Rectal Suppository as a Versatile Platform for Delivering Physicochemically Diverse Antiretrovirals for HIV Prevention

by

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When reflecting on new Human Immunodeficiency Virus (HIV-1) infections across the globe, we find that men who have sex with men (MSM) are disproