ~62kD in size. Scrambled non-specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. Treatment with 4nmol at both 48 and 72 hour treatments partially down-regulated the glycosolated forms of CCR5, but do not appear to have any effect on the unglycosolated forms. However, treatment with 10nmol siRNA at 48 hours almost completely eliminates the glycosolated form of CCR5 with little of no effect on the unglycosolated CCR5. Importantly, treatment with 10nmol siRNA for 72 hour completely eliminates glycosolated CCR5 and significantly down-regulates unglycosolated forms. Results representative of two different experiments with duplicate samples.

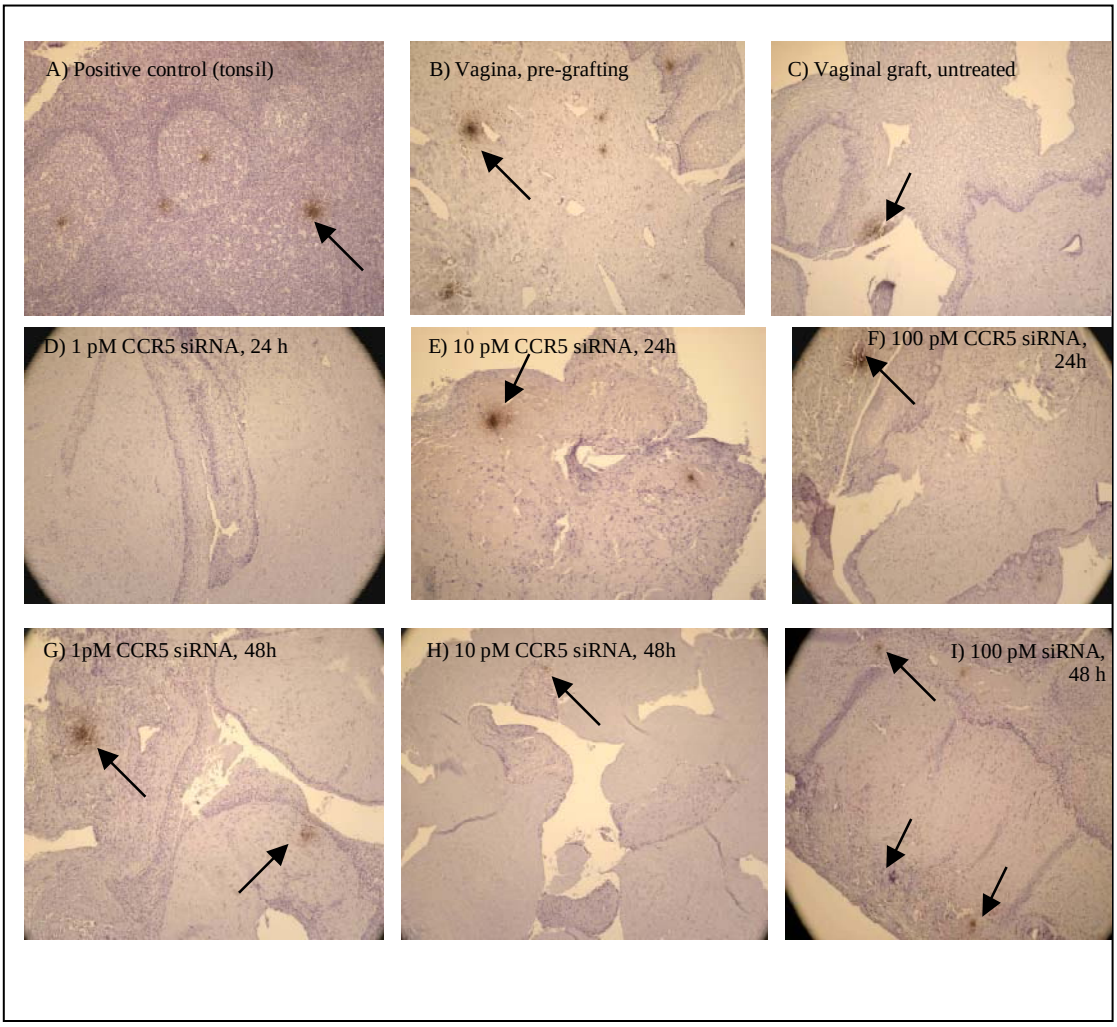


Figure 20: Detection of CCR5 expression in vaginal xenografts by immunohistochemistry.

Vaginal xenografts were treated with varying concentrations of CCR5-siRNA for either 24 hr or 48 hr prior to immunohistochemical staining. Untreated human tonsil (A) was used as a postive control and pre-grafted vaginal tissue (B) was used to verify the person was not homozygous for the CCR5Δ32 mutation. Vaginal grafts were either left untreated (C) or treated with either 1pM (D), 10pM (E) or 100 pM(F) for 24 hr; or 1 pM (G) 10pM (H), or 100pM (I) for 48 h prior with CCR5-specific siRNA. Arrows indicate characteristic pockets of CCR5 positive cells. All magnifications shown are 10X with some sections (D, F and I) having been subjected to the wide angle mode of the camera.

control for donor-to-donor variations, grafted tissue is distributed as evenly as possible across all treatment groups.

3.2.5 Immunohistochemistry for detection of CCR5 in vaginal grafts:

Paraffin embedded samples of harvested vaginal grafts were cut into 4 µm thickness tissue sections and stained with hematoxylin and eosin staining to examine morphology. The tissue sections were stained for cell surface protein CCR5 using mouse monoclonal antibody (Santa Cruz Biotechnology) using the VectorStain Elite kit (Vector Laboratories, Burlingame, CA) as per the manufacturer's instructions. Briefly, all tissue sections were baked at 55º C for one hour to evaporate the paraffin. The samples were then dehydrated and rehydrated by incubation with xylene and a graded alcohol series to prevent non-specific binding. Endogenous peroxidases were destroyed by immersion with 3% hydrogen peroxide in deionized water for 10 minutes. After the appropriate antigen retrieval (recommended by the antibody manufacturer) samples were blocked with 10% horse serum (Vector Labs) in PBS for one hour at room temperature in a humidified chamber. All antibodies were diluted in 1% horse serum in PBS per the manufacturer's instructions with a total volume of 150 µl was added to experimental samples and incubated overnight. Samples were washed twice for five minutes each with incubations were performed for 30 minutes at room temperature. Color visualization of the complex was achieved by incubating the tissue sections with diaminobenzidine tetrahydrochloride PBS, incubated with a biotinylated anti-mouse secondary antibody (Vector Labs) for 60 minutes. After two more washes in PBS, the streptavidin-peroxidase conjugate containing Ni^{2+} for 5 minutes. Lastly,

tissue samples were briefly rinsed in water and counterstained with hematoxylin for 10 minutes. Stained tissue was examined using a Olympus microscope at the magnifications indicated.

3.2.6 Preparation of Tissue Lysates:

Tissue lysates were collected from human tissue/xenografts using the FastPrep® Homogenizer (Bio101 Systems), according to the manufacturer's instructions. Briefly, each frozen tissue section was diced, 500 µl of ice cold RIPA buffer (150mM Sodium Chloride,1% NP40, 0.5% DOC, 1% SDS, 50mM Tris, 1mM PMSF, 5µg/ml Aprotinin, 1µg/ml Pepstatin-A, 2 µg/ml Leupeptin and 200 µM Sodium Orthovandate) was added to each matrix tube and homogenized at a setting of 6.0 m/s^2 for 30 seconds. Tissue lysates were then subjected to sonication followed by an incubation on ice for 45 minutes and centrifuged at 16,000 g for 10 minutes at 4ºC. Total cellular protein concentration was determined by performing a Modified Lowry Protein Assay (Pierce, Rockford, IL.) according to the manufacturer's instructions and the samples were read using a Beckman Coulter DU800 spectrophotometer.

3.2.7 Western Blot for detection of CCR5:

50 µg of total cellular proteins from each tissue lysate were run on a 4/12% stacking SDS polyacrylamide gel using the BioRad Mini Protean II (BioRad) and transferred to nitrocellulose (Protran BA 83, Whatman Schleicher & Schuell, Dassel, Germany). Each membrane was than incubated for an hour at room temperature in 5% milk in TBS, washed three times at room temperature in TBS-T for ten minutes each then incubated in primary monoclonal mouse antibody (Santa Cruz Biotechnology, Santa

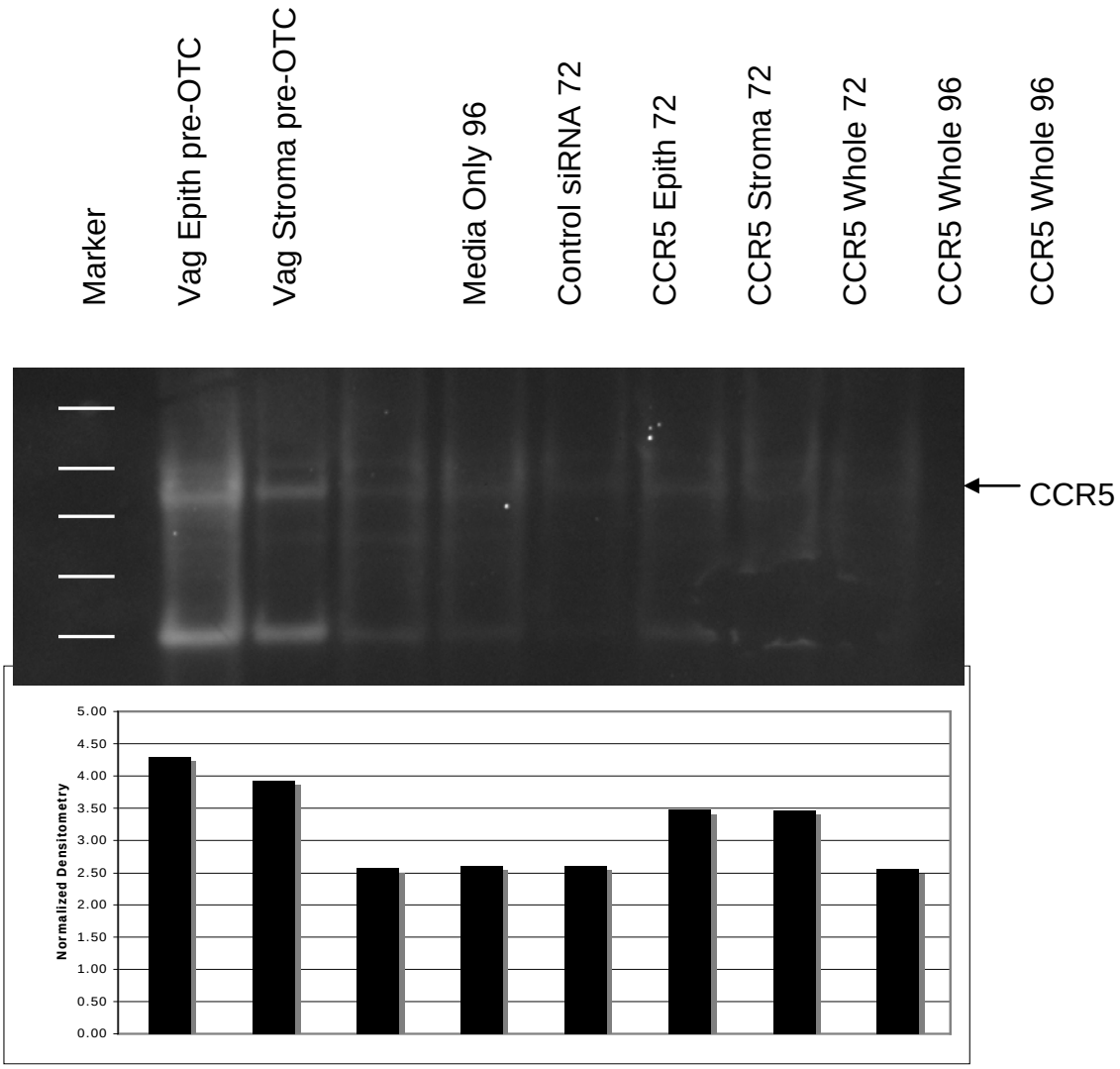


Figure 21:Western Blot of OTC CCR5-siRNA treated vaginal tissue

Western Blot showing no down-regulation of CCR5 as compared to control siRNA in human vaginal tissue lysates. Using the organ tissue culture system vagina tissues were treated with 10nmol CCR5-siRNA and incubated for either 72 or 96 hours after which lysates were collected as described in Materials and Methods. Unglycosolated (immature) forms of CCR5 are ~49 kD and glycosolated (mature) forms are ~62kD in size. Scrambled non-specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. Normalization graph shows no down-regulation in CCR5-siRNA treated tissue as compared to control. Results are representative of two different experiments with duplicate samples.

Cruz, CA) specific for human CCR5. Following incubation with primary antibody each blot was washed three times as previously described and incubated with secondary mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA) labeled with horseradish peroxidase tag. After incubation with secondary antibody each blot was washed three times as previously described and developed using SuperSignal West Dura Chemiluminescent Detection Kit (Pierce, Rockford, IL.) per the manufacturer's instruction on a Alpha Imaging system following the manufacturer's instructions. Subsequent to the chemiluminescent detection of CCR5, each membrane was stripped and incubated with a goat polyclonal primary antibody specific for cyclophilin A for normalization with all subsequent steps being performed as described above.

3.3 Results:

3.3.1 Immunohistochemistry shows CCR5 expression in vaginal xenografts:

To verify conservation of the normal morphology of grafted vaginal tissue, hematoxylin and eosin (H&E) staining was performed prior to grafting the tissue and after the healing process of the graft. For every graft performed, all tissue sections showed maintenance of the normal architecture with the squamous epithelial and basal layers clearly visible (data not shown). To visualize CCR5 expression in the xenografts and to analyze down-regulation of CCR5 in response to siRNA treatment, we performed immunohistochemical staining of untreated and treated grafted vaginal tissue. As shown in Figure 20, a positive signal for CCR5 in the avidin-biotin complex system used was indicated by a color change to brown, which was visible in the no treatment, 1 pM CCR5-siRNA, 10 pM or 100 pM CCR5-siRNA treated tissue sections that were treated for 24 and 48 hours. All observations were made at 10X

magnification. Since we do not have the proper microscope with software to analyze and quantify immunohistochemistry we decided not to utilize this method of detection for future experiments. However, these results confirmed the variation observed from xenograft to xenograft and helped the guide our troubleshooting process. From the imunohistochemical staining we decided to decrease the time duration from tissue pick up to surgery (from eight to three hours) to ensure the immune cell population was retained in tissue before implantation as well as to increase the CCR5-siRNA concentrations.

3.3.2 FITC-tagged siRNA penetrates human vaginal tissue *in vivo*:

To determine the kinetics of absorption and to verify that the liposome-delivered siRNA would be absorbed through the squamous epithelia of the vaginal xenografts and have the potential to reach the target macrophages and Langerhan cells, we treated vaginal xenografts with non-specifc FITC-siRNA. Vaginal tissue was treated with 4nM non-specific FITC-siRNA for 2, 4, 12, 24 or 48 hours and cryosections were examined by fluorescent microscopy. By 2 hours post-treatment a positive green FITC signal was observed in the FITC-siRNA treated xenografts, as indicated by the white arrows in Figure 15. Some of these cells in the 2 hour treatment xenografts show positive FITC signal in the cytoplasm of the cells as is evident by the green signal directly adjacent to the blue DAPI stained nucleus. As indicated by the white arrows in Figure 15, all of the xenografts treated for 4, 12 and 24 hour visibly show a positive green signal, which is clearly observed in the cytoplasm of the cells. In these xenografts there is also an increased intensity in brightness of the FITC signal, with

the brightest signal being observed at 24 hours and at 48 hours the signal starts to fade. There was no positive signal in the untreated group. However, the red arrows indicate autofluorescence emanating from the connective tissue in the stroma, presumably type I collagen, which studies have shown that many connective tissues, specifically collagen, will autofluoresce when excitated in all wavelengths.

3.3.3 FITC-nanoparticles penetrate human vaginal tissue *in vivo* but not lentiviral vector system:

To establish an alternate and a potentially more efficient siRNA delivery method we treated vaginal xenografts with FITC-nanoparticles or sodium fluorescein control for either 6 or 24 hours and examined the cryosections by fluorescent microscopy. The nanoparticles were made of polyethyleneglycol and have had a FITC molecule conjugated the nanoparticles. As shown in Figure 16, by 6 hours post-treatment the FITC-nanoparticle group we observed the FITC-nanoparticles (green) had penetrated the tissue but did not appear to have entered the cells. However, in the 24 hour FITC-nanoparticle treated tissue there was clear penetration into the tissue as well as entry into the cytoplasm as indicated by the white arrows. There was no positive signal from the sodium fluorescein negative controls and untreated groups. Red arrows indicate tissue autofluorescence originating from the connective tissue, presumably type I collagen. We also treated vaginal tissue with a non-specific lentiviral vector system, which upon successful entry into cells and if active transcription is occurring a green fluorescent protein (GFP) is expressed. As shown in Figure 17, there is no positive GFP signal observed in the vaginal tissue.

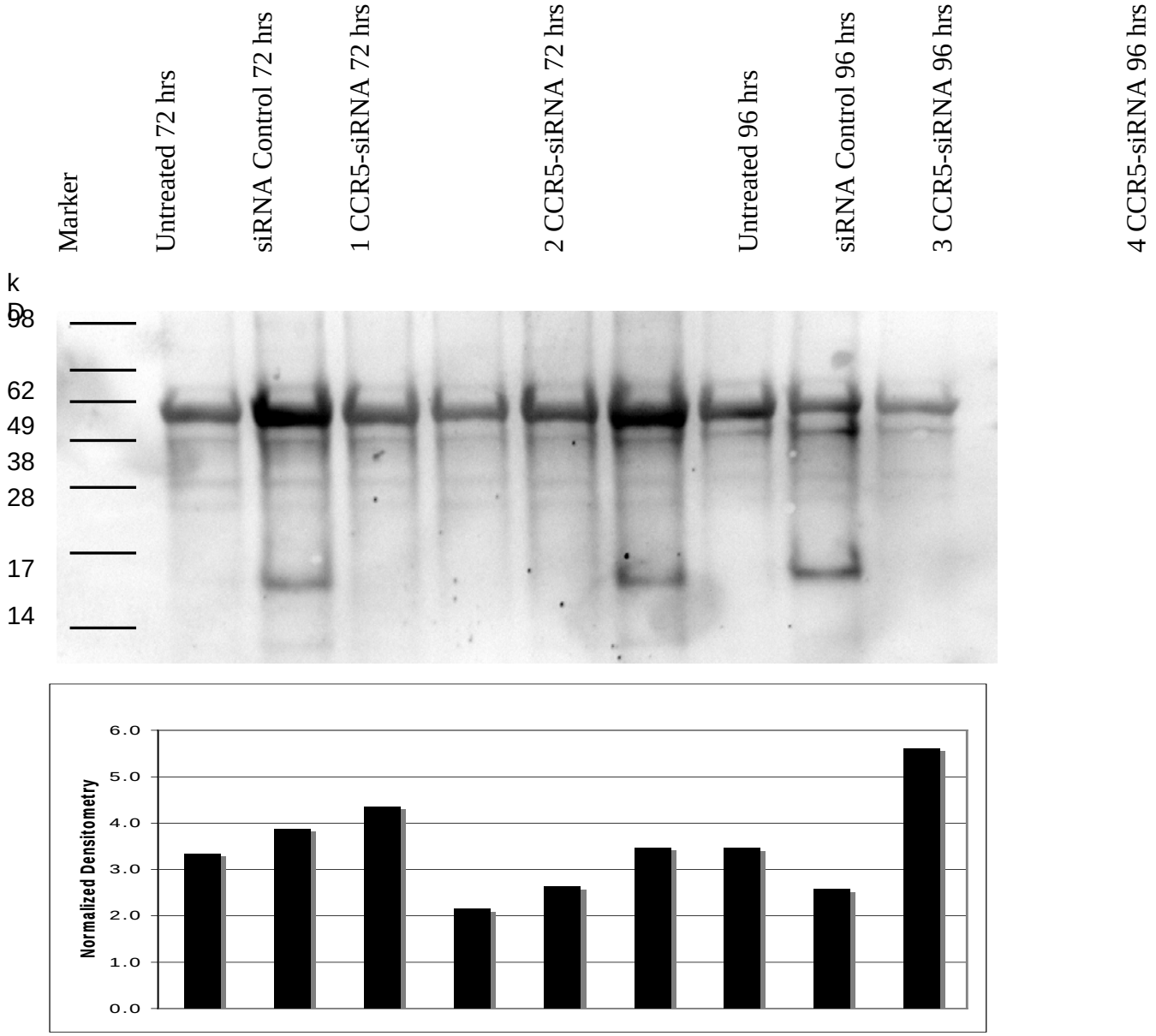


Figure 22: Western Blot of CCR5 siRNA treated Xenografts

Western Blot with normalization showing no down-regulation of CCR5 in human vaginal xenograft tissue lysates. Using our laboratories human vaginal xenograft system, xenograft tissues were treated with 10nmol CCR5-siRNA and incubated for either 72 or 96 hours after which tissue lysates were collected as described in Materials and Methods. Unglycosolated (immature) forms of CCR5 are ~49 kD and glycosolated (mature) forms are ~62kD in size. Untreated was included as a control to verify the person was not homozygous for the Δ32 mutation. Scrambled non -specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway.

Number of Mice	Donor/ # Xenografts	siRNA Treatment Groups (n=number of mice per group)	Harvest Time (hrs)	Observations and Conclusions
3	Ungrafted mice	Mouse blood & spleen harvested	N/A	No antibody cross reactivity
10	Donor A/10	No Treatment, Liposome Only, 1pM, 10pM or 100pM (For all groups n=1)	24 and 48	No down-regulation
7	Donor B & C/7	No Treatment (n=1), Liposome Only (n=1), 1pM (n=1), 10pM (n=2) or 100pM (n=2)	48	No down-regulation
5	Donor D/5	No Treatment (n=1), 1pM (n=1), 10pM (n=1) or 100pM (n=2)	48	No down-regulation
8	Donor E & F/8	No Treatment, 1pM, 10pM or 100pM CCR5 siRNA For all groups n=1	24 and 48	No down-regulation
3	Donor G/3	No Treatment (n=1) and 4nM (n=2)	48	No down-regulation
10	Donor H/10	No Treatment (n=2) and 4nM (n=8)	24 and 48	No down-regulation
2	Donor I/2	No Treatment (n=2)	N/A	CCR5 WB detection
6	Donor J/6	No Treat (n=2), 4 nmol FITC-siRNA (n=4) or FITC-siRNA only (n=2)	24 and 48	FITC in tissue & visible in cytoplasm
12	Donor K/ 12	No Treat (n=2) or 4nmol FITC-siRNA (n=10)	0, 2, 4 & 12	FITC in tissue & visible in cytoplasm at 4 & 12 hr
10	Donor L/10	No Treat (n=2), 2nmol (n=4) or 4 nmol CCR5 siRNA (n=4)	24 and 48	No down-regulation
5	Donor M/5	No Treat (n=1), 4 nmol CCR5 (n=2) or 4nmol Control siRNA (n=2)	24 and 48	No down-regulation
10	Donor N/10	No Treat (n=3), 4 nmol CCR5 (n=4) or Control siRNA (n=3)	48	No down-regulation
6	Donor O/6	No Treat (n=1), FITC-Nanoparticles (n=3) or Sodium Fluorescien (n=2)	6 and 12	FITC in tissue not in cytoplasm
9	Donors O, P/9	No Treat (n=1); FITC-nanoparticles (n=1) or Sodium Fluorescin (n=1); 6 xenografts did not form	24	FITC in tissue & visible in cytoplasm
9	Donor Q/9	No treat (n=2); 10nmol CCR5 (n=5) or Irrelevant siRNA (n=2)	72 & 96	Partial down-regulation
6	Donor R/6	No treat (n=1); HIV-1 day 0(n=2); day 3(n=1) or day 7(n=2)	0, 72 & 168	Detection of Gag/Pol transcript
3	Donor R/3	*L. plantarum* CVN+ (n=3)	72	Detection of CVN
10	Donor R/10	No treatment (n=2), *L. plantarum* CVN+ (n=4); *L. plantarum* WT (n=4)	168	Not analyzed
Total of 134 mice	**Total of 131 xenografts**			

Table 2: Human Vaginal Xenograft Summary.

3.3.4.Successful validation of pooled CCR5-siRNA in P4-R5 cells.

To determine the effectiveness and ability of the liposomal delivered pooled CCR5-siRNA we treated P4-R5 cells with either 4 nmol or 10 nmol pooled CCR5-siRNA or non-specific scrambled siRNA for 48 or 72 hours after which cell lysates were collected and analyzed by Western Blot. Non-specific scrambled control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. In Figure 18, the top band corresponding to ~62kD is the glycosolated (mature) form of CCR5 and the bottom band corresponding to ~49 kD is the unglycosolated (immature) forms of CCR5. As shown in Figure 18, treatment with 4nmol pooled CCR5-siRNA for 48 and 72 hour treatments partially down-regulated the glycosolated forms of CCR5, but do not appear to have any effect on the unglycosolated forms. However, treatment with 10nmol CCR5-siRNA for 48 hours almost completely abolishs the glycosolated form but has little effect on the unglycosolated CCR5. Importantly, treatment with 10nmol CCR5-siRNA for 72 hours completely eliminates the glycosolated CCR5 and partially down-regulates unglycosolated forms.

3.3.5. No down regulation of CCR5 observed in human vaginal tissue using the *ex vivo* OTC system

To determine if CCR5 could be down regulated in human vaginal tissue lysates, we used the OTC system. The vagina tissues were treated with 10nmol CCR5-siRNA and incubated for either 72 or 96 hours after which lysates were collected as described in Materials and Methods. Unglycosolated (immature) forms of

CCR5 are ~49 kD and glycosolated (mature) forms are ~62kD in size. Scrambled non-specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. The epithelia and stroma were separated to help ascertain if down regulation was occurring in the entire tissue or just in one portion of the tissue. This could be biologically relevant to the tranmission of HIV-1. However, upon normaliztion of the Western Blot with cyclophilin A, both the 72 and 96 hour treatments with CCR5-siRNA showed no down-regulation in the epithelia, stroma or whole tissue.

3.3.6. Treatment of human vaginal xenografts with CCR5-siRNA

To detemine if we could successfully down regulated CCR5 in xenograft tissue, we treated xenograft with 10nmol CCR5-siRNA, incubated 72 or 96 hours followed by collection of tissue lysates. Western blot did not show down-regulation of CCR5 in human vaginal xenograft tissue lysates as compared to scrambled controls. Unglycosolated (immature) forms of CCR5 are ~49 kD and glycosolated (mature) forms are ~62kD in size. Scrambled non-specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. Untreated controls were included to confirm that individual donor was not homozygous for CCR5Δ32 mutation and that the individual did express CCR5.

Transfection Reagent	Concentrations Tested	Observed
Lipofectamine 2000	2, 3, 4 or 5 ug/10 nmol siRNA	2 and 3 ug low transfection efficiency; 4 and 5 ug toxic < 80% cell death
Fugene 6	2, 3, 4 or 5 ug/10 nmol siRNA	2, 3 and 4 ug low transfection efficiency; 5 ug toxic < 65% cell death
Arrestin	2, 3, 4 or 5 ug/10 nmol siRNA	All non-toxic >15% transfection efficiency
DharmaFect 1	2, 3, 4 or 5 ug/10 nmol siRNA	2 and 3 ug low transfection efficiency; 4 and 5 ug toxic < 60% cell death
DharmaFect 2	2, 3, 4 or 5 ug/10 nmol siRNA	2, 3 and 4 ug low transfection efficiency; 5 ug toxic < 60% cell death
DharmaFect 3	2, 3, 4 or 5 ug/10 nmol siRNA	2 and 3 ug low transfection efficiency; 4 and 5 ug toxic < 60% cell death
Dharmafect 4	2, 3, 4 or 5 ug/10 nmol siRNA	2 and 3 ug low transfection efficiency; 4 and 5 ug toxic < 60% cell death

Table 3: Summary of different transfection reagents and concentrations tested.

3.3.7 Succesful infection of HIV-1 (BaL) in vaginal xenografts without reconstituting with PBMCs.

To confirm that we could successfully infect human vaginal xenografts without reconstituting the xenograft with peripheral blood mononuclear cells we inoculated five xenografts with 10 ul of undiluted HIV-1 (BaL), harvested the xenografts on day 0, 3 and 7 and performed RT-PCR for the viral gag/pol transcripts. It was necessary to harvest xenografts at these time points to verify de novo viral transciption. This is based on our laboratories previous published work, which showed a positive signal was observed on day 0 (which was residual HIV-1 inoculum), disappears on day 3 and returns on day 7, indicating de novo viral transcriptional activity. As shown in Figure 18, the gag/pol is detected on day 0, almost completely disappears on day 3 and increases in intensity on day 7, indicating viral replication and active viral transcription is occurring.

Discussion:

We used human cell lines, vaginal tissue and human vaginal xenografts to determine if CCR5 could be down regulated using a cationic liposome delivery system. We started with Lipofectamine 2000 (Invitrogen) as our liposomal complex delivery system for our CCR5-siRNA and tested several different transfection reagents and concentrations, as shown in Table 3, to determine the best ratio of siRNA to transfection reagent to achieve maximum delivery of our siRNA molecules to the cells and tissues.

After testing several different siRNAs targeting CCR5 we decided to use the validated SMARTpool siRNA from Dharmacon. To determine the effectiveness and ability of the liposomal delivered pooled CCR5-siRNA to down regulate CCR5, we

treated P4-R5 cells with either 4 nmol or 10 nmol pooled CCR5-siRNA or non-specific scrambled siRNA for 48 or 72 hours. Then cell lysates were collected and analyzed by Western Blot. Non-specific scrambled control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. The Western Blot data establishes that both the glycosolated (mature, ~62kD) and unglycosolated (immature, ~49 kD) forms of CCR5 can be detected with our antibody. The 4nmol pooled CCR5-siRNA treatment incubated for 48 and 72 hours resulted in partially down-regulating the glycosolated forms of CCR5, but does not appear to have any effect on the unglycosolated forms when compared to the control siRNA. However, a 48 hour treatment with 10nmol CCR5-siRNA almost completely eliminates the glycosolated form but has little effect on the unglycosolated form of CCR5. Notably, a 72 hour treatment with 10nmol CCR5-siRNA completely eliminates the glycosolated CCR5 and partially down-regulates unglycosolated forms as determined by Western Blot analysis. This data indicates that CCR5 can be successfully down regulated in P4-R5 cells using the pooled CCR5-siRNA from Dharmacon using a single application. The validation of an efficient siRNA targeting CCR5 was necessary before proceeding to the *ex vivo* organ tissue culture system and the *in vivo* human vaginal xenograft system.

Since we were able to down-regulate CCR5 in P4-R5 cells, which are adherent HeLa cells that do not normally express CCR5, we needed to determine if we could down regulate CCR5 in human vaginal tissue containing human lymphocytes. To this end, an *ex vivo* organ tissue culture (OTC) system was utilized. Using the OTC, fresh

vaginal tissues were treated with 10nmol liposomal CCR5-siRNA or scrambled control siRNA, incubated for either 72 or 96 hours and then tissue lysates were collected. At the time of harvesting, the stroma and epithelia were separated in one of the CCR5-siRNA treated tissues from both 72 and 96 hour time points. This was necessary because it was not known if the siRNA would penetrate through all of the layers of the squamous epithelia. By separating the epithelia from the stroma it could be deduced if CCR5 was being down-regulated in either or both of the tissue layers. Scrambled non-specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. As seen in Figure 21, at 72 and 96 hour post-treatment CCR5 was not down-regulated in either the epithelia, stroma or whole tissue as compared to the scrambled control siRNA. Prior to normalization we thought that there was down reulgation of CCR5, but after normalization we believe that the high concentrations of Lipofectamine combined with the high concentrations of CCR5-siRNA was toxic to the tissue.

The human vaginal xenograft model system was used to determine kinetics and absorption of liposome delivered siRNA or nanopartilces into human vaginal tissue for a potential use as a microbicide. To determine if the siRNA would be absorbed through the squamous epithelia of the vaginal xenografts and have the potential to reach the target macrophages and Langerhan cells, we treated vaginal xenografts with non-specifc FITC-siRNA. Vaginal tissue was treated with 4nM non-specific FITC-siRNA for 2, 4, 12, 24 or 48 hours and cryosections were examined by immunofluorescent microscopy. By 2 hours post-treatment a positive green FITC signal was observed in the FITC-siRNA treated xenografts. Some of these cells in the

2 hour treatment xenografts show positive FITC signal in the cytoplasm of the cells as is evident by the green signal directly adjacent to the blue DAPI stained nucleus. All of the xenografts treated for 4, 12 and 24 hour visibly show a positive green signal, which is clearly observed in the cytoplasm of the cells. In these xenografts there is also an increased intensity in brightness of the FITC signal, with the brightest signal being observed at 24 hours and at 48 hours the signal starts to fade. There was no positive signal in the untreated group. However, there was autofluorescence emanating from the connective tissue in the stroma, presumably type I collagen, which studies have shown that many connective tissues, specifically collagen, will autofluorescence when excitated in all wavelengths.

To establish an alternate and a potentially more efficient siRNA delivery method we treated vaginal xenografts with FITC-nanoparticles or sodium fluorescein control for either 6 or 24 hours and examined the cryosections by immunofluorescent microscopy. The nanoparticles were made of polyethyleneglycol and have had a FITC molecule conjugated the nanoparticles. By 6 hours post-treatment the FITC-nanoparticle group we observed the FITC-nanoparticles (green) had penetrated the tissue but did not appear to have entered the cells. However, in the 24 hour FITC-nanoparticle treated tissue there was clear penetration into the tissue as well as entry into the cytoplasm as indicated by the white arrows. There was no positive signal from the sodium fluorescein negative controls and untreated groups. Autofluorescence was again observed originating from the connective tissue, presumably type I collagen.

We were able to show that after 4 hours liposomal-siRNA and after 24 hours nanoparticles can adequately traverse the multiple layers of the squamous epithelia in human vaginal xenograft tissue. Where a portion of the HIV CCR5-positive target cells are localized. Surprisingly, originally when the xenografts were treated with 4nM CCR5-siRNA there was no decrease in expression of CCR5 by immunohistological staining and Western Blot analyzes. Since we currently do not have a method for quantifying immunohistochemcial stained tissue sections we decided to perform Western Blot analysis to detect CCR5 protein levels. To this end it was also necessary to determine the adequate dose of CCR5-specific siRNA required for treatment of vaginal tissue to abolish expression of CCR5.

Based on the FITC-siRNA absorption data we decided to start with our laboratories human vaginal xenograft system prior to normalization of the CCR5-siRNA OTC experiments. Xenograft tissues were treated with 10nmol CCR5-siRNA and incubated for either 72 or 96 hours after which tissue lysates were collected as described in Materials and Methods. Unglycosolated (immature) forms of CCR5 are ~49 kD and glycosolated (mature) forms are ~62kD in size. Scrambled non-specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. The xenograft CCR5-siRNA treatments were only performed once and need to be repeated but based on the Western Blot analysis and normalization data in Figure 22 there was no down-regulation of CCR5 in the xenografts. Interestingly, three of the five xenografts treated with CCR5-siRNA and the scramble controls appear to have some upregulation of CCR5, indicating the siRNA itself may be affecting the pathway. A microarray needs to be performed to

help assess changes in other genes in the RNAi pathway are being affected or possibly if regulatory genes for CCR5 recycling is being affected. Based on these results that there was no down regulation of CCR5 in the xenograft tissue it may be necessary to increase the concentration of CCR5-siRNA and change the delivery vehicle to nanoparticles. The xengrafts are metabolically active and contain the same proteins and enzymes found in normal vaginal tissue. Even though the siRNA is encapsulated inside liposomes it may be possible for the CCR5-siRNA to be degraded after liposomal fusion with the outer most layer of the squamous epithelia. The nanoparticles may provide more protection from siRNA degradation and may be less toxic, however, more testing is required to confirm this hypothesis. Utilizing nanoparticle has its advantages such as they are can be stored at room temperature and based on our results the nanopartles also penetrate human vaginal epithelia. The disadvantage to utilizing nanoparticles is the cost. It may also be more appropriate target other surface molecules on the vaginal epithelia that are known to bind to HIV-1, such as gp340 and syndecan-1. Targeting other molecules and testing different combinations of surface molecules could be performed at lower concentrations both siRNA and delivery vehicle whether it be liposomes, nanoparticles or other vehicle.

4. Lactobacilli secreting Cyanovirin: Probiotics can they work?

Veronica Holmes, Yana Thaker, Li Ming, Bharat Ramratnam and Mary K. Howett

The following chapter is written as an independent manuscript to allow for an easy transition from thesis format to submission format after combined with data from non-human primates, which occurred in parallel at Brown Univeristy.

4.1 Introduction:

The primary route of transmission for human immunodeficiency virus type 1 (HIV-1) is vaginal and rectal sexual intercourse. Some scientists believe that a prophylactic vaccine could potentially prevent sexual transmission of HIV-1, however, no clinically effective anti-HIV-1 vaccine has been successful in providing protective immunity. While consistent and reliable condom use is effective in preventing sexual transmission of HIV-1, this largely male-controlled method is not widely accepted among many at risk groups. (Tsai, 2004) In the absence of an effective preventative-vaccine, topical microbicides can provide a useful and cost-effective means of preventing new infections.(Pusch, 2005)

In 1997 a novel protein, Cyanovirin-N (CVN), was discovered to have potent anti-HIV-1, anti-HIV-2 and anti-SIV activity.(Boyd, 1997) CVN is an 11-kD protein, which is produced by the cyanobacterium, *Nostoc ellipsosporum*. This protein has been shown to be resistant to physico-chemical degradation, multiple freeze-thaw cycles, heat and can withstand treatment with denaturants, detergents and some organic solvents. (Bewly, 1998; Boyd, 1997) E*x vivo* experiments utilizing human cervical tissue and *in vivo* experiments utilizing adult female cynomolgus macaques (*Macaca fasciularis*) showed treatment with low doses of CVN in a gel formulation prevented infection from HIV-1 and SIV, respectively, when treatments occurred up to an hour before inoculation. (Tsai, 2004) Another recent study has shown that

combining CVN with the peptide 12p1, which was isolated from a phage display library, has a synergistic effect increasing the anti-HIV-1 activity of both compounds allowing lower dosages to be used without decreasing efficacy.(McFadden, 2007) The antiviral activity of CVN is due to the irreversible multivalent interactions between CVN and high mannose residues on the HIV-1 envelope glycoprotein, gp120.(Tsai, 2004; Botos, 2002; Shenoy, 2002) It is these molecular interactions that prevent fusion of the virus particle with the target cells and make CVN an ideal candidate for investigation as a potential microbicide.

One approach for delivering microbicides is to use probiotics, specifically recombinant normal flora in the vaginal environment. According to the WHO probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host".(FAO/WHO, 2002) In 1907 the Russian scientist, Elie Metchnikoff, hypothesized that replacing or diminishing the number of putrefactive bacteria in the gut with lactic acid bacteria could normalize bowel health and prolong life.(Anukam, 2007) Recently, a new probiotic product, Activa®, has been introduced into the marketplace by Dannon yogurt. Activa® is a lowfat yogurt containing large quantities of a living unique bifidobacterium culture, *Bifidus regularia*™. Dannon proclaims that it is this unique bifidobacterium which slows intestinal transit of food thereby optimizing the function of the gastrointestinal tract. (Dannon, 2008) For many years gynecologists have advised women to eat yogurt for its beneficial nutritional and probiotic properties. Daily ingestion of yogurt containing live lactobacilli has been shown to decrease vulvovaginal *Candida* infections by three fold.(Hilton, 1992)

The predominate normal flora found in the vagina is lactobacilli. These bacteria are gram-positive rods, primarily facultative anaerobes that generally have a fastidious growth environment. They prefer an acidic environment and help create one by producing lactic and other acids.(Reid 2001) Byproducts produced from lactobacilli metabolism have antagonistic effects against urinary and vaginal pathogens. These byproducts consist of biosurfactants that inhibit adhesion; the acids, bacteriocins and hydrogen peroxide inhibit growth; the coaggregation molecules block the spread of pathogens. (Reid, 2001)

Recent studies have shown that exogenously applied lactobacilli or indigenous lactobacilli can reduce the risk of urinary tract infections, where lactobacilli depletion is associated with bacterial vaginosis and yeast vaginosis.(Reid, 2001; Reid, 1995; Raz, 1993; Gupta, 1998) Ingested lactobacilli, specifically *L. rhamnosus* GR-1 and *L. reuteri* RC-14, have been shown to influence the vaginal environment by being delivered to the vagina following oral ingestion via the natural passage through the intestine and along the perineal and vulval skin.(Reid and Bruce 2001; Anukam 2007) An imbalance in or a depletion of normal flora in the vaginal environment has been associated with an increased risk of acquiring opportunistic infections, including HIV-1. (Chang 2003; Taha 1998) Recently, recombinant lactobacilli as a delivery method for microbicidal molecules and compounds has attracted a lot of attention and may provide a unique opportunity to prevent the transmission of HIV-1. The first recombinant lactobacilli tested was a recombinant *L. jensenii* Xna, which secreted CVN and a two domain soluble CD4 (2D CD4) molecule.(Chang, 2003) *In vitro*

testing of this lactobacilli showed a small decrease in HIV-1 binding.(Chang, 2003) As seen in Figure 23, our collaborator has combined two different expression cassettes, one for intracellular expression and a second for maximum secretion of CVN by lactobacilli.(Pusch, 2006) Subsequent to the design of the CVN construct our

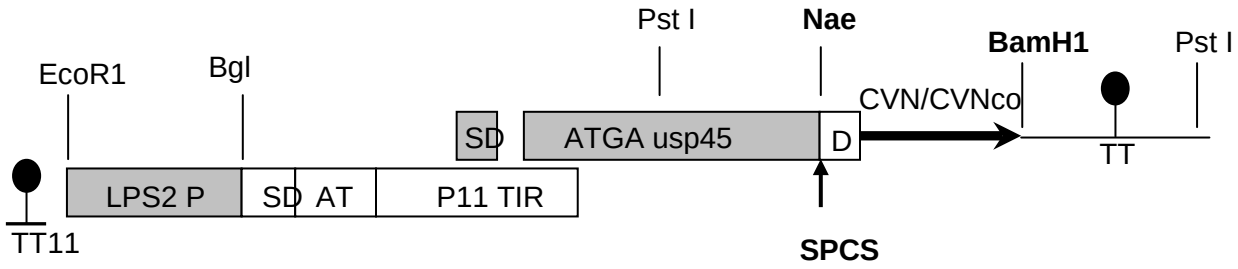


Figure 23: Cyanovirin (CVN) construct transformed into *L. plantarum*.

Expression cassettes for intracellular expression (pTSV11-CVN) and secretion into the medium (pTSV2-CVN) were combined to create the above CVN construct. Restriction sites used for cloning are in bold. ATGA Usp45 leader indicates gene fusions from the leader sequence of the lactococcal secreted protein Usp45, followed by its original signal peptidase cleage site DTNSD (D) for enhanced scretion (pTSV2-D-CVN). Vertical black arrows indicate the signal peptidase cleavage site (SPCS), followed by the propeptide sequence DTNSD (pTSV2-D-CVN/pTSV2-D-CVNco). CVNco indicates codon optimization of CVN for expression in different recombinant lactobacilli. TT1 and TT2 represent the transcription terminators; LPS2 P is the bacteriophage promotor; SD, Shine-Dalgarno motif; ATG and ATGA (start/stop) are the translation initiation start codons; P11 TIR, translation initiation region from L. lactis promotor 11. (Pusch, 2005)

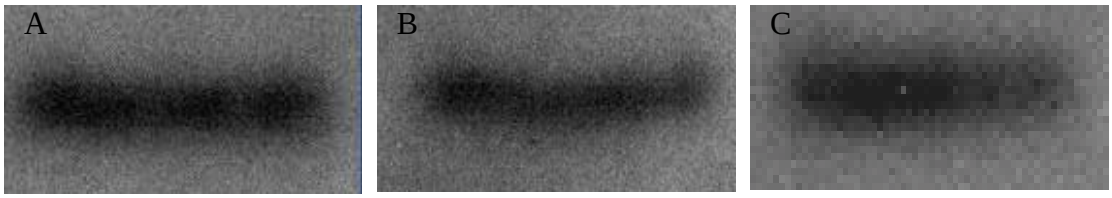


Figure 24: Western Blot detection of CVN secretion from different strains of lactobacilli.

One gastrointestinal (GI) strain, *L. plantarum*, and two vaginal strains, *L. jensenii* and *L. gasseri* were transformed with the CVN construct from Figure 1. Western Blot analysis shows that both *L. plantarum* (A) and *L. gasseri* (C) secreted more CVN as compared to *L. jenseii* (B). However, since *L. gasseri* does not produce hydrogen peroxide we chose *L. plantarum* as the first lactobacilli delivery system to test. Attempts were made to transform another strain, *L. crispatus*, but this strain could not be transformed with our CVN construct.(Unpublished results by Dr. Bharat Ramratnam, Brown University)

collaborator transformed *L. plantarum, L. jensenii* and *L. gasseri* that all secrete CVN as detected by Western Blot analysis, as shown in Figure 24.(Ming and Ramratnam, unpublished data) In this report we describe a system for utilizing one of the recombinant lactobacilli, *L. plantarum,* to synthesize and secrete an anti-HIV-1 compound reconstituted on human vaginal tissue and human vaginal xenografts.

4.2 Material & Methods:

4.2.1 Determination of generation time and growth rates of recombinant and wild type lactobacilli.

The recombinant lactobacilli that secretes cyanovirin, *L. plantarum* CVN+, utilized was designed and supplied by Dr. Bharat Ramratnam at Brown University (Providence, Rhode Island) and was always grown in Man, Rogosa and Sharpe (MRS) media or agar (Difco) with erythromycin (10 ug/ml) added for selection of recombinant bacteria. The wild type lactobacilli, *L. plantarum*, was purchased from American Type Culture Collection (Manassas, VA) and was always grown in MRS media. To determine the growth rate of each strain of lactobacilli MRS was inoculated from glycerol stocks and the optical density was measured on a DU 800 spectrophotometer (Beckman Coulter Fullerton, CA) at specified time points. To determine generation times either wild type or recombinant *L. plantarum* was grown overnight in MRS media and then diluted to $1x10^{-4}$, $1x10^{-5}$ or $1x10^{-6}$ in MRS media. After which each dilution was plated on MRS agar for WT *L. plantarum* or MRS agar

with erythromycin (10 ug/ml) added for selection of recombinant *L. plantarum* and incubated at 37º C until colonies appeared (approximately 7.5 hours). Following incubation the colonies on each plate were counted and the total number of bacteria was counted utilizing a hemocytometer, to obtain generation times and colony forming units (CFU). Calculation of generation time was determined using the following formula:

$$n = \frac{\log(N) - \log(N_0)}{\log 2} \qquad \text{and to get hours} \qquad t/n$$

Where N is the number of cells after growth and N_0 is the initial number of cells. Once n is determined then divide t, which is the time in between N_0 and N by n to get the hours.

4.2.2 Reconstitution of lactobacilli on human vaginal tissue in OTC:

The organ tissue culture model system (Gupta, 2002) using human vaginal tissue was utilized to determine if the lactobacilli would grow and adhere to normal vaginal tissue. Briefly, vaginal tissue obtained from women undergoing reconstructive surgeries for non-cancerous reasons were collected immediately prior to performing the experiment. Only grossly normal samples released by surgeon were used. All samples were from tissues that would normally be discarded. Tissue from a single donor was divided into 6 contiguous 6.0 mm circular pieces by an Acu-punch® biopsy scalpel. The cut tissue sections were washed three times in serum-free and antibiotic-free DMEM (Mediatech, Herndon, VA). The circular pieces of tissue with the epithelial layer oriented on top, were placed into the top chamber of 12-well Transwell

dish. A sterile 5% solution of agarose in Hank's medium was added to the area surrounding the tissue and allowed to solidify creating a tight seal around the tissue. Antibiotic free DMEM supplemented with 10% fetal bovine serum (1 ml) was added to the bottom chamber to keep the Transwell membrane in contact with the medium and feed the tissue from underneath. Also, 500 µl of MRS media containing either recombinant *L. plantarum* CVN+, wild type *L. plantarum* or *L. lactis* grown to an O.D. of 1.8 was added to the top chamber and incubated in a humidified chamber at 37º for 24, 48 or 72 hours. At each time point 300 µl of MRS media was removed from the top chamber and 300 µl of fresh media was added, tested for a decrease in pH and cyanovirin secretion as well as Gram stained.

4.2.3 Detection of lactobacilli from bacteria cultures and on human vaginal tissue by Gram stain.

Lactobacilli were grown on normal human vaginal tissue using the organ tissue culture system described above or as bacterial cultures. At the specified time points (24, 48 or 72 hours) lactobacilli was collected from each inoculated tissue and Gram stains were performed to detect the bacteria. Each bacterial smear was heat fixed, incubated for 20 seconds with crystal violet and washed with distilled water. After which Gram's iodine solution was applied to each smear, incubated for 1 minute and rinsed with 95% ethanol to decolorize. To stop the reaction of the alcohol each smear was washed with distilled water for 5 seconds and incubated with safranin for 20 seconds followed by washing with distilled water. All smears were examined using an Olympus IX-81 microscope and all magnifications are shown are 60X.

4.2.4. Detection of cyanvirin by indirect antibody ELISA.

The indirect ELISA was performed using the Protein Detector ELISA HRP Microwell Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, a 96 well plate was coated with 100 µl of either supernatant from inoculated cultures or vaginal xenografts and incubated for one hour at room temperature. Following incubation the plate was emptied and 300 µl of the bovine serum albumin/blocking solution was added to each well, incubated at room temperature for 15 minutes after which the plate was emptied and washed four times with wash solution supplied in the kit. Subsequently, 100 µl of secondary antibody was applied to each well, incubated for one hour at room temperature, emptied and wash five times. Followed by 100 µl of substrate solution being added to each well, after development of color 100 µl of stop solution was added to each well and the plate was read on a plate reader at 405 nm.

4.2.4 Western Blot Analysis of CVN secretion from bacteria cultures.

Experiments were performed as previously described.(Pusch, 2005) Briefly, bacterial cultures were grown to an optical density of 1.8, centrifuged at 1000 RPMs for 10 minutes at 4ºC. Culture supernatant was removed, filtered through a 0.45 µm syringe filter and subjected to a Lowry protein assay to determine total protein levels. Proteins (50 ug) were then resolved on a 15% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred onto a Hybond polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA). The membrane was probed with a rabbit polyclonal antibody (provided by Barry O'Keefe, NCI) and immunodetection was performed using the

peroxidase-based ECL Plus detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.

4.2.5 HIV-1 Cell-free Inactivation Assay.

Experiments were performed as previously described.(Urdaneta, Wigdahl et al. 2005) Briefly, one day prior to the assay, P4-R5 HeLa cells (Charneau, Mirambeau et al. 1994) were seeded into 12-well culture dishes at a concentration of $8x10^4$ cells per well and incubated overnight at 37°C with 5% CO_2. The cells used in this assay, P4-R5, are HeLa cells that have been engineered to express HIV-1 receptors (CD4, CCR5 and CXCR4) and have an integrated HIV-1 promoter fused to a B-Galactosidase (β-gal) reporter gene.(Charneau, Mirambeau et al. 1994) This allows β-gal expression to be measured when the HIV-1 promoter has been activated. On the day of the assay 5 μl of a high titer stock ($10^{7.17}$ tissue culture infective dose 50/ml) ($TCID_{50}$) of HIV-1 (strain IIIB; Advanced Biotechnologies, Inc., Columbia, MD) was treated with an equal volume (5 μl) of the MRS culture media and incubated for ten minutes at 37°C. This MRS media incubated with HIV-1 was collected from recombinant *L. plantarum* or wild type *L. plantarum* cultures grown to an O.D. of 3.0, after which 1M sodium hydroxide was added dropwise to increase the pH of the media to 7.0. To stop the reaction, 990 μl of RPMI media (supplemented with 10% FBS) was added to the virus/media mixture and 300 μl of this dilution was applied to the P4-R5 cells in triplicate. Following a two-hour incubation at 37°C with 5% CO_2, 2 ml of fresh DMEM media (supplemented with 10% FBS and with half the concentration of puromycin 250 ug/ml) was added to each well. After incubation for 46 hours at 37°C with 5% CO_2, cells were washed twice with phosphate buffered saline, lysed and

incubated for one hour at room temperature with reaction buffer supplied in Galacto-Star™ β-Galactosidse Reporter Gene Assay System (Applied Biosystems, Foster City, CA), per the manufacturer's instructions. Following the incubation β-gal expression was quantified utilizing Fluoroskan Ascent FL Luminometer (Thermo Scientific, Waltham, MA). β-gal expression was measured in relative luminescence units per second (RLU/s). A one-way ANOVA followed by Dunnett's (compare all versus control) post-test was performed using GraphPad Software to determine statistical significance.

