VAGINAL POLYMERIC FILM CONTAINING COMBINATION OF ANTIRETROVIRALS FOR PREVENTION OF HIV-1 SEXUAL TRANSMISSION: IMPACT OF CO-DELIVERY

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The HIV epidemic is an ongoing global health challenge. Prevention strategies are one means to combat HIV. Part of prevention incudes behavior modifications, circumcision, vaccine product development and oral and topical pre-exposure prophylaxis (PrEP). Topical PrEP products, also referred to as topical microbicides, are designed for vaginal or rectal application for the purpose of prevention of HIV-1 sexual transmission. Products being designed are coitally or pericoitally dependent or provide sustained release of the active agent for long term protection. The potency of antiretrovirals (ARVs) in HIV therapy promoted the exploration of their use as topical microbicides. Dapivirine (DPV) and Tenofovir (TFV) are replication inhibitors which have been explored as topical microbicides most widely in clinical trials. A 1% TFV vaginal gel applied in a coitally dependent fashion was shown to reduce HIV acquisition by 39%. However, other trials have not been able to show similar successful results with use of the same TFV gel. Therefore, to improve efficacy, combinations of ARVs are being pursued as topical microbicides. The development and characterization of a polymeric vaginal film

containing DPV and TFV is presented in this dissertation. Drug – drug compatibility and a solid state solubility for polymer selection process are described. Physical and chemical characterization of the film demonstrated acceptable film properties. Efficacy testing *in vitro* showed the film to be effective at preventing HIV infection. Finally, stability testing proved the film to be stable at different environmental conditions for 6 months. Moreover, as ARVs drug tissue level is important for their bioactivity, the impact of the co-delivery of TFV and DPV on their tissue accumulation and distribution was assessed in an excised human ecto-cervical model. Results showed that the film was able to release both agents from the film for subsequent delivery into the target tissue. Interestingly, the data showed that the combination film product resulted in achieving higher DPV tissue amounts in the stroma whereas no impact on TFV tissue accumulation was observed. Collectively, these results provide evidence of the ability of polymeric films to accommodate ARVs combinations and illustrate the impact of co-delivery on drug tissue accumulation and distribution.

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HIV	Human Immunodeficiency Virus
CVM	Cervico-Vaginal Mucus
EM	Electron Microscopy
MPT	Multiple Particle Tracking
DCs	Dendritic Cells
STIs	Sexual Transmitted Infections
TER	Trans-Epithelial Resistance
BV	Bacterial Vaginosis
LCs	Langerhans Cells
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-integrin
PrEP	Pre-Exposure Prophylaxis
N-9	Nonoxynol-9
DPV	Dapivirine
TFV	Tenofovir
FTC	Emtricitabine
MVC	Maraviroc
TDF	Tenofovir Disoproxil Fumarate
RIs	Replication Inhibitors

NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NtRIs	Nucleotide Reverse Transcriptase Inhibitors
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
SHIV	Simian/Human Immunodeficiency Virus
CVL	Cervico-Vaginal Lavage
MO-DCs	Monocyte-Derived Dendritic Cells
PK	Pharmacokinetics
ARV	Antiretroviral
CAP	Cellulose Acetate Phthalate
EC_{50}	Half Maximal Effective Concentration
IC_{50}	Half Maximal Inhibitory Concentration
API	Active Pharmaceutical Ingredient
PEG	Polyethylene Glycol
HPMC	Hydroxypropyl Methyl Cellulose
HEC	Hydroxy Ethyl Cellulose
PVA	Polyvinyl Alcohol
PVP	Polyvinylpyrrolidone
Na CMC	Carboxymethyl Cellulose Sodium
VCF	Vaginal Contraceptive Film
PSS	Polystyrene Sulfonate
AZT	Zidovudine

PCL	Poly Epsilon-Caprolactone
IVR	Intravaginal Ring
UHPLC	Ultra High Performance Liquid Chromatography
RH	Relative Humidity
DMEM	Dulbecco's Modified Eagle Medium
BSA	Bovine Serum Albumin
$TCID_{50}$	Half Maximal Tissue Culture Infective Dose
IHC	Immunohistochemistry
SEM	Scanning Electron Microscopy
SMST	Standard Microbicide Safety Test
PEM	Polarized Epithelial Model
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
HAART	Highly Active Antiretroviral Therapy
TFA	Trifluoroacetic Acid
TFV-DP	Tenofovir Diphosphate
VFS	Vaginal Fluid Simulant
O.C.T	Optimum Cutting Temperature
MTBE	Methyl Tert-Butyl Ether
T_g	Glass Transition Temperature
HSV	Herpes Simplex Virus

PREFACE

Finishing my training and work in graduate school was never possible without the support and help of many people. I would like to thank everyone who helped me along the way during the time I spent in graduate school.

My greatest gratitude goes to my advisor Dr.Lisa Rohan. Her support, help, mentorship were beyond any expectation I had. From a volunteer in her lab to a graduate student under her supervision, Dr.Rohan gave me every chance and opportunity possible in order for me to realize my full potential as an independent scientist.

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This journey would not have been possible without the unyielding support of my parents (Nezar and Samar) and family (Reem, Hadil, Hazar, Mohammed and Mulham). Their continued support for me to pursue my dreams is invaluable. My father taught me that education is the greatest asset one can acquire in life and he always made sure that he did everything he could for me to get the best education possible.

My life as a graduate student was full of ups and downs, but no matter what I always found comfort, support, help and love in my dear wife Asmaa. She believes in me and always is there for me. I thank her from the bottom of my heart and wish that I could be the best person to her as I can possibly be.

This work is dedicated to my children Naya and Nezar

1. INTRODUCTION

1.1 HIV "The Epidemic"

Despite progress in combating the human immunodeficiency virus (HIV) epidemic in the past decade, HIV remains a major health challenge. By the end of 2011, 34 million people were living with HIV globally. There is, however, a disproportionate distribution of people living with HIV between countries. The hardest hit area remains Sub-Saharan Africa with one in twenty adults living with HIV. AIDS related death accounted for 1.7 million deaths globally in 2011. Nevertheless as a sign of the progress made, the number of new infections in 2011 was 20% lower than in 2001 [1].

The vast majority of new HIV infections are through sexual transmission. Although behavior change and advocating condom use can have a positive impact on new HIV infection rates, these strategies have not been sufficient to significantly decrease HIV infection rate. Behavior change is a complex and lengthy process that is faced with different cultural and social norms across the world. Condom use is also associated with steep challenges that arise from social, cultural and economic factors which differ for each country. Data showed that male circumcision is effective at reducing the risk of HIV infection [2, 3]. In 2007 the World Health Organization (WHO) recommended voluntary medical male circumcision as a way to reduce HIV infection in males.

Women represented 49% of people living with HIV worldwide in 2011. Yet they are affected disproportionately in the hardest hit areas such as Sub-Saharan Africa where women accounted for 58% of people living with HIV in 2011 (Figure 1.1). This feminization of the epidemic prompted focus on prevention strategies for women. A woman's ability to protect herself from HIV infection is hindered by socio-economic norms. Furthermore, due to anatomical factors, women are more susceptible to HIV sexual transmission [1].

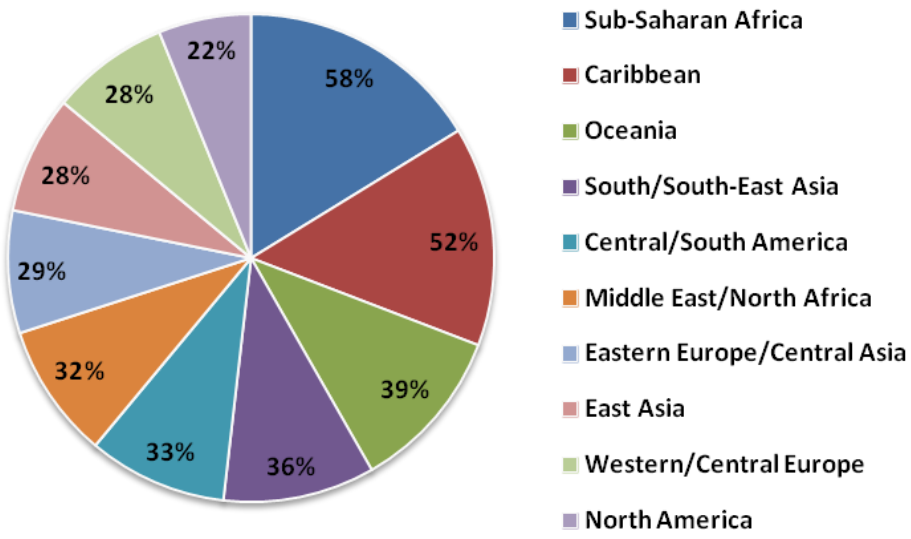

Figure 1.1 Women's share of people living with HIV by region at the end of 2011

1.2 HIV-1 Female Sexual Transmission

1.2.1 The Female Genital Mucosa

Anatomical sites in the female lower genital tract include: vagina, ectocervix and endocervix. The ability for HIV-1 penetration and infection has been established at each of these sites [4]. The vaginal and ecto-cervical mucosa is comprised of a multilayered non-keratinized stratified squamous epithelium covering lamina propria and vascularized tissue. The multilayer epithelium is further subdivided into: basal, parabasal, intermediate, and superficial [5]. As the cells migrate from the basal layer upwards to the superficial layer they become flatter. In contrast, the endocervix is covered with a single layer of mucus secreting columnar epithelium resting on a vascularized lamina propria [6].

The surface of the epithelium in the female genital mucosa is covered with a hydrophilic mucus layer called the glycocalyx. This layer provides a protective barrier against foreign pathogen invasion. It consists mainly of glycoproteins (mucins) and water. Mucus is a semipermeable barrier that allows the exchange of some materials such as nutrients, water, and hormones while being impermeable to most bacteria and many pathogens. Mucus is continuously secreted and shed or degraded. The robust barrier mechanism of mucus includes entrapment and immobilization of pathogens before they reach the epithelial surface [7]. To establish infection, cell free and cell-associated HIV-1 must penetrate the mucus barrier that coats and adheres to vaginal epithelia during sexual intercourse. Mucus limits the number of virions that reach the epithelium. Through this mechanism mucus can reduce the probability of infection [8].

1.2.2 HIV-1 Entry to the Female Genital Mucosa

HIV-1 sexual transmission can occur by vaginal or rectal routes. The probability of HIV-1 transmission per exposure act via the rectal route (1 in 200 – 1 in 300) is higher than the vaginal route (1 in 200 – 1 in 2000) [4]. Vaginal intercourse carries a relatively low risk of HIV-1 transmission per coital act. The female genital tract is equipped with natural defense mechanisms to infection such as the epithelial barrier, acidic pH, and hydrogen peroxide secreted by innate microflora. The risk of infection increases when any of these natural defenses is compromised. Despite these natural defenses HIV-1 can penetrate and establish an infection in the female genital tissue. The main events in the HIV-1 transmission process are: viral entry to the mucosa, infection of target cells, and dissemination to regional lymph nodes to initiate systemic infection [9]. HIV-1 transmission and target cells are discussed below however it is important to mention that for the establishment of productive infection a local expansion of the initially infected cells occurs by an influx of CD4+ T-cells leading to the formation of the so-called founder cell population [10]. This population is thought to produce a sufficient number of new virions to support viral dissemination to draining lymph nodes.

The process of transmission starts when semen containing cell free or cell associated virus is deposited in the vagina. HIV-1 infection can be established by both free and cell-associated virus therefore both forms can successfully penetrate the mucosa [11, 12]. Some of the deposited cell associated or free virus is trapped in cervico-vaginal mucus (CVM). Using 3D visualization and volume modeling Maher et al. showed that mucus was able to provide some protection to the epithelium by trapping HIV-1 virions which were shown to be suspended in

mucus above the columnar endocervical epithelium [13]. The mechanisms by which mucus prevents viruses such as HIV-1 from infecting mucosal surfaces are not fully understood. However, it is generally believed that by steric obstruction mucus can exclude particles that are larger than the effective size of its pores. Depending on the methods used, mucus pore size estimates range from approximately 100 nm to 500–800 nm, and even 1,000–10,000 nm or larger using various conventional electron microscopy (EM) procedures [14]. Using non-mucoadhesive nanoparticles and multiple particles tracking (MPT), the mucus pore size in fresh undiluted human mucus was found to be in the range of approximately 50–1800 nm [14]. However, a study with cervico-vaginal mucus obtained from healthy women with dominant lactobacilli flora demonstrated efficient trapping of HIV-1 in the mucus [15]. In this study the trapping properties of mucus were attributed to the presence of lactic acid secreted from lactobacilli the predominant innate mircoflora. Regardless of the mechanism, HIV-1 trapping in the mucus may increase viral mucus residence time which can have a positive or negative outcome. Immobilization of the virus can allow for it to be attacked by innate defenses, conversely it may provide more time for the virus to interact with the mucosa which can foster transmission.

Several target cells of HIV-1 have been identified in the sub-epithelium. Those include CD4+ T-cells, macrophages and dendritic cells (DCs). For this reason the initial entry events leading to HIV-1 reaching and infecting sub-epithelial target cells are of great interest. Nonetheless, the exact mechanism(s) by which HIV-1 enters the female genital mucosa are not fully understood. It is proposed that HIV-1 mucosal tissue entry may occur via several pathways

including: physical breach or abrasions in the epithelium, direct infection of epithelial cells, transcellular transport across epithelial cells or transcytosis, sequestration or endocytosis in epithelial cells, and uptake by or infection of intraepithelial immune cells [4, 11, 16, 17]. It is highly likely that HIV-1 entry occurs through a combination of pathways with a varying degree of contribution of each pathway.

The integrity of the epithelium lining the genital mucosa is critical to the natural defense against pathogen invasion. Any breach in the epithelial barrier opens routes for HIV-1 to invade the mucosa. The epithelium could be disrupted due to physical abrasions caused by sexual trauma, inflammation induced by sexually transmitted infections (STIs) such as bacterial vaginosis (BV) or other ulcer inducing infections [18-20]. Interestingly, it was found that direct exposure of genital epithelial monolayer to HIV-1 decreases the trans-epithelial resistance (TER) across the epithelium. This reduction was due to a significant decrease in tight junction protein expression thus leading to increased permeability indicating an impairment in barrier function [21]. Increased permeability of the epithelial barrier could allow transmission of normally excluded pathogens [22]. Any gaps or loss of barrier in the epithelium provide the virus with access to intra- and sub- epithelial cellular targets (such as T-cells and Langerhans cells) in addition to gaining access to basal and parabasal epithelial cells which are thought to be more susceptible to HIV-1 binding, endocytosis, or transcytosis [4, 11]. Additionally the presence of trauma, infection or inflammation leads to the secretion of pro-inflammatory cytokines and chemokines which in turn help in recruiting HIV-1 target immune cells (such as T-cells) [16].

Another proposed process of HIV-1 entry is transcytosis. In this process, the HIV-1 virus crosses from the apical to the basal side of the epithelial cells. Cell surface glycosphingolipids, sulphated lactosylceramide expressed by vaginal epithelial cells and galactosylceramide expressed by ecto-cervical epithelial cells were implicated in fostering trancytosis [23]. Nevertheless, it was reported, using cultured primary genital cells, that the efficiency of transcytosis seem to be low and could only account for a small portion of HIV-1 entry to the genital mucosa [24]. Direct infection of epithelial cells is another suggested mechanism of HIV-1 entry and penetration of female genital mucosa. This pathway however is controversial as data has not been shown to be consistent with regard to direct infection of epithelial cells by HIV-1 [25, 26]. Another mechanism of HIV-1 entry involving epithelial cells is endocytosis [4, 11]. Also several reports have shown that HIV-1 could bind to epithelial cells from the lower female genital tract via a variety of cell surface molecules [4, 17]. What role this binding plays in HIV-1 entry into and infection of female genital mucosa is unclear.

Once HIV-1 is in the epithelium it comes in contact with intra-epithelial immune cells mainly CD4+ T-cells and Langerhans cells (LCs). CD4+ T-cells are the primary target of HIV-1 virus infection. The infection of these cells is fusion mediated and involves the CD4 receptor and chemokine co-receptors CCR5 or CXCR4. The presence of these cells in the epithelium suggests that they could play a role in HIV-1 entry to the genital mucosa [9]. Additionally, intra-epithelial LCs may be involved in HIV-1 entry to the genital mucosa. Yet the role of LCs in viral entry is still debated. It was demonstrated that vaginal intra-epithelial LCs commonly expresses CD4 and CCR5 receptors [27]. This finding suggests that intra-epithelial LCs could be infected with HIV-

1 and become a transmitter of infection to underlying cellular targets. On the other hand studies have shown that vaginal intra-epithelial LCs can sequester HIV-1 virus through multiple receptors [28]. One receptor that is potentially involved in HIV-1 endocytosis into vaginal LCs is the C-type lectin langerin. This receptor binds to HIV-1 surface glycoprotein gp120 in a mannose dependent fashion. Langerin was identified to be efficient in binding to HIV-1 leading to virus sequestration into epidermal LCs [29]. The infection or sequestration of HIV-1 in intra-epithelial immune cells can allow for its transfer to sub-epithelial target cells where infection is propagated.

1.2.3 HIV-1 Target Cells

After HIV-1 penetrates the epithelium of the female genital mucosa, it encounters mononuclear target cells in the sub-epithelium. Those targets include CD4+ T-cells, dendritic cells and macrophages.

The HIV-1 infection process starts with viral entry through a fusion dependent manner through different cell surface receptors. Once viral RNA is in the host cell, the reverse transcriptase enzyme converts the single - stranded viral RNA to double-stranded proviral DNA. The newly formed DNA integrates into the host cell DNA via viral integrase enzyme. When the host cell receives an activation signal, the proviral DNA utilizes host RNA polymerase to create copies of the HIV-1 genomic material, as well as shorter strands of mRNA which are used as a blueprint to make long chains of HIV-1 proteins. Viral enzyme protease cuts the long chains of the viral proteins into smaller individual proteins. As the HIV proteins come together with copies

of the viral RNA, a new virus particle is assembled. The newly assembled virus buds out from the host cell moving to infect a new cell [30].

It has been established that the primary target of HIV-1 is lymphocytes (CD4+ T-cells) [11, 18]. The stroma of the human female genital tract is rich with CD4+ T-cells [31]. Analysis of the type of CD4+ T-cells in human cervico-vaginal tissue *ex vivo* revealed that they are mostly effector memory cells with broad expression of CCR5 [32]. HIV-1 can directly infect CD4+ T-cells using viral surface epitops (gp120, gp41) and cell surface receptors CD4 and CCR5 [33]. CD4+ T-cells can also be infected through cell mediated transmission. Efficient HIV-1 infection of CD4+ T-cells mediated by DCs, macrophages and other infected CD4+ T-cells has been demonstrated [28]. This cell mediated transmission occurs through a virological synapse which is a complex that forms at the interface between HIV-1 infected and uninfected cells. Its assembly is driven by gp120 interaction with CD4 and CCR5 or CXCR4 coreceptors and depends upon stable cell-cell junctions maintained by adhesion molecules [34, 35].

Another cellular target of HIV-1 infection is dendritic cells. DCs consist of an array of subsets that differ in origin, maturation state and location. There are a considerable number of studies implicating DCs in HIV-1 mucosal transmission [4, 28]. The major role of DCs in HIV-1 mucosal transmission is enhancement of infection [28]. DCs express the C-type lectin DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin) receptor. HIV-1 attachment to DC-SIGN leads to virus endocytosis and preservation without productively infecting DCs. HIV-1 bearing DCs can subsequently transfer endocytosed HIV-1 to CD4+ T-cells prior to viral degradation in the endosome [28]. Additionally, productive infection of DCs

has been demonstrated *in vitro*. However, this matter remains debatable and warrants further investigation [4]. Studies showed that lower female genital tract DCs are the first to capture HIV-1 within the first 2 hours of viral exposure [9].

Macrophages residing in the cervico-vaginal stroma are also considered a target of HIV-1. Although their infection and role are still ambiguous, infection of macrophages was illustrated in human cervical explant culture [36]. Macrophages are also thought to be mediator in transmitting HIV-1 to CD4+ T-cells [28, 37].

1.3 Prevention of HIV-1 Sexual Transmission by Topical Microbicides

As the HIV epidemic continues to spread with no cure or vaccine available, prevention has become a primary focus in combating the epidemic. Specific to this work, significant effort has been directed into prevention of sexual transmission of HIV. Behavioral change and promoting safe sexual practices including condom use are effective but have proven to be insufficient as they face cultural norms and socioeconomic factors. Male circumcision has also been recommended as an effective way to reduce risk of HIV infection. Additionally, there is a feminization of HIV epidemic especially in the hardest hit areas such as sub-Saharan Africa where women account for the majority of people living with HIV. Thus there is an urgent need for a female controlled method for prevention of HIV sexual transmission.

1.3.1 Definition and Classification

Topical microbicides or topical pre-exposure prophylaxis (PrEP) are products that can be applied vaginally or rectally for the purpose of prevention of sexually transmitted infections including sexual transmission of HIV-1 [38]. It has been estimated that a 60% effective microbicide can prevent millions of new infections each year [39].

An ideal topical microbicide product must be safe and effective [40]. The product must not harm the natural defenses that exist in the genital tract. It also must not induce any type of inflammation or immune response that could lead to an influx of immune cells which are cellular targets of HIV-1. In addition, a topical microbicide product must be acceptable and affordable [41]. Since topical microbicides are designed to prevent sexual transmission of HIV-1, their acceptability by both partners is essential to ensure user compliance. Finally, as the HIV epidemic severity is highest in developing countries, it is important that a low cost topical microbicide is made available for distribution in these parts of the world. Hence feasibility of purchase in developing countries with low economic and purchasing powers is needed.

Topical vaginal microbicides are classified by their primary mechanism of action [12, 20, 40, 42-45]. Vaginal defense enhancers work by strengthening or supporting the natural defense mechanisms in the vagina. Such products help maintain the vaginal pH in an acidic range (examples are BufferGel and Acidform) or facilitate colonization of vaginal flora with lactobacilli. Surfactants or detergents are another class of agents tested as microbicides. Surfactants were the first to be evaluated for topical HIV prevention in human clinical studies. Their mechanism of action involves nonspecifically disrupting the viral envelope by solublizing

membrane proteins. One example of a surfactant which was evaluated as a topical microbicide is nonoxynol-9 (N-9), a vaginal contraceptive agent. N-9 failed in clinical testing due to safety concerns that led to an increase in HIV infections [46]. A third category of topical microbicides are polyanionic polymers such as cellulose sulfate and the natural polymer carrageenen. These agents exert their anti-HIV activity by nonspecifically binding to viral membrane and blocking fusion events. Other classes of microbicide candidates have specific mechanism of action against HIV-1. Entry or fusion inhibitors (such as Cyanovirin-N, Griffithsin, Maraviroc) target viral membrane epitopes (gp120, gp41) or cell receptors (CD4, CCR5, and CXCR4) to prevent the sequence of viral binding, fusion, and entry that leads to cell infection. Another promising class of specific anti-HIV agents is replication inhibitors. Current replication inhibitors (such as Tenofovir and Dapivirine) work by inhibiting HIV-1 reverse transcriptase and preventing viral replication. There are two sub-classes of replication inhibitors: nucleotide/nucleoside and non-nucleoside reverse transcriptase inhibitors [47]. Replication inhibitors are further discussed in section 1.3.2. Finally, most recently integrase and protease inhibitors are being evaluated as candidates for use in microbicide products [48, 49]. Integrase inhibitors such as Raltegravir and Elvitegravir are in early testing and development as candidate topical microbicides. They bind to the viral integrase enzyme active site and block viral DNA strand transfer to the host genomic material [33]. On the other hand the protease inhibitors saquinavir and darunavir are among a group of drugs from this class being tested as candidate topical micrbicides. Protease inhibitors block viral protease enzyme activity that is required for post-translational processing of precursor viral proteins into functional proteins for the budded virion to be infectious.

As the drug candidates used in topical microbicides evolved in terms of their specificity to HIV-1, another classification system is used that categorizes microbicides in three classes based on their specificity to HIV-1. These classes are non-specific, moderately specific and HIV-specific. Nonspecific microbicides include detergents and buffering agents; moderately specific microbicides include anionic polymers, dendrimers, and small polyanionic molecules. Both frequently target more than one pathogen. The HIV-specific microbicides include entry, integrase, protease, and reverse transcriptase inhibitors [44, 50].

1.3.2 The Replication Inhibitors Dapivirine & Tenofovir

Replication inhibitors (RIs), or reverse transcriptase inhibitors, were the first HIV specific antiretrovirals (ARVs) to enter topical microbicides clinical trials. Their basic mechanism of action is blocking the replication of viral RNA to proviral DNA. There are two classes of RIs: nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs).

Tenofovir (TFV or PMPA), (9-[(R)-2-(phosphonomethoxy) propyl] adenine monohydrate), is an NtRTIs (Figure 1.2). It blocks reverse transcription by competing with natural nucleotide incorporation in the forming proviral DNA chain. That results in the termination of DNA synthesis because TFV lacks the 3-hydroxyl group necessary to form the ester bond with the next nucleotide [48, 49]. However, for TFV to be active it must undergo two phosphorylation steps. Of note, NRTIs require three phosphorylation steps for activation [47-49]. One key feature of TFV that may render it a suitable candidate as topical microbicide is that its

active form has a long cellular half-life which may support prolonged anti-HIV activity even with coital independent dosing regimens [47, 51]. It should be noted that TFV is used in HIV therapy as an ester prodrug called tenofovir disoproxil fumarate (TDF) [52]. Two oral products manufactured by Gilead Sciences Inc. contain either TDF only (VIREAD®) or in combination with the NRTI emtricitabine (FTC) (TRUVADA®) are used in HIV therapy.

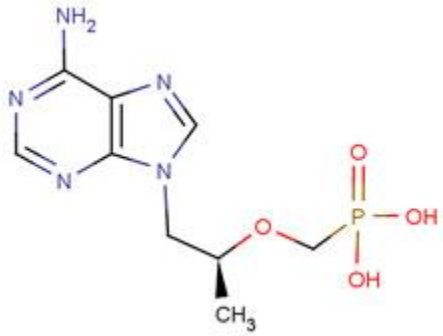

Figure 1.2 Chemical structure of tenofovir (TFV)

As a topical microbicide, a 1% TFV vaginal gel was tested *in vitro* and *ex vivo* and showed effectiveness in preventing HIV-1 infection [53]. The gel was evaluated in macaque animal models and showed complete protection of animals from SHIV (simian human

immunodeficiency virus) infection [54]. TFV gel was shown to provide protection for an extended period of time. Dobard et al demonstrated that 1% TFV gel applied vaginally provided protection to 4/6 macaques against SHIV infection when virus exposure was performed 3 days after gel application [51].

In clinical trials, a two week course of 1% TFV vaginal gel used twice daily was safe and well tolerated in sexually abstinent and sexually active HIV-negative and positive women [55]. In another clinical study, TFV gel applied once daily was shown to increase the anti-HIV activity of cervico-vaginal lavage (CVL) in a dose dependent manner. This activity was shown to persist in the presence of semen [56]. However, in clinical efficacy trials, TFV evaluated as topical microbicide or oral PrEP has not shown consistent results [57]. In a phase IIb study (CAPRISA004), 1% TFV vaginal gel provided 39% reduction in HIV-1 acquisition when used in a coital dependent manner [58]. This was the first proof of concept that an ARV based topical microbicide could be used for HIV prevention. However, in another clinical trial (VOICE) the same gel was not effective in preventing HIV acquisition when used once daily in a coitally independent application [59]. TFV has also been evaluated in clinical trials as oral PrEP. In a clinical trial (called iPrEx) the daily oral use of TDF/FTC combination was able to reduce HIV acquisition by 44% in men who have sex with men (MSM) and transgender women [60]. The same oral TDF/FTC combination was tested in high risk HIV negative women for efficacy. The study was stopped due to futility as the number of infections was equal between the placebo and the active groups [57]. The Partners PrEP study showed that TDF/FTC daily oral use reduced HIV acquisition by 73% compared to placebo in HIV serodiscordant couples [57]. Currently,

two an ongoing phase III clinical trials (FACTS001 and CAPRISA008) are evaluating the 1% TFV vaginal gel in heterosexual women for efficacy with coitally dependent use to confirm results of CAPRISA004. In addition to its anti-HIV activity, TFV was shown to block herpes simplex virus (HSV) infection as well [61].

Dapivirine (DPV, TMC120) (4[4-[(2,4,6-trimethylphenyl)amino-2-pyrimidinyl]amino]benzonitrile), is an NNRTI (Figure 1.3). Developed and evaluated in clinical trials as therapeutic agent, DPV exhibited low bioavailability when administered orally therefore it is only being pursued as topical microbicide product. NNRTIs are noncompetitive inhibitors that bind with high affinity to an allosteric binding pocket on the reverse transcriptase enzyme thereby blocking enzyme activity and halting HIV-1 replication [62]. Specifically, NNRTIs bind to a single site, which is ~10 angstroms away from the polymerase active site, on the p66 subunit of the viral reverse transcriptase enzyme p66/p51 heterodimer [63]. This binding induces conformational changes in the enzyme and causes parts of it to be rigid. The two events lead to an alteration of the substrate-enzyme binding mode and/or affect the translocation of the double strand leading to non-competitive inhibition of the enzyme [62]. Resistance to NNRTIs is associated with mutations of the amino acids that make up the binding pocket on the p66 subunit. Because of their mechanism of action, NNRTIs are generally lipophilic compounds and unlike NRTIs/NtRTIs, no phophorylation is required for NNRTIs to be active [47].

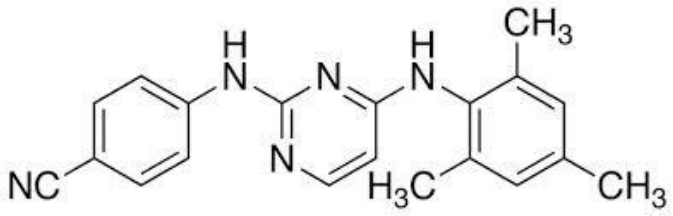

Figure 1.3 Chemical structure of dapivirine (DPV)

DPV has been shown *in vitro* and *ex vivo* to possess anti-HIV activity when used in nanomolar levels against various HIV-1 isolates and a wide range of NNRTI-resistant HIV-1 isolates [64]. DPV was able to prevent HIV-1 infection by cell free or cell associated virus in co-cultures of monocyte-derived DCs (MO-DCs) and CD4+ T-cells after 24 hours treatment [65]. Pre-treatment of cell free HIV-1 with DPV blocked subsequent infection of MO-DCs and CD4+ T-cells co-cultures [66]. The findings in this study support the notion that NNRTIs can permeate cell free HIV-1 and may be able to inactivate the virus before its penetrates the epithelium or reaches its target cells [47]. Evaluation of DPV in a dual chamber model of cervical epithelial cells confirmed its ability to block HIV-1 infection of MO-DCs and CD4+ T-cells by cell associated virus [67]. In a non-polarized ecto-cervical tissue explant model, DPV was shown to inhibit HIV-1 infection in concentrations as low as 0.1 nM with complete inhibition of infection at 10 nM as determined by the detection of proviral DNA [64]. A vaginal gel containing DPV was shown to be successful in preventing HIV-1 vaginal transmission by cell associated HIV in a humanized severe combined immunodeficient (hu-SCID) mouse model [68]. A gel formulation

of C^{14}-DPV applied once daily in rabbits or macaques resulted in high concentrations of DPV in vaginal and cervical tissues for >24 hours with low plasma concentration [69]. DPV was also formulated in a vaginal ring to be used as a sustained release topical microbicide [70].

In clinical trials, there have been eight completed clinical trials evaluating safety, pharmacokinetics (PK) and acceptability of the DPV vaginal gel, and six completed clinical trials evaluating safety and PK of the DPV vaginal ring (Appendix I). In healthy HIV negative women DPV vaginal gel applied twice daily for 42 days was shown to be safe and well tolerated [71]. Another phase I study evaluated vaginal gels, containing a range of DPV concentrations (25, 50 and 150 μM), applied twice daily for 7 days in HIV negative and positive women. Results of the study showed that all concentrations tested were well tolerated with low systemic absorption [72]. Two phase I studies evaluating the PK of DPV vaginal gel showed good DPV distribution in the female lower genital tract with low systemic absorption associated with gel use over the study period [73, 74]. Intravaginal rings containing DPV were evaluated in two Phase 1 studies for safety and PK over a seven day application period. DPV ring formulations showed safety and effective delivery of DPV to the lower genital tract. Systemic absorption of DPV was found to be low with levels <50 pg/ml [75]. In another Phase 1 study which compared matrix and reservoir DPV vaginal rings, 28 day use was well tolerated and did not induce any serious or severe adverse events [76]. Currently, two phase III trials are ongoing evaluating the long term safety and efficacy of the DPV vaginal ring applied for 4 weeks.

1.3.3 Combination Microbicides

In clinical trials, several topical microbicide products failed to significantly reduce HIV acquisition. The only success of a topical microbicide in efficacy testing is the TFV vaginal gel as tested in the CAPRISA004 trial. However, the efficacy of the product was low (39% reduction in infection rates). In order to improve the efficacy of topical microbicides, several strategies such as use of combinations have been adopted. It is expected that a topical microbicide product containing a combination of anti-HIV drug candidates will have an improved efficacy [38]. The benefit of combination microbicides is increased barrier to infection particularly if the drug candidates block HIV infection at different stages of the viral cycle with distinct mechanism of action. Additionally, a reduction in the dose required for each drug may be possible if they act additively or synergistically. This could theoretically reduce the potential for toxicity and resistance [38, 48, 49, 77].

To date many antiretroviral (ARV) drug combinations have been evaluated [78]. A combination of cellulose acetate 1, 2-benzenedicarboxylate (CAP), a polymer that nonspecifically blocks HIV-1 entry, and UC781, an NNRTI, was tested *in vitro* and resulted in significant synergistic and complementary effects against HIV-1 infection [79]. This resulted in meaningful dose reductions for each compound. Maraviroc (MVC), a CCR5 antagonist, was shown *in vitro* to have additive effect when combined with replication inhibitors including TFV [80]. Similarly, an additive effect was demonstrated *in vitro* in the combination of MIV-150 (NNRTI) and carrageenan. The 50% effective concentration (EC_{50}) showed the combination to be 10 times more potent than carrageenan only [81]. A gel containing a combination of 2.5%

TFV and 1% IQP-0528 was tested *in vitro* and *ex vivo* in ecto-cervical and colorectal tissues [82]. Results showed that *in vitro* both drugs exhibited a 50% effective dose in nanomolar levels and the gel was able to protect the tissues from HIV-1 infection. In animal models, Parikh et al. showed that a vaginal gel containing 1% TFV and 5% FTC applied twice-weekly fully protected macaques from a total of 20 exposures to simian-human immunodeficiency virus [54]. In rhesus macaques, a MIV-150/zinc acetate combination gel was shown to fully protect the animals as opposed to partial protection by either active agent individually [83]. Other combinations have been developed and/or evaluated as potential combination microbicide products [84-88]. Recently, a study by Schader et al. showed that the combination of TFV and DPV exerts additive effect against wild type HIV-1 and synergistic effect against NNRTI resistant HIV-1 tested *in vitro* in TZM-bl cell line [89]. Furthermore, it was shown that different HIV-1 strains and subtypes grown in tissue culture containing suboptimal concentrations of DPV and TFV had fewer NNRTI resistance mutations as compared to when the tissue culture contained suboptimal concentrations of DPV only [90].

1.3.4 Status of Topical Microbicides in Clinical Trials

The first topical microbicide product to be evaluated in clinical trials contained the surfactant Nonoxynol-9. The product failed due to safety concerns as the product was proven toxic to the genital mucosa [46]. Other non-specific topical microbicide products (such as Carraguard, cellulose sulfate, Buffergel and PRO2000) failed in clinical trials due lack of efficacy [91-96]. However, as research and development of topical microbicides moved towards

the use of specific anti-HIV agents represented by ARVs, the first sign of success emerged. The phase IIb clinical trial (CAPRISA 004) showed that a 1% TFV vaginal gel administered 12 hours before and after vaginal sexual intercourse reduced HIV-1 acquisition by an overall 39% and by 54% in women with high gel adherence [58]. However, as described previously in section 1.3.2 data regarding TFV from other efficacy trials have been conflicting. The other advanced ARV being evaluated in clinical trials is DPV. Multiple clinical trials have proven the safety of DPV vaginal gel and ring as discussed in section 1.3.2.

Currently, there are multiple phase III clinical trials ongoing evaluating the efficacy of the TFV gel and the DPV vaginal ring (Appendix II). Additionally, several phase I safety trials are ongoing evaluating safety of other products including combination microbicide.

1.4 Delivery of Topical Vaginal Microbicides

A successful safe and effective topical microbicide product fundamentally hinges on delivery system design. Efficacy, safety, and acceptability will be influenced by the dosage form choice and design attributes of the delivery system. Formulation of topical microbicide products faces numerous challenges.

1.4.1 Challenges and Considerations

Several factors determine dosage form choice and design. The physical and chemical properties of the active pharmaceutical agent (API), drug candidate mechanism of action and the biological environment each contribute to formulation of the dosage form [38, 97, 98].

Although each product faces its own specific challenges related to its design and function, drug delivery to the vagina is generally met with common obstacles. The vagina is naturally equipped with defense mechanisms to protect against foreign pathogens or exogenous substances. These mechanisms represent a barrier to drug delivery to the vagina. The vaginal fluid contains water, mucus, enzymes, proteins, and cellular debris. All these components have the ability to interact with the API or dosage form causing loss in activity, destabilization of the API, and impact on dosage form retention and distribution [5]. As explained in section 1.2.2, the presence of mucus in the vagina works as a natural barrier to HIV-1 transmission. Water insoluble compounds must not only be able to dissolve in vaginal fluids but also cross the aqueous mucus barrier. The delivery system chosen for such APIs must be able to enhance or overcome this solubility issue. Use of cyclodextrins and nanoparticles has been demonstrated as a remedy for the dissolution of water insoluble compounds in vaginal fluids [99, 100]. Thickness of the epithelium of the mucosal tissue plays a role in the permeability and tissue accumulation of APIs. This is specifically an issue for APIs that work inside the tissue or on cellular levels. Cyclic changes, age, use of hormonal contraception and other factors all impact the thickness of the epithelium [101]. Other biological factors in the vagina such as acidic pH, presence of lactobacilli and enzymatic activity must be considered for the delivery of topical microbicides.

From a drug development stand point, topical microbicide development requires consideration of several factors. Understanding of the mechanism of action of the API is essential to identity the appropriate delivery systems which would achieve the right delivery and targeting for optimal API function. For topical microbicides, delivery systems for ARVs must be

able to deliver these compounds to the site of action in the tissue because ARVs work on cellular level. On the other hand pre-formulation studies are important to conduct in order to determine parameters such as API stability, water solubility and anti-HIV activity. These parameters are necessary to understand in order to optimize the delivery system attributes so that product safety and efficacy are maximized. For example if stability in aqueous environment is an issue for an API, use of excipients to stabilize the API or use of a solid dosage form may be required. Additionally, retention of API anti-HIV activity after formulation must be assured. Therefore, delivery systems that require heat or high shear in their fabrication may be challenging to use especially with biopharmaceuticals. One of the considerations for topical microbicide products is the retention and distribution of the dosage form in the vaginal lumen. This is important to assess because it has implications for an array of outcomes ranging from local tissue PK to consumer acceptability. Hydrogels, depending on their formulation, show the ability to spread and cover most of the cervico-vaginal surface [102-104]. That could be beneficial for distributing the API across the cervico-vaginal tissue in order to provide protection against HIV-1 infection. However the ability of hydrogel to spread in the vaginal lumen is often associated with product leakage which could hinder acceptability and lead to loss of API. Conversely, while vaginal rings do not have leakage issues they rely on the ability of the API to distribute throughout the cervico-vaginal tissue which may be limited due to the inherent properties of the API [105] and the limited volume of fluids available in the vagina. Another important factor to consider in topical microbicide delivery is safety. The delivery system should not induce any immune response or cause harm to the natural defenses and components of the vaginal ecosystem including natural

microflora [106]. An induction in immune response leads to recruitment of HIV-1 target cells. The delivery system should not cause disruption of the epithelium which would allow for easier access for HIV-1 to reach its target cells.

Additional factors are to be considered for combination microbicide delivery. The degree of complexity increases when designing a delivery system for combination microbicides. For instance the compatibility of the two API together must be evaluated. Co-delivery of APIs may cause a change in local tissue concentrations by impacting transport and clearance of both APIs. The delivery system should account for any impact of co-delivery on local tissue drug levels in order to maximize efficacy particularly as changes in drug tissue levels could affect product efficacy especially with the use of ARVs.

Finally, consumer acceptability of any microbicide product is essential to the success of the product. Therefore, formulation of the same in API in different dosage form may be needed in order to increase consumer acceptability and adherence to use of the product [107].

1.4.2 Vaginal Polymeric Films for Topical Microbicides Delivery

Several dosage forms have been clinically investigated for use as drug delivery systems for topical microbicide [5, 108, 109]. Hydrogels have been the most widely studied for vaginal delivery of topical microbicides. However, this dosage form is largely associated with messiness, product leakage, and requires an applicator. In addition, the relatively large gel dosing volume can potentially cause dilution or alteration of vaginal fluids leading to loss of natural antiviral activity [105]. Water soluble polymeric films offer an alternative delivery strategy for topical

microbicides. Films are solid dosage forms that dissolve once placed on a moist mucosal surface. This dosage form offers fast and accurate dose administration and can be administered without an applicator. Other advantages of vaginal films include the potential for discreet use and decreased product volume [5, 105, 109]. The low product volume of vaginal films reduces the potential for product leakage with use. From a product development stand point films are convenient reproducible dosage forms that can be used to stabilize drugs that are susceptible to degradation in aqueous environments. Previous results from acceptability studies with vaginal films showed that women preferred films over other vaginal dosage forms [110-113]. A recent acceptability study of vaginal films, soft-gel capsules, and tablets as potential topical microbicide delivery systems showed that films were highly acceptable [114]. The film was chosen more often than a soft-gel capsule (SGC) or tablet as the preferred dosage form for use (39% and 37% vs. 25%, respectively). More women preferred to use films (61%) rather than the SGC (53%) or tablet (49%).

A typical film formulation is comprised of the active pharmaceutical ingredient (API), film forming polymers, plasticizers, and other excipients such as disintegrants [115]. For vaginal application, polymers used are usually water soluble such as cellulose derivatives, polyvinyl alcohol, pullulan, gums (xanthan, locust bean, and guar) and chitosan. The type of polymer used and its molecular weight can significantly impact the properties of the film such as mechanical strength, disintegration rate and dissolution. Additionally, plasticizers are used in film formulation to provide flexibility to allow the film to endure mechanical stress such as that encountered during use. Glycerin, sorbitol, propylene glycol, and citrate derivatives are examples

of plasticizers used in film formulations. The amount and type of plasticizer used in the formulation can impact the mechanical properties of the film. Other excipients used in film formulation may vary depending on its use. For instance, disintegration agents such as polyethylene glycol (PEG) are used to provide fast disintegration and quick drug release [116].

An example of a marketed vaginal film is the Vaginal Contraceptive Film (VCF). Polymeric films have been studied for vaginal administration of antifungal and antibacterial drug candidates [117-119]. Topical microbicide products formulated as polymeric films for vaginal application have been developed and evaluated [120-125]. A water dispersible microbicidal cellulose acetate phthalate film was also developed as a topical microbicide [121]. The film was shown to rapidly inactivate HIV-1 and HSV. A vaginal film containing 300 mg Polystyrene sulfonate (PSS), a noncytotoxic antimicrobial contraceptive agent, was developed. The PSS films showed activity against HIV-1 comparable to PSS drug substance [122]. A bioadhesive vaginal film containing zidovudine (AZT), an NRTI, has been developed [126]. Film bioadhesion was shown to proportionally correlate with the percentage of hydroxypropyl methyl cellulose (HPMC) in the film formulation. Proteins and peptides with anti-HIV activity were also formulated into vaginal films. One example is RC-101, a synthetic microbicide analog of retrocyclin, which has been shown *in vitro* to have activity against HIV-1. A film formulation containing 100 µg RC-101 per film unit was developed [124]. The film was shown to be active against HIV-1 both in *in vitro* and *ex vivo* studies and maintained *in vitro* bioactivity for at least 6 months.

1.4.3 Pharmacokinetics: The Importance of Mucosal Tissue Drug Levels to Efficacy

Optimizing drug delivery systems for microbicides requires attention to a host of parameters as described previously. Pharmacokinetic parameters in terms of distribution and clearance are what ultimately dictate local drug tissue concentration in the mucosa. The delivery system of an anti-HIV agent needs to achieve the goal of delivering the API to the intended site of action in a concentration and duration sufficient to outweigh and outlast the virus so prevention of HIV-1 transmission is blocked [107]. Since the replication inhibitors DPV and TFV are both active within the tissue/cells, local PK analysis is essential to better understand the delivery efficiency of these drugs from the dosage form to the site of action [127].

Optimizations (including the excipients used) made to the delivery system can change the local mucosal drug concentration. Grammen et al. developed an *in vitro* system to assess the impact of formulation excipients on microbicide drug permeation [128]. The study showed that TFV had a much higher flux across HEC-1A cell line than DPV when delivered from an aqueous based gel. That is likely due to the aqueous solubility of TFV. However, DPV flux could be enhanced 30 fold by use of excipients such as polyetheylen glycol (PEG) and cyclodextrins in the gel. A change in flux or permeation will impact local tissue concentrations. Clinical data also exist illustrating how the delivery system impacts local tissue PK parameters. A phase I clinical study evaluated the PK of two DPV vaginal rings (reservoir and matrix) [76]. Results showed that both rings were able to successfully deliver DPV to the lower genital tract with concentrations 4 logs greater than the reported EC_{50}. However, the maximum concentrations and drug exposure were significantly higher with matrix ring use as compared to reservoir ring use.

Both DPV and TFV require delivery into the female lower genital tract tissues giving their mechanism of action. It is vital from a drug delivery stand point to understand the impact of the delivery system and its formulation on local mucosal tissue drug concentration. This will help to assess the functionality of the delivery system to effectively deliver the active agents to target and conduct the proper modifications to maximize efficacy of such required delivery. The relationship of cellular or tissue concentration to product efficacy was exemplified in pharmacokinetic studies with TFV vaginal gel. Hendrix et al. illustrated that there is a positive correlation between TFV intracellular concentration and the likelihood for infection with higher TFV concentrations resulting in lower infection rates [127]. More recently, analysis of results from CAPRISA004 showed that a relationship between HIV-1 incidence rate and TFV concentration in aspirated cervico-vaginal fluid (CVL) [129]. In the low-exposure group ($[TFV]_{VF}$<1000 ng/mL) HIV incidence rate did not differ from the placebo group whereas the rate was significantly lower in high-exposure group ($[TFV]_{VF}$>1000 ng/mL). Although the relationship between tissue and CVL drug concentrations is yet to be made, the positive correlation between TFV levels in CVL and efficacy provides an example of how drug levels *in vivo* correlates with overall efficacy.

1.5 Hypothesis and Specific Aims

Reverse transcriptase inhibitors have gained a great deal of attention recently. They are currently considered to be the most promising ant-HIV agents being studied in the clinic. RTIs prevent HIV-1 infection by blocking replication of viral RNA to DNA in the target cells. HIV-1

has several cellular targets (such as CD4+ T-cells, dendritic cells, and macrophages) in the cervico-vaginal tissue. Mostly, these target cells exist in the sub-epithelial tissue. Therefore a drug delivery system of RTIs must be able to deliver them to the cervico-vaginal tissue efficiently. Dapivirine (DPV) and Tenofovir (TFV) are the most advanced of the RTIs candidates currently being developed as topical microbicides. They differ in aqueous solubility with DPV being hydrophobic and TFV being hydrophilic. From a drug delivery stand point; once the drug is released from its dosage form it becomes available for permeation into the target tissue. The process of permeation can be affected by several factors including but not limited to dosage form design and drug chemical properties. As a result of permeability kinetics, drug tissue concentrations and distribution are determined. This is of great importance to topical microbicides containing DPV and/or TFV due to the need for both drugs to be present in the tissue site of action in levels sufficient to prevent HIV-1 infection. Polymeric vaginal films offer a promising delivery system for topical microbicide drug candidates including RTIs. Polymeric films provide portability, do not require an applicator, and have no leakage or messiness. All of these benefits can increase user acceptability and compliance. In addition, as a solid dosage form polymeric films decreased the dilution effect and more efficiently releases drug payload resulting in higher drug concentration at the tissue surface. This can theoretically drive the permeation of the drug into the tissue. That is why polymeric films are potentially advantageous to RTIs delivery. However, as combination microbicides are being considered for clinical evaluation, it is important to assess how co-delivery can influence the tissue permeation and accumulation of each drug candidate.

We hypothesize that dapivirine and tenofovir can be co-delivered using a water soluble polymeric vaginal film. We further hypothesize that co-delivery of dapivirine and tenofovir will change their tissue accumulation and distribution in the target mucosal tissue due to subsequent changes in drug concentration gradients.