4.2.6 Reconstitution of human vaginal xenograft model system with recombinant lactobacilli:

Six to fourteen week old female NOD/LtSz-*scid/scid* mice (Jackson Laboratory, Bar Harbor, ME) were maintained under specific pathogen-free environmental conditions, in accordance with all Drexel University's Institutional Review Board and Institutional Animal Care and Use Committee's rules and bylaws. Vaginal tissue was obtained from patients undergoing reconstructive surgeries for non-cancerous reasons. The vaginal tissue samples were cut into split-thickness grafts, ~ 2cm x 2cm x 0.5 mm in size, consisting of the vaginal epithelial layer with minimal stroma. The tissues were rolled into a tubular conformation with the epithelial layer directed inward and placed within the subcutaneous space on the backs of the NOD/SCID mice. Estrogen pellets were also implanted so that the circulating peripheral blood estrogen levels were 200 pg/ml, the average normal level in pre-menopausal women. This allowed sustained growth of the human vaginal xenografts. Grafts were allowed to heal for ~21

days after implantation, at which time the xenografts were inoculated with 10 ul of either wild type *L. plantarum* or recombinant *L. plantarum* CVN+ and incubated for 72 hours. At the 72 hour time point the xenografts were either harvested (as decribed below) or challenged with 10 ul of HIV-1 BaL (Advanced Biotechnologies, Columbia, MD) and incubated for 7 days. Following the incubation the mice were euthanized, the xenografts were removed, washed with sterile phosphate buffered saline (PBS). The resulting wash PBS was centrifuged with the supernatant to undergo indirect ELISA to detect CVN secretion and the pellet was to be used for Gram stain, as described above. The HIV-1 challenged xenografts were to undergo homogenization, total tissue RNA extraction and RT-PCR for HIV-1 Gag/Pol and Tat/Rev transcripts. However, this analysis will occur at our collaborator's laboratory at Brown University, Rhode Island due to the unfortunate passing of our laboratory's Principle Investigator. To control for donor-to-donor variations, grafted tissue was distributed as evenly as possible across all inoculation groups.

***4.3 Results*:**

4.3.1 Wild Type and recombinant *L. plantarum* grow at different rates.

To determine the growth rate of both recombinant and WT *L. plantarum* bacterial cultures were grown in MRS media with optical density measurements of the inoculated cultures being performed every thirty minutes. As shown in the growth curves in Figure 25 the WT *L. plantarum* doubled approximately every hour as compared to the recombinant *L. plantarum*, which doubled approximately every three hours. This doubling time was also confirmed by estimating the generation times utilizing the formula described in the Materials and Methods section. It was

determined that the estimated generation time for recombinant *L. plantarum* was 2.51 hours as compared to the WT which had a generation time of 1.06 hours.

4.3.2 Detection of Lactobacilli by Gram stain, subsequent pH changes and CVN secretion.

To verify the growth of the lactobacilli along with the resultant pH change and CVN secretion, WT or recombinant *L. plantarum* was grown in the MRS media for 24, 48 or 72 hours. At the specified time points 2 ml was removed from each culture subjected to Gram stain and the remaining volume was filtered. The subsequent filtered product was subjected to pH measurement, Western Blot and indirect ELISA for the detection of CVN. As shown in Figure 26A and 26B both WT and recombinant *L. plantarum* are detectable by Gram stain and Figure 26E shows that the pH of the cultures changed from 6.5 to 4.5 indicating growth and lactic acid secretion. The Western Blot analysis in Figure 27 shows that CVN was detected in all of the time points collected (24, 48 and 72 hours) for the recombinant *L. plantarum* but not the negative control *L. lactis*. To determine the minimum detection limits for CVN from bacterial cultures we utilized an indirect ELISA. Figure 28A shows the minimum detection limits was in the nanogram range for CVN concentration using the indirect ELISA method and the resulting standard curve. From the standard curve in Figure 28A we were able to determine the minimum volume required from filtered supernatant that allowed for detection of our secreted CVN, which corresponded to 0.1 to 1 µl from recombinant *L. plantarum* culture supernatant, as shown in Figure 28B.

4.3.3 Re-coloniozation of human vaginal tissue in OTC with lactobacilli.

No previous studies have been published which have grown or reconstituted recombinant *L. plantarum* on human vaginal tissue *ex vivo*. To determine that WT and recombinant *L. plantarum* could be grown on human vaginal tissue we utilized an *ex vivo* organ tissue culture system (OTC) as previously described. (Gupta 2002) The vaginal tissue was placed epithelia side up in a transwell dish, surrounded by agarose, treated with either WT or recombinant *L. plantarum* cultures and incubated

Figure 25: Recombinant and WT L. plantarum grow at different rates.

Figure 26: Gram stain and pH change in vitro and ex vivo.

Detection of both recombinant (A) and WT (B) *L. plantarum* from bacteria cultures as well as recombinant (C) and WT (D) *L. plantarum* from inoculated organ tissue cultures by Gram stain at 24, 48 and 72 hours after inoculation. In all samples a pH change from 6.5 to 4.5 was detected after 48 hours post-inoculation. Results of all Gram stains were similar and pictures are representative all experiments. Pictures shown are 48 hour post-inoculation and in 60X magnification.

for either 24, 48 or 72 hours. The OTC system allows the tissue to be oriented in a manor that allows for treatment/inoculation with the bacteria. In this system the only exposed portions of the tissue are the squamous epithelia cells, which are the cells that the lactobacilli naturally adhere to in the vaginal environment. As shown in Figure 26C and 26D both the WT and recombinant *L. plantarum* was detected by Gram stain by 48 hours post-inoculation with a subsequent change in pH from 6.5 to 4.5 as seen in Figure 26E. Results were similar after 72 hours post-inoculation (data not shown). There was no change in pH detected in the recombinant *L. plantarum* OTC cultures after 24 hours but low quantities of bacterial were detected by Gram stain (data not shown).

4.3.4 Recombinant *L. plantarum* secreted cyanovirin decrease cell-free HIV-1 infectivity *in vitro*.

To verify the cyanovirin secreted from the recombinant *L. plantarum* retained its anti-HIV-1 activity we tested filtered bacterial culture supernatant against cell-free HIV-1. Recombinant or WT *L. plantarum* was grown in MRS media until the optical density reached approximately 3.0, after which the cultures were centrifuged and the supernatants were collected, filtered and the pH adjusted to 7.0 to ensure the pH would not be a contributing factor in decreasing HIV-1 infectivity. Three separate experiments with triplicate samples were performed and in each experiment a statistically significant ($p<0.01$) decrease in anti-HIV-1 activity was observed in the recombinant *L. plantarum* grown for 48 hours (O.D. 3.0) relative to uninfected control, as shown in Figure 29.

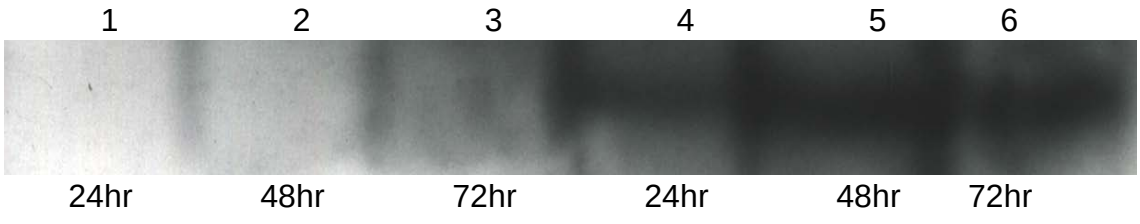


Figure 27: Cyanovirin secreted from recombinant *L. plantarum* as detected by Western Blot.

WT *L. lactis* (lanes 1, 2, 3) and recombinant *L. plantarum* (4, 5, 6) were grown in Man, Rogosa and Sharpe media at 37º for 24, 48 and 72 hours. At each time point 3 ml of media was removed from inoculated cultures, centrifuged, filtered and subject to Western Blot. Cyanovirin was detected in all of the CVN positive *L. plantarum* lanes while the *L. lactis* are negative for cyanovirin. Results shown are for the 48 hour collection time point.

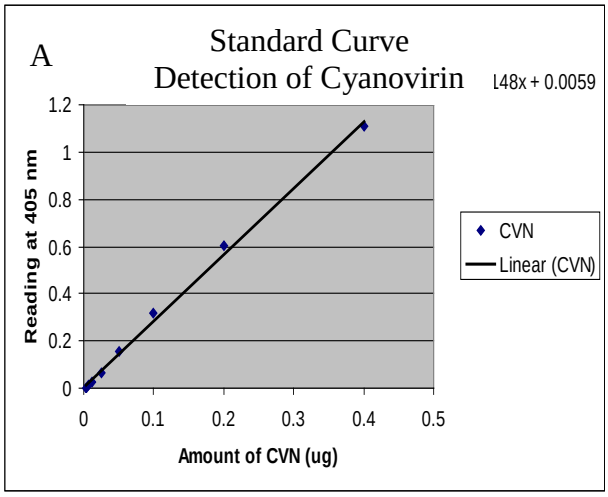
4.3.5 Expected re-colonization of recombinant *L. plantarum in vivo* and the effect on HIV-1 infectivity.

Our previous *ex vivo* experiments have shown that both recombinant and WT *L.plantarum* can be grown on human vaginal tissue and subsequently detected, but we did not know if both bacteria could be grown and re-colonize in the lumen of our human vaginal xenograft model system. To determine this we inoculated the lumens of vaginal xenografts with 10 ul of either recombinant or WT *L.plantarum* from bacterial cultures that were grown for 48 hours (O.D. 3.0) and incubated for 72 hours. Following the incubation the xenografts were either harvested or challenged with HIV-1, with the HIV- 1 challenged vaginal xenografts being allowed to incubate for an additional 7 days for viral replication. These xenografts have not been analyzed due to the unfortunate passing of our laboratory's Principle Investigator, therefore this

analysis will occur at our collaborator's laboratory at Brown University. However, based on our *ex vivo* results we are expecting that both the recombinant and WT *L.plantarum* to have reconstituted the xenografts and secreted CVN.

4.4 Discussion

The bacterial species that colonize the human body are both numerous and diverse. These bacteria or normal flora inhabit the skin, the entire digestive system and urogenital tract in women. Many of these normal flora contribute to the healthy development of the immune system, prevent infection from pathogenic and opportunistic microbes as well as maintain the intestinal barrier function. Illness, HIV-1 infection, poor nutrition, use of antibiotics and other factors can affect this delicate balance and cause emergence of harmful bacterial. An imbalance in or a



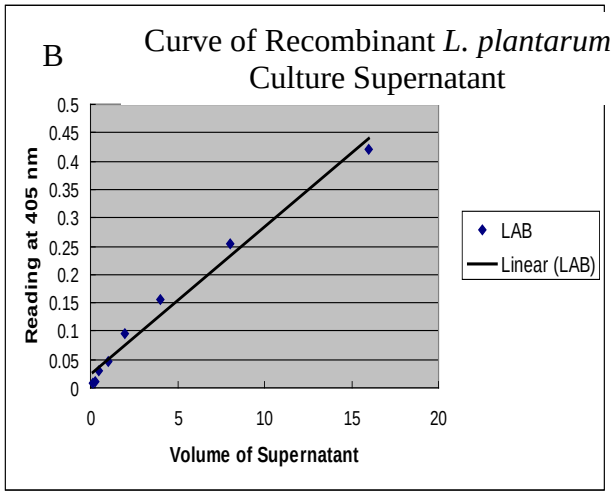


Figure 28: Minimum detection limits of CVN from recombinant L. plantarum by indirect ELISA.

WT or recombinant *L. plantarum* was grown in MRS media until the O.D. reached 1.8. At which time cultures were collected, centrifuged, filtered and subject to indirect ELISA. A standard curve was used to determine the concentration of CVN produced by the inoculated recombinant *L. plantarum* cultures. As shown in the standard curve (A) the sensitivity of the lower detection limits for the CVN concentration by indirect ELISA is in the nanogram range, which corresponds to volume of recombinant *L. plantarum* supernatant (B) of 0.1 to 1 µl.

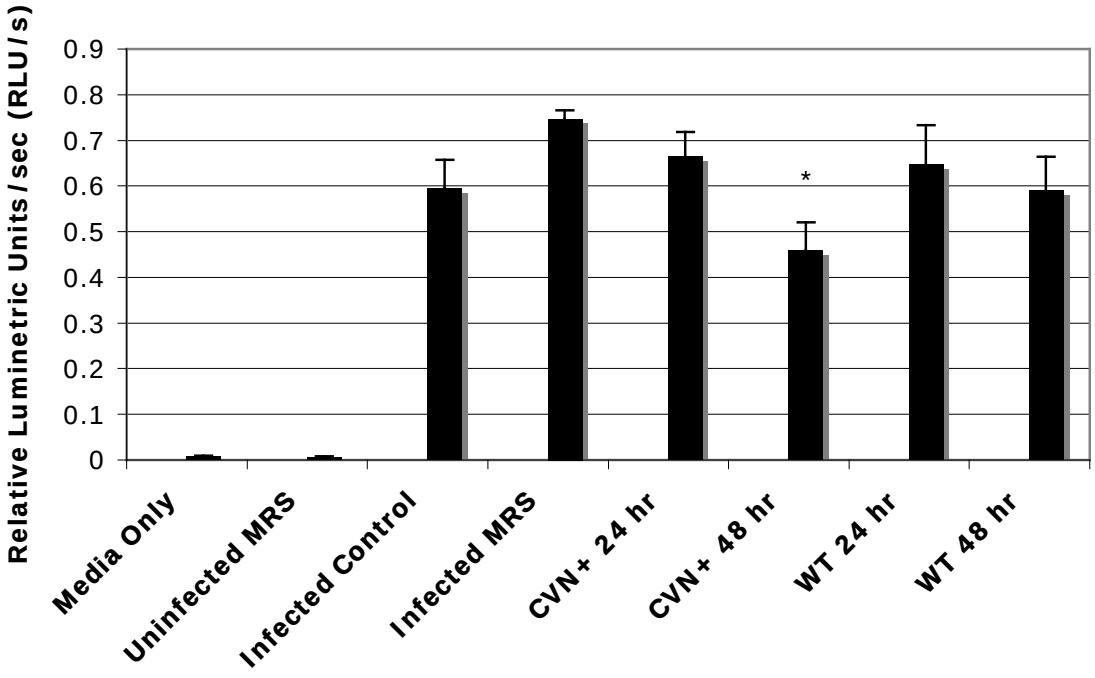


Figure 29: Secreted cyanovirin decreases cell-free HIV-1 activity *in vitro*.

HIV-1 was pre-treated with supernatant from either MRS media, WT or recombinant *L. plantarum* after the pH was adjusted to 7.0, prior to infection of P4-R5 indicator cells which express β-gal if infected. Media only and uninfected MRS groups show baseline expression of β-gal; infected control and infected MRS act as viral infectivity controls; CVN+ 24 hr and 48 hr represent the recombinant *L. plantarum* supernatant grown for 24 or 48 hours, respectively; and WT 24 hr and 48 hr represent the wild type *L. plantarum* supernatant grown for 24 or 48 hours respectively. Results are representative of 3 different experiments with n=3/group with similar outcomes. *=$p<0.01$

depletion of normal flora in the vaginal environment has been associated with an increased risk of acquiring opportunistic infections, including HIV-1. (Chang, 2003; Taha, 1998) It has been shown that the daily ingestion of yogurt containing live lactobacilli has been shown to decrease vulvovaginal *Candida* infections by three fold.(Hilton, 1992) The normal flora found in the vagina environment is lactobacilli. These bacteria prefer an acidic environment and help create one by producing lactic and other acids.(Reid 2001) Byproducts produced from lactobacilli metabolism have antagonistic effects against urinary and vaginal pathogens.

There are many challenges that confront the development of an effective HIV-1 microbicide. The microbicide must be able to target and neutralize a genetically diverse population of viruses. Mucosal administration of the microbicide must not cause adverse effects such as immune cell infilitration in the host or sexual partner and

must be able to maintain sufficient muscosal concentrations of active compound over extended periods of time. Some microbicides that require application immediately prior to coital acts may lower the frequency of compliance and complicate evaluations of efficiacy. Unfortunately, in many societies cultural norms prevent women from taking the necessary steps to apply a vaginal microbicide immediately before intercourse. There must be alternatives to topical microbicides because even the most efficacious topical microbicide may be of limited value if it can not easily be used. Thus, a vital need exists to develop efficient systems that allow for the delivery of microbicides to mucosal surfaces over extended periods of time.