This hypothesis will be addressed through the following specific aims:

Specific aim 1: Formulation of a water soluble vaginal film containing the hydrophobic reverse transcriptase inhibitor dapivirine.

Specific aim 2: Development and evaluation of a water soluble vaginal film containing a combination of dapivirine and tenofovir.

Specific aim 3: Assessment of impact of co-delivery on dapivirine and tenofovir tissue accumulation and distribution in human ecto-cervical tissue model *ex vivo*.

2. FORMULATION OF WATER SOLUBLE VAGINAL FILM CONTAINING HYDROPHOBIC DAPIVIRINE

2.1 Introduction

As discussed in chapter 1, Dapivirine (DPV) is a potent replication inhibitor that is being developed as a topical vaginal microbicide product. It is a tight binding non-nucleoside reverse transcriptase inhibitor (NNRTI). The first example of protection using *in vitro* models with NNRTIs was with UC781, a potent anti-HIV drug candidate with hydrophobic nature [100, 130-132]. As a topical microbicide product, DPV has been formulated and evaluated in vaginal gel and intravaginal ring (IVR) dosage forms [68-70, 133-135]. Malcolm et al. showed that a core type silicone elastomer vaginal ring containing DPV exhibits zero-order release for 71 days with about 140 μg DPV released daily [135]. DPV has also been incorporated in various surface engineered poly epsilon-caprolactone (PCL) based nanoparticles. Negatively charged nanoparticles were stable up to one year at 5 - 40°C [136]. In clinical trials, DPV vaginal gel showed good safety profile and low systemic absorption of DPV [71-74]. Similarly, DPV intravaginal rings were evaluated in two Phase 1 studies for safety and PK over a seven day application period and showed safety and effective delivery of DPV to the lower genital tract with low systemic absorption [75, 76].

For a topical microbicide to succeed it has to be highly acceptable for use by consumers. Therefore user acceptability and compliance must be considered during the development of topical microbicides. It is likely that several dosage forms with different attributes will be needed

to provide women with more choices which will likely result in improved adherence. Vaginal polymeric films for drug delivery are widely studied for delivery of various drugs [137]. The advancement in polymer science has allowed for the development of films for a range of pharmaceuticals. The merits and acceptability of polymeric films as a dosage form have been discussed in chapter 1.

To address the dissertation hypothesis regarding polymeric films ability to deliver a combination of TFV and DPV it is first necessary to establish the capability of a water soluble polymeric film to accommodate the hydrophobic drug candidate DPV. Therefore the aim of the studies described in this chapter was to develop and characterize a film containing the NNRTI DPV for vaginal use to prevent HIV-1 sexual transmission. Comparative studies were carried out with drug substance and film formulation to demonstrate that the formulation process did not result in significant loss of antiviral activity. *In vitro* drug release experiments were conducted to ensure rapid release of DPV from the film. Drug release is important in that it is required for drug permeation upon insertion *in vivo*.

2.2 Methods

Dapivirine (DPV) was provided by the International Partnership for Microbicides (IPM). DPV is a white micronized powder with molecular weight of 329.4 and pKa of 5.8. It is water insoluble (LogP = 5.27 at pH 9) and has a 2.5 mg/mL solubility in propylene glycol. UC781 is also a water insoluble NNRTI. Given that the potent anti-HIV activity of UC781 has been well established [132, 138] it was used as a control in *in vitro* bioactivity studies.

2.2.1 Dapivirine Film Formulation

The film formulation was composed of PVA, HPMC 4000, PEG 8000, propylene glycol, and glycerin. Solvent casting methods were used for film manufacture. Briefly, an aqueous film solution containing the excipients and DPV (in a dispersed state) was prepared. The solution was cast onto a polyester substrate attached to the hot surface of an automatic film applicator (Elcometer® 4340) using either a spreading blade with reservoir. After casting, film solution was allowed to dry for 20 minutes before it was removed from the substrate. Once film sheets were obtained they were cut using a die press into 1" x 2" individual unit doses.

2.2.2 Dapivirine-Excipient Compatibility

Weighed amounts of DPV were placed in glass vials and mixed with 320 µL propylene glycol. Polymer solution (1.68 ml) composed of polyvinyl alcohol (PVA), hydroxypropyl methyl cellulose (HPMC) 4000, and polyethylene glycol (PEG) 8000 was added to each vial and vortexed. The vials were sealed under nitrogen and protected from light using aluminum foil. The vials were placed in conditions of 30°C / 65% relative humidity (RH), 40°C / 75% RH or 50°C. Drug content was monitored for 14 days at all conditions to assess DPV stability. DPV drug content was determined using an ultra-high performance liquid chromatography (UHPLC) method. The method utilizes a Waters Acquity BEH C18 1.7µ 2.1x50mm. The mobile phase used was (A) 0.08% TFA in water (B) 0.05% TFA in Acetonitrile (ACN). A gradient method was used where % of mobile phase (B) changes from 10 – 80 – 10 over 15 minutes run time.

2.2.3 Physical and Chemical Characterization

DPV film weight, thickness and appearance was recorded. Tensile strength as a measure of films strength was assessed using a texture analyzer (TA-XT.Plus) connected to a data acquisition and analysis software. Tensile strength was calculated using the following equation: Tensile strength = Force (N) / Cross sectional area of the film (cm^2). Residual water content of the films was measured using Karl-Fisher apparatus (Metrohm, 758 KFD Titrino). Film disintegration testing was conducted by submerging a film in 3 ml water and mixing by rotation using an orbital shaker. Disintegration was monitored visually as the time for complete film structural loss. For determination of DPV drug content, films were dissolved in a 50% ACN solution and heated for 5 minutes at 50˚C. An aliquot was taken from the solution and analyzed using the previously described UHPLC method. Dissolution of DPV films was evaluated using a class IV USP apparatus (SOTAX CP7) using a 60 mL reservoir and a flow rate of 16 mL/min. The dissolution media used was distilled water containing 1% cremophor. DPV solubility in the dissolution media is 40 μg/mL. Film dissolution was monitored under sink conditions for 60 minutes and DPV was detected by UV spectrometer at 310 nm.

2.2.4 Stability Assessment

DPV films were held for 24 months at 30°C / 65% RH and for 6 months at 40°C / 75% RH. Testing of the films was conducted at predetermined time points. At each time points weight, thickness, appearance, tensile strength, water content, and drug content were tested. In addition, disintegration, dissolution, Lactobacillus compatibility and *in vitro* anti-HIV activity (TZM-bl cell based model) were tested at specific time points throughout the stability protocol.

2.2.5 Compatibility with Lactobacillus

The standard microbicide safety test was used as previously described [139, 140] to assess Lactobacillus compatibility with DPV vaginal film. Briefly, bacterial suspensions were prepared in ACES buffer and the films were then dissolved and mixed in the suspensions. The suspensions were then incubated for 30 min at 37°C. Samples were taken at zero time and again after 30 min. Viability was determined by standard plate count. A sample is considered compatible if the reduction in viability was < log10.

2.2.6 Dapivirine Tissue Permeability

Human excised cervical tissue was obtained from University of Pittsburgh Health Sciences Tissue Bank under IRB Protocol PRO09110431. Tissue samples were from healthy volunteers undergoing routine hysterectomy for non-cervical issues. A section of each tissue was retained for histological evaluation. Excess stromal tissue was removed and the epithelial layer isolated using a Thomas-Stadie Riggs tissue slicer by a longitudinal slice through the entire

tissue. Tissue thickness was measured by placing the tissue between two pre-measured slides. Tissue permeability studies were performed in a Franz cell system. Isolated epithelial sections of the tissue were placed between the donor and receptor compartments of the Franz cell with the epithelial side of the tissue oriented toward the donor compartment. The tissue-loaded Franz cells are maintained in an environment at 37°C throughout the experiment. The receptor was filled with 10% PEG 300 in Krebs buffer (pH=4.0). DPV drug substance solubilized in the receptor medium (100 μg/mL) or DPV film (sufficient weight of film to obtain 100 μg/mL) solubilized in receptor medium (400 μL) was added to the donor chamber. At predetermined time points, 200 μL was removed from the receptor chamber and replaced with the same volume of fresh medium. The collected samples were analyzed for drug content using previously described UHPLC methods.

2.2.7 Dapivirine Film *In Vitro* Anti-HIV Activity

2.2.7.1 HIV-RT and Cell-Based HIV-1 Replication Assay

Weighed pieces of DPV films were dissolved in either DMEM (for cell-based assays) or 1X RT-buffer [for *in vitro* reverse transcriptase (RT) assays] at 1:10 w/v. Multiple dilutions of the starting solutions were then prepared and used. For the RT assay, the ability of each film dilution to inhibit the RNA-dependent DNA polymerase activity of HIV-1 RT was determined using poly(rA)-oligo(dT) and [3H-TTP] as previously described [100]. Cell-based single HIV-1 replication cycle assays for direct antiviral activity of the film formulations used P4R5 indicators cells as described above.

2.2.7.2 TZM-bl Assay

Anti-HIV activity testing using TZM-bl assay was performed as previously described [141]. DPV films or placebo films were dissolved in 2 mL of saline. Ten-fold serial dilutions up to 1:107 of the original 2 ml sample of the DPV film were made. The placebo film was handled in a similar manner. As a control, the drug substance was used. DPV was dissolved in DMSO and serial dilutions beginning at 1000 nM were made using the TZM-bl medium (DMEM with 10% BSA supplemented with antibiotics). The respective dilutions were added in triplicate to plated TZM-bl cells. HIV-1BaL was added next and the assay was cultured for 48 hours. Infection was detected by adding BrightGlo (Promega) a chemiluminescent developer of luciferase to each well. Control wells comprising cells alone or cells with HIV-1 only served as background and maximal luciferase activity, respectively. Efficacy was calculated as % inhibition of infection by the following formula:

$$\% \text{ inhibition} = (\text{Treated well-cells alone}) / (\text{HIV-1 only-cells alone}) \times 100$$

The effective dose at 50% was calculated using GraphPad Prism software.

2.2.7.3 Polarized Explant Model (PEM)

Freshly obtained cervical tissue was incubated in a solution consisting of equal amounts of Penicillin-Streptomycin solution and Fungizone (Mediatech, Inc, Manassas, VA) for 10 minutes at room temperature, then rinsed three times with RPMI media prior to use. Defined pieces of tissue were clamped mechanically between two neoflon cell chambers. The receptor chamber was filled with RPMI/10% serum/1% Penicillin-Streptomycin solution. One fourth of a

DPV film was weighed and dissolved in 400 µL of RPMI, and this solution was added on top of the tissue in the donor chamber. The tissue was then incubated with this RPMI-film preparation for 1 h. After incubation, the film solution was removed from the epithelial surface of the tissue, and the surface was rinsed several times with RAFT medium to remove any residual film. A total of 400 µL consisting of RAFT medium, virus inoculum, and C^{14}-PEG 4000 (tissue integrity tracer) was added to the donor chamber, and the tissue was incubated for 4h to allow HIV infection/transmission to occur. The virus used was the dual-tropic HIV-1 89.6. After 4h, aliquots of both donor and receptor chamber medium were taken and reserved for further analysis (scintillation spectrometry, qRT-PCR of HIV-1 RNA, infectivity).

2.2.7.4 Cervical Explant Model

In this model both toxicity and anti-HIV-1 activity were assessed and set up as previously described [36, 53]. Briefly, explants were placed with the luminal side up in a transwell. The edges around the explants were sealed with Matrigel™. The explants were maintained with the luminal surface at the air-liquid interface. The lamina propria was immersed in medium. The explants were set-up in duplicate for each treatment. For toxicity testing, DPV or placebo films were dissolved in 2 mL of culture medium and 100 µL were applied to the apical side of the explants for 18 hours. The next day, explants were washed and viability was evaluated using the MTT assay and histology. For anti-HIV activity, 100 µL of dissolved DPV or placebo film were mixed with 5×10^5 tissue culture 50% infectious dose ($TCID_{50}$) of HIV-1_{BaL} and added to the apical side of the explants. Eighteen hours after application the explants were washed and fresh

medium was applied to the basolateral compartment. Every 3 to 4 days over a 3 week period, supernatant was collected and stored at -80°C for HIV-1 p24 analysis and fresh medium was added back. Stored supernatants were tested for HIV-1 replication using an HIV-1 $p24_{gag}$ ELISA. End of study explants were fixed in 10% buffered and processed by routine paraffin embedding for histology to determine product efficacy by immunohistochemical (IHC) analysis for HIV-1 p24 antigen. For toxicity evaluation, slides were stained with hematoxylin and eosin. De-paraffinized, rehydrated sections were digested in 0.1 mg/mL Proteinase K for 15 min, washed, and incubated at room temperature for 1 hour with anti-p24 (dilution 1:6). Visualization was achieved with the DAKO Corp.'s LSAB-2 kit with the universal alkaline phosphatase system. Sections were counterstained in Mayer's hematoxylin. Negative controls were tissue sections from each explant incubated with normal mouse ascitic fluid.

2.3 Results

2.3.1 Development and Evaluation of DPV Vaginal Film

2.3.1.1 Film Formulation

DPV was formulated in a polyvinyl alcohol (PVA) based vaginal film (Figure 2.1). PVA is used in vaginal film formulations as demonstrated by recent patent of vaginal films [137]. It is the main polymer in the VCF marketed film [142]. PVA constituted 38.3% (w/w) of the film. Other excipients in the formulation included 19.1% hydroxypropyl methyl cellulose (HPMC), 25.5% polyethylene glycol (PEG) 8000, 7.9% glycerin and 7.9% propylene glycol.

In order to study the impact of drug loading on film properties two loading doses of DPV were evaluated: 0.5 and 1.25 mg DPV per 1”x2” film unit. These dosing levels were chosen based on the concentration of DPV in the vaginal gel formulation evaluated in clinical trials [71-74].

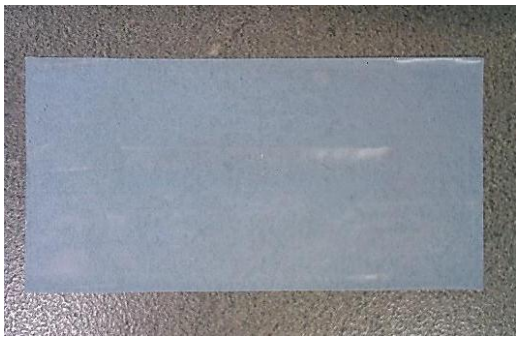

Figure 2.1 Picture of a 2”x1” DPV vaginal film

2.3.1.2 Dapivirine-Excipient Compatibility Study

Once a film formulation prototype was selected a compatibility study of DPV with the excipients used in the film formulation was performed. DPV was mixed with the excipient mixture (propylene glycol, PVA, HPMC4000, and PEG8000) and stored at different conditions as described in the methods. Drug content analysis results showed that no drug loss. Stability of DPV in the excipient mixture was an indication of its compatibility with the excipient (Figure 2.2).

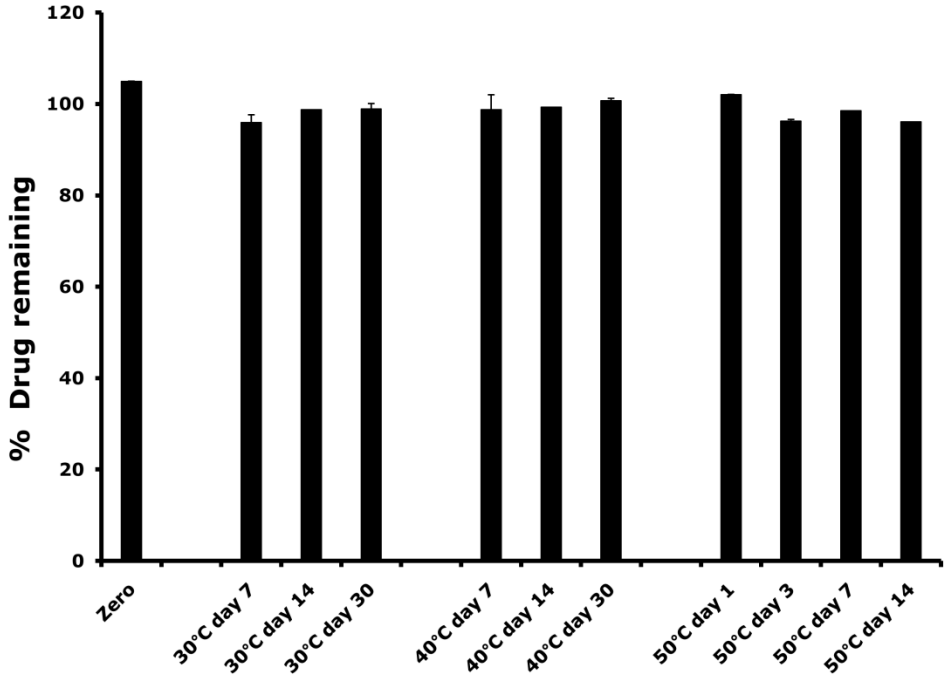

Figure 2.2 DPV compatibility profile with the excipients used in the film formulation. DPV stability in the excipients mixture was indicative of its compatibility with the excipients

2.3.1.3 Physical and Chemical Characterization

Dapivirine films were soft, flexible and semi-transparent. A visual evaluation by scanning electron microscope (SEM) showed a uniform film surface which is an indication that, after drying, the residual water content was sufficient to maintain the polymer hydration. The SEM also confirmed suitable drug dispersion in the film's polymeric network as no signs of drug aggregation or precipitation were observed (Figure 2.3).

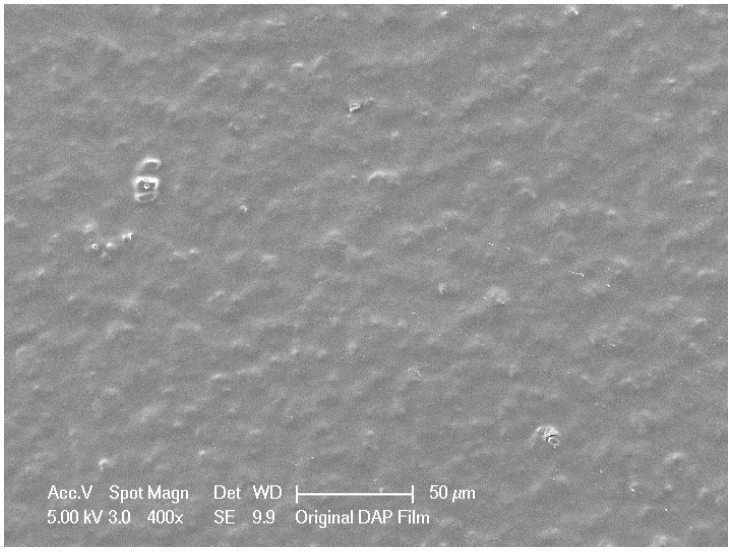

Figure 2.3 SEM picture of 1.25 mg DPV film

The thickness of the films was set to 70 μm thick which was suitable to achieve the required doses. Visual disintegration studies showed the quick dissolving nature of the films (< 10 minutes). Table 2.1 shows DPV film characteristics with both dosing levels 0.5 mg and 1.25 mg. Dosing level was found not to alter film properties. DPV loading in the film was based on the dosing levels of DPV in a vaginal gel that were evaluated clinically and shown to be safe and well tolerated [71, 74]. In DPV gel clinical trials, participants used 2.5 mL of gel products with increased DPV concentrations up to 0.05%. Hence, given the volume of gel administered the total maximum mass delivered was 1.25 mg DPV per dose.

Table 2.1 DPV film physical and chemical characteristics. All values presented as mean ± SD

Parameter	1.25 mg DPV Film	0.5 mg DPV Film
Weight (mg)	91.23 ± 4.93 (n = 6)	94.7 ± 10.01 (n =14)
Thickness (mm)	0.073 ± 0.005 (n = 6)	0.063 ± 0.007 (n = 14)
Disintegration (min)	6.33 ± 0.577 (n = 3)	4.33 ± 0.58 (n = 3)
Water content % (w/w)	1.38 ± 0.17 (n = 3)	2.4 ± 0.19 (n = 3)
Tensile strength (N/cm^2)	538.24 ± 57.17 (n = 3)	777.59 ± 19.99 (n = 3)
Drug Content % (w/w)	1.4 ± 0.03 (n = 42)	0.546 ± 0.016 (n = 5)

Additionally, DPV film (1.25 mg/film) dissolution was evaluated in a class IV USP dissolution apparatus. Results showed that greater than 50% of the loaded DPV was released from the film within 10 minutes (Figure 2.4). These dissolution results confirm the quick dissolving property of the film. From an *in vivo* application stand point, it is hypothesized that the rapid release of DPV from the film will enhance the concentration of DPV at the tissue surface and thus drive diffusion of DPV into the target tissue.

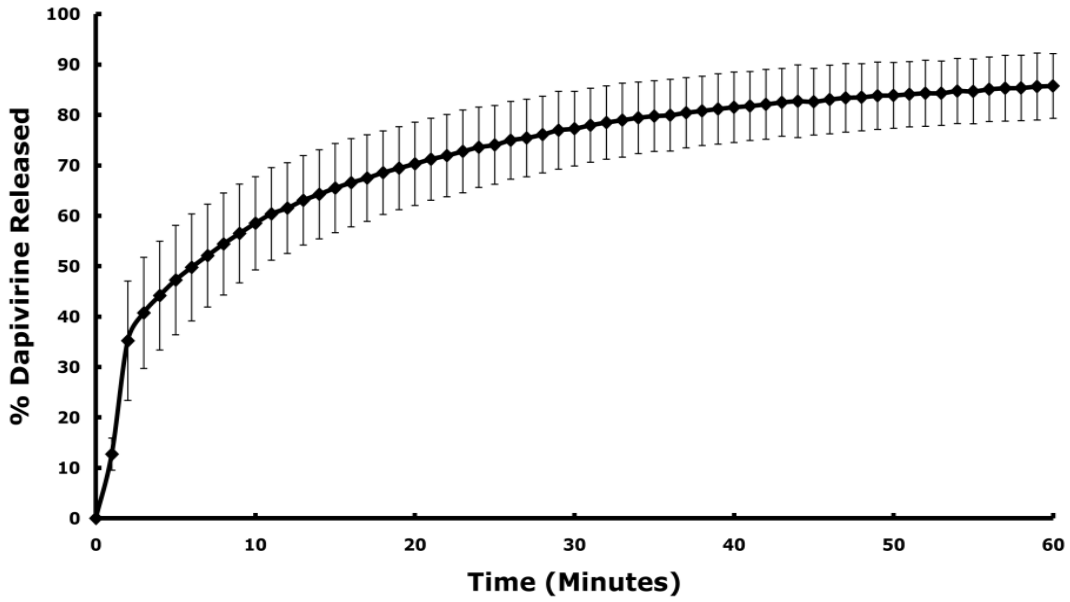

Figure 2.4 1.25 mg DPV film dissolution. Rapid DPV release from the film was observed with >50% loaded drug released in less than 10 minutes. Data presented as mean±SD (n=3)

2.3.1.4 *In Vitro* Assessment of DPV Film anti-HIV Activity

DPV film (1.25 mg/film) was tested for anti-HIV activity in the TZM-bl assay. The EC_{50} obtained for DPV in this assay was of 38.7 nM (Figure 2.5). The placebo film had minimal anti-HIV activity which returned to baseline activity after a 1:100 dilution. This activity observed in the placebo film is more than likely due to the presence of the polymer excipients.

Furthermore, DPV film (0.5 mg/film) was tested for its ability to inhibit HIV-RT and cell-based HIV-1 replication *in vitro*. Inhibition of cell-based HIV-1 replication by DPV film (0.5 mg/film) is over 200-fold more potent than inhibition of RT activity (Figure 2.6). This observation is probably due to the hydrophobic nature of DPV.

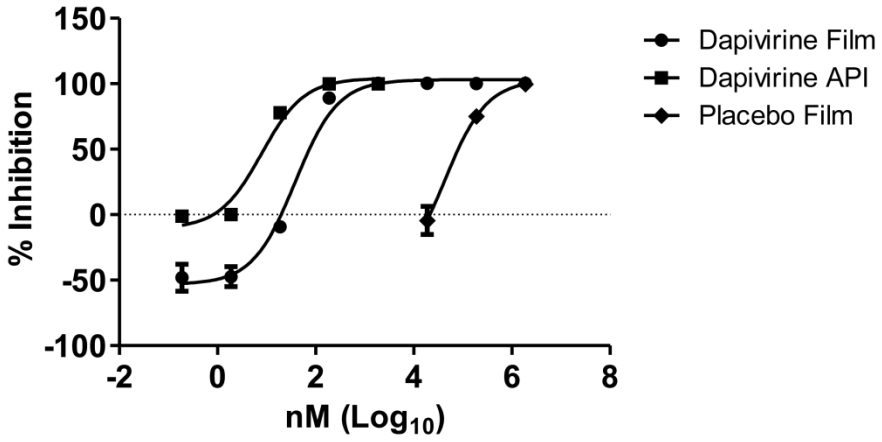

Figure 2.5 Placebo and DPV containing film anti-HIV activity in vitro in TZM-bl cell line

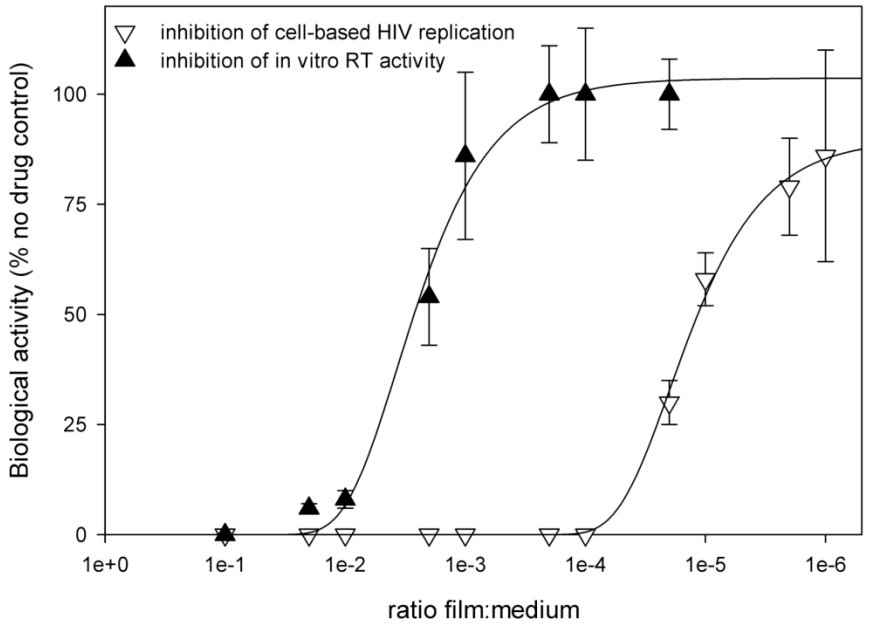

Figure 2.6 DPV film activity in vitro in cell-based HIV replication assay and in vitro RT activity assay. DPV showed inhibition of both HIV-1 replication and RT activity

2.3.1.5 Compatibility with Lactobacillus

Lactobacillus is a main component of the vaginal microflora. By maintaining the vaginal acidic pH and producing hydrogen peroxide it represents a defense mechanism against foreign pathogens including HIV-1 [143]. Therefore it is important to ensure that DPV film does not alter Lactobacillus or its function.

The compatibility of DPV film with Lactobacillus was assessed using the Standard Microbicide Safety Test (SMST). Results showed that DPV film did not induce any deteriorating effects on several strains of Lactobacillus as no loss of bacterial viability was observed (Table 2.2).

Table 2.2 Evaluation of DPV film compatibility with Lactobacillus. Results present are the averages of all experiments using the organisms. A positive value indicates there was a slight increase in the viability. These organisms were tested at different time intervals. The films passed all safety tests; the standard for passing the test is there cannot be a loss of viability ≥ 1 $\log_{10}$.

Lactobacillus strain tested	Log Difference (T30 minute Plate Count - T0 minute Plate Count)
L. crispatus ATCC 33197	0.245
L. jensenii ATCC 25258	0.0587
L. jensenii LBP 28Ab	0.158

2.3.1.6 Stability Assessment

After obtaining a prototype DPV film with acceptable characteristics, the film stability was evaluated. DPV film (1.25 mg/film) was stored at 30°C / 65% RH and 40°C / 75% RH for stability assessment. Based on the physical and chemical assessments conducted DPV film was

shown to be stable for a period of 24 months at 30°C / 65% RH and for at least 6 months at the accelerated conditions (40°C / 75% RH). Figures 2.7 and 2.8 show DPV film drug content, and dissolution obtained for each condition over the stability time period.

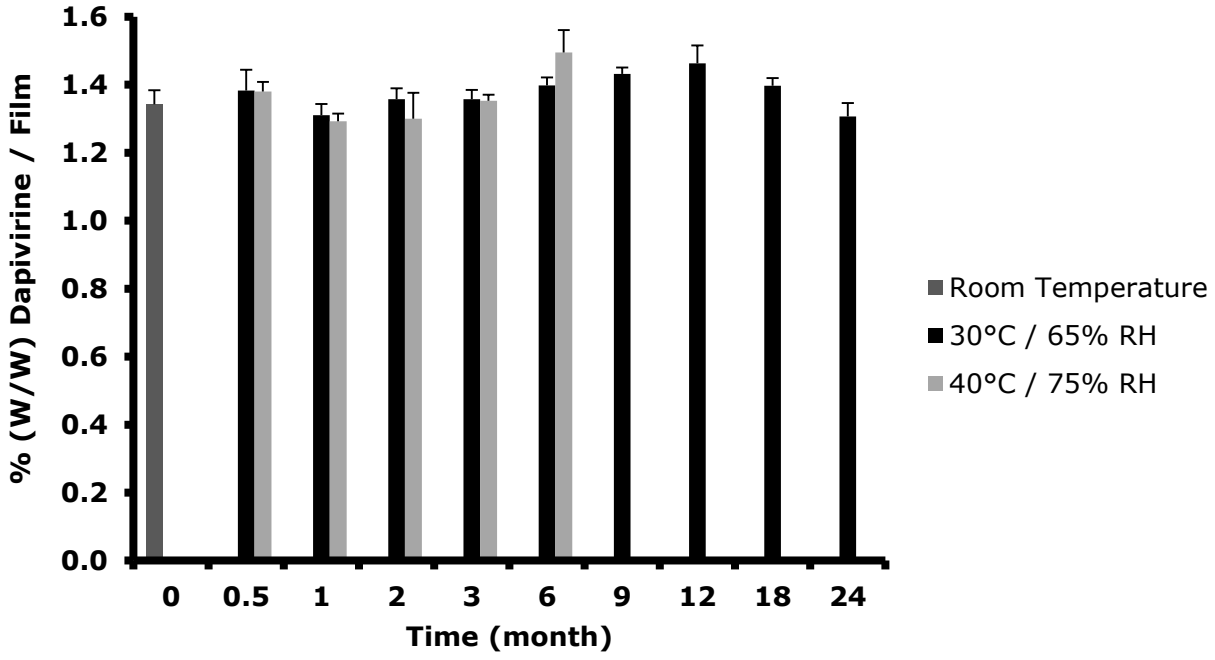

Figure 2.7 Drug content of DPV film (1.25 mg/film) over the time course of stability testing. No change in DPV film content was observed

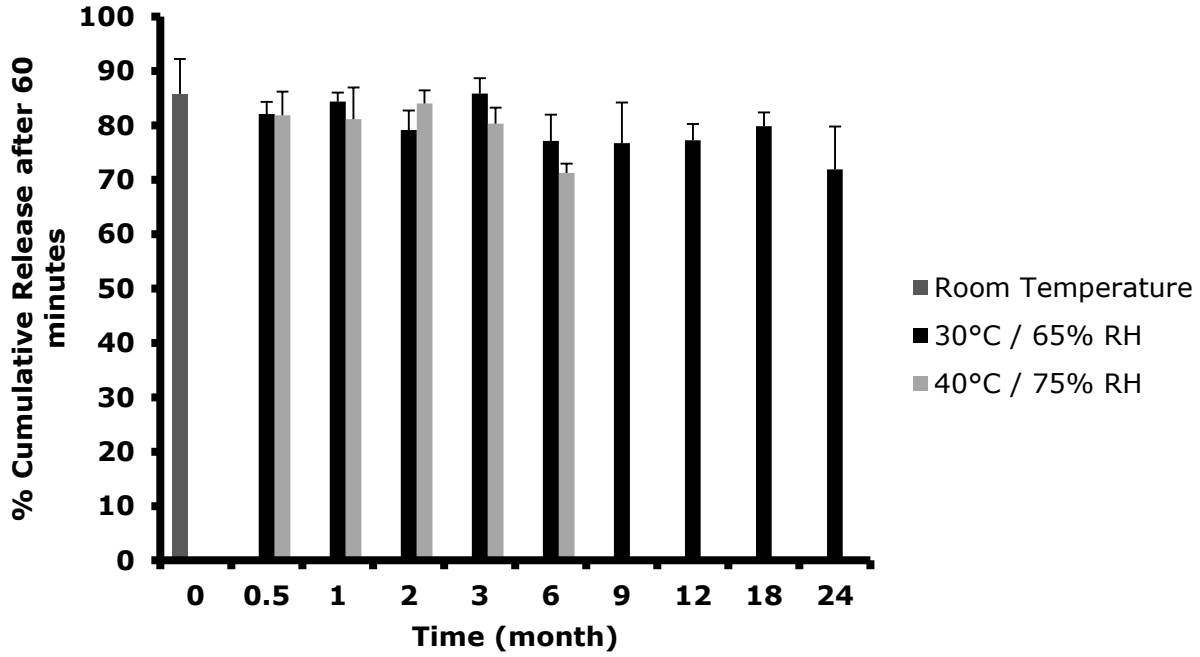

Figure 2.8 Dissolution of DPV film (1.25 mg/film) over the time course of stability testing. No change in DPV % cumulative release at 60 minutes was observed

In addition, the DPV film maintained anti-HIV activity over the stability period in the two conditions. No cellular toxicity was observed at any time point over the stability period as tested in the TZM-bl assay. Furthermore, compatibility with Lactobacillus was monitored throughout the stability assessment period. In the Lactobacillus film compatibility testing no breakdown of the film into toxic products was detected over time evident by the retained Lactobacillus compatibility over the period of stability testing.

2.3.2 Dapivirine Permeability in Human Excised Cervical Tissue

The permeability of DPV into human excised cervical tissue was studied for both film formulated and unformulated DPV in a Franz cell model. Calculated permeability parameters are shown in (Table 2.3). Both drug substance and film formulated DPV were able to permeate through the epithelium of excised human cervical tissue under the experimental conditions described. There was no significant difference observed between permeability coefficients obtained for formulated or unformulated DPV.

Table 2.3 Calculated permeability parameters of DPV drug substance. Apparent permeability values Papp (cm/sec) for DPV drug substance and 1.25 mg DPV film. For each tissue sample, permeability testing was done in triplicate. Values presented as mean ± SD

	1.25 mg Dapivirine film		Dapivirine drug substance	
Tissue #	**Mean**	**SD**	**Mean**	**SD**
1	1.66E-06	6.22E-07	3.66E-06	9.76E-07
2	2.19E-06	2.81E-07	2.1E-06	1.78E-06
3	1.15E-06	2.53E-07	3.04E-06	8.29E-07

2.3.3 *Ex Vivo* Assessment of Anti-HIV Activity

To further confirm the bioactivity of DPV film and ensure that the film formulation did not lead to loss of anti-HIV activity, the bioactivity of DPV film was tested in two *ex vivo* models: an excised human cervical tissue explant model and polarized explant model (PEM). For the explant model tissues were treated with 100 µl of film containing 1.25 mg DPV film dissolved in 2 ml of medium, 100 µl of placebo film dissolved in 2 ml of medium, or left untreated (control). Overall, both DPV and placebo film showed bioactivity with a reduction in HIV-1 p24 levels (Figure 2.9-A). However, all of the explants exposed to the DPV film were protected as compared to 6 of 8 explants protected by the placebo film. These were confirmed by IHC staining for HIV-1 p24 in tissues (Figure 2.9-B). In the PEM model, a piece of cervical tissue is clamped between two chambers mechanically. DPV film was able to block HIV-1 transmission. The number of HIV-1 copies as measured by qRT-PCR in the receptor compartment was below detection limits indicating blocking of HIV-1 transmission in the explant tissue (Table 2.4).

Table 2.4 Effects of 1.25 mg DPV film on HIV-1 transmission in the polarized explant model (PEM)

PEM Sample	HIV donor (copies/ml)	HIV receptor (copies/ml)	HIV explants culture (copies/ml)
No Treatment	2×10^8	9.2×10^2	1.19×10^5
1.25 mg DPV Film	2×10^8	Below Detection	Below Detection

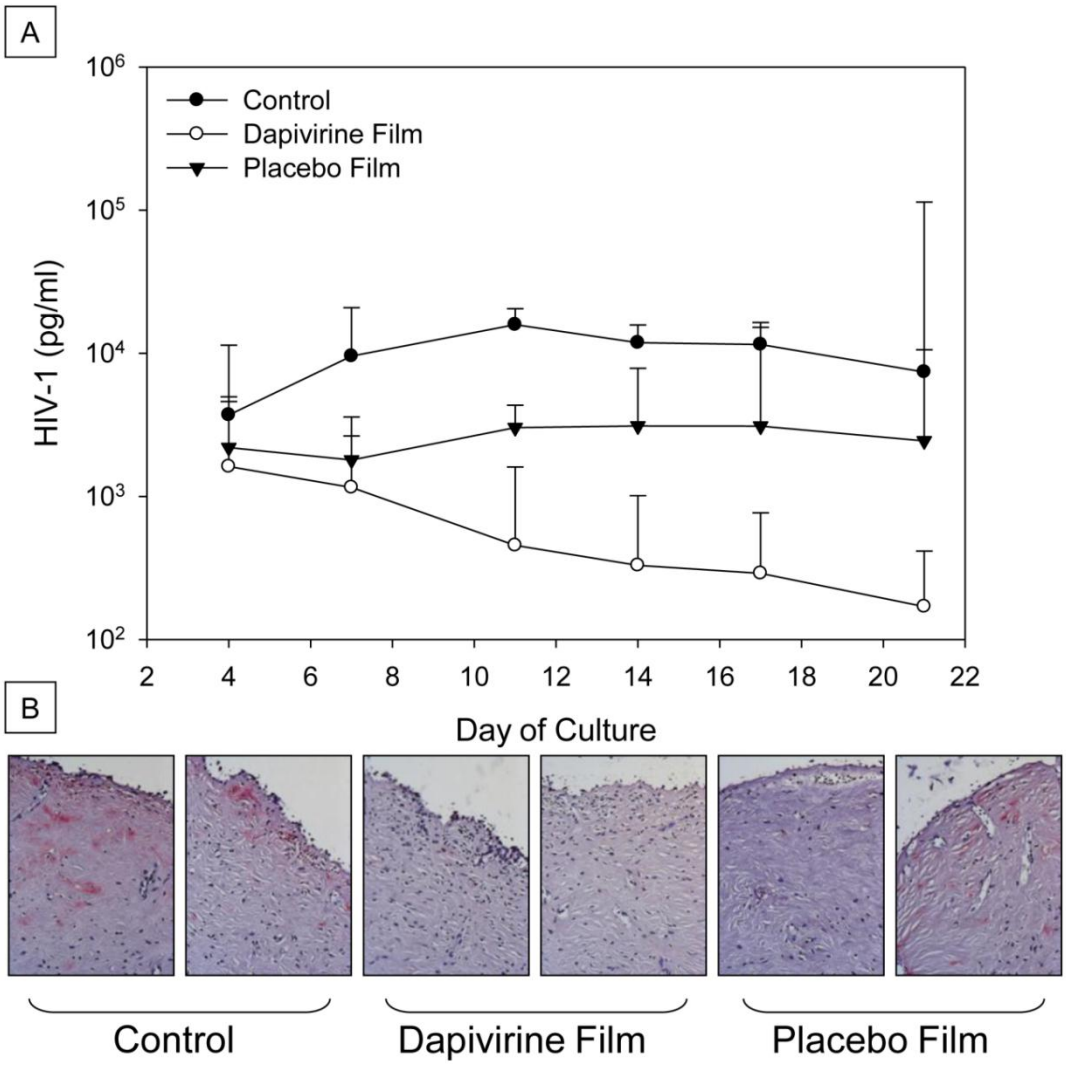

Figure 2.9 Bioactivity of 1.25 mg DPV film when tested in the excised human cervical tissue explant challenge model. P24 staining (in red) is the hallmark for infection. (A) P24 levels over 21 days (B) Representative histology pictures of tissues after 21 day of infection studies

2.4 Discussion and Conclusion

The rational design of a mixture of excipients and active drug agent constitutes a pharmaceutical formulation. Film formulations are composed mainly of a base polymer platform and a plasticizer to provide flexibility to the film. Other excipients that could be used include a humectant to enhance soft texture, and a disintegrant to provide rapid film disintegration once in contact with fluids [115]. Fundamentally, the primary excipient in a film formulation is the film forming polymer(s). Various polymers have been used in vaginal film formulations such as polyvinyl alcohol (PVA) and cellulose derivatives. PVA is the polymer base which is used in several currently marketed vaginal film products. For this reason PVA was used as the main film forming polymer in the DPV film formulation. The use of another polymer was required in order to increase the viscosity of the polymer melt during the manufacturing process. As discussed later in this section higher viscosity was required to provide a uniform dispersion of DPV in the film solution. HPMC was used as a co-polymer. The mixture of PVA/HPMC provided the viscosity necessary for DPV dispersion and produced a smooth and flexible film. Sorbitol, propylene glycol, glycerin and combinations of these were evaluated as plasticizers for the film formulation. It was found that use of glycerin enhanced film flexibility, mechanical properties and also enhanced film softness. Given the hydrophilic nature of the polymeric composition (PVA/HPMC) and the hydrophobic nature of DPV, the drug was incorporated as a dispersion in the film product. Propylene glycol was used to facilitate the dispersion of DPV into the polymeric film. In addition to its role as a dispersing agent, propylene glycol served as a secondary plasticizer in the film. Rapid disintegration was targeted to ensure quick drug release

therefore PEG 8000 was included in the film formulation as a disintegrant. After preparing the film polymeric solution, solvent casting method was used to manufacture the films. Film manufacture involved pouring an 8” film sheet which was then cut into individual 1” x 2” film units. Content uniformity studies were conducted to ensure adequate distribution of drug was achieved both across the film sheet as well as within each individual film.

The initial phase of solvent casting methods for film manufacture involves preparation of a solution (containing the drug and excipients) for casting. Hence it was important to establish that DPV did not have any incompatibility with the excipients of choice in the formulation while in the solution state where degradation is more likely to occur. A DPV excipient compatibility study was conducted with the excipients identified for utilization in the film formulation. DPV showed stability in the excipient mixture for 14 days at three different conditions (30°C, 40°C and 50°C). The compatibility study results assured DPV-excipient compatibility in the solution phase of the manufacturing process.

As rapid film disintegration was targeted to ensure quick drug release, film disintegration and dissolution studies were conducted. Both disintegration and dissolution studies confirmed rapid drug release from the film formulation. The film disintegration rate as observed visually was less than 10 minutes. Dissolution studies showed that >50% of DPV film load is released in less than 10 minutes. However, it should be noted that due to the hydrophobic nature of DPV it was necessary to include cremophor (a nonionic surfactant) in the dissolution medium to maintain sink conditions throughout the dissolution experiment. As a solid dosage form, residual water content is important to determine in order to ensure film structural stability over time. The

amount of residual water in the film should be kept at levels required to maintain film flexibility without compromising the stability of the film. The developed DPV film had water content at levels of lower than 5% of film weight. After establishing acceptable film characteristics the stability of the film was assessed for 24 months at 30°C/65%RH and 6 months at 40°C/75%RH. The data confirmed that the film is stable in terms of physical, chemical and biological activity.

As the film formulation and manufacture process includes mixing and heating, it was important to ensure that the shear and heat stress did not alter the anti-HIV activity of DPV. The anti-HIV activity of DPV films was tested *in vitro* in a HIV-RT assay, cell-based HIV-1 replication assay and TZM-bl assay. In all models used, DPV formulated in the film showed potent anti-HIV activity. Inhibition of cell-based HIV-1 replication was over 200-fold more potent than inhibition of RT activity. This observation is likely due to the hydrophobic nature of DPV. Film dissolution leads to precipitation and substantial loss of bioactivity in pure aqueous media such as that used in the RT assay. However, cells provide a hydrophobic environment in the context of the cell membrane which can aid in sequestration of bioactive drug thereby leading to substantially more potent inhibition in cell-based replication assays. It should be noted that the 1.25 mg DPV film anti-HIV activity was not tested in HIV-RT and cell-based HIV-1 replication assays. However, as stated in the results the 0.5 mg DPV film showed inhibition of HIV-1 in both assays. The 1.25 mg DPV film showed potent activity in the TZM-bl model. Therefore it is to be expected that the 1.25 mg DPV film would show potent inhibition of HIV-1 in the HIV-RT and cell based HIV replication assays. The TZM-bl assay also confirmed that DPV films maintained potent anti-HIV activity.

Dapivirine works by interrupting the viral cycle at the replication step, thus it is desirable that DPV enters the tissue to reach the target cells which lie in the sub-epithelial layers. The stratified multilayer vaginal epithelium may represent a barrier for DPV permeation. For this reason, the permeability of DPV in both the formulated and unformulated state was evaluated using excised cervical tissue in a Franz-cell model to establish the permeability profile for DPV. No significant differences were found between the apparent permeability coefficients obtained for unformulated DPV as compared to those obtained for film formulated DPV. This data suggests that quick disintegration allows for the rapid release of DPV from the film resulting in permeability profiles similar to those for unformulated drug. These studies are important in that they establish the ability of DPV, formulated into a polymeric film, to permeate into the target tissue to be available for inactivation of HIV-1 viral replication. It should be noted that this data also suggests that DPV delivered from a film may lead to systemic absorption. That was observed in clinical studies with DPV vaginal ring and gel. The extent of the systemic absorption of DPV delivered from the film as opposed to the other dosage forms warrants evaluation.

Although permeability studies showed that DPV was able to permeate target tissues, it is important to establish that this results in adequate anti-HIV activity. *Ex vivo* tissue models are widely used to understand HIV-1 transmission and evaluate anti-HIV activity of drug candidates [144]. For this reason, the anti-HIV activity of DPV films was studied in two *ex vivo* tissue models using human cervical explant tissue. The first is the polarized explant model which has been thoroughly described in the literature [36, 53]. This model uses a transwell system in which the tissue is sealed using matrigel establishing the polarization between the apical and basolateral

compartments. DPV film was shown to prevent HIV-1 infection in the tissue and that observation was confirmed with IHC staining for HIV-1 P24 in tissue. Additionally, developed and validated a polarized explant model (PEM) that can be used to study HIV-1 infection of cervical tissue as well as the anti-HIV activity of microbicide product candidates. In this model the tissue is sandwiched and mechanically sealed between a donor and receptor compartments. The mechanical seal provides a more thorough barrier, thus tissue permeation is more specifically confined to the exposed area. DPV film was shown to exhibit inhibition of HIV-1 infection in this model as well.

It is important that a topical microbicide product not to be harmful to the natural vaginal environment. It should not exert any toxic or harmful effects on the natural defenses of the local environment including the microflora, specifically lactobacillus as explained in section 2.3.1.5. DPV film compatibility with lactobacillus was evaluated. No loss of bacterial viability or formation of toxic products was observed. Additionally, no cellular toxicity of the film has been detected in the TZM-bl cell model. These studies provide evidence of safety of the film product.

The choice of a dosage form for topical microbicides is dictated by several factors such as chemical characteristics of the drug candidate and user acceptability and compliance. For topical vaginal microbicides, hydrogels have been the main dosage form being used as they have a history of use in female products and an established user acceptability profile. However, hydrogels are usually associated with drawbacks that could suppress topical microbicide usage and hinder the deployment of topical microbicides because of reduced user acceptability. Thus alternative dosage forms are required. One dosage form option for vaginal delivery of drug

substances is a polymeric film. Films are easy to use, require no applicator, cause no messiness or leakage due to the small dose volume, and are inexpensive to manufacture. Given that microbicide products are specifically being targeted for undeveloped countries such as sub-Saharan Africa where the HIV-1 epidemic is the worst, the economic feasibility of a product is critical to its effectiveness. More importantly, for the delivery of DPV to the tissue as a necessity for its function, polymeric films offer a suitable delivery system for DPV. It is anticipated that the rapid release and small volume of the film would create *in vivo* a high concentration gradient of DPV in the vaginal lumen which would drive DPV permeation into the mucosal tissue.

We presented in this chapter, the feasibility of formulating a hydrophobic anti-HIV drug (dapivirine) into a water soluble polymeric film. DPV was successfully formulated as a vaginal film which provides rapid drug release, lacks toxicity to the innate microflora *lactobacilli* and maintains stability and bioactivity for 24 months. The film capacity to provide protection from HIV-1 infection was established in *in vitro* and *ex vivo* evaluations. This film product was available for studies towards understanding the role of drug release on tissue accumulation comparing single entity and combination film products. Particularly, the film developed in this chapter was used to elucidate mechanisms of DPV accumulation and distribution in human ecto-cervical tissue (chapter 4). Given the high potency of DPV combined with advantages of the film dosage form, it will be valuable to evaluate this promising drug/delivery system in the clinic.