One approach for delivering microbicides with the capability of synthesizing an antiviral compound is to use probiotics, specifically recombinant normal flora in the vaginal environment. A recent study tested a recombinant lactobacilli, *L. jensenii* Xna, which secreted CVN and a two domain soluble CD4 (2D CD4) molecule.(Chang, 2003) *In vitro* testing of this lactobacilli showed a small decrease in HIV-1 binding.(Chang, 2003) We have also decided to test a recombinant lactobacilli, *L. plantarum*, that secretes CVN, which was developed by our collaborator, Dr. Bharat Ramratnam at Brown University. CVN has been shown to have effective antiviral (HIV-1, HIV-2 and SIV) activity.(Boyd, 1997)

To determine the feasibility of using recombinant lactobacilli, *L. plantarum,* as a microbicide we measured the growth rates of both WT and recombinant *L. plantarum* and measured CVN secretion. This was necessary to evaluate if the recombinant *L. plantarum* would grow and compete for resources with the normal flora in the vaginal environment. As shown in the growth curves in Figure 25 the WT

L. plantarum grew at a much quicker rate doubling approximately every hour as compared to the recombinant *L. plantarum*, which grew at a much slower rate doubling approximately every three hours. The recombinant *L. plantarum* would not be able to compete for food and resources with the WT lactobacilli already colonizing the vagina. Growth curves need to be performed on the other recombinant lactobacilli, *L. jensenii* or *L. gasseri*, which may grow at quicker rates that are closer to their comparable WT strains and therefore would be more likely to be able to compete for resources.

To verify the recombinant *L. plantarum* was secreting CVN, WT or recombinant *L. plantarum* was grown in the MRS media for 24, 48 or 72 hours. The supernatant was then subjected to Western Blot and indirect ELISA for the detection of CVN. The Western Blot analysis in Figure 27 shows that CVN was detected in all of the time points collected (24, 48 and 72 hours) for the recombinant *L. plantarum* but not the negative control. The detection of CVN from very small volume of supernatant was very important because when utilizing the human vaginal xenograft model system the largest instilling volume is 10 ul maximum. To determine the minimum detection limits for CVN from bacterial cultures we utilized an indirect ELISA. Figure 28A shows the minimum detection limits was in the nanogram range for CVN concentration using the indirect ELISA method and the resulting standard curve. From the standard curve in Figure 28A we were able to determine the minimum volume required from filtered supernatant that allowed for detection of our secreted CVN, which corresponded to 0.1 to 1 µl from recombinant *L. plantarum*

culture supernatant, as shown in Figure 28B. These results indicated that once the vaginal xenografts were recolonized with the recombinant *L. plantarum*, we could detect CVN secretion and determine its effect on HIV-1 infectivity.

The recombinant *L. plantarum* CVN microbicide would protentially provide benefits to a women that is infected with HIV-1 and is being treated with highly active antiretroviral therapy (HAART). It is known that HAART causes imbalances in the normal flora of people who are required to take it for survival and specifically many times women have recurrent vaginal yeast infections. A recombinant *L. plantarum* microbicide, which secretes CVN, given regularly to a HIV-1 infected women to recolonize the vaginal environment could decrease vaginal yeast infections but also decrease cell-free HIV-1 in vaginal secretions.

Before beginning the *in vivo* testing, the anti-HIV-1 activity of the CVN secreted from the recombinant *L. plantarum* was assessed. Filtered recombinant or WT *L. plantarum* culture supernatant with the pH adjusted to 7.0 was tested against cell-free HIV-1. The pH had to be adjusted to ensure the pH would not be a contributing factor in decreasing HIV-1 infectivity. Three separate experiments with triplicate samples were performed and in each experiment a statistically significant ($p<0.01$) decrease in anti-HIV-1 activity was observed in the recombinant *L. plantarum* grown for 48 hours (O.D. 3.0) relative to uninfected control, as shown in Figure 29. These results show the CVN secreted from the recombinant *L. plantarum* retained its anti-HIV-1 activity *in vitro.*

To date there are no published reports of recolonizing human vaginal tissue *ex vivo* or *in vivo* with recombinant lactobacilli. To determine if the WT and

recombinant *L. plantarum* could be grown on human vaginal tissue we utilized an *ex vivo* organ tissue culture system (OTC) as previously described. (Gupta 2002) Vaginal tissue treated with either WT or recombinant *L. plantarum* cultures and incubated for either 24, 48 or 72 hours. This system allows the lactobacilli to only be exposed to the squamous epithelial cells, which the lactobacilli naturally adhere to in the vaginal environment. As seen in Figure 26C and 26D both the WT and recombinant *L. plantarum* was detected by Gram stain by 48 hours post-inoculation with a subsequent change in pH from 6.5 to 4.5 as seen in Figure 26E. Results were similar after 72 post-inoculation (data not shown). There was no change in pH detected in the recombinant *L. plantarum* OTC cultures after 24 hours but low quantities of bacterial were detected by Gram stain (data not shown). The change in pH detected indicates the bacteria were growing on the human tissue and secreting lactic acid into the surrounding environment similar to the vaginal environment.

Our data have shown that we were able to grow and detect the recombinant lactobacilli, the secreted CVN retatined its anti-HIV-1 activity *in vitro* and recolonize human vaginal tissue *ex vivo*. Also, we have shown in Figure 18 (chapter 3), that we were able to successfully infect human vaginal xenografts without the need of reconstituting the xenografts with human peripheral blood mononuclear cells, as was required previously for this HIV-1 xenograft model system. Subsequently, to determine if the lactobacilli would colonize the lumen of the vaginal xenografts the xenografts were inoculated with recombinant lactobacilli and upon harvesting were washed with PBS and subjected to Gram stain. Afterward the wash was sent to Brown

University for detection of CVN by indirect ELISA, a Gram stain was also performed but only a small population of lactobacilli was detected (data not shown) but more xenografts need to inoculated to ensure the lactobacilli detected was not residual bacteria from the initial inoculum. To determine if the recombinant lactobacilli would decrease HIV-1 infectivity we inoculated the lumen of vaginal xenografts and challenged with HIV-1. Upon harvesting, the vaginal xenografts were sent to Brown University for analysis due to the closing of Dr. Mary K. Howett's laboratory at Drexel University.

A study determining the kinetics of vaginal and oral delivery of the drug misoprostol, which is used to prevent stomach ulcers and for inducing labor, demonstrated that vaginal delivery provided a superior delivery method as compared to the oral administration for the induction of labor.(Zieman 1997) Another study demonstrated that vaginally administered indomethacin, which is used to treat many different types of arthritis and to stop preterm births, was more effect than oral indomethacin for halting preterm labor.(Abramov 2000) Vaginal drug delivery has been routinely used by clinicans to deliver bromocriptine to treat high prolactin levels in women suffering from nausea and vomiting making treatment with an oral medication difficult. These studies combined with the FITC-siRNA absorption data from chapter 2 (Figures 15 and 16) in this thesis demonstrate that vaginal tissue can absorb medications and small molecules. Therefore it is possible for the secreted CVN from the recombinant lactobacilli to be absorbed by the tissue in the vaginal environment and may travel to the lymph nodes providing another avenue to irrevesibly bind HIV-1.

Vaginal normal flora have been shown to effect HIV-1 viral load within vaginal secretions of HIV-1 positive women.(Hitti 2008) In an observation cohert study that collected cervico-vaginal lavages from HIV-1 positive women showed that lactobacilli, which secreted hydrogen peroxide, had an effect on HIV-1 viral load in vaginal secretions.(Hitti 2008) The women whose vaginal normal flora changed from bacterial species that cause bacterial vaginosis(BV) to lactobacilli experienced a five fold decrease in HIV-1 viral load; and the women whose normal flora changed from lactobacilli to the bacterial species that cause BV experienced a three fold increase in HIV-1 viral load.(Hitti 2008) A change in vaginal normal flora women has also been associated with bacterial vaginosis and an increased risk of HIV-1 infection.(Chang 2003; Taha 1998)

Previous work that has been performed in our laboratory has shown that the human vaginal xenografts do not retain the normal flora from the patient (Welsh and Howett, unpublished results), thus, making the xenograft system an ideal model system to evalute the recombinant *L. plantarum* as an anti-HIV-1 microbicide. These experiments provide (and will provide) proof-of-principle in determining if the amount of cyanovirin produced and secreted can sufficiently decrease HIV-1 activity *in vivo* to reduce its transmission. Consequently, this will also help in determining if recombinant probiotics can be utilized as a microbicide. Future experiments to be performed using the vaginal xenograft model system will include testing of other recombinant lactobacilli, which secrete CVN; combining these recombinant and WT bacteria in the initial inoculum to detemine if the recombin ant lactobacilli can

compete with the WT in an environment completely devoid of bacteria; creating different recombinant lactobacilli that can secrete other microbicidal compounds or peptides (e.g. 5P12-RANTES, fucans or 12p1 peptide); and combining recombinant lactobacilli that secrete different anti-HIV-1 compounds as a way of delivering and testing combination microbicides with different modes of action. Live microbial microbicide delivery strategies, as outlined in this thesis, potentially have many advantages over gel delivered topical microbicides, such as, deliverying long lasting protection due to bacterial colonization decreasing the need for immediate precoital application and low cost to manufacturer once normal flora bacteria has been transformed.

5. Overall Discussion and future directions

The sexually transmitted disease epidemic has been compared to an iceberg, what is seen is just a minuscule portion of a much larger problem, most of which is unseen. (CA policy Review 2007) At the end of December 2007, worldwide 34 million people were infected with human immunodeficiency virus type 1 (HIV-1) (UNAIDS 2007), approximately 20 million people were infected with human papillomavirus (HPV) (CDC 2007) and at least 45 million people, in the United States alone, are infected with herpes simplex virus type 2 (HSV-2).(CDC 2006) There are no cures for these persistent and recurrent infections just pharmacological maintenance regimes or surgical treatments. If diagnosis occurs early in infection, management of the subsequent chronic disease can be the only option. These diseases can potentially cause major physical and psychological limitations in the daily lives of people who are infected. In our society, chronic diseases are prevalent and among the

most costly health problems. Many chronic diseases are considered preventable.(insert ref) The cost of preventing these diseases is significantly less than post-infection treatment. The Center for Disease Control (CDC) has estimated that the cost of prevention (Safer Choice Program, school based STI and pregnancy program) is 37% of the cost of treatment.(CDC, 2008) A recent government report estimated the numbers of newly acquired STIs in teenagers in California for 2005 was 1.12 million with a resulting combined cost of treatment of $1.11 billion. (CA Policy Rev,2007) The cost estimate included lifetime medical costs based on incidence rates, undiagnosed and untreated disease and treatment costs. This estimate does not include indirect costs, such as lost wages due to time off of work or productivity. (CA Policy Rev, 2007) In the United States direct medical costs for treating STIs totals $15.3 billion annually. Sixty congressman co-sponsored a resolution to Congress requesting all levels of government to provide additional funding for screening and increased programming for education focused on the risks and the prevention of sexually transmitted diseases.(Congresswomen Jones, Ohio)

Although the impact to families has been highly publicized, the impact of sexually transmitted diseases to our society and the world population is often overlooked. These diseases are the root of several of our society's biggest problems, infertility, cervical cancer and AIDS. Several strategies must be employed simultaneously at both the population and individual levels to combat and overcome these diseases. Microbicides, vaccines, condom use, education and raising the socio-economic status of women and the poor all need to be addressed before we can defeat these diseases.

The development of an effective anti-HIV-1 vaccine has proven to be a difficult task. The traditional methods for producing vaccines, using attenuated virus, is too dangerous with HIV-1 because some virions may retain or regain adequate replication potential to produce new infectious virions.(Robinson 2007; Whitney 004) Another setback encountered in the anti-HIV-1 vaccine program was antibodies that were produced which targeted viral envelope protein gp120, a protein known to have highly variable sequences and high levels of glycosylation.(Robinson, 2007; Richmann 2003; Wei, 1995) Once antibodies bound to the specific gp120 molecules rapid selection would occur on these variable sequences causing escape mutants, plus these antibodies were ineffective on other HIV-1 quasispecies found in the HIV-1 population. An alternate approach taken by vaccine researchers is to elicit a effector T-cell response utilizing several different methods, such as: using recombinant viral vectors to delivery viral proteins; using DNA vaccines to delivery viral proteins; delivering adjuvanted proteins and peptides; or combining two or more modalities for antigen delivery in heterologous prime/boost regimens.(Robinson 2007) The problem with many of the above approaches is effective delivery of DNA, protein or peptide to the appropriate antigen presenting cells without activating a memory immune response that could be due to a previous exposure to the delivery method. The proliferation of HIV-1 infections combined with the lack of an anti-HIV-1 vaccine further highlights the dire need for new preventative measures to stop transmission, like topical microbicides (for review see (Balzarini and Van Damme 2007; van de Wijgert and Shattock 2007).

Summary of Conclusions by Chapter:

Chapter 2: Formulated Sodium Dodecyl Sulfate (SDS) Hydrogel. We hypothesized that our formulated SDS Hydrogel would have potent anti-viral activity against enveloped and non-enveloped sexually transmitted viruses.

- Histological examination indicates SDS Hydrogel is non-toxic to human vaginal, cervical and foreskin tissues *ex vivo*.
- SDS Hydrogel inactivates cell-free and cell-associated HIV-1 *in vitro* at low concentrations and with rapid kinetics.
- SDS Hydrogel inactivates HSV-2 *in vitro* at low concentrations and with rapid kinetics.
- SDS Hydrogel decreases de novo HPV-11 transriptional activity *in vitro* by 90%.
- Formulations of SDS Aquadapt hydrogel appear safe for epithelia application and effective for virus inactivation.

Chapter 3: Liposomal delievered CCR5-siRNA microbicide. We hypothesized that down-regulating human chemokine receptor 5 on HIV-1 target cells (CD4+ T-cells, Langerhan cells and tissue macrophages) normally found in the vaginal environment would decrease and prevent HIV-1 binding and the subsequent infection both *in vitro* and *in vivo*.

- Liposomal delivered pooled CCR5-siRNA down regulates CCR5 *in vitro* on human P4-R5 HeLa cells.

- Fluorescent, non-specific siRNA enters the cytoplasm of human vagainl xenograft epithelium. Efficiency of liposomal delivery is greater than siRNA nanoparticle delivery.

Chapter 4: Lactobacilli the secreting anti-HIV-1 compound, cyanovirin (chapter to be inserted in revision). We hypothesized that HIV-1 transmission would be decreased by using a recombinant probiotic lactobacilli with the ability to secrets cyanovirin (CVN), an anti-HIV-1 compound, known to decrease HIV-1 binding and transmission *in vitro* and *in vivo*.

- Recombinant lactobacilli secreting CVN grow at a slower rate than wild type lactobacilli *in vitro* and *ex vivo*.
- Recombinant lactobacilli secrete cyanovirin into surrounding media *in vitro*.
- Cyanovirin secreted from recombinant lactobacilli decreased HIV-1 activity *in vitro*.
- Recombinant lactobacilli can be reconstituted on human vaginal epithelial tissue in *ex vivo* organ tissue culture system.

During the sexual revolution in the 1960's and 1970's over the counter spermicides gained enormous popularity and gave rise to the novel idea of a microbicide. These popular topical spermicides are alternatives to oral birth control systems and are available in foams, creams, films and as condom coatings.(Howett Kuhl 2005) A microbicide is defined as any compound that can be applied inside the vagina to prevent the transmission of sexually transmitted diseases. In microbicidal

research and development there is the dream of “the ideal microbicide” which must encompass all of the following characteristics: non-toxic, non-inflammatory, undetectable, broad spectrum activity against bacterial and viral sexually transmitted infections, efficacy against cell-free and cell-associated HIV-1, no effect on the structural integrity of vaginal and cervical mucosal epithelium, stability at tropical temperatures, resistant to acidic pH and allow for the retention or reconstitution of lactobacilli. (Balzarini 2007; Howett & Kuhl 2005) To make it attractive for use, it must be odorless, colorless, tasteless, low cost and readily accessible.(Balzarini and Van Damme 2007) In addition to the difficult standards set by the legend of the “ideal microbicide”, as explained above, there are also the practical requirements set by the buying public that must also be taken into consideration when developing a new microbicide. It ultimately depends on the willingness of women to use a specific microbicide. In practical use, if women find the formulation unacceptable and avoid using the microbicide, then the best microbicide will be no more effective than a placebo. This scenario leaves no chance to prevent the transmission of these incurable viruses that are continually plaguing our society. For a microbicide to be successful it must also be made accessible to the people who need it the most, the poorest in our society and in the developing world. Once a compound has successfully completed clinical trials and enters the marketplace, it must first be given to community liaisons, such as, doctors and tribal leaders, to ensure the microbicide is properly presented in a culturally acceptable manner in the societies where it is introduced. It is very probable that there will not be a microbicide “silver bullet”, in other words one microbicide may not be universally acceptable for all individuals in all situations. Hopefully,

microbicidal research will produce several different microbicides with different modes of action that can be used under different circumstances. For a microbicide to empower women, scientists must take into account diverse cultural sensibilities around the world when developing a microbicide. Once a successful microbicide is approved by the FDA for public use it is the end of the beginning, companies and universities must continue to develop new applications (e.g. vaginal films or rings) for entry into clinical trials.

A clinical trial is a research study on human volunteers to answer specific health questions with the results being submitted to the FDA in a new drug application.(FDA 2008) The experimental compound is administered to human subjects where safety and efficacy is determined. The different classifications of clinical trials are treatment, prevention, screening and quality of life trials. Microbicide clinical trials are classified as prevention clinical trials because they examine ways to prevent diseases using medicines, vitamins, vaccines, prophylaxis or lifestyle changes.(FDA 2008) Traditionally, there are three phases to clinical trials but the FDA has fast tracked HIV-1 microbicides because of the urgent need and the clinical development challenges that a candidate microbicide must endure. The two main challenges a candidate microbicide must deal with in clinical trials are the low seroconversion rate and condom counseling. (FDA Memoramdum 2003) Ethical conduct necessitates safe sex and condom counseling combined with low seroconversion rates (the measurable endpoint of efficacy) both contribute to the very

large sample size (several thousand) required to provide adequate power when determining if the effect of the microbicide is statistically significant.