Acknowledgment

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The data presented was published as a research article in "Drug Delivery and Translational Research" journal, an official journal of the Controlled Release Society [145]. Citation of the article is:

"Akil, A., et al., Development and characterization of a vaginal film containing dapivirine, a non-nucleoside reverse transcriptase inhibitor (NNRTI), for prevention of HIV-1 sexual transmission. Drug Delivery and Translational Research, 2011. 1(3): p. 209-222."

3. DEVELOPMENT AND EVALUATION OF DAPIVIRINE/TENOFOVIR COMBINATION VAGINAL FILM

3.1 Introduction

The search for an effective topical microbicide for prevention of HIV-1 sexual transmission is well underway. Research on strategies and approaches to enhance the efficacy of topical microbicides is warranted. In pursuit of that, researchers have turned to antiretroviral (ARV) combinations as an answer. The great degree of success associated with highly antiretroviral active therapy (HAART) in reducing HIV infection progression and mortality rate [146-148] has inspired research in the area of topical microbicides to include combination products. In addition to improved efficacy, benefits of combination microbicides include: increased barrier to infection, fewer chances of viral resistance, and possible reduction in the effective dose of each drug which could theoretically reduce the potential for toxicity [77]. A number of published scientific reports evaluated and demonstrated the benefits of different combinations of anti-HIV drug candidates for use in topical microbicides. Examples and details of those reports are discussed in chapter 1.

Tenofovir (TFV) and dapivirine (DPV) are the two most advanced ARVs in topical microbicide clinical trials [43, 149]. DPV and TFV both are reverse transcriptase inhibitors but have different mechanisms of action. TFV is a nucleotide analog (NtRTI) whereas DPV is a tight binding non-nucleoside molecule (NNRTI) [47]. During transcription of viral DNA, when taken up, TFV is unable of forming the ester bond with the next nucleotide/nucleoside and thus

terminates the reverse transcription process. Whereas DPV binds to hydrophobic pocket on the viral reverse transcriptase enzyme preventing conformational changes required for enzymatic activity resulting in abrogation of the replication process. In combination TFV and DPV have been shown to exert additive effects against wild type HIV-1 and synergistic effects against NNRTI resistant HIV-1 [89]. As the two most studied and leading ARVs in topical microbicide clinical trials, combined with the impressive profile of their anti-HIV activity and the benefit of their combination, the assessment of the development of a TFV and DPV combination as a topical microbicide product is warranted.

The mechanism of action of TFV and DPV demands that both be present in the target tissue. Drug delivery systems used for TFV and DPV alone or in combination must be able to deliver the drugs to the tissue. Polymeric vaginal films present a promising drug delivery system for the combination of TFV and DPV. The low volume of films coupled with a designed rapid drug release profile allow for high concentration gradient formation which is the force underlying drug permeation into the tissue. Additionally, films have advantages such as accurate dosing and portability [5, 105, 109]. Acceptability studies with vaginal films showed that women preferred films over other vaginal dosage forms [110-114]. Vaginal films have been implemented as a dosage form for a variety of drugs [117-119, 121, 124-126, 150]. Studies in chapter 2 describe a vaginal film containing DPV which was developed and shown to be effective in blocking HIV-1 infection in both *in vitro* and *ex vivo* studies.

The studies in this chapter were aimed toward the development of a vaginal film containing the combination of TFV and DPV. Formulation development, characterization and product stability testing are described for the combination product.

3.2 Methods

3.2.1 Drug-Drug Compatibility

The compatibility of TFV and DPV was evaluated in the solid and liquid state. For evaluations of compatibility in the solid state, 1.25 mg DPV and 40 mg TFV were mixed in a glass vial. Vials were then stored for 12 weeks at three conditions: 25°C, 40°C and 65°C. For evaluations of compatibility in the liquid state, a bulk solution containing TFV and DPV (TFV/DPV ratio of 32:1) was prepared using 50% acetonitrile. The bulk solution was then dispensed into glass vials which were stored for 12 weeks at the same conditions used for solid state samples.

The concentration of each drug in the samples was monitored over time to assess compatibility. Solid state samples were dissolved in 50% acetonitrile and then analyzed using ultra-high performance liquid chromatography (UHPLC) whereas liquid state samples were analyzed by UHPLC without any processing. The UHPLC method for TFV determination utilized a reversed phase column (Acquity BEH C18 1.7 µm, 2.1×50 mm) with an isocratic mobile phase system composed of 10 mM dibasic potassium phosphate KH_2PO_4 and 2 mM t-Butylammonium bisulfate (tBAHS), (pH 5.7) : Methanol (90:10). TFV is detected by UV spectrometer at 210 nm. The UHPLC method for DPV determination utilized a reversed phase

column (Acquity BEH C18 1.7 µm, 2.1×50 mm) with a mobile phase system composed of 0.08% triflouro acetic acid (TFA) in water and 0.05% TFA in acetonitrile. A gradient method was used for drug elution where % of mobile phase (B) changed from 10 – 80 – 10 over 15 minutes run time. DPV is detected by UV spectrometer at 290 nm.

3.2.2 Solid Phase Solubility

To investigate the ability of polymers to inhibit TFV crystal formation a solid phase solubility study was conducted. Different polymer/drug ratios were tested by mixing TFV with varied polymer amounts. The polymer/drug ratio studied were 2:1, 4:1 and 6:1. A 1% (w/v) TFV solution in water was prepared by dissolving 0.05 g in 5 mL water (pH>4.5 using sodium hydroxide). Various polymers were added to the drug solution to achieve the specified range of polymer/drug ratio. Polymers screened were: sodium carboxy methyl cellulose (Na CMC), hydroxypropyl methyl cellulose (HPMC), hydroxyl ethyl cellulose (HEC), polyvinyl alcohol (PVA), and polyvinyl pyrrolidone (PVP). The amounts of the polymer added were 0.1, 0.2 and 0.3 g. Once solutions were made, they were spread onto a glass slide and allowed to dry overnight at room temperature leaving a film layer on the glass slide. The film layer was then examined with light microscopy for visual detection of crystal growth.

3.2.3 Film Formulation

The combination film formulation was composed of hydroxypropyl methyl cellulose (HPMC), hydroxyethyl cellulose (HEC), carboxymethyl cellulose sodium (Na CMC) and glycerin. Solvent casting methods were used for film manufacture. Briefly, an aqueous film solution containing the excipients and both drugs (TFV and DPV) was prepared. The solution was cast onto a polyester substrate attached to the hot surface of an automatic film applicator (Elcometer® 4340) using a doctor blade. The thickness of the blade was set to 115 µm. The film sheet was allowed to dry for 15 minutes at 70°C before it was removed from the substrate. Once film sheets were obtained they were cut using a die press into 2" x 2" individual units.

3.2.4 Physical and Chemical Characterization

Film weight, thickness and appearance were recorded. Tensile strength as a measure of film mechanical strength was assessed using a texture analyzer (TA-XT.Plus) connected to a data acquisition and analysis software. Puncture strength was calculated using the following equation: Puncture strength = Force (g) / Cross sectional area of the film (cm^2). Residual water content of the films was measured using Karl-Fisher apparatus (Metrohm, 758 KFD Titrino).

Drug content of the film was determined by means of ultra-high performance liquid chromatography (UHPLC). The film unit was dissolved in 40 mL 50% acetonitrile solution. After vortexing for 10 min, the solution was analyzed using UHPLC for determination of DPV content. An aliquot of the solution was diluted 20 times with 5% acetonitrile and analyzed using UHPLC for TFV content. The UHLPC methods used are described in the section 3.2.1.

Dissolution of the combination film was evaluated using class IV USP apparatus (SOTAX CP7) connected to a fraction collector. A 100 mL of 1% cremephor in water was used as the dissolution medium. The flow rate was set to 16 mL/min and the temperature was 37°C. Samples were collected at predetermined time points over 60 min run time. TFV and DPV content in the samples was determined by UHPLC methods described in the section 3.2.1.

3.2.5 Anti-HIV Activity

Anti HIV activity was studies using a TZM-bl cell based model as described in chapter 1 (section 2.2.7.2).

3.2.6 Compatibility with Lactobacillus

Compatibility with Lactobacillus was evaluated as per methods described in chapter 1 (section 2.2.5).

3.2.7 Stability Assessment

The stability of the TFV/DPV film was assessed for 12 months at 25°C / 60% RH and 6 months at 40°C / 75% RH. Testing of the film was conducted at 1, 2, 3, 6 and 12 months. At each time point weight, thickness, appearance, puncture strength, water content, and drug content were tested. In addition, disintegration, film dissolution, Lactobacillus compatibility and *in vitro* anti-HIV activity were tested.

3.3 Results

3.3.1 Tenofovir – Dapivirine Compatibility

The chemical compatibility of TFV and DPV was evaluated in solid and liquid states to ensure that the combination of these drugs can be formulated in one dosage form. The study was conducted at room temperature and stressed conditions in terms of temperature and humidity. Stability of the drugs as an indicator of compatibility was assessed by monitoring of drug content of both drugs over 12 weeks. As data showed, there was no significant loss of TFV (Figure 3.1) or DPV (Figure 3.2) observed in any sample in either solid or liquid states when exposed to the conditions tested. Results showed the amount of TFV and DPV to remain stable over time.

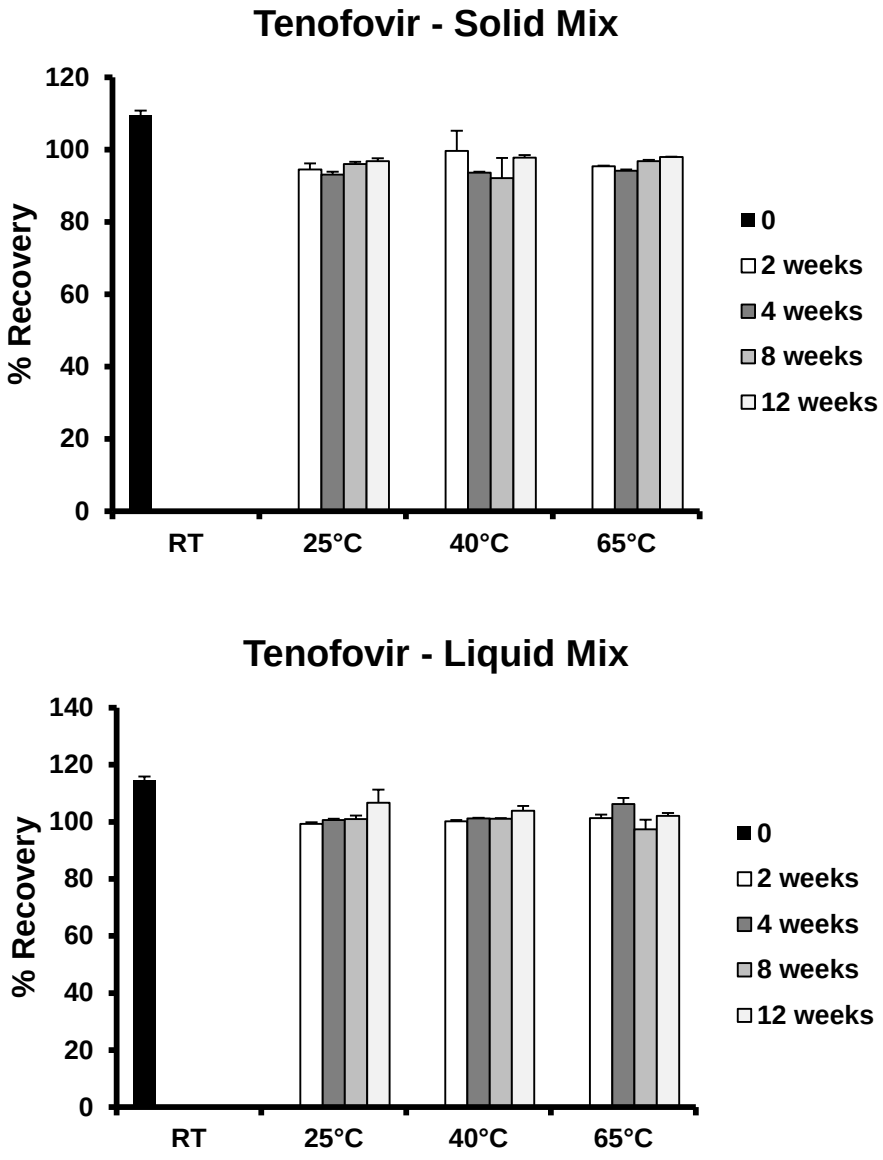

Figure 3.1 TFV stability in solid and liquid mix samples. Stability was ensured by comparing the % TFV recovered to the original TFV amount added to the sample

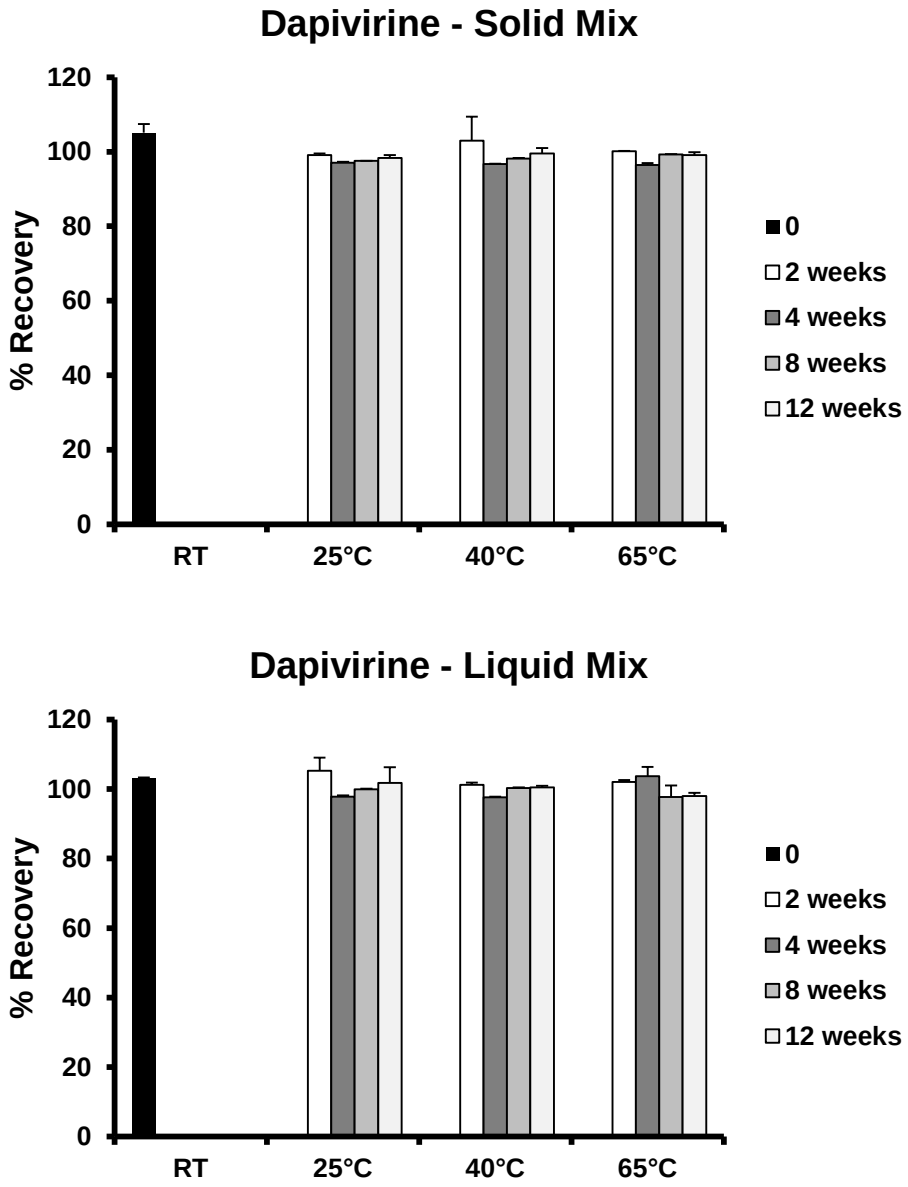

Figure 3.2 DPV stability in solid and liquid mix samples. Stability was ensured by comparing the % DPV recovered to the original DPV amount added to the sample

3.3.2 TFV/DPV Film Formulation Development

The target loading dose for each drug candidate was: 1.25 and 40 mg per film unit (2”x2”) for DPV and TFV respectively. Target loading dose levels were based on the current dosing levels used clinically in the respective vaginal gel product for each drug [55, 58, 73, 74]. However at the 40 mg level, TFV was shown to exhibit crystallization in the film over time. It was found that 20 mg TFV could be formulated into a film without crystallization. To address the crystallization issue, film forming polymer in the formulation was chosen based on evaluation of the polymers ability to prevent TFV crystallization. Polymer choice also considered the film desired appearance and physical characteristics (such as peelability, flexibility, and brittleness).

To identify the appropriate polymer(s) choice for prevention of TFV crystallization a solid phase solubility study was conducted. Six polymer types at varying concentrations were mixed with TFV in solution. Different polymer/drug ratios were tested to study the impact of increasing polymer concentration on the inhibition of TFV crystallization. Light microscopy was used to monitor crystallization. The results of the study are shown in table 3.1. Na CMC was shown to inhibit TFV crystallization at all concentrations tested. PVA was able to inhibit TFV crystallization when used at higher concentration. Other polymers tested showed inhibition only at the highest concentration tested with the exception of PVP-K30 which resulted in crystallization at all concentrations tested. Figure 3.3 provides example images from the microscopic evaluations performed in the solid phase solubility study.

Table 3.1 A solid phase solubility study of TFV in several potential film forming polymers was conducted to determine the optimal polymer for use in TFV film formulation

Polymer	Polymer/Drug Ratio	TFV
Na CMC (Sodium carboxy methyl cellulose)	2	No crystal
	4	No crystal
	6	No crystal
HPMC (hydroxypropyl methyl cellulose)	2	Crystal
	4	Crystal
	6	No crystal
HEC (hydroxyl ethyl cellulose)	2	Crystal
	4	Crystal
	6	No crystal
PVA (polyvinyl alcohol)	2	Crystal
	4	No crystal
	6	No crystal
PVP-K90 (polyvinyl pyrrolidone)	2	Crystal
	4	Crystal
	6	No crystal
PVP-K30 (polyvinyl pyrrolidone)	2	Crystal
	4	Crystal
	6	Crystal

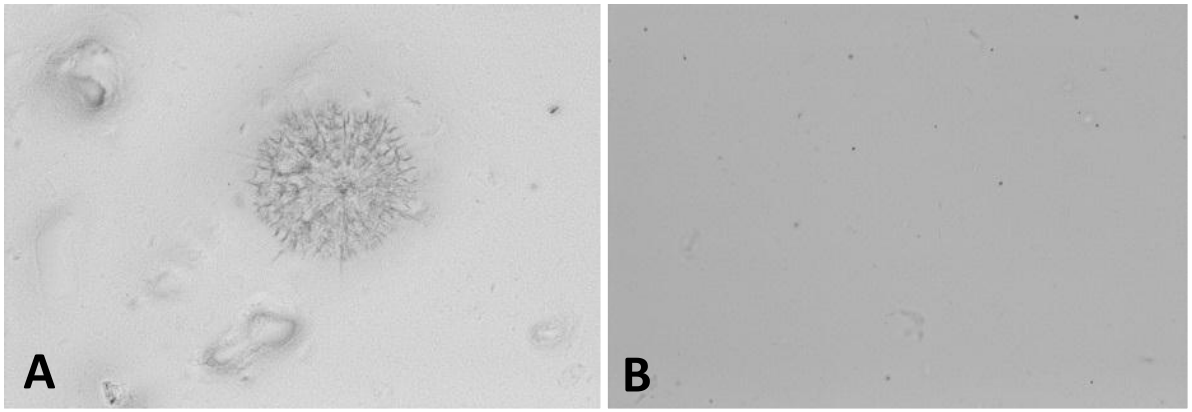

Figure 3.3 Example of microscopic results from the solid phase solubility study. A polymer/drug ratio of 2 (HPMC/TFV) did not prevent crystal growth (A) whereas a ratio of 6 (HPMC/TFV) showed no crystal growth in the film layer (B)

The data generated from the solid phase solubility study was used to direct polymer excipient choice for the TFV/DPV film formulation. Different combinations of the polymers selected were used to make multiple film formulations. The prototype films were exposed to stress conditions (temperature of 50°C) for 15 days to monitor TFV crystallization. A formulation containing the combination of 5% HPMC, 2% Na CMC, and 5% HEC with 2% glycerin as a plasticizer showed no TFV crystallization in the film for 15 days at 50°C.

3.3.3 Physical – Chemical Characterization

The developed combination film was characterized in terms of chemical, physical and biological attributes. Table 3.2 shows the physical and chemical characteristics obtained for the optimized TFV/DPV film formulation.

Table 3.2 Physical and chemical characteristics of TFV/DPV film

Parameter	TFV/DPV Film	
Weight (mg)	312.43 ± 6.89 (n=12)	
Thickness (um)	90 ± 10 (n=12)	
% Water Content (w/w)	9.14 ± 0.64 (n=3)	
Puncture Strength (g/cm^2)	27.09 ± 2.22 (n=3)	
% Drug Content (w/w)	**TFV**	7.18 ± 0.24 (n=3)
	DPV	0.396 ± 0.01 (n=3)

Analysis by means of UHPLC showed that the film's drug content was accurate when compared to the theoretical target dose for each drug. TFV loading accuracy in the film was 111.15 % in whereas DPV loading accuracy was 98.40 % of the theoretical target dose.

The dissolution of TFV/DPV film was assessed using a USP class 4 flow through system. 1% cremophor in water was used as the dissolution medium to provide sink conditions for both TFV and DPV. Results showed that the release of TFV and DPV from the film was rapid. Figure 3.4 shows the release profile of DPV and TFV from the combination film. The majority of the

loaded TFV was released within 30 min with 96.01 ± 1.08 % released whereas only 51.32 ± 7.19 % DPV was released within the same time frame. TFV solubility in the medium was measured to be ~7.5 mg/mL whereas DPV solubility in the medium is ~40 μg/mL. This difference in solubility explains the more rapid TFV release from the film compared to the release of DPV from the same film.

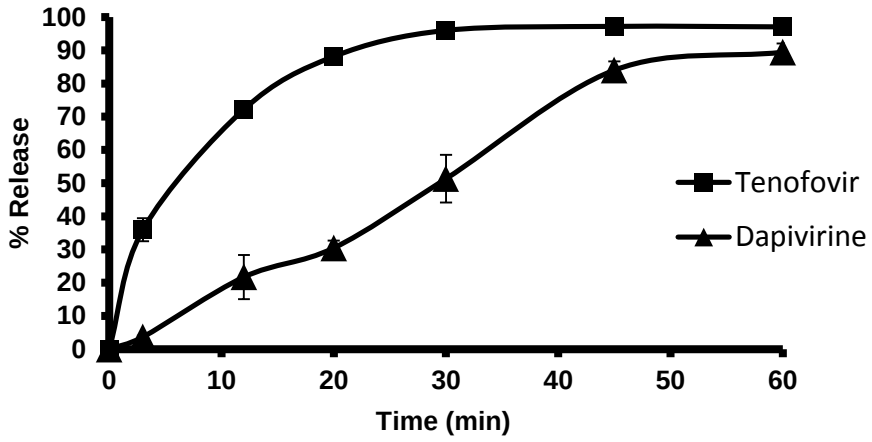

Figure 3.4 TFV/DPV film dissolution as tested in class IV USP apparatus

3.3.4 Anti-HIV Activity

TFV/DPV film anti-HIV activity was assessed *in vitro* using a TZM-bl cell based model. The film showed potent anti-HIV activity. HIV-1 infection inhibition was evaluated for the combination film and compared to its respective single entity film. TFV EC_{50} in the combination

film was found to be 550 nM whereas the single entity TFV film had an EC_{50} value of 6170 nM. The EC_{50} for DPV was about 30 nM in both the combination and DPV film (Figure 3.5). There was no loss in cell viability as a result of cells exposure to the combination film.

3.3.5 Compatibility with Lactobacillus

The compatibility of the combination film with Lactobacillus was assessed using the Standard Microbicide Safety Test (SMST). The film did not exhibit toxic effect on several strains of Lactobacillus as no loss of bacteria viability was observed (Table 3.3).