The first candidate microbicide to reach Phase III clinical trials was the spermicidal non-ionic surfactant nonoxynol-9 (N-9), which inactivated HIV-1 *in vitro* by disrupting the outer membrane but failed to prevent sexual transmission *in vivo*.(Van Damme, Ramjee et al. 2002; Davis and Doms 2004) In murine studies designed to evaluate the toxicity of N-9, cervical epithelial disruption and immune cell infiltration was observed by two hours post-treatment.(Catalone, Kish et al. 2004) Indicating that N-9 drastically increases inflammation providing additional lymphocyte targets for HIV-1.(Catalone, Kish et al. 2004) Women using N-9 were shown to have higher HIV-1 infection rates, almost certainly because N-9 disrupted the epithelial cell membranes in the genital tract that serve as a protective barrier against viral and bacterial infection and increased inflammation.(Van Damme, Ramjee et al. 2002; Van De Wijgert and Coggins 2002; Davis and Doms 2004) Exploratory analysis showed that the treatment group had an increase in genital tract lesions with an epithelial breach, which was linked to a higher incidence of HIV-1 seroconversion.(Balzarini 2007)

Another candidate microbicide to reach Phase III clinical trials was C31G. This compound is also a surfactant that is comprised of an equimolar mixture of two surface-active amphoteric agents, buffered with citric acid.(Balzini) Twenty three months into phase III clinical trials an independent monitoring board recommended halting the trial because too few women were becoming infected with HIV to provide the statistical power needed to show a difference between C31G and the placebo.

(Mascolini 2007; Peterson, Nanda et al. 2007) A separate clinical study has since shown that many patients were hesitant to use C31G because of the associated burning sensations.(Balzini and Wyrick, 2002) C31G is no longer being developed as a vaginal microbicide (van de Wijgert and Shattock 2007) but continues to be tested as a spermicide.

The development of the Ushercell microbicide, a cellulose sulfate polyanionic compound which blocked viral entry into cells, has also been halted in phase III clinical trials. CONRAD and Polydex Pharmaceuticals were developing this compound. Interim analysis of 1300 women in Benin, South Africa, Uganda and India indicated higher HIV-1 infection rates in women using the cellulose sulfate compound as compared to placebo.(Honey 2007) Based on these results, an independent data monitoring committee decided to stop the study. There were more seroconversions in the Ushercell group. Of the 1,425 women enrolled in the trial, 41 seroconverted 25 of which were using Ushercell and 16 using placebo. (IAS Conf Sydney 2007; Microbicide Quarterly March 2007) An additional exploratory analysis, that excluded women because they stopped using Ushercell, revealed that there were more infections among women using Ushercell as compared to the placebo and the difference was statistically significant.(IAS Conf Sydney 2007)

The first candidate microbicide to complete phase III clinical trials, Carraguard, contains galactose-linked polysaccharides (carrageenan) from red seaweek *Gigartina skottsbergii*.(Balzini 2002) Carraguard blocks viral entry into cells and blocks the trafficking of HIV-1 infected macrophages from the vagina to the

lymph nodes.(Balzini 2002, Perotti 2003; Schaeffer 2000) The Population Counsel was developing Carraguard. Unfortunately, Carraguard failed in phase III trials because the microbicide did not prevent a statistically significant level of HIV-1 infections when compared to placebo.(2008) There were over 4,200 women who completed this study in South Africa, of which there were 134 seroconversions in the microbicide group as compared to 151 seroconversion in the placebo group. In contrast to Carraguard, our prototypic SDS Hydrogel differs because it kills sexually transmitted viruses (HIV-1, HPV and HSV-2) instead of blocking infection by physically binding to virus surfaces.

There are two schools of thought in microbicidal research and development. The first group asserts that the viruses must be killed to prevent infection and transmission. A disadvantage with this school of thought is that many of the compounds examined and tested have been strong compounds that also caused damage to the human epithelia at the concentrations required for anti-viral activity. The second group asserts that blocking the virus from binding to receptors allows the virus to be expelled from the vaginal environment before the infection and transmission occurs. A disavantage with this school of thought is that binding the virus can be reversible overtime and if the virus is not expelled from the vaginal environment the infectious virions may still have the capacity for infection. Much of microbicide research has been focused on preventing HIV-1 infection and one of the problems in the HIV-1 microbicide field is more times than not the two schools of thought are not combined. Recently, the groups are moving closer together realizing that it is more advantageous to utilize both schools of thought with many different

modes of action for developing a non-toxic and efficiacous microbicides. Combining microbicides with different modes of action to prevent HIV-1 infectivity is where the field of HIV-1 microbicide research headed.

Microbicide research is a very challenging field because of the species specificity of sexually transmitted viruses, the private nature of how they are transmitted and the social and cultural norms that surround such private acts. One of the many obstacles in this challenging field is no one knows which tissues and cells are infected first. It is not known if it is vaginal, cervical, endometrial or uterian tissue or all of the tissues. Also, it is not known which are the first cells that become infected no one knows if it is T cells, dendritic cells that bind and capture HIV-1 using mannose receptors (DC-SIGN) and transport the infectious virions to the lymph nodes where they are presented to the target cells. In addition, new receptors (gp340, syndecans, $\alpha 4\beta 7$ integrins, galactosyl ceramide) are constantly being discovered that bind HIV-1 and allow HIV-1 to remain in the vaginal environment instead of being expelled and the virion can remain infectious for an extended period of time. Another challenge in microbicide research is the vaginal environment and how HIV-1 gains access to its target cells. During coital acts tears and rips can occur in the vaginal wall, which allow sexually transmitted viruses (specifically HIV-1 and HPV) to gain access to their target cells. Also, in the vaginal enviroment there are vaginal rugae that increase the surface area of the vagina by expanding to accommodate intercourse and there are no tight junctions in vaginal and cervical tissues allowing the immune cells to move in and out of the mucosa. Based on these challenges I propose the following

model. During coital acts infected seminal fluid enters the vaginal environment (vaginal, cervical and uterian tissue all being exposed) and the infected virions can do one or more following simulanteously: bind T-cells (resulting in infection); bind DC-Sign being captured by dendritic cells are then transported back to the lymph node and presented to target cells; are trancytosed through the epithelia to target cells in stroma; bind gp340, syndecans or other glycoproteins remaining infectious until an immune target cell moves into the vaginal environment and encounters the infectious virion while trying to clear any foreign pathogens left behind following intercourse. After coital acts the vaginal rugae contract possibly trapping infected seminal fluid, which has been shown to increase HIV-1 infectivity, thereby preventing some of the infectious virions from being expelled.

The work set forth in this thesis examined three vastly different microbicides each with very different modes of action. The SDS Hydrogel has broad spectrum activity and inactivates HIV-1, HSV-2 and disrupts HPV-11 infectivity. The liposomal delivered short interfering RNA (siRNA) targeting human chemokine receptor 5 (CCR5) may potentially inhibit binding of M-tropic HIV-1 forms from binding to the co-receptor, thus, preventing subsequent infection. Lastly, the pro-biotic cyanovirin secreting lactobacilli microbicide works specifically to block infection by binding the HIV-1 glycoprotein 120 (gp120). The first of these microbicides has broad-spectrum activity against bacteria and viruses while other two microbicides are specific only for HIV-1, for example, attacking specific stages of the HIV-1 lifecycle. Although these microbicides work very differently from each other the final objective of this work always remained the same, to use current technologies

to prevent the transmission of sexually transmitted viruses.

SDS Hydrogels

Anonic surfactants, such as sodium dodecyl sulfate (SDS) and other alkyl sulfates, possess chaotropic properties in addition to their surfactancy. Detergents are amphipathic in nature, containing a polar head group at one end and long hydrophobic carbon chain at the other end. The polar group forms hydrogen bonds with water molecules, while the hydrocarbon chains aggregate via hydrophobic interactions.(Leninger 2003) SDS has previously been shown to disrupt enveloped viruses and in cell culture will dissociate membranes into their lipid and protein components.(Becker, Helenius et al. 1975; Howett, Neely et al. 1999) Importantly, the surfactant and chaotropic properties of alkyl sulfates give SDS very effective anti-microbial and anti-viral activity. As far back as 1952, SDS was combined with sodium perborate for use as a douche to treat vaginal infections. (Karnaky 1952; O'Brien and Thoms 1955) In intact mucosal tissues, surface keratinization, vaginal exudate and cervical mucus form a barrier layer not easily disrupted by SDS. Sodium dodecyl sulfate and other alkyl sulfates are Generally Recognized As Safe (GRAS) by the FDA (FDA 2006), are regularly used as a common component in topically applied consumer products for skin and mucosa (e.g. body lotions, baby wipes, toothpaste and shampoo) and as food additives (e.g. marshmallows and gelatin). Our laboratory has previously shown that at concentrations ranging from 0.01% to 1% SDS kills HIV-1, HPV, herpes simplex virus (HSV) types 1 and 2 (Howett, Neely et al. 1999; Krebs, Miller et al. 1999; Krebs, Miller et al. 2000; Krebs, Miller et al. 2002; Howett and

Kuhl 2005; Urdaneta, Wigdahl et al. 2005; Hartmann, Wigdahl et al. 2006), *Chlamydia trachomatis* (Achilles, Shete et al. 2002) *Neisseria gonorrhea* as well as Gram positive and Gram negative bacteria (Hartmann and Howett, unpublished results). As a result of our laboratories previously published reports we decided to test three different concentrations of SDS in the Hydrogel vehicle. The three different concentrations tested for anti-viral activity were 0.1%, 0.5% and 1% SDS in the Hydrogel vehicle.

To determine toxicity to issues that would be exposed to an SDS-based microbicide, human vaginal, cervical and newborn foreskin tissues were exposed *ex vivo* to either placebo, 0.1%, 0.5% or 1% SDS Hydrogel formulations and incubated for 3, 5, 16 or 24 hours. The hematoxylin and eosin staining shows that even the highest concentration of 1% SDS Hydrogel did not cause apparent disruption in the squamous epithelium of the vaginal tissue at the histologic level even after 24 hours of continued exposure. Furthermore, there was no decrease in the thickness of the squamous epithelia in the SDS Hydrogel treated groups as compared to the no treatment groups. A recent study by Ghosh and Blankschtein has shown that SDS in glyercol reduces pore number density and radius, decreasing SDS micelle penetration into the stratum cornuem and therefore decreasing the perturbation of the skin barrier as compared to SDS in an aqueous solution. (Ghosh and Blankschtein 2007) Viscous gels containing SDS may also decrease micelle penetration into the stratrum corneum, thereby, further decreasing toxicity. Another factor contributing to the formulated SDS's low toxicity is that SDS is bound to the patented Hydrogel, SA Aquadapt, which allows the SDS to retain its potent anti-viral activity while decreasing the

exposure points of intact epithelia. A very important property of the Hydrogel is its design. These gels are designed to remain adhered to mucosal surfaces, specifically vaginal epithelia in our instance, for three hours and then expelled or "sloughed off" from the vagina. Between the closing of vaginal space, extrusion of cervical mucus and vaginal cell exudates the vagina is a "self emptying" orifice. Since SDS is bound to the Hydrogel the SDS will also be expelled after the three hours further decreasing the chances for irritation and immune cell infiltration. Previous studies in our laboratory have shown that SDS in aqueous cell culture media inactivated HIV-1 in one minute. This inactivation data and adding additional lag time for application of the SDS Hyrdogels before coital acts occur, were the main contributing factors in determining the "sloughing off" time point. Our *ex vivo* histological data using human tissues show that 1% SDS Hydrogel is non-irritating at the macroscopic levels. However, further testing will be required to prove to the FDA that our prototypic 1% SDS Hydrogel is non-toxic to the potential sites of exposure, mainly cervical, vaginal and penile tissues. To support the safety of a potential microbicide and to evaluate acute, subchronic and chronic toxicity the FDA requires studies be performed in at least two animal species, with one species being a non-rodent. Several different models can easily be utilized to test for tissue toxicity. The first model that should be employed is the rabbit vaginal irritation model system (RVI). This model system is ideally suited for histological analysis that will examine tissue morphology and architecture to determine any subsequent changes in the epithelia due to treatment with the SDS Hydrogels. However, the exposed targets in RVI is columnar epithelum

not squamous epithelium. RVI studies conducted via an NIH contracted study have shown that 1% aqueous SDS scores equal to placebo buffer alone in a 10 day RVI. The work to be preformed in this RVI system would need to include at least three different doses of the SDS Hydrogels to indicate the upper, middle and lower limits for toxicity. Preferably the doses would include a higher SDS concentration (2% - 4%) that will serve to indicate the upper range were toxicity is known to occur; the prototypic 1% SDS Hydrogel active dose, per the data in this thesis; and a much lower dose (0.05% - 0.1%) SDS concentration that has been shown to have limited anti-viral activity and will serve to show the lower range. The rabbit vaginal irritation system will allow the toxicity and absorption or toxicokinetic studies to be performed simultaneously to evaluate SDS Hydrogel ADME (absorption, disposition, metabolism and excretion) profile using the same animals. At the appropriate time points, blood shall be taken and after euthanization of the rabbits additional rabbit organs (at a minimum the blood, liver, kidneys and spleen) should be harvested to determine if the SDS Hydrogels are systemically absorbed. The FDA recommends the use of the rabbit vaginal irritation model to study toxicity and irritation after application of the compound. This model system can also be used for intravaginal repeated exposure testing using the prototypic 1% SDS Hydrogel. Several studies in mouse have shown an inflammatory response after single (Catalone 2004) and repeated exposures to N-9.(Fichorova, 2004; Nirthuthisard 1991) and benzalkonium chloride (BZK) (Fichorova, 2004), a cationic detergent and component of the Protectaid™ contraceptive sponge. To ensure there is no irritation or toxicity due to repeated exposures, which will occur during Phase I clinical trials, of the SDS

Hydrogels one intravaginal application per day should be applied over the course of 10 days or longer. The SDS Hydrogels are designed to be expelled from the vagina after three hours, therefore, at the four hour time point (prior to the next application) a vaginal lavage should be performed and the resulting fluid should analyzed for an increase in cytokine activity. It is important to restate that the cellular architecture of rabbit vaginal tissue differs from human vaginal tissue. The majority of the rabbit vaginal tissue is comprised of columnar epithelia, which is more sensitive and similar to human rectal epithelia. Due to the increased sensitivity of columnar epithelia, if no disruption is detected after application of a compound it is assumed that no disruption will occur in the hardier squamous epithelia of the human vaginal tissue. A second model system that can be used is the non-human primate models that are housed and cared for by the National Center for Research Resources. There are several different non-primate model systems that are used for toxicity and efficacy testing. The three most common models are the pigtail macaque, Rhesus macaque and the cynomolgus macaque, which all require challenging with either simian immunodeficiency virus (SIV) or an SIV/HIV-1 chimera.(Kim 2006; D'Cruz, 2007; Subbarao 2007; Kish-Catalone 2007) There are several inherent problems with using non-human primates, they are very expensive to purchase and/or maintain, difficult to handle and there is chance of the infection being transmitted to the animal technician. Another problem is that in some of the colonies, specifically the pigtailed macaques, are used repeatedly for many different compounds. They therefore may have over-sensitized or damaged the cervical or vaginal epithelia. An alternative model system for testing toxicity and

absorption is the porcine model system. Several studies have shown that pigs have similar vaginal tissue architecture and permability.(Squier 2008; D-Cruz 2007)

Our laboratory has previously shown effective HSV-2 inactivation by 0.1% SDS diluted in cell culture media.(Howett, Neely et al. 1999) We performed HSV-2 plaque assays with SDS Hydrogels to determine the effectiveness of the formulated SDS Hydrogels against this enveloped virus. In two separate experiments with duplicate samples all of the SDS Hydrogel treatment concentrations showed strong anti-HSV-2 activity. Treatment concentrations as low as 0.05% to 0.5% were effective at inactivating HSV-2 *in vitro,* as was indicated by abolishing the virus' ability to generate plaques in African Green monkey kidney cell monolayers. This data is consistent with our laboratories previously published reports that establish that an aqueous SDS solution completely inactivated HSV-2 by dissolving the lipid envelope and denaturing viral proteins.(Howett, Neely et al. 1999)

An ideal microbicide must be active against both cell-free and cell-associated HIV-1, which is found in semen and vaginal secretions. We tested our formulated SDS Hydrogels against both forms. Our laboratory has previously shown that SDS diluted in cell culture media inactivates cell free HIV-1 within 1 minute.(Howett, Neely et al. 1999; Urdaneta, Wigdahl et al. 2005) To verify that our formulated SDS Hydrogels have the same efficacy against HIV-1 as SDS in cell culture media, five separate experiments with triplicate samples were performed to determine anti-viral activity. Experiments were conducted using both cell-free and cell-associated forms of HIV-1. In each experiment highly statistically significant anti-HIV-1 activity ($p<0.0001$) was observed in all concentrations of SDS Hydrogel against both infectious forms relative

to untreated HIV-1 control. HIV-1 infectivity experiments were performed using P4-R5 MAGI HeLa cells to test if the formulated SDS Hydrogels had anti-HIV-1 activity against both cell-free and cell-associated virus the 0.5% and 1% SDS Hydrogels completely inactivated cell-associated HIV-1. Furthermore, our data shows that all concentrations of the formulated SDS gels were highly active against cell free HIV with a highly statistically significant p value of 0.0001. In the cell free HIV-1 infectivity assays it was observed in all experiments that the placebo slightly increased HIV-1 infectivity. However, this pro-infectivity effect was completely ablated in the presence of even the lowest concentrations of SDS in the Hydrogel formulations. SDS has such potent anti-viral activity that at 1% and 0.5% SDS Hydrogel completely inactivated HIV-1 and at the lowest concentration (0.1%) of the SDS formulation viral infectivity decreased >95%. The modest proinfectivity of the placebo that was observed *in vitro* may not be seen *in vivo*. It may be due to the bioadhesiveness of the Hydrogel placebo or to the ability of the gel to exclude the virus therefore slightly increasing the effective concentration. Viral infectivity may be artificially increased due to pre-treating the virus with the formulated Hydrogel placebo and then applying infectious mixture to the cells. In normal vaginal tissue there is a protective coating of mucus that is not found on the cells in culture. This problem may be overcome by treating the cells with cervical mucus, thereby providing the protective layer, after which the cells could be pre-treated with a coating layer of Hydrogel placebo and subsequently adding infectious virus. The proinfectivity of the Hydrogel placebo is negliable relative to the large decrease in virus infectivity seen in the presence of SDS.

The data from both cell-free and cell-associated HIV-1 experiments combined with the data from the HSV-2 experiments prove that the SDS Hydrogels have anti-viral activity against sexually transmitted enveloped viruses. Recent studies show that semen increases HIV-1 infectivity by as much as 50 fold (Munch 2007) and aids in epithelial tissue penetration.(Maher 2005) In light of these reports, continued studies should be performed to determine if our prototypic SDS Hydrogel is as efficacious when either HIV-1 or HSV-2 is diluted in semen, vaginal secretions or a combination of both. After which *in vivo* experiments using our laboratory's human epithelial vaginal xenograft model system need to be performed to determine the effectiveness of our formulated prototypic SDS Hydrogel against HIV-1 and HSV-2. The experiments to be performed *in vivo* should also include testing using HIV-1 and HSV-2 diluted in cell culture media, semen, vaginal secretions or a combination of both.

To determine if the SDS Hydrogel formulations had activity against non-enveloped virus, HPV-11 infectivity assays were performed followed by RT- PCR using nested primers targeted towards the *de novo* viral mRNA transcript, E1^E4. This viral transcript is the most abundant transcript detected during the HPV-11 life cycle and infection. Therefore, its detection by RT-PCR would be indicative of active *de novo* viral transcription. Our results show that treatment with 0.5% or 1% SDS Hydrogels resulted in a 10 fold decrease in the levels of viral transcript, E1^E4. The initial concentration 0.1% (final concentration 0.05%) SDS Hydrogel did not decrease HPV-11 activity (data not shown). The RT-PCR results show that formulated SDS Hyrdogel decreases HPV-11 infectivity by 90% *in vitro*. This is evident by the loss of the E1^E4 band in the 10^{-5} columns in the 0.5% and 1% SDS Hydrogel experimental

groups as compared to the 10^{-6} column in the Placebo and HPV experimental control group, which show viral infectivity. Our laboratory's previous studies show that ≤1% SDS disrupts HPV and releases DNA (Kish and Howett, unpublished results). This is a unique feature of SDS that sets it apart from other candidate microbicides with a more limited scope of action. The next steps should include experiments being performed utilizing our laboratory's human vaginal xenograft model system, to determine the effectiveness of our formulated prototypic SDS Hydrogel in decreasing the ability of experimentally generated HPV-11, HPV-16 or both to form experimental papillomas. Our laboratory has recently and successfully infected human foreskin and cervical tissue *ex vivo* with an experimentally generated HPV-16.(Alquist, 2006; Lao and Howett, unpublished results) It is important to show and further strengthens our case for the phase I clinical trials, to be able to successfully show that our prototype SDS Hydrogel had the same activity *in vivo* against HPV-16, one of the high risk papillomaviruses associated with cervical cancer, as HPV-11.

The prototypic SDS Hydrogel works by killing the viruses thereby preventing the problem of infectious virus or infected seminal fluid remaining in the vaginal environment even if captured by dentritic cells or trapped in contacted vaginal rugae.

Liposomal delievered CCR5-siRNA microbicide

Chemokines or chemotactic cytokines, are small proteins that are involved in activation and trafficking of leukocytes to sites of inflammation as well as dysregulation of these processes.(Kuhmann 2008; Opperman 2004; Yoshie, 2001) These proteins are divided in two subfamilies, CC- and CXC-chemokines, depending

on the location of the first two conversed cysteines. Chemokine receptors are G protein coupled receptors (GPCR) that have seven transmembrane domains with three extracellular loops and four intracellular loops.(Lederman 2006) Chemokine receptor 5 (CCR5) is a human receptor with an extracellular amine terminal tail and an cytoplasmic carboxy terminal tail.(Opperman 2004; Lederman 2006; Kuhmann 2008) CCR5 normally binds MIP-1ą, MIP-1β and RANTES chemokines. By competing for binding with HIV-1, MIP-1α, MIP-1β and RANTES have been shown to be major suppressive factors produced by $CD8^+$ T-cells [bind pat #9]. There is a proposed two-step model for CCR5 binding and recycling. After a ligand binding, CCR5 undergoes a conformational change that activates the bound intracellular G protein. When the G protein is activated it disassociates and activates phospholipase C producing two second messengers, inositol 1,4,5 triphosphate and diacylglycerol.(Lederman 2006) Both C and G protein-coupled receptor kinases phosphorylate the serine residues found in the carboxy terminus tail of CCR5, which in turn recruits β-arrestins. These β-arrestins have clathrin binding sites, which connect CCR5 to the clathrin network. Once this complex binds the clathrin network receptor endocytosis is initiated. In the subsequent endosome, CCR5 and bound ligand are separated, dephosphorylation of CCR5 occurs and the CCR5 receptor is recycled to the cells surface. (Lederman 2006, 12-16)

A 1996 Hemophilia Growth and Multicenter AIDS Cohort study (Dean et al. 1996) discovered that mutations in CCR5 conferred some resistance in humans to HIV-1 (R5) infection. This mutation was found to be present at a frequency of approximately 10% in the Caucasian population in the United States and Europe but is

not found in Asian, African, Middle East or American Indian populations.(Galvani 2003; Dean 1996; Samsone 1996) It was originally believed that this mutation in CCR5 arose from the Bubonic Plaque, which struck and ravaged Europe in the mid 1300's. The mutation was traced back through descendents of survivors of the "Black Death". Nonetheless, this is controversial, some researchers attribute the mutation to selective pressurers from smallpox infections.(Galvani et al. 2003) An alternate hypothesis on the origins of this mutation is that the "Black Death" was not really the bubonic plaque but rather a viral hemorrhagic fever that was undetectable by the methods of that time.(Duncan et al. 2005) It is further hypothesized that this viral hemorrhagic fever would have caused the same bulbous swelling in the lymph nodes, which was one of the main indicators for diagnoses of the bubonic plaque. Also, people infected with viral hemorrhagic fevers develop visible black blotches due to the formation of subcutaneous hemorrhages directly under the skin, for which the "Black Death" is named. The most notable discrepancy in the original hypothesis is that the responsible bacterium of the plague, *Yersinia pestis,* does not use CCR5 as a receptor. Other scientists believe that the selective pressure that caused the CCR5Δ32 mutation occurred long before the "Black Death" devastated Europe, due to its detection in a Bronze Age skeleton.(Duncan, 2004; Hummel 2004)

This mutation was named CCR5Δ32 due to a 32 base pair deletion that encodes the second extracellular protein loop between transmembrane domains four and five of the seven transmembrane architecture.(Dean, Carrington et al. 1996; Agarwal, Zainab Van Horn et al. 2004; Oppermann 2004) CCR5Δ32 encodes a

truncated protein that is retained in the endoplasmic reticulum, which is not detectable and unstable on the cell surface.(Benkirane, Jin et al. 1997; Agrawal, VanHorn-Ali et al. 2004) Homozygous individuals expressing CCR5Δ32/CCR5Δ32 mutation are resistant to HIV-1 infection by M-Tropic strains and heterozygous individuals expressing CCR5Δ32/wt alleles progress at a much slower rate to AIDS.(Dean, Carrington et al. 1996) People who are homozygous with this mutation seem phenotypically normal except for an increased susceptibility to contracting a fatal form of West Nile Virus infection. For these reasons CCR5 has become a very attractive target of investigation to many researchers.

A promising approach has been to create synthetic chemokines molecules that compete with HIV-1 for the CCR5 co-receptor. The most successful of the synthetic chemokines to inhibit binding of HIV-1 is PSC-RANTES and 2-RANTES, which is a RANTES analog chemically identical to native RANTES except for the substitution of a nonanoyl group, thioproline and cyclohexylglycine for the first three N-terminal amino acids of the native protein.(Lederman, Veazey et al. 2004) This synthetic PSC-RANTES was vaginally applied to rhesus macaques and upon challenging with SHIV, a Simian Immunodeficiency Virus and Human Immunodeficiency Virus chimera, it was shown that 12 out of the 15 monkeys treated were protected by 100 μM treatment.(Lederman, Veazey et al. 2004)

Several studies have shown that short interfering RNA (siRNA) has successfully down-regulated CCR5, which had an effect on HIV-1 entry and replication.(27,28,29) In siRNA treated U87-CCR5 cells, engineered to express CCR5, a 48% decrease in CCR5 expression was observed followed by a 55% decrease

in viral entry, which resulted in inhibition of HIV-1 replication.(Martinez, Gutierrez et al. 2002) Another study showed treating infected macrophages with siRNA targeting both CCR5 and HIV-1 p24 antigen subsequently down-regulated CCR5 and precluded viral entry for fifteen days.(Song, Lee et al. 2003) It has also been shown that down-regulating CCR5 in MAGI-CCR5 cells decreased CCR5 expression by ten fold, which in turn decreased viral replication by three to seven fold.(Qin, An et al. 2003) A recent study has shown that liposomal delivered siRNA as a microbicide can protect mice from a lethal form of Herpes Simplex Virus 2 (HSV2) infection when treated two to four hours prior to viral challenge.(Palliser, Chowdhury et al. 2005)

We used human cell lines, vaginal tissue and human vaginal xenografts to determine if CCR5 could be down regulated using a cationic liposome delivery system. There are many cationic liposome mediated delivery systems but they all have a number of common features.(Sioud and Sorensen 2003) Typically, there is a positively charged head group that incorporates one or more positively charged nitrogen atoms allowing for the interaction between the transfection reagent and the negatively charged sugar-phosphate backbone of the nucleic acid molecule, which will be incorporated into the liposome.(Sioud and Sorensen 2003; Dalby, Cates et al. 2004) The charged head group is normally linked to one, two or three hydrocarbon chains by a spacer allowing for more potential for structural variations in these molecules. (Sioud and Sorensen 2003; Dalby, Cates et al. 2004) We choose Lipofectamine 2000 (Invitrogen) as our liposomal complex delivery system for our CCR5-siRNA.

Optimization of the concentrations of Lipofectamine to siRNA was performed to ensure the maximum delivery of our siRNA molecules to the cells and tissues.

After testing several different siRNAs targeting CCR5 we decided to use the validated SMARTpool siRNA from Dharmacon. The SMARTpool plan makes use of Dharmacon's SMARTselection program, which pinpoints siRNAs that have an excellent possibility of effective and specific silencing. To increase the probability of silencing messenger RNA (mRNA) even more, the SMARTpool combines four of the SMARTselection designed siRNAs into a single pool. The SMARTpool platform has been validated in HeLa, HEK 293 and NIH3T3 cell lines.

To determine the effectiveness and ability of the liposomal delivered pooled CCR5-siRNA to down regulate CCR5, we treated P4-R5 cells with either 4nmol or 10nmol pooled CCR5-siRNA or non-specific scrambled siRNA for 48 or 72 hours. Then cell lysates were collected and analyzed by Western Blot. Non-specific scrambled control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. The Western Blot data establishes that both the glycosolated (mature, ~62kD) and unglycosolated (immature, ~49 kD) forms of CCR5 can be detected with our antibody. The 4nmol pooled CCR5-siRNA treatment incubated for 48 and 72 hours resulted in partially down-regulating the glycosolated forms of CCR5, but does not appear to have any effect on the unglycosolated forms when compared to the control siRNA. However, a 48 hour treatment with 10nmol CCR5-siRNA almost completely eliminates the glycosolated form but has little effect on the unglycosolated form of CCR5. Notably, a 72 hour treatment with 10nmol CCR5-siRNA completely eliminates the glycosolated CCR5

and partially down-regulates unglycosolated forms as determined by Western Blot analysis. This data indicates that CCR5 can be successfully down regulated in P4-R5 cells using the pooled CCR5-siRNA from Dharmacon using a single application. The validation of an efficient siRNA targeting CCR5 was necessary before proceeding to the *ex vivo* organ tissue culture system and the *in vivo* human vaginal xenograft system. This validation has to be performed every time a new shipment of pooled siRNA is ordered because there can be lot to lot or batch to batch variation in the efficiency of the siRNA. Also, if we did not observe down-regulation of CCR5 we would have tested each siRNA in the pool to find out which one was the most effective and which siRNA was the least effective. This may have been necessary because the strongest siRNA may overshadow the effectiveness of the other siRNA in the pool. After testing each siRNA individually we would have eliminated the less effective siRNA ensure uniform distribution of the effective siRNA to all cells.

Since we were able to down-regulate CCR5 in P4-R5 cells, which are adherent HeLa cells that do not normally express CCR5, we needed to determine if we could down regulate CCR5 in human vaginal tissue containing human lymphocytes. To this end, an *ex vivo* organ tissue culture (OTC) system was utilized. Using the OTC, fresh vaginal tissues were treated with 10nmol liposomal CCR5-siRNA or scrambled control siRNA, incubated for either 72 or 96 hours and then tissue lysates were collected. At the time of harvesting, the stroma and epithelia were separated in one of the CCR5-siRNA treated tissues from both 72 and 96 hour time points. This was necessary because it was not known if the siRNA would penetrate through all of the

layers of the squamous epithelia. By separating the epithelia from the stroma it could be deduced that CCR5 was being down-regulated in either or both of the tissue layers. Down-regulation of CCR5 in the epithelia but not in the stroma could have an effect on HIV-1 infectivity. Scrambled non-specific control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. At 72 and 96 hours post-treatment CCR5 was not down-regulated in the epithelia, stroma or whole tissue as compared to the scrambled control siRNA.

To confirm that the normal morphology and architecture of the xenograft vaginal tissue was preserved before performing experiments, hematoxylin and eosin (H&E) staining was performed on vaginal tissue prior to grafting and after the healing process. For every xenograft performed, all tissue sections retained the normal architecture with the squamous epithelial and basal layers being clearly visible. The histological results of the vaginal xenografts demonstrated that the xenograft system was operational at Drexel University, thus, allowing siRNA experiments to proceed.

The human vaginal xenograft model system was used to determine kinetics and absorption of liposome delivered siRNA or nanopartilces into human vaginal tissue for the potential use as a microbicide. To determine the kinetics of absorption and to verify that the liposome-delivered siRNA would be absorbed through the squamous epithelia of the vaginal xenografts and have the potential to reach the target macrophages and Langerhan cells, we treated vaginal xenografts with non-specifc FITC-siRNA. Vaginal tissue was treated with 4nM non-specific FITC-siRNA for 2, 4, 12, 24 or 48 hours and cryosections were examined by immunofluorescent microscopy. By 2 hours post-treatment a positive green FITC signal was observed in

the FITC-siRNA treated xenografts. Some of these cells in the 2 hour treatment xenografts show positive FITC signal in the cytoplasm of the cells as is evident by the green signal directly adjacent to the blue DAPI stained nucleus. All of the xenografts treated for 4, 12 and 24 hour visibly show a positive green signal, which is clearly observed in the cytoplasm of the cells. In these xenografts there is also an increased intensity in brightness of the FITC signal, with the brightest signal being observed at 24 hours and at 48 hours the signal starts to fade. There was no positive signal in the untreated group. However, there was autofluorescence emanating from the connective tissue in the stroma, presumably type I collagen, which studies have shown that many connective tissues, specifically collagen, will autofluoresce when excitated in all wavelengths.

To establish an alternate and a potentially more efficient siRNA delivery method we treated vaginal xenografts with FITC-nanoparticles or sodium fluorescein control for either 6 or 24 hours and examined the cryosections by immunofluorescent microscopy. The nanoparticles were made of polyethyleneglycol and have had a FITC molecule conjugated the nanoparticles. By 6 hours post-treatment the FITC-nanoparticle group we observed the FITC-nanoparticles (green) had penetrated the tissue but did not appear to have entered the cells. However, in the 24 hour FITC-nanoparticle treated tissue there was clear penetration into the tissue as well as entry into the cytoplasm as indicated by the white arrows. There was no positive signal from the sodium fluorescein negative controls and untreated groups.

Autofluorescence was again observed originating from the connective tissue, presumably type I collagen.

We were able to show that after 4 hours liposomal-siRNA and after 24 hours nanoparticles can adequately traverse the multiple layers of the squamous epithelia in human vaginal xenograft tissue. Where a portion of the HIV CCR5-positive target cells are localized. Surprisingly, originally when the xenografts were treated with 4nM CCR5-siRNA there was no decrease in expression of CCR5 by immunohistological staining and Western Blot analyzes. Immunohistochemical staining was performed on untreated and CCR5-siRNA treated grafted vaginal tissue, to detect and visualize CCR5 expression in the xenografts. A positive signal for CCR5 in the avidin-biotin complex system used was indicated by a color change to brown, which was visible in the CCR5-siRNA treated and untreated tissue sections that were treated for 24 and 48 hours. Since we currently do not have a method for quantifying immunohistochemcial stained tissue sections we decided to perform Western Blots to detect CCR5 protein levels. To this end it was also necessary to determine the adequate dose of CCR5-specific siRNA required for treatment of vaginal tissue to abolish expression of CCR5.