Table 3.3 Compatibility of TFV/DPV film with Lactobacillus. After 30 min incubation of the bacteria with the films no loss of growth was observed indicating no harmful impact of the film on the bacteria

Lactobacillus strain tested	Log Difference (T30 minute Plate Count - T0 minute Plate Count)
L. crispatus ATCC 33197	0.125
L. jensenii ATCC 25258	-0.085
L. jensenii LBP 28Ab	-0.150

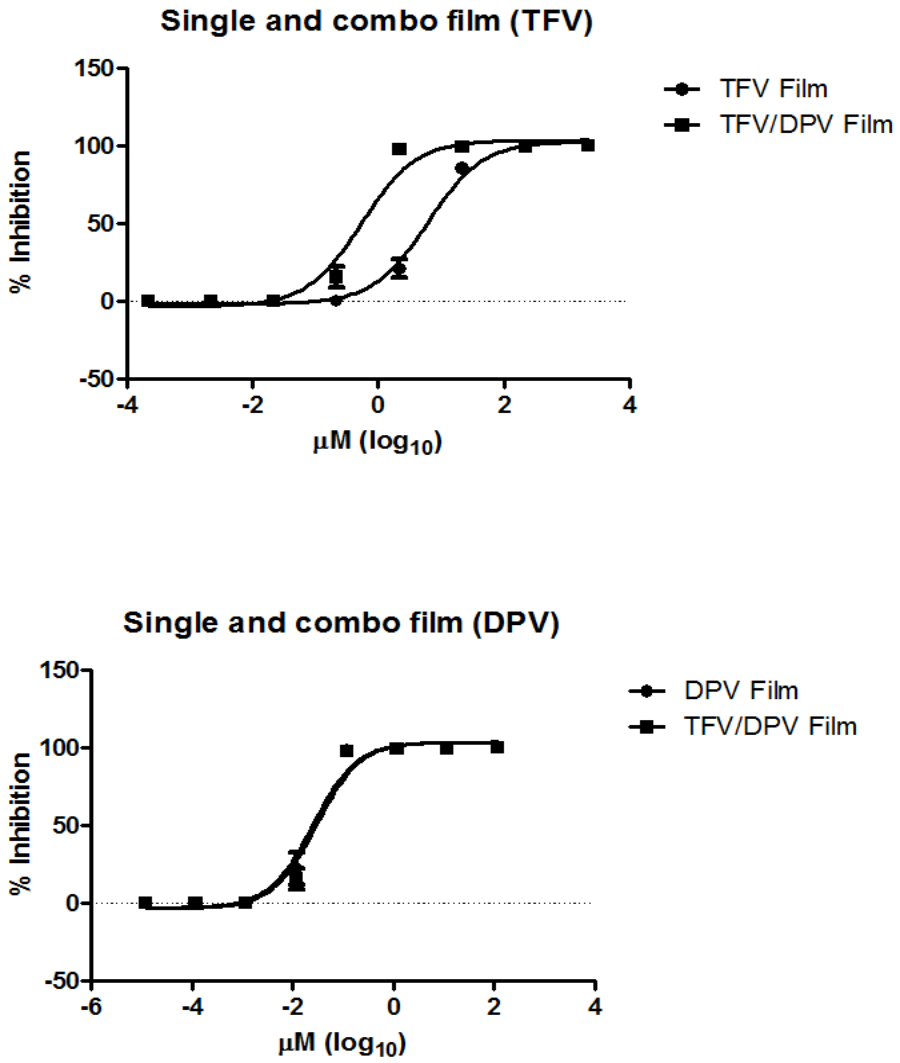

Figure 3.5 Anti-HIV activity of TFV and DPV in single entity and combination films as tested in TZM-bl cell based model

3.3.6 Stability Assessment

The stability of the developed TFV/DPV film was evaluated for 6 months at 40°C/75% RH and 12 months at 25°C/60% RH. In addition to weight, thickness, water content, and puncture strength, film dissolution, drug content, compatibility with Lactobacillus and anti-HIV activity were evaluated. Figure 3.6 shows the drug content results for DPV and TFV during the duration of the stability study. There was no difference in TFV and DPV content in the film over the stability period tested confirming that both TFV and DPV were stable in the film. *In vitro* ant-HIV activity testing did confirm as well that the film maintained its anti-HIV activity over 6 months as was indicated by their EC_{50} values (Table 3.4). Results showed that all other parameters tested for the film were stable throughout the stability study.

Table 3.4 Anti-HIV activity of TFV and DPV in the combination film over 6 months stability monitoring

		Tenofovir EC_{50} (µM)	Dapivirine EC_{50} (nM)
Time Zero		0.55	29.18
3 months	25°C/60%RH	1.41	76.42
	40°C/75%RH	1.26	68.19
6 months	25°C/60%RH	0.27	14.53
	40°C/75%RH	0.35	18.89

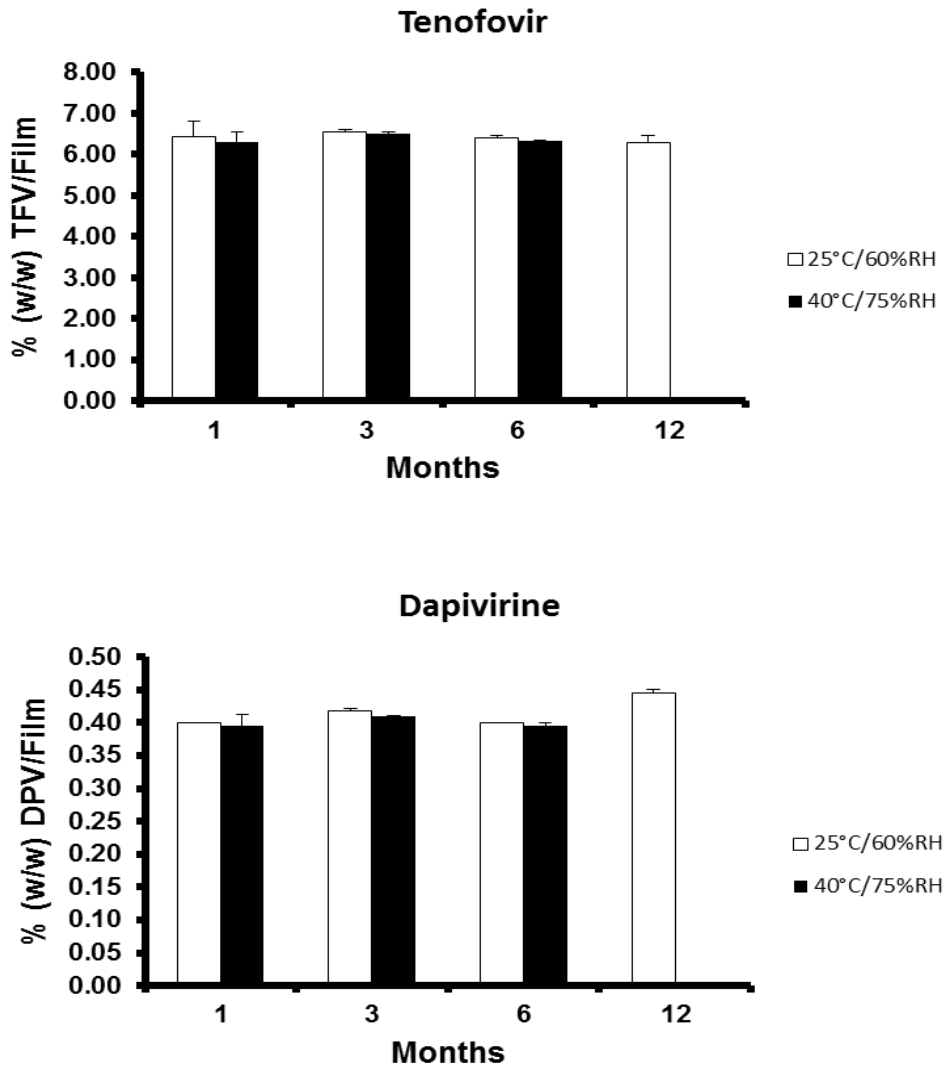

Figure 3.6 Drug content results of TFV/DPV film over 12 months of stability monitoring

3.4 Discussion and Conclusion

The development of a topical microbicide containing a combination of ARVs entails an added layer of complexity in order to incorporate two active agents into one dosage form. For a combination product to be successfully formulated it is important first to assess the chemical compatibility of the drug candidates with each other. Such information is important to establish that the two active agents can be formulated into the same dosage form. Therefore to formulate TFV and DPV into a polymeric film the compatibility of both drug candidates with each other was evaluated. The stability of the two drug candidates in both the solid and liquid state was monitored at different conditions by measuring the DPV and TFV content in the samples over 12 weeks. Data showed that TFV and DPV are chemically compatible with each other as their drug content in solid and liquid state samples was stable at all conditions tested. No degradation products were identified from the chromatograms at any time point evaluated.

A typical vaginal film formulation is composed of film forming polymer(s), plasticizer, and API [115]. Polymer choice is critical to film attributes and properties. Most importantly, excipients should be chosen which maintain the physical and/or chemical stability of the active agent. The formulation of DPV and TFV combination into a single film product faced challenges given the opposing solubility of these two drug candidates. Because of the hydrophilic nature of TFV, it was solubilized in the hydrophilic polymers of the film whereas the hydrophobic drug DPV remained dispersed in the solid state. However, after solvent casting the amount of water (solvent) in the film decreases drastically thus potentially causing over-saturation of TFV solubility in the film matrix that could cause TFV to precipitate out. TFV precipitation could lead

to drug crystallization in the film. For that reason, the polymer selection process was based primarily on the ability of each individual polymer to prevent TFV crystallization. Several polymers used in film formulation showed ability to prevent TFV crystallization at different polymer concentrations. Sodium CMC showed complete inhibition of TFV crystallization at all concentrations tested. Other polymers prevented TFV crystal growth at the higher concentrations included HEC, HPMC and PVA. The ability of a polymer to inhibit API crystallization is dependent on the magnitude of its interaction with the active agent [151, 152]. TFV is a weak acid and its solubility in water is >150 mg/mL when pH is greater than 4.5. At that pH level sodium CMC works as a weak base which allows for acid-base bond formation between the polymer and TFV. Hydrogen bonding is another possible mechanism by which TFV can interact with the polymers screened. CMC, HEC, HPMC and PVA all are capable of hydrogen bonding. The interaction between TFV and the polymers can lead to the formation of a boundary layer in which the polymer accumulates on the surface of TFV preventing aggregation and crystallization.

The developed TFV/DPV film was characterized in terms of its physical, chemical and biological properties. The characterization of the film included: weight, thickness, residual water content and puncture strength. The residual water content of the film is an important parameter. The residual water maintains polymers hydration and network formation thus consequently plays a role in the films physical appearance and attributes. Therefore in the TFV/DPV film developed the residual water content was kept at levels required for polymer hydration. The residual water content of both films was less than 10% of film weight which maintained good film flexibility.

Films with residual water content of more than 10% failed to stabilize TFV and prevent crystal growth. The mechanical property of puncture strength was measured for the TFV/DPV film. The puncture strength exhibited by both films was deemed appropriate for films to handle stress. The film was flexible and able to withstand folding induced stress without breaking.

Dissolution testing of TFV/DPV film showed that greater than 50% of the TFV and DPV loading levels were released within 30 minutes. In molar values, by 30 min, ~ 60 μM TFV and ~ 1.9 μM DPV was released from the film. The released amounts of both drugs are at levels which are much higher than the reported *in vitro* IC_{50} for TFV and DPV (~2 μM for TFV and less than 10 nM for DPV) [64, 153].

The anti-HIV activity of TFV and DPV was evaluated in a TZM-bl cell based model after film formulation to ensure that the formulation or manufacturing did not lead to loss of activity. The film showed potent anti-HIV activity indicating that TFV and DPV activity was not altered due to formulation. It is noteworthy to point out that TFV exhibited more potent anti-HIV activity in the combination film as compared to the single entity film illustrated by the decrease in EC_{50} value for TFV in the combination film. It is likely that this observation is due to the presence of DPV which has significantly greater anti-HIV activity than TFV. A topical microbicide product should not be toxic to the vaginal environment. In the TZM-bl cell model, the TFV/DPV film did not alter cell viability indicating no toxicity towards the cells. Additionally, a compatibility assessment of the film with Lactobacillus was conducted. The results showed no sign of any toxic effects of the film on the Lactobacillus. The stability of the

TFV/DPV film was evaluated for 12 months. Data collected confirmed that the film is stable as no significant changes in its physical, chemical or anti-HIV activity were observed.

The work presented in this chapter showed that a vaginal film containing a combination of TFV/DPV was successfully developed. The thorough *in vitro* testing showed that the film has acceptable characteristics and exerts potent anti-HIV activity with no sign of toxic impact on the major components of the normal vaginal flora. This developed film will be used in studies conducted chapter 4 towards understanding the impact of co-delivery of TFV and DPV on tissue accumulation and distribution.

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4. THE IMPACT OF DAPIVIRINE AND TENOFOVIR CO-DELIVERY ON THEIR TISSUE ACCUMMULATION AND DISTRIBUTION IN HUMAN ECTO-CERVICAL TISSUE

4.1 Introduction

As reverse transcriptase inhibitors, the mechanism of action of DPV and TFV demands their presence in the mucosal tissue or cells. From a drug delivery stand point, the efficacy of a topical microbicide containing DPV and/or TFV depends in part on the ability to deliver the drugs into the mucosal tissue. The relationship of cellular or tissue concentration to product efficacy was illustrated in the pharmacokinetic studies with TFV vaginal gel discussed in section 1.4.3. The studies conducted in chapter 3 resulted in the development of a polymeric film containing a combination of both DPV and TFV. As a solid dosage form films have a small volume which minimizes the dilution effect with vaginal administration *in vivo*. Theoretically, that would create a higher concentration of DPV and TFV upon release from the film on the mucosal tissue surface which would drive tissue permeation by diffusion of the film load of TFV and DPV. However, an understanding of the impact of the co-delivery of both drugs on their tissue accumulation is needed.

Multiple pre-clinical and clinical studies established that DPV permeates and accumulates in various cells and tissues. DPV was shown to permeate and accumulate in a HEC-1A cell line [128]. After 1 hour incubation period, 56% of loaded DPV was present inside the cells. Similar results were obtained by das Neves et al. where free DPV was shown to permeate

and accumulate in a CaSki cell monolayer and pig vaginal mucosa after a 4 hour exposure period [154]. Nanoparticle formulations were found to alter the permeability and retention of DPV in the two models. Several clinical pharmacokinetic studies with DPV microbicide gel and ring showed that DPV accumulates locally in the female lower genital mucosa [71, 73-76]. In two phase I clinical studies, DPV vaginal rings (200 and 25 mg DPV) applied for 7 days delivered DPV to the lower genital tract mucosal tissue while exhibiting low systemic absorption [75]. The mean concentration of DPV in vaginal and cervical tissue biopsies was found to be higher for the 25 mg DPV ring (1.5-3.5 μg/g) than for the 200 mg DPV ring (0.3-0.7 μg/g). In another phase I clinical study, DPV vaginal gels (0.001, 0.005 and 0.02%) applied for 10 consecutive days showed demonstrated low systemic absorption and good distribution in the lower genital tract as analysis of all biopsies taken from the vagina and cervix showed presence of DPV [73].

Evidence also exists for permeation of TFV into mucosal tissue. Animal studies and human clinical trials have demonstrated that TFV delivered in a gel dosage form permeated and accumulated in tissues of the lower genital tract. A pharmacokinetic study conducted in rabbits showed that a once daily application of a 1% TFV gel led to high TFV accumulation in vaginal fluid and tissue [155]. In macaques, vaginal application of TFV gel resulted in rapid TFV distribution in cervix and vagina [156]. In animals exposed to a 1% TFV gel, drug concentration in tissue remained detectable for 24 hours after dosing. This macaque data was confirmed in human clinical trials where data showed that a single dose and multiple doses of a 1% TFV vaginal gel exposure resulted in high genital tract concentrations for at least 24 hours post-dose with minimal systemic absorption [157]. Tenofovir diphosphate (TFV-DP), the active

metabolite, was detectable in approximately 40% of all tissue samples collected in the study. In a separate clinical study, vaginal dosing of 1% TFV gel (40 mg per dose) was shown to be superior to oral dosing of TFV (300 mg per tablet) in terms of accumulation of TFV in lower genital tract tissue [158]. TFV-DP in vaginal tissue was quantifiable in ≥90% of women following vaginal dosing. Only 19% of vaginal tissue biopsies obtained from women following oral dosing were shown to have quantifiable TFV-DP. Additionally, TFV-DP in vaginal tissue was ≥130 fold higher with vaginal dosing compared to oral dosing.

In each of the studies described above either TFV or DPV was evaluated as a single entity product. To advance a film containing DPV and TFV to the clinic it is important to establish the ability of the film to deliver both agents to the target tissue and understand the impact of their co-delivery on their tissue accumulation. Therefore, the aim of the studies conducted in chapter 4 was to evaluate DPV and TFV tissue accumulation and distribution in human ecto-cervical tissue after tissue exposure to a vaginal film containing DPV and TFV.

4.2 Methods

4.2.1 Tissue Exposure Studies

Human ecto-cervical tissue was obtained from University of Pittsburgh Health Sciences Tissue Bank under IRB protocol PRO09110431. Tissue samples were from healthy volunteers undergoing routine hysterectomy for non-cervical reasons.

4.2.1.1 Tenofovir/Dapivirine Vaginal Film

In these studies flow through diffusion cells were used. Excised human ecto-cervical tissue was placed in between donor and receptor compartments. Before tissue placement excess stromal tissue was removed using a Thomas Stadie Riggs tissue slicer. The test article introduced into the donor compartment was a single 6mm biopsy punch of TFV/DPV (20/1.25 mg) film, DPV (1.25 mg) film or TFV (20 mg) film with 450 µL vaginal fluid stimulant (VFS). The receptor medium used was Dulbecco's Modified Eagle Medium (DMEM) at a flow rate of 50 µL/min. Excised human ecto-cervical tissue was exposed to each film for 6 hours and the cells were water jacketed to maintain a temperature of 37°C. 3 mL receptor sample was collected throughout the experiment every hour for 6 hours. After the exposure period, the tissue was cut in half. One half was processed for histological evaluation and epithelial thickness measurement whereas the second half was used for the determination of TFV and DPV concentration by LC/MS/MS. The tissues used for determination of DPV and TFV content were embedded into blocks using Optimal Cutting Temperature (O.C.T) compound and sectioned by cryostat. Fifteen (20 µm) sections were cut followed by cutting of (200 µm) sections for the remainder of the tissue.

4.2.1.2 Dapivirine Vaginal Film

In these studies Franz cells were used where excised human ecto-cervical tissue was placed between the donor and receptor compartments. Before tissue placement excess stromal tissue was removed using a Thomas Stadie Riggs tissue slicer. The receptor medium used was vaginal fluid simulant (VFS) at (pH=4.2). Four different product application scenarios were modeled by altering the test article introduced into the donor compartment as follows:

Test article 1- Single 6mm biopsy punch of DPV (1.25 mg) film + 450 µL VFS

Test article 2- Double 6mm biopsy punches of DPV (1.25 mg) film + 450 µL VFS

Test article 3- 100 µL aliquot of DPV (1.25 mg) film dissolved in 0.5 mL VFS

Test article 4- 100 µL aliquot of DPV (1.25 mg) film dissolved in 1.5 mL VFS

The tissue was exposed to each test article for 6 hours. Franz cells were water jacketed to maintain a temperature of 37°C throughout the exposure period. 200 µL sample from the receptor was collected every hour for 6 hours. After the exposure period, the tissue was cut in half and one half was processed for histological evaluation and epithelial thickness measurement whereas the second half was used for the determination of DPV concentration by LC/MS/MS. The tissues used for determination of DPV content were embedded into blocks using an O.C.T compound and sectioned by cryostat into 100 µm sections. In a separate set of experiments the sectioning protocol was modified to elucidate DPV epithelial distribution. The protocol was modified such that the tissues were sectioned into 15 (20 µm) sections followed by (200 µm) sections for the remainder of the tissue.

4.2.1.3 C^{14} – Dapivirine

In these studies excised human ecto-cervical tissue was placed between the donor and receptor compartments of a neoflon two compartment diffusion cell. Prior to tissue placement the excess stromal tissue was removed using a Thomas Stadie Riggs tissue slicer. The receptor medium used was VFS at (pH=4.2). Three different product application scenarios were modeled by altering the test article introduced into the donor compartment as follows:

Test article 1- 5 µL of C^{14}-DPV solution (250 µCi/mL) + 445 µL VFS

Test article 2- 10 µL of C^{14}-DPV solution (250 µCi/mL) + 440 µL VFS

Test article 3- 15 µL of C^{14}-DPV solution (250 µCi/mL) + 435 µL VFS

The purpose of testing three different test articles was to assess the impact of increased concentration of the radio labeled drug in the donor compartment on the distribution of the drug in the tissue. The tissue was exposed to each loading scenario for 6 hours. The experimental setup was maintained at a temperature of 37°C. After the exposure period, the tissue was cut in half and one half was processed for histological evaluation and epithelial thickness measurement whereas the second half was impeded in O.C.T blocks and cut by a cryostat into sections. Autoradiography was conducted on tissue sections.

4.2.2 Determination of Drug Concentration in Human Ecto-Cervical Tissue Sections

4.2.2.1 Dapivirine

Tissue samples (sections) from the exposure study were homogenized. Tissue homogenates were then transferred to extraction tubes. A liquid extraction with a mixture of methanol, acetonitrile and MTBE (methyl-tert-butyl ether) was used to extract DPV. After drying under nitrogen, final samples were reconstituted in 500 µL of the injection solvent (6:2:2) (acetonitrile:water:100 mM ammonium formate buffer). Samples were injected onto an ultra-high performance liquid chromatography (UHPLC) connected to a triple quad mass spectrometer (with electric spray ionization source) for analysis. The column used was a Phenomenex Hyperclone 3u BSD C8 150x4.6 mm. A gradient method was used for separation which consisted of two mobile phases (A: 12.5 mM NH_4FA Buffer in 60% acetonitrile and B: 25 mM NH_4FA Buffer in 80 % acetonitrile). Mobile phase gradient was setup so that the percentage of acetonitrile increased from 60% to 80% over 1.5 minutes, held at 80% for 2 minutes then equilibrated back to 60% for the rest of the run. A 40 µL injection volume was used and the run time was 6 min with a flow rate of 1 mL/min. A positive SRM scan was used to monitor: 330.2 → 158 for DPV and 334 → 145.1 for d^4-DPV (internal standard). The standard curve prepared over the range of 0.2-50 ng/mL was determined to be linear. The concentration of DPV in unknown samples was determined by the plot of concentration vs area (ratio of DPV/ d^4-DPV) with the equation: $y = ax + b$ where y is the area and x is the concentration (ng/mL).

4.2.2.2 Tenofovir

Tissue samples (sections) from the exposure study were homogenized. Tissue homogenates were then transferred to extraction tubes. TFV was extracted from the homogenate using a methanol liquid extraction. Final samples were reconstituted in 100 µL of a 0.1% formic acid solution. Samples were injected onto a UHPLC/MS/MS (with electric spray ionization source) for quantification of TFV. The column used was an Agilent ZORBAX XDB-C18 5 µm, 4.6x50 mm. A gradient method was used for separation which consisted of two mobile phases (A: 0.1% formic acid in water and B: 0.1% formic acid in methanol). Mobile phase gradient was setup so that the percentage of methanol increased from 5% to 50% over 2 minutes, held at 50% for 0.5 minute then equilibrated back to 5% for the rest of the run. A 10 µL injection volume was used and the run time was 5 min with a flow rate of 0.5 mL/min. A positive SRM scan was used to monitor: 288 → 176.1 for TFV and 293 → 181.1 for C^{15}-TFV (internal standard). The standard curve prepared over the range of 5-250 ng/mL was determined to be linear. The concentration of TFV in unknown samples was determined by the plot of concentration vs area (ratio of TFV/ C^{15}-TFV) with the equation: $y = ax + b$ where y is the area and x is the concentration (ng/mL).

4.2.3 Autoradiography

In a dark room, slides of tissue sections were taken from -20°C and allowed to come to room temperature. Once at room temperature the slides were processed using KODAK NTB autoradiography emulsion which was pre-heated to 45°C in a water bath. The slides were then air dried for 15 minutes. Once dry, slides were stored at 4°C for 20 hours in a black box. After the incubation period, the slides were developed and fixed per manufacturer instructions using KODAK developer D-19 and KODAK fixer.

4.2.4 *In Vitro* Release

A diffusion cell system connected to an automated fraction collector was used for these studies. A cellulose membrane (Spectra/Por 1 MWCO 6000-8000 Da, Diameter 33 mm) was placed between the donor and receptor compartments. A single 6mm biopsy punch of TFV/DPV, TFV or DPV film was placed into 450 μL VFS in the donor compartment. The receptor compartment medium used was 1% cremophor in water. The run time was 6 hours and 37°C was maintained by a water jacket. 500 μL from the receptor was sampled every hour for 6 hours. Sample analysis of DPV and TFV was conducted using a UHPLC. The methods for TFV and DPV content analysis were previously described in section 3.2.1.