Based on the FITC-siRNA absorption data we decided to start with our laboratories human vaginal xenograft system prior to normalization of the CCR5-siRNA OTC experiments. Xenograft tissues were treated with 10nmol CCR5-siRNA and incubated for either 72 or 96 hours after which tissue lysates were collected as described in Materials and Methods. Unglycosolated (immature) forms of CCR5 are ~49 kD and glycosolated (mature) forms are ~62kD in size. Scrambled non-specific

control siRNA was included to show any effect that treatment or introduction of siRNA would have on the RNAi pathway. The xenograft CCR5-siRNA treatments were only performed once and need to be repeated but based on the Western Blot analysis and normalization data in Figure 22 there was no down-regulation of CCR5 in the xenografts. Interestingly, three of the five xenografts treated with CCR5-siRNA and the scramble controls appear to have some upregulation of CCR5, indicating the siRNA itself may be affecting the RNAi pathway. A microarray needs to be performed to help assess changes in other genes in the RNAi pathway are being affected or possibly if regulatory genes for CCR5 recycling is being affected. Based on these results that there was no down regulation of CCR5 in the xenograft tissue it may be necessary to increase the concentration of CCR5-siRNA and change the delivery vehicle to nanoparticles. The xengrafts are metabolically active and contain the same proteins and enzymes found in normal vaginal tissue. Even though the siRNA is encapsulated inside liposomes it may be possible for the CCR5-siRNA to be degraded after liposomal fusion with the outer most layer of the squamous epithelia. The nanoparticles may provide more protection from siRNA degradation and may be less toxic, however, more testing is required to confirm this hypothesis. Utilizing nanoparticle has its advantages such as they are can be stored at room temperature and based on our results the nanopartles also penetrate human vaginal epithelia. The disadvantage to utilizing nanoparticles is the cost. It may also be more appropriate target other surface molecules on the vaginal epithelia that are known to bind to HIV-1, such as gp340 and syndecan-1. Targeting other molecules and testing different

combinations of surface molecules could be performed at lower concentrations both siRNA and delivery vehicle whether it be liposomes, nanoparticles or other vehicle.

Using siRNA technology to prevent the transmission of STI, specifically HIV-1, is something many researchers have tried. Unfortunately, targeting HIV-1 only is effective for short periods of time and results in escape mutants. (Boden, 2006) The HIV-1 population in an infected individual is a genetically diverse population of quasispecies, which has contributed to virus' evolution and why targeting the HIV-1 is not the best strategy. That would be adding another selective pressure that the virus can easily overcome. However, a much better strategy is to target a human gene that HIV-1 needs as a co-receptor, such as CCR5. If our experiments are successful then this study could provide proof-of-principle for using siRNA targeting human genes as a microbicide but also for prevention of other infectious diseases. Until the projected cost of producing an siRNA microbicide could be decreased this microbicide would be targeted towards developed nations and is not designed for immediate use before coital acts. This microbicide would need to be administered several times a week to ensure CCR5 down regulation.

Recombinant Normal Flora as a Microbicide

The bacterial species that colonize the human body are both numerous and diverse. These bacteria or normal flora inhabit the skin, the entire digestive system and urogenital tract in women. Many of these normal flora contribute to the healthy development of the immune system, prevent infection from pathogenic and opportunistic microbes as well as maintain the intestinal barrier function. Illness, HIV-1 infection, poor nutrition, use of antibiotics and other factors can affect this

delicate balance and cause emergence of harmful bacterial. An imbalance in or a depletion of normal flora in the vaginal environment has been associated with an increased risk of acquiring opportunistic infections, including HIV-1. (Chang, 2003; Taha, 1998) It has been shown that the daily ingestion of yogurt containing live lactobacilli has been shown to decrease vulvovaginal *Candida* infections by three fold.(Hilton, 1992) The normal flora found in the vagina environment is lactobacilli. These bacteria prefer an acidic environment and help create one by producing lactic and other acids.(Reid 2001) Byproducts produced from lactobacilli metabolism have antagonistic effects against urinary and vaginal pathogens.

There are many challenges that confront the development of an effective HIV-1 microbicide. The microbicide must be able to target and neutralize a genetically diverse population of viruses. Mucosal administration of the microbicide must not cause adverse effects such as immune cell infilitration in the host or sexual partner and must be able to maintain sufficient muscosal concentrations of active compound over extended periods of time. Some microbicides that require application immediately prior to coital acts may lower the frequency of compliance and complicate evaluations of efficiacy. Unfortunately, in many societies cultural norms prevent women from taking the necessary steps to apply a vaginal microbicide immediately before intercourse. There must be alternatives to topical microbicides because even the most efficacious topical microbicide may be of limited value if it can not easily be used. Thus, a vital need exists to develop efficient systems that allow for the delivery of microbicides to mucosal surfaces over extended periods of time.

One approach for delivering microbicides with the capability of synthesizing an antiviral compound is to use probiotics, specifically recombinant normal flora in the vaginal environment. A recent study tested a recombinant lactobacilli, *L. jensenii* Xna, which secreted CVN and a two domain soluble CD4 (2D CD4) molecule.(Chang, 2003) *In vitro* testing of this lactobacilli showed a small decrease in HIV-1 binding.(Chang, 2003) We have also decided to test a recombinant lactobacilli, *L. plantarum*, that secretes CVN, which was developed by our collaborator, Dr. Bharat Ramratnam at Brown University. CVN has been shown to have effective antiviral (HIV-1, HIV-2 and SIV) activity.(Boyd, 1997)

To determine the feasibility of using recombinant lactobacilli, *L. plantarum,* as a microbicide we measured the growth rates of both WT and recombinant *L. plantarum* and measured CVN secretion. This was necessary to evaluate if the recombinant *L. plantarum* would grow and compete for resources with the normal flora in the vaginal environment. As shown in the growth curves in Figure 25 the WT *L. plantarum* grew at a much quicker rate doubling approximately every hour as compared to the recombinant *L. plantarum*, which grew at a much slower rate doubling approximately every three hours. The recombinant *L. plantarum* would not be able to compete for food and resources with the WT lactobacilli already colonizing the vagina. Growth curves need to be performed on the other recombinant lactobacilli, *L. jensenii* or *L. gasseri*, which may grow at quicker rates that are closer to their comparable WT strains and therefore would be more likely to be able to compete for resources.

To verify the recombinant *L. plantarum* was secreting CVN, WT or recombinant *L. plantarum* was grown in the MRS media for 24, 48 or 72 hours. The supernatant was then subjected to Western Blot and indirect ELISA for the detection of CVN. The Western Blot analysis in Figure 27 shows that CVN was detected in all of the time points collected (24, 48 and 72 hours) for the recombinant *L. plantarum* but not the negative control. The detection of CVN from very small volume of supernatant was very important because when utilizing the human vaginal xenograft model system the largest instilling volume is 10 ul maximum. To determine the minimum detection limits for CVN from bacterial cultures we utilized an indirect ELISA. Figure 28A shows the minimum detection limits was in the nanogram range for CVN concentration using the indirect ELISA method and the resulting standard curve. From the standard curve in Figure 28A we were able to determine the minimum volume required from filtered supernatant that allowed for detection of our secreted CVN, which corresponded to 0.1 to 1 µl from recombinant *L. plantarum* culture supernatant, as shown in Figure 28B. These results indicated that once the vaginal xenografts were recolonized with the recombinant *L. plantarum*, we could detect CVN secretion and determine its effect on HIV-1 infectivity.

The recombinant *L. plantarum* CVN microbicide would protentially provide benefits to a women that is infected with HIV-1 and is being treated with highly active antiretroviral therapy (HAART). It is known that HAART causes imbalances in the normal flora of people who are required to take it for survival and specifically many times women have recurrent vaginal yeast infections. A recombinant *L. plantarum*

microbicide, which secretes CVN, given regularly to a HIV-1 infected women to recolonize the vaginal environment could decrease vaginal yeast infections but also decrease cell-free HIV-1 in vaginal secretions.

Before beginning the *in vivo* testing, the anti-HIV-1 activity of the CVN secreted from the recombinant *L. plantarum* was assessed. Filtered recombinant or WT *L. plantarum* culture supernatant with the pH adjusted to 7.0 was tested against cell-free HIV-1. The pH had to be adjusted to ensure the pH would not be a contributing factor in decreasing HIV-1 infectivity. Three separate experiments with triplicate samples were performed and in each experiment a statistically significant ($p<0.01$) decrease in anti-HIV-1 activity was observed in the recombinant *L. plantarum* grown for 48 hours (O.D. 3.0) relative to uninfected control, as shown in Figure 29. These results show the CVN secreted from the recombinant *L. plantarum* retained its anti-HIV-1 activity *in vitro.*

To date there are no published reports of recolonizing human vaginal tissue *ex vivo* or *in vivo* with recombinant lactobacilli. To determine if the WT and recombinant *L. plantarum* could be grown on human vaginal tissue we utilized an *ex vivo* organ tissue culture system (OTC) as previously described. (Collens 20001) Vaginal tissue treated with either WT or recombinant *L. plantarum* cultures and incubated for either 24, 48 or 72 hours. This system allows the lactobacilli to only be exposed to the squamous epithelial cells, which the lactobacilli naturally adhere to in the vaginal environment. As seen in Figure 26C and 26D both the WT and recombinant *L. plantarum* was detected by Gram stain by 48 hours post-inoculation with a subsequent change in pH from 6.5 to 4.5 as seen in Figure 26E. Results were

similar after 72 post-inoculation (data not shown). There was no change in pH detected in the recombinant *L. plantarum* OTC cultures after 24 hours but low quantities of bacterial were detected by Gram stain (data not shown). The change in pH detected indicates the bacteria were growing on the human tissue and secreting lactic acid into the surrounding environment similar to the vaginal environment.

Our data has shown that we were able to grow and detect the recombinant lactobacilli, the secreted CVN retatined its anti-HIV-1 activity *in vitro* and recolonize human vaginal tissue *ex vivo*. Also, we have shown in Figure 18 (chapter 3), that we were able to successfully infect human vaginal xenografts without the need of reconstituting the xenografts with human peripheral blood mononuclear cells, as was required previously for this HIV-1 xenograft model system. Subsequently, to determine if the lactobacilli would colonize the lumen of the vaginal xenografts the xenografts were inoculated with recombinant lactobacilli and upon harvesting were washed with PBS and subjected to Gram stain. Afterward the wash was sent to Brown University for detection of CVN by indirect ELISA, a Gram stain was also performed but only a small population of lactobacilli was detected (data not shown) but more xenografts need to inoculated to ensure the lactobacilli detected was not residual bacteria from the initial inoculum. To determine if the recombinant lactobacilli would decrease HIV-1 infectivity we inoculated the lumen of vaginal xenografts and challenged with HIV-1. Upon harvesting, the vaginal xenografts were sent to Brown University for analysis due to the closing of Dr. Mary K. Howett's laboratory at Drexel University.

Vaginal normal flora has been shown to effect HIV-1 viral load within vaginal secretions of HIV-1 positive women.(Hitti 2008) In an observation cohert study that collected cervico-vaginal lavages from HIV-1 positive women showed that lactobacilli, which secreted hydrogen peroxide, had an effect on HIV-1 viral load in vaginal secretions.(Hitti 2008) The women whose vaginal normal flora changed from bacterial species that cause bacterial vaginosis (BV) to lactobacilli experienced a five fold decrease in HIV-1 viral load; and the women whose normal flora changed from lactobacilli to the bacterial species that cause BV experienced a three fold increase in HIV-1 viral load.(Hitti 2008) A change in vaginal normal flora women has also been associated with bacterial vaginosis and an increased risk of HIV-1 infection.(Chang 2003; Taha 1998)

Previous work that has been performed in our laboratory has shown that the human vaginal xenografts do not retain the normal flora from the patient (Welsh and Howett, unpublished results), thus, making the xenograft system an ideal model system to evalute the recombinant *L. plantarum* as an anti-HIV-1 microbicide. These experiments provide (and will provide) proof-of-principle in determining if the amount of cyanovirin produced and secreted can sufficiently decrease HIV-1 activity *in vivo* to reduce its transmission. Consequently, this will also help in determining if recombinant probiotics can be utilized as a microbicide. Future experiments to be performed using the vaginal xenograft model system will include testing of other recombinant lactobacilli, which secrete CVN; combining these recombinant and WT bacteria in the initial inoculum to detemine if the recombinant lactobacilli can compete with the WT in an environment completely devoid of bacteria; creating different

recombinant lactobacilli that can secrete other microbicidal compounds or peptides (e.g. 5P12-RANTES, fucans or 12p1 peptide); and combining recombinant lactobacilli that secrete different anti-HIV-1 compounds as a way of delivering and testing combination microbicides with different modes of action. Live microbicide delivery strategies, as outlined in this thesis, potentially have many advantages over gel delivered topical microbicides, such as, deliverying long lasting protection due to bacterial colonization decreasing the need for immediate precoital application and low cost to manufacturer once normal flora bacteria has been transformed.

My future interests in microbicidal research would focus on common surface receptors that multiple viruses may utilize for the initial infection of genital epithelia and if those receptors could be down regulated to prevent infection. Semen, vaginal secretions and cervical mucus all play roles in both the protection and contribute to the infectivity of viruses. Researchers are now beginning to study the mechanisms and model systems need to be developed, standardized and validated using these biological fluids. Another area of interest is co-infection studies, for example, is co-infection by two or more different strains of high risk HPV a pre-requisite for progression to cervical cancer. Viral probiotics and recombinant probiotics are both very interesting and an avenue worth exploring. Recombinant viruses and their effects on the human immune system in the vaginal environment need to be studied to determine if they can safely be utilized.

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List of Abbreviations

ABC	Abstinence, Be faithful, Condom use
ADME	Absorption, disposition, metabolism and excretion
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
BZK	benzalkonium chloride
CA	California
CAS	Chemical Abstract Service
CCR5	Chemokine Receptor 5
CCR5Δ32	Chemokine Receptor 5 mutation with 32 base pair deletion
CD4	Cluster of Differentiation-4

CDC	Center for Disease Control
cDNA	Complementary Deoxyribonucleic Acid
CIN	Cervical Intraepithelial neoplasia
CMC	Critical micellar concentration
CVN	Cyanovirin
CXCR4	Chemokine (C-X-C motif) Receptor 4
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ER	Endoplasmic reticulum
FAO	UN Food and Agriculture Organization
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
gD	Glycoprotein D, HSV
GP41	Glycoprotein 41
GP120	Glycoprotein 120
GPCR	G Protein-Coupled Receptor
GRAS	Generally Recognized As Safe
H&E	Hematoxylin and Eosin
HIV-1	Human immunodeficiency virus type 1
HSV-2	Herpes simplex virus type 2
HPV	Human papillomavirus
L1	Late 1, HPV major capsid protein

L2	Late 2, HPV minor capsid protein
LAT	Latency active transcript
MAGI	Multinuclear Activation of a Galactosidase Indicator Discussion
MIP	Macrophage Inflammatory Protein (e.g., MIP-1α)
mRNA	Messenger RNA
N-9	Nonoxynol 9
NOD/SCID	Non-obese diabetic severely compromised Immunodeficient mice
O-T-C	Over the counter
OTC	Organ tissue culture system
P53	Tumor suppressor protein 53
PCR	Polymerase chain reaction
PIC	Pre-integration complex
pRB	Retinoblastoma protein
PSC-RANTES	N^a-(*n*-nonanoyl)-*des*-Ser^1-EL-thioproline^2, L-α-cyclohexyl-glycine^3 Regulated on Activation, Normal T Expressed and Secreted
RISC	RNA induced silencing complex
RLU	Relative Luminescence Units
RNA	Ribonucleic Acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute media

RT-PCR Reaction	Reverse Transcription – Polymerase Chain
SDS	Sodium Dodecyl Sulfate
siRNA	Short Interfering RNA (corrected spelling)
SHIV	Simian Immunodeficiency Virus and Human Immunodeficiency virus chimera
SIV	Simian Immunodeficiency Virus
STI	Sexually transmitted infections
TCID	Tissue Culture Infective Dose
UNAIDS	Joint United Nations Programme on HIV/AIDS
VP16	Viral Protein – 16
WHO	World Health Organization

Vita

Veronica Holmes decided to further her education after a ten year career in accounting. She was given the opportunity to purse a career that would be exciting and interesting and at the same time have the potential to help thousands of people through her research. Veronica attended Immaculata University, Immaculata, Pennsylvania, from 1998 to 2002, where she graduated, *Cum laude* with honors in December 2002. She was then appointed to a Teaching Assistantship at Temple University, Philadelphia, PA, from January to August 2003, where she taught several laboratories including human physiology and non-major general biology. In September 2003 Veronica joined the graduate program in the Department of Bioscience and Biotechnology at Drexel University, Philadelphia, PA.

While at Drexel Univeristy Veronica had the pleasure of working with several different professors but ultimately joined the laboratory of Dr. Mary K. Howett, the Department Head. Under the tutelage of Dr. Mary K. Howett, Veronica received training utilizing infectious disease models, *in vitro*, *ex vivo* and *in vivo*, as well as in virology, molecular biology and cell biology. Veronica's work relied heavily on the Dr. Howett's patented human vaginal xenograft model system, making Veronica one of five people in the world who has been trained and has utilized this *in vivo* model system for studying the prevention of sexually transmitted viruses in human tissue.