4.2.5 Statistical Analysis

For comparison of drug tissue concentrations and *in vitro* release data, a Student T-test was used to compare the difference in mean values between single entity and combination film exposure groups ($p < 0.05$ was considered significant). One-Way ANOVA was used to compare difference in mean values of drug accumulation across the epithelium ($p < 0.05$ was considered significant).

4.3 Results

4.3.1 Drug Accumulation in Human Ecto-Cervical Tissue after Exposure to DPV/TFV Vaginal Film

Human ecto-cervical tissue was exposed to a TFV/DPV, DPV or TFV vaginal film *ex vivo*. Flow-through diffusion cells were utilized for these studies in order to closely simulate *in vivo* application of the product. Analysis of drug content in tissues was conducted using UHPLC/MS/MS. Results showed that, after 6 hours, TFV and DPV were present in the tissue whether the exposure was using a single entity or combination film. (Table 4.1). However, when comparing the tissue concentrations of DPV between the single entity and combination film groups, the data showed that tissue exposure to the combination film resulted in significantly higher DPV accumulation ($p < 0.05$). The amount of DPV in the tissue exposed to the combination film was approximately twice as much as of that found in the tissue exposed to the single entity film (0.5 and 1.1 µg DPV for single entity and combination film groups respectively).

Table 4.1 Concentration and amount of DPV in human ecto-cervical tissue after 6 hour exposure to DPV film or DPV/TFV film.

*** p<0.05 (Student t-Test)**

	DPV Film	DPV/TFV Film
Concentration in Tissue (µg/g)*	14.21 ± 4.13 (n=6)	31.03 ± 12.63 (n=7)
Amount in Tissue (µg)*	0.50 ± 0.22 (n=6)	1.10 ± 0.28 (n=7)

Since the epithelium represents a rate limiting barrier to diffusion, its thickness plays a role in drug tissue accumulation. Further analysis revealed that there is no difference in DPV levels in the epithelium as a result of the exposure to either the single entity or the combination films (Figure 4.1). Thus, it was concluded that the observed significant difference in DPV whole tissue amounts could be correlated with DPV stromal localization.

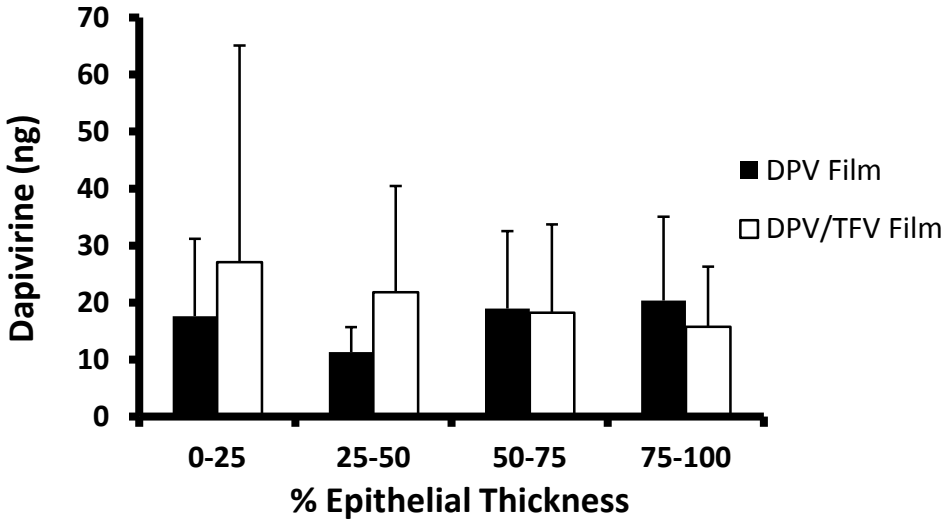

Figure 4.1 DPV amount throughout the epithelium of human ecto-cervical mucsoa

With regard to TFV, no significant difference in TFV whole tissue amounts was observed between the single entity and the combination film groups after the 6 hours exposure period (Table 4.2). Evaluation of TFV amounts localized in the epithelium only showed no significant difference in TFV epithelial amounts between the two film groups (Figure 4.2).

Table 4.2 Concentration and amount of TFV in human ecto-cervical tissue after 6 hour exposure to TFV film or TFV/DPV film

	TFV Film	TFV/DPV Film
Concentration in Tissue (µg/g)	33.88 ± 8.67 (n=7)	34.92 ± 14.98 (n=6)
Amount in Tissue (µg)	1.46 ± 0.53 (n=7)	1.45 ± 0.75 (n=6)

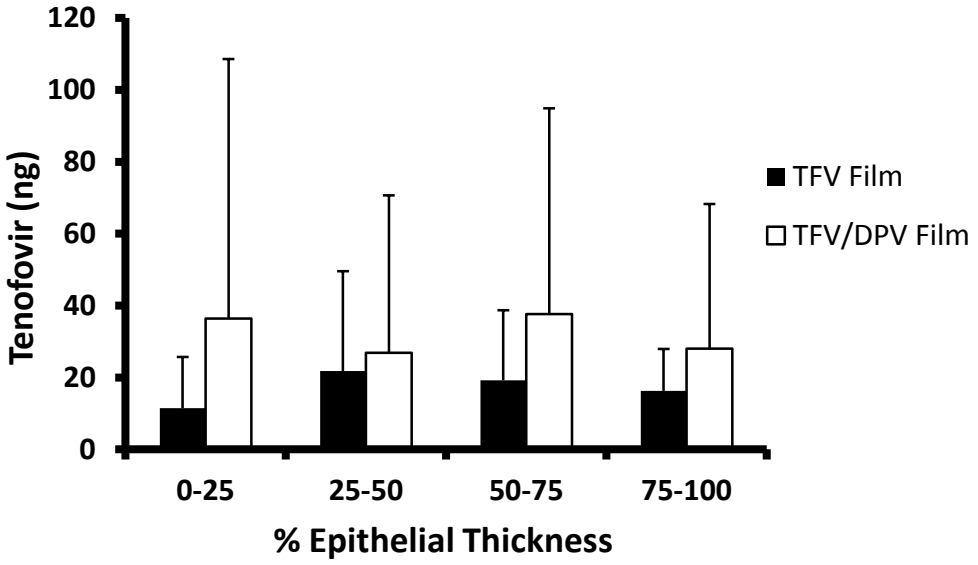

Figure 4.2 TFV amount throughout the epithelium of human ecto-cervical mucsoa

4.3.2 Drug Accumulation in Human Ecto-Cervical Tissue after Exposure to DPV Vaginal Film

In order to elucidate why DPV accumulated higher when co-delivered with TFV it was important to first understand the mechanism of DPV tissue permeation when delivered from a film. For that purpose human ecto-cervical tissue exposure studies to a DPV vaginal film using a simulated *in vivo* situation were conducted. Vaginal fluid simulant was utilized in these studies as the medium in the donor and receptor compartments. Different scenarios associated with varied DPV concentrations in the donor were investigated as described in the methods section 4.2.1.2. The various scenarios, chosen for evaluation, simulate potential film behavior following *in vivo* administration.

Results showed that DPV could be found throughout the ecto-cervical tissue (Figure 4.3). This indicates that the film was able to release drug allowing for its permeation into the tissue. DPV tissue permeation was found to be dependent on the concentration of DPV in the donor. The highest exposure concentration resulted in the greatest DPV tissue concentration (98.68 µg/g) (Table 4.3). This suggests that DPV tissue permeation is primarily due to passive diffusion. By examining the amount of DPV per tissue section from the epithelium to the stroma it was noticeable that DPV amounts were highest at a level of roughly 200 – 500 µm. This level can be correlated with the location of the basal layer of the epithelium and the beginning of the stroma. Considering that major HIV-1 targets (represented by CD4 T-cells) exist in the sub-epithelium, DPV accumulation at the basal layer of the epithelium is likely to be desirable.

Table 4.3 Tissue concentration of DPV (µg/g) in human ecto-cervical tissue after different exposures to DPV vaginal film

Scenario	DPV Tissue Concentration (µg/g)
Single 6mm punch	47.45 ± 25.40
Double 6mm punch	62.54 ± 24.08
Aliquot of film/1.5 mL	97.56 ± 6.67
Aliquot of film/0.5 mL	98.68 ± 44.22

Increased Conc. Gradient

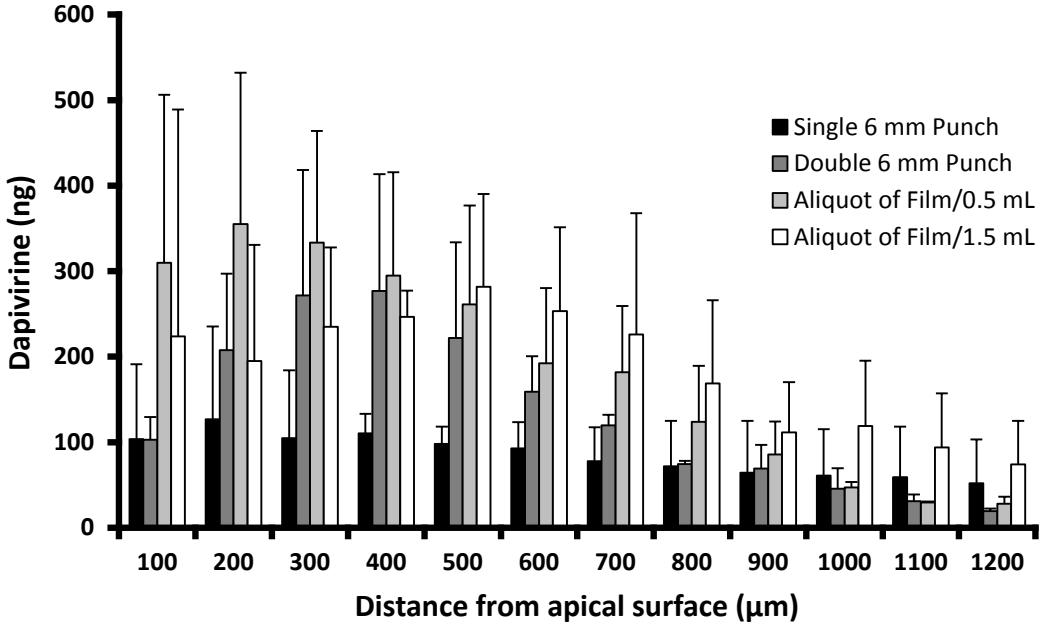

Figure 4.3 Amount of DPV (ng) per 100 µm section throughout human ecto-cervical tissue after 6 hours exposure to DPV vaginal film

When looking at DPV amount in the epithelium only, no drug gradient was identified (Figure 4.4). There was no significant difference in DPV amounts throughout the epithelium.

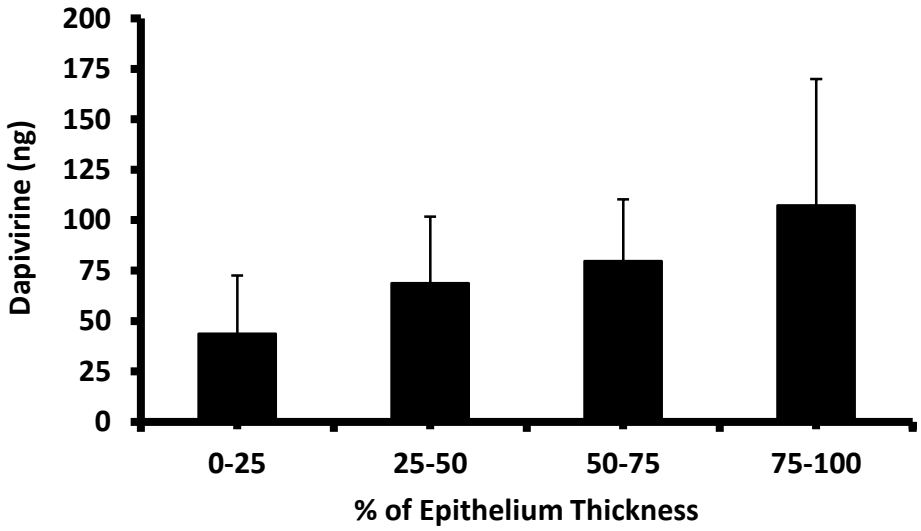

Figure 4.4 Dapivirine amount (ng) throughout the epithelium across human ecto-cervical mucosa

It is noteworthy to mention that a tissue permeability study with DPV vaginal film was conducted within the studies described in chapter 2. The experimental setup of the permeability study in chapter 2 was similar to the tissue exposure studies described in this section. The permeability study in chapter 2 was conducted to assess the impact of formulation on DPV tissue permeation by comparing the permeability of film formulated and unformulated DPV. Whereas the purpose of the studies described in this section was to elucidate mechanism of DPV tissue

permeation and accumulation after exposure to the DPV vaginal film. However, the results of the permeability study in chapter 2 showed that film formulation did not have an impact on DPV tissue permeability owing to the quick release of DPV from the film. Additionally, these results suggest that the film formulation excipients did not cause a deterioration of the epithelium as a barrier. On the other hand, the findings of the tissue exposure studies in this section showed that the quick release characteristic of the film allowed for DPV permeation into the tissue as indicted in the presence of the drug across the tissue. Taken together, the findings from the two studies showed that the film formulation of DPV does not change its tissue permeation while being able to deliver DPV to the tissue.

4.3.3 Human Ecto-Cervical Tissue Exposure to C^{14}-DPV

In order to visualize the distribution of DPV in human ecto-cervical tissue, C^{14} labeled DPV was used. Tissue exposure studies were conducted in a similar experimental setup as that used for DPV film exposure studies. The donor compartment contained three different concentrations (2.78, 5.56 and 8.33 µCi/mL) of C^{14}-DPV in VFS. After a 6 hour exposure period, the tissues were sectioned and autoradiography was performed. As shown in figure 4.5 the radio labeled DPV diffused through the epithelium. With increasing donor C^{14}-DPV concentration accumulation of C^{14}-DPV (represented by a band of black dots) tissue localization became more evident. Visual data obtained from autoradiography confirms concentration dependent localization of DPV in the tissue observed in the findings of the previously described tissue exposure studies with the DPV film.

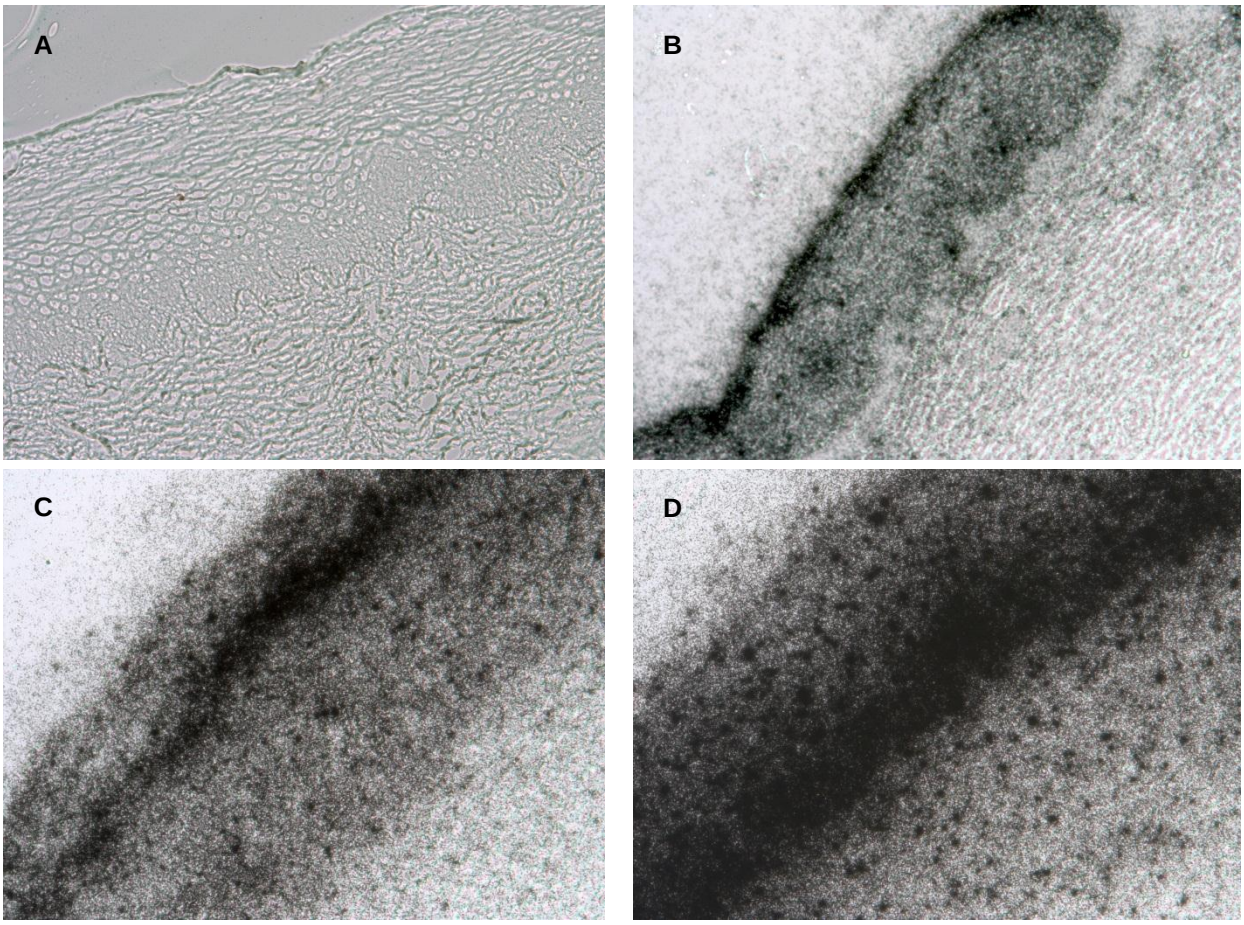

Figure 4.5 C^{14}-DPV distribution throughout human ecto-cervical tissue after 6 hour exposure period to different concentrations of the radioactive material. (A) negative control (B) 2.78 (C) 5.56 (D) 8.33 µCi/mL

4.3.4 DPV/TFV Combination Film *In Vitro* Release

Since DPV tissue permeation was found to be driven by passive diffusion, it can be hypothesized that the difference in DPV tissue concentration, after tissue exposure to single entity or combination film products, can be attributed to a faster DPV release from the combination film. To test this hypothesis *in vitro* drug release was tested in a similar setup to the tissue exposure studies. *In vitro* drug release experiments used the same donor as the tissue exposure studies. However, the receptor utilized in the *in vitro* drug release studies was 1% surfactant in water. The use of this receptor medium was necessary from a technical stand point to create the sink condition effect produced by the tissue in the exposure studies. The results showed that DPV was released faster from the combination film than the single entity film. By 4 hours the % released of DPV from the combination film was 6.15±1.42 compared to 4.42±0.88 from the single entity film. This observed difference was statistically significant ($p < 0.05$). At the end of the experiment (6 hours) the % released of DPV was 9.44±2.17 and 6.33±0.83 for the combination and single entity film respectively which was also a significant difference ($p < 0.05$). Figure 4.6 shows DPV release plotted as % released over time. The release of TFV from the single entity and the combination film was not different. At the end of the experiment (6 hours) the % released of TFV was 39.73±2.19 and 41.09±1.26 for the combination and single entity film respectively. The plot of % release of TFV over time is shown in figure 4.7.

The obtained values of % drug released after 6 hours was low considering the quick release profile of the film as described in chapter 3. It is important to emphasize that the USP system used in *in vitro* release studies in chapter 3 has large volume of medium in continuous

circulation, compared to the diffusion cell system used in studies in this section. Whereas in the cell diffusion system, the donor contains only 450 μL medium which makes the process of DPV solubilization slow and does not provide enough volume to solubilize the entire TFV amount present in the 6mm film punch.

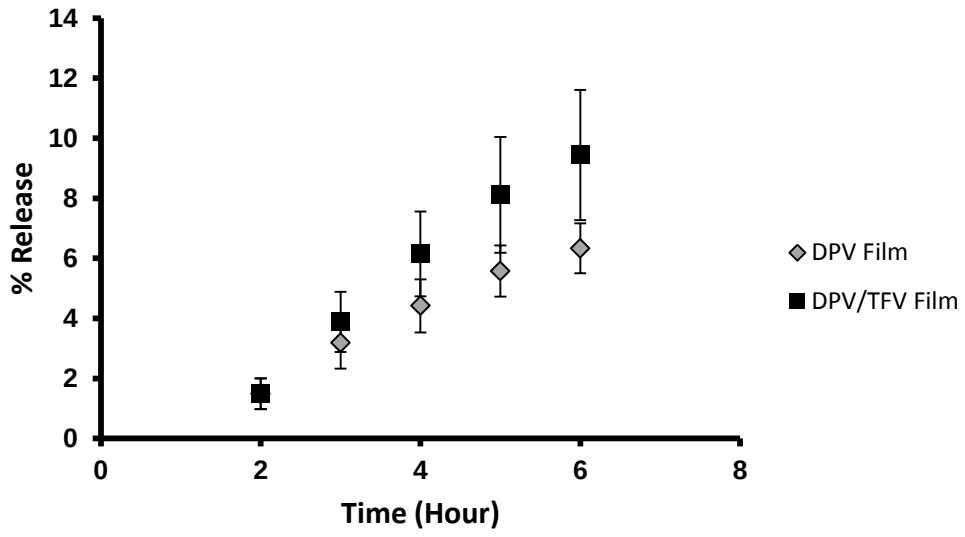

Figure 4.6 DPV release from the DPV and DPV/TFV films

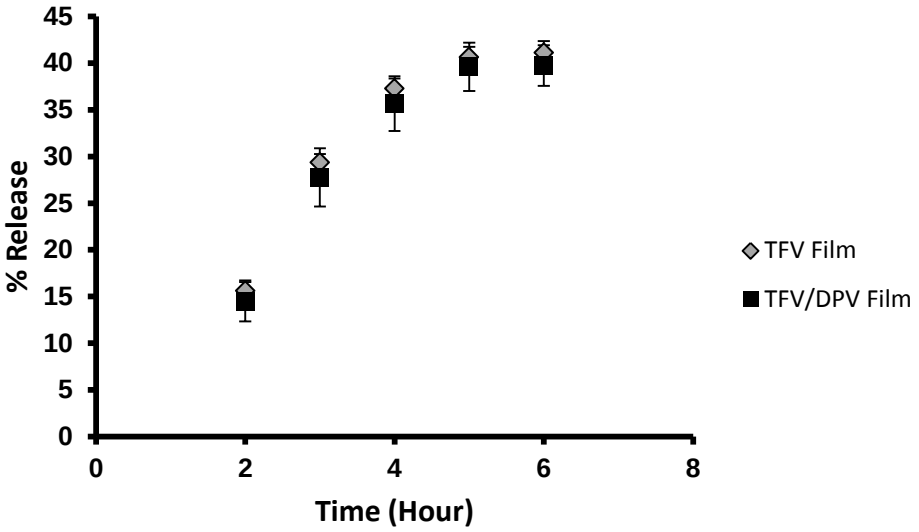

Figure 4.7 TFV release from the DPV and TFV/DPV films

4.4 Discussion and Conclusion

As combinations of ARVs are being considered for topical combination microbicides, it is imperative to conduct comprehensive pre-clinical testing to understand the impact of the combination on safety and efficacy of the product. From a delivery efficiency stand point, co-delivery of two active agents introduces an added layer of complexity that requires additional testing. Specifically, it is important to understand whether co-delivery impacts the tissue accumulation of the individual ARVs.

The studies conducted in this chapter were aimed to understand the impact of co-delivery of DPV and TFV from a vaginal film on their tissue accumulation and distribution. The dosing levels of DPV and TFV in the film are in line with levels being evaluated in clinical trials. The experimental setup chosen used flow through diffusion cells to simulate blood flow *in vivo* and maintained sink condition. Single entity and combination films of DPV and TFV were compared. Film formulation excipients were identical so any impact of formulation on the permeability of both drugs into the tissue was similar. The results confirmed the ability of the films to deliver DPV and TFV to the tissue whether formulated individually or in combination. In theory, a solid dosage, polymeric film minimizes dilution effect of the active agents concentration released from the film. This creates a higher concentration of the active agent on the tissue surface which drives drug tissue permeation. Further mathematical interpretation of the obtained data which scales the data from the amount of DPV in the film punch used in the exposure study to the total film DPV content shows that following 6 hours of tissue exposure to a whole film unit, approximately 152 or 334 nM DPV would be delivered from single entity or combination films respectively. These values represent levels which are much higher than the reported EC_{50} for DPV (1 nM in CEM T-cells) [65]. Similar calculations for TFV show that by 6 hours the tissue exposed to an entire film unit would contain roughly 0.45 μM TFV delivered from either the single entity or combination film. This value is within the reported TFV IC_{50} range of 0.04-8.5 μM [159].

With regard to the impact of the combination on DPV and TFV tissue accumulation, the data showed that there was a significant difference of DPV concentration between tissues exposed to the single entity film as compared to the combination film. Further analysis of DPV amounts in the epithelium showed no difference between the two film groups which indicates that the observed difference in whole tissue amounts between groups is primarily due to differences in DPV accumulation in the stroma. Theoretically, since most HIV-1 target cells are located in the stroma, the increased DPV stromal accumulation may lead to better protection of HIV-1 target cells located there. This finding further supports use of the TFV/DPV combination film as a topical microbicide. On the other hand increased DPV stromal accumulation may lead to higher systemic absorption of DPV as increased drug amounts would be in closer proximity to blood circulation. Systemic absorption could lead to or increase chances for selection of drug resistant HIV-1 strains. Therefore the potential consequences of increased DPV stromal accumulation on efficacy and systemic absorption should be further evaluated. With regard to TFV, the co-delivery of TFV and DPV in a film formulation did not have any influence on TFV tissue accumulation. TFV tissue concentrations were similar between the single entity and combination film exposed tissues. The epithelial TFV amounts were also similar between the two film groups suggesting no advantage from a TFV tissue accumulation stand point for the combination product.

To understand why DPV accumulation was higher after tissue exposure to the combination film, it was first necessary to understand how DPV permeates into ecto-cervical tissue after exposure to DPV film. From an application stand point, vaginal films could be

inserted folded or unfolded and later disintegrate or form a gel mass depending on the formulation excipients and amounts of fluid present at the time of application. The loading in the donor compartment in the exposure studies with DPV film was designed so that it simulated these potential scenarios. Results showed that DPV accumulation in the tissue was concentration dependent which is consistent with passive diffusion. As a hydrophobic molecule, it is expected that DPV permeates into the tissue through transcellular pathway passive diffusion. The sectioning of the tissue allowed for clearer elucidation of where DPV accumulates in the tissue. It was found that DPV generally accumulated equally throughout the tissue from the epithelium to the stroma. However, DPV highest amounts were found to be around 200 - 500 µm deep into the tissue. The thickness of the epithelium was variable among tissues used in the study and ranged between 100 – 200 µm. By comparing thickness of the epithelium with DPV accumulation pattern one can see that DPV formed a reservoir at the basal layer of the epithelium and through the upper part of the stroma. For further examination of DPV amount in the epithelium, the exposed tissues were sectioned into 20 µm sections for the first 300 µm of the tissue which goes beyond the epithelial thickness in most cases. The data showed that there is no difference in DPV amounts throughout the epithelium. A slight trend towards increased DPV amounts at the basal layer can be seen which is consistent with the observation of DPV reservoir formation as described previously.

Visualization of DPV accumulation in human ecto-cervical tissue was made possible using C^{14}-DPV. Tissue exposure studies were conducted using unformulated C^{14}-DPV. Three donor C^{14}-DPV concentration levels were tested to show the impact of increased concentration. The data confirmed radio labeled DPV accumulation throughout the tissue with highest accumulation at the basal epithelial cells or upper stroma.

Since DPV enters the tissue mainly by passive diffusion, it is logical to assume that the increased DPV tissue concentration after exposure to the combination film is a result of a higher DPV concentration in the donor. A higher concentration in the donor would mean that the release of DPV from the combination film is faster than its release from the single entity film. This assumption was tested by assessing the *in vitro* release of DPV and TFV from the single entity and combination films in a diffusion cell system similar to that used in the exposure studies. The data showed that no difference in TFV release existed between the single entity and combination film. Whereas DPV release from the combination film was faster than its release from the single entity film. The increased DPV release from the combination film can be attributed to several factors. Since TFV is a hydrophilic compound with weak acidic properties, it is expected that TFV would be released quickly from the film upon contact with fluids (VFS used the experiment). This was demonstrated experimentally through *in vitro* release studies where it can be observed that by two hours the % TFV released was approximately 10 times higher than that of DPV (Figure 4.6 and 4.7). Considering that TFV is a week acid, its rapid release leads to a decrease in the pH of the medium. DPV chemical structure contains two amine groups which can be protonated in an acidic pH environment leading to an increased solubility of DPV in the

medium. Increased DPV solubility can enhance DPV release from the film matrix. Another potential contributing factor to increased DPV release from the combination film is the high loading level of TFV in the film. Since TFV is a major component of the overall film network, its rapid release results in a decreased barrier for DPV diffusion from the polymeric network. Either of these scenarios (increased solubility in the medium and/or increased diffusivity through the film polymeric network) would lead to more rapid release of DPV from the combination film. Consequently yielding a higher DPV concentration on the apical surface of the tissue would lead to increased DPV tissue diffusion and higher tissue accumulation.

In conclusion, TFV and DPV delivered from a vaginal polymeric film were shown to permeate into human ecto-cervical tissue after *ex vivo* exposure to a film containing either drug or both in combination. Co-delivery of TFV and DPV had no impact on TFV tissue accumulation but resulted in significant increased DPV tissue accumulation. This increased tissue accumulation was found to be associated with faster DPV release from the combination film. The mechanism by which DPV permeates through the tissue is passive diffusion. DPV was shown to form a reservoir at the base of the epithelium or upper stroma. It is warranted to study whether this observed increased in DPV tissue concentration will result in enhanced efficacy and/or systemic exposure. The data presented sheds light on the impact of co-delivery of ARV combinations from a polymeric film on tissue levels and points out to the importance of pre-clinical studies to evaluate delivery efficiency and factors that impact it.

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5. DISCUSSION OF MAJOR FINDINGS AND FUTURE DIRECTIONS

5.1 Major Findings, Implications and Limitations

Although slower than a decade ago, the continued rise in new HIV infections worldwide poses a serious health challenge. It also points to the fact that available prevention strategies from educational interventions to promoting safe sexual practices have not been enough to bring about a significant decline in HIV infection rates. On the other hand, vaccination against HIV infection has not been successful and AIDS (the outcome disease of an HIV infection) is still incurable. Therefore, research on all three fronts to combat the HIV epidemic is warranted.

Since sexual transmission is the primary source of new HIV infections, prevention methods focused on sexual transmission are in pursuit. Topical microbicides are part of the broader pre-exposure prophylaxis (PrEP) products which are designed for use in a healthy HIV negative population. The work presented and discussed in this dissertation is focused in the area of topical vaginal microbicide product development.

In its early stages topical microbicide research has produced several candidate products that were evaluated in clinical trials. Unfortunately, none of the products has proven successful. However, the introduction of HIV specific antiretrovirals (ARVs) to topical microbicide research led to the first success in human trials. A phase IIb clinical trial (CAPRISA004) the product 1% TFV gel was able to reduce HIV acquisition by 39% when administered within 12 hours before sexual intercourse and within 12 hours after [58]. However, this result has not been consistently shown and has yet to be validated in a phase III clinical trial. Meanwhile, the use of ARVs

combinations in topical microbicides has become a research focus toward improvement of product efficacy. This concept has been extrapolated from the HIV therapy field where ARV combination therapy has resulted in significant improvement of clinical outcomes.

One of the aspects related to topical microbicide product development is dosage form choice. Dosage form choice is a multifaceted process dictated by factors ranging from the active agent(s) properties to user acceptability. For some ARVs being evaluated for use in topical microbicides, one aspect which is particularly important is the ability of the dosage form to deliver the drugs into the genital mucosal tissue. With this said, given the emphasis on combination product development the impact of co-delivery on ARVs tissue accumulation needs to be considered for development of efficient and functional dosage forms given that drug tissue accumulation, for ARVs, can influence their ability to block HIV-1 infection.

The work conducted in this dissertation was based on the hypothesis that a polymeric film can serve as a delivery system for a combination of DPV and TFV and that the film will be able to deliver both agents to the tissue. However, co-delivery of DPV and TFV will lead to a change in their accumulation in human ecto-cervical tissue due to associated concentration gradient changes. The studies conducted to address the hypothesis included the development of a polymeric vaginal film containing DPV and TFV and the evaluation of DPV and TFV accumulation in human ecto-cervical tissue after exposure to the combination film. The major findings of the dissertation are summarized below.

5.1.1 Polymeric Films for Topical Delivery of Antiretrovirals (ARVs)

Considering that no marketed vaginal film product contains a hydrophobic molecule and the majority of polymers approved or have history of use in vaginal products are water soluble, it was needed to ensure the ability of formulating DPV in a water soluble film. Due to its hydrophobicity, DPV was to be dispersed in the film formulation. Thus the polymer selection was guided by the ability of the polymeric mix to maintain DPV in a uniformly dispersed state throughout the manufacturing process and in the solid film unit after solvent evaporation. A combination of PVA and HPMC was found to be suitable for incorporation of DPV into the film. The type and ratio of PVA and HPMC can be manipulated to change the viscosity of the polymeric film solution, disintegration and dissolution of the film. The other main excipient used for film formulation is the plasticizer. After screening multiple plasticizers, glycerin was chosen for use as a plasticizer in the film formulation based on its ability to provide the film with flexibility and enhance its smoothness. DPV was dispersed in propylene glycol and subsequently incorporated in the film formulation. In the case of the combination film, the attempt to incorporate TFV into the developed DPV film was unsuccessful due to the inability of the PVA-HPMC polymeric network to inhibit TFV crystallization at the target loading dose. For that reason, a solid phase solubility study of TFV in several polymers was conducted to identify polymers capable of stabilizing TFV in the film formulation. Using the results of the solid phase solubility study, several polymeric compositions were made and stressed at high temperature to test the ability of each composition to prevent TFV crystallization in the film matrix.

A combination of Na CMC, HPMC and HEC was found to be successful in producing a film matrix which could successfully solubilize TFV. It is highly likely that the interaction of the polymer with TFV is the primary factor associated with inhibition of TFV crystallization. Since the plasticizers function in the film formulation is to maintain a loose and flexible network, too much network flexibility could allow for TFV molecules to move more freely which may lead to drug crystallization. Glycerin amount (the plasticizer of choice) in the formulation was optimized to balance network flexibility and drug stability.

A comprehensive evaluation of the films was conducted. Physical parameters (weight, thickness, appearance), chemical parameters (residual water content, disintegration, dissolution, drug content), mechanical parameters (tensile or puncture strength), toxicity and anti-HIV activity all were assessed. Both films exhibited acceptable characteristics. Their *in vitro* toxicity and anti-HIV activity were established in TZM-bl cell based model which is one of the most widely used in HIV research. This model utilizes a genetically engineered HeLa cell line that expresses CD4, CXCR4 and CCR5 and contains Tat-inducible Luciferase and β-Gal reporter genes. These cells are readily infected by a wide array of HIV-1 isolates. The film formulation did not alter the anti-HIV potency of TFV or DPV in the TZM-bl cells. The EC_{50} of both drugs formulated was similar to their EC_{50} as unformulated drugs. Drug release from the films was rapid. In either film, more than 50% of the loading dose was released within 30 minutes into an aqueous medium. It is to be noted that the specification for quick release were set for testing with a flow through class IV USP apparatus. Film dissolution and drug release is a multistep process. As a solid dosage form, the steps dictating drug release from a film dosage form are

disintegration, deaggregation and solubilization. The slowest step in the process is the rate limiting step. For water soluble polymeric films, such as the ones described in this dissertation, it is more than likely that all three steps of drug release occur simultaneously. The drug release profiles of DPV film (chapter 2) and TFV/DPV film (chapter 3) show a quick initial release of drug followed by a reduction in the release rate. Hence this release profile is consistent with first-order release kinetics. In either film product, once the film is in contact with the dissolution medium the polymers of the film hydrate which results in polymer relaxation. At the same time, polymer erosion occurs. The two processes of polymer relaxation and hydration are the primary factors which drive film disintegration and deaggregation. In addition to polymer relaxation and erosion, drug solubilization also occurs. The combined effect of the three processes creates, in the first part of drug release, a polymeric matrix with high concentration of the active agent. This matrix is surrounded by a boundary layer of the medium. In a class IV USP apparatus, the continuous flow of medium ensures low concentration of the active agent at the boundary layer – medium interface which, coupled with the high concentration of the active agent in the matrix – boundary layer interface, forms a steep concentration gradient in the boundary layer. Drug release is driven by the concentration gradient in the boundary layer. This concentration gradient is initially high which is visualized in the release profile of DPV film and TFV/DPV film showing rapid drug release. With the depletion of the drug from the matrix over time, the concentration gradient in the boundary layer decreases resulting in a reduction in drug release rate and solubility becomes the rate limiting step of the process. The dependence of the release rate on the concentration gradient can be described as a first-order release process. In the

TFV/DPV film the release of TFV is faster than that of DPV due to the higher TFV solubility in the release medium. Rapid drug release from the product is an essential attribute for topical microbicides intended to be used in a coitally dependent fashion. That would ensure that the drug reaches its target before HIV and prevent infection.

Finally, the stability of the two film products was evaluated. Both films demonstrated stability physical, chemical and pharmacological stability. 24 months stability data for the DPV film and 12 months for the TFV/DPV film was acquired. Stability testing of the TFV/DPV film is ongoing and planned for 24 months.

Overall, the data demonstrated the feasibility of use of polymeric films as a dosage form for ARVs as topical microbicides. The advancement in polymer science allows for great versatility in film design in order to tailor it to the needs of the product and its application. As solid dosage forms, films offer an excellent alternative to hydrogels for topical microbicide development. Importantly, the developed DPV film was recently evaluated in a macaque animal model for safety and pharmacokinetics and is currently being evaluated for safety and pharmacokinetics in a phase I human clinical trial outside of the scope of this dissertation.

5.1.2 Co-delivery of ARVs: Impact on Tissue Accumulation

Since ARVs work at the cellular level, their tissue accumulation and distribution are key to their bioactivity and hence success in topical microbicides. The tissue penetration and accumulation of ARVs in genital mucosa can be affected by the delivery system or dosage form chosen. Further drug tissue localization is a reflection of the delivery efficiency of the product.

As hypothesized, the polymeric film successfully delivered DPV and TFV into the tissue when presented either in combination or individually. As demonstrated in studies with the film products, the release of the drugs from the dosage form was quick. Rapid release from the product results in increased drug concentration at the tissue surface which drives permeation of the drug into the tissue through diffusion processes. DPV is hydrophobic and is expected to permeate the tissue via transcellular pathway. In this pathway, DPV preferably partitions to the lipid bi-layer of the cell membrane where it gets solubilized creating a concentration gradient in the cell membrane driving its diffusion through the cell. TFV is hydrophilic and is expected to permeate the tissue primarily through the paracellular pathway. The spaces between the epithelial cells are aqueous and molecules soluble in water, such as TFV, can diffuse through the epithelium via these inter-cellular channels.

Another major finding from the tissue exposure studies was that co-delivery of DPV and TFV using the film dosage form at a loading level of 20/1.25 mg (TFV/DPV) resulted in a significant increase in DPV accumulation in the tissue. TFV tissue accumulation was not affected by co-delivery. To dissect the mechanism for increased DPV tissue accumulation, it was essential to understand the mechanistic pathway by which DPV accumulates in the tissue. Data generated show that the higher the concentration at the tissue surface, the higher the accumulation of DPV in the tissue. This is consistent with the passive diffusion process which was anticipated for DPV tissue permeation. For the combination film to cause a higher DPV tissue accumulation the prediction was that the combination film is releasing higher DPV amounts which would translate into an increased concentration of DPV at the tissue surface. To

evaluate that, *in vitro* release studies were conducted in diffusion cells similar to those used in the tissue exposure studies. As predicted, the release of DPV from the combination film was faster than that for DPV only film.

The third finding from these studies was that both TFV and DPV distribute evenly throughout the epithelium following a 6 hour exposure of the tissue to the film product. The distribution of the drugs across the epithelium provides a consistent barrier for protection from HIV infection.

An additional important finding from the exposure studies related to DPV tissue distribution. Despite the fact that DPV distributed across the tissue from the epithelium to the stroma it showed the highest amounts at the basal epithelial layer through the upper stroma. That was confirmed using radio labeled DPV and autoradiography. This drug distribution pattern matches the relative distribution of HIV-1 target cells in the genital mucosa. CD4 T-cells (HIV-1 main target cells) have been found to form a cluster band directly beneath the epithelium and infiltrating the epithelium to some extent [31]. Based on the mechanism of action of DPV, the co-localization of DPV with cellular targets may be beneficial from a bioactivity stand point.

5.1.3 Implications for Topical Microbicides Development

As current research and development of topical microbicides is moving towards the use of HIV specific ARVs in topical microbicide products, the work presented in this dissertation contributes to the advancement of topical microbicides by providing the first ever ARV containing vaginal film for evaluation in clinical trials as topical microbicide product for HIV-1

sexual transmission prevention. Furthermore, considering that ARVs combinations are being pursued as a strategy to enhance topical microbicide products effectiveness, the developed combination (TFV/DPV) film product is positioned as a pipeline product for further preclinical and clinical assessment. This is the first combination film product containing two ARVs. Both the DPV and TFV/DPV film products have been tested in a comprehensive algorithm that evaluated their physical, chemical properties, toxicity and anti-HIV activity. The results showed that the products developed within the scope of this work are ready for further development and warrant further evaluation.

From a dosage form perspective, the findings of this work validate polymeric films as a delivery platform that can be utilized in the design of topical microbicide products. The data showed the versatility and potential application of polymeric films for vaginal delivery of ARVs. The findings confirmed that films are able to deliver two chemically different active agents, whether formulated individually or in combination, to the target tissue. Combining these data with studies showing that women preferred and rated films high and acceptable for vaginal use, this dosage form provides a promising platform for administration of anti-HIV drugs. The addition of the film dosage form to the existing platforms currently being used for vaginal administration of microbicide drug candidates provides an additional product option to users. Considering the diversity of target female populations who would use a topical microbicide product, greater options may lead to enhanced compliant product use which will positively impact product efficacy.

Published reports have established the benefit of the TFV/DPV combination by demonstrating synergistic activity against NNRTI HIV resistance strains. The findings of the work in this dissertation provide, to our knowledge, the first demonstration of the benefits of the TFV/DPV combination from a drug delivery perspective. The observed enhancement of DPV release from the combination film was found to be correlated with enhanced tissue concentrations. From a pharmacokinetic perspective this observation is important as it has been shown that for ARVs, drug tissue levels are linked to product efficacy. Hence this data provides further rationale for development of this combination.

5.1.4 Limitations

With regard to film product development, although the films developed within the scope of this dissertation showed acceptable properties, the lack of universal standard target specifications for vaginal films makes the development process somewhat subjective to one's perspective of what is acceptable. Additionally, no clinically relevant target loading dose or release profile has been identified for the field or for vaginal administration of DPV or TFV. Thus the development process was guided by the requirements to produce a film that proves stable, acceptable, safe and effective in the available models for testing.

The tissue exposure studies provided valuable information on the accumulation and distribution of TFV and DPV in lower female genital mucosa. For technical reasons, the tissues used were all frozen and thawed at the time of experiments. This may have compromised the effect of transport and permeation processes. However, previously no impact of freezing and

thawing on the diffusion pathway of small molecules was found [160, 161]. The impact of co-delivery of TFV and DPV was found to be dependent on drug exposure level at the tissue surface. However, since no target tissue concentration for effectiveness has been identified for TFV and DPV to date, the relevance of this finding to a marketable product with large scale implementation may require re-evaluation once the optimal drug loading levels are identified.

5.2 Proposed Future Studies

This research has identified several future studies which can contribute to further development of the TFV/DPV film as well as expand concepts developed regarding drug tissue accumulation. Below is a list of proposed studies which can enhance the findings from work conducted within this dissertation:

1- The stability study of TFV/DPV film must be completed. 12 months of data has been collected to date, however, the study is planned for 24 months and acquiring 24 months stability data would support advancement of this product to clinical testing.

2- Testing the efficacy of the TFV/DPV film in an *ex vivo* cervical tissue explant model would provide further confirmation of the impact of these findings on anti-HIV activity. It will be important to correlate bioactivity with findings from tissue exposure studies to understand if increased DPV tissue accumulation is associated with enhanced efficacy.

3- The tissue exposure studies should be further expanded to include different exposure periods, more tissue replicates, and use of fresh tissue. All of which would further validate the model and the results. Different exposure periods would provide data essential to evaluate the kinetics of drug penetration and the impact of the co-delivery. This information is important especially in the extent of coital use. Additionally, it would be informative to conduct comparative tissue exposure studies of DPV film and gel products in order to understand the impact of dosage form differences on tissue drug accumulation.

4- It is valuable to validate the findings of the tissue exposure studies conducted *ex vivo* with *in vivo* data from an animal study (Macaque). These studies would be possible to conduct by administering the film product to macaques prior to necropsy. Whole reproductive tract would be collected after animal sacrifice and drug levels could be then analyzed in the mucosal tissue.

5- Given that animal and human pharmacokinetic studies are being conducted with the developed DPV film from this dissertation, it would be feasible to develop a mathematical pharmacokinetic model to predict the *in vivo* delivery efficiency of the film. A recent study with the TFV vaginal gel presented an example of the development of a multi-compartment pharmacokinetic model to study the delivery of TFV from the gel and understand formulation and physiological parameters impacting product performance [162].

Appendix I

Dapivirine Clinical Trials (October 2013)

TRIAL NAME	DESCRIPTION	STATUS
IPM 001	Dapivirine ring safety (Ring-001)	Completed
IPM 008	Dapivirine ring safety (Ring-002)	Completed
IPM 018	Dapivirine ring safety & PK (Ring 002 & 003)	Completed
IPM 011	Placebo ring acceptability & safety	Completed
IPM 024	Dapivirine ring safety & PK (Ring-004)	Completed
IPM 013	Dapivirine ring safety & PK (Ring-004)	Completed
IPM 015	Dapivirine ring safety (Ring-004)	Completed
IPM 027 (The Ring Study)	Dapivirine ring long-term safety & efficacy (Ring-004)	Ongoing
IPM 007	Seroconverter protocol	Ongoing
MTN-020 (ASPIRE)	Dapivirine ring safety & effectiveness (Ring-004)	Ongoing
IPM 003	Dapivirine gel safety & PK (Gel-002)	Completed
IPM 004	Dapivirine gel PK (Gel-002)	Completed
IPM 005B	Dapivirine gel safety (Gel-002)	Completed
IPM 012	Dapivirine gel PK & safety (Gels 4750 & 4789)	Completed
IPM 020	Dapivirine gel safety (Gels 4759 & 4789)	Completed
IPM 014A	Dapivirine gel safety & acceptability (Gel 4759)	Completed
IPM 014B	Dapivirine gel safety & acceptability (Gel 4789)	Completed
MTN-012 / IPM 010	Dapivirine gel male tolerance (Gel 4759)	Completed

Appendix II

Ongoing Topical Microbicides Clinical Trials (October 2013)

TRIAL NAME	PHASE	POPULATION	CANDIDATE(S)
FACTS 001	III	2900 Women	1% TFV gel
IPM 027	III	1650 Women	4-week vaginal DPV ring
MTN 020	III	3476 Women	4-week vaginal DPV ring
CAPRISA 008	III	700 Women	1% TFV gel
MTN 017	II	186 Transgender, Men who have sex with men (MSM)	Daily oral TDF/FTC, Reformulated rectal 1% TFV gel
MTN 005	I/II	252 Women	Placebo vaginal ring
Project Gel	I	240 MSM	1% TFV rectal gel, HEC placebo rectal gel
MTN 008	I	105 Women	1% TFV gel
CONRAD 120	I	36 Women	1% TFV vaginal ring
CONRAD 118	I	54 Women	1% TFV gel, Antifungal cream, Antimicrobial gel, Contraceptive ring
CONRAD 117	I	48 Women	TFV/FTC fast dissolve vaginal tablet, TFV-only fast dissolve vaginal tablet
CONRAD 114	I	72 Women	1% TFV gel, Depo-provera, Oral contraceptive
NNRTI Microbicide Gel	I	30 Women	Carageenan-based placebo gel, MIV-150/Zinc acetate vaginal gel
IPM 026/MTN 013	I	48 Women	4-week vaginal DPV ring, 4-week vaginal DPV-MVC ring, 4-week vaginal MVC ring
MTN 011	I	48 Men, Women	1% TFV gel
MTN 014	I	28 Women	Reformulated rectal 1% TFV gel, Reformulated vaginal 1% TFV gel
CONRAD A10-114	I	72 Women	1% TFV gel
Vaginal Microbicide Adherence	Other	70 Women	Dye Stain Assay, UV-Light, Wisebag
Sex Workers Study	Other	267 Women	HEC placebo vaginal gel
MTN 003B	Other	518 Women	Daily oral TDF/FTC, Oral TDF

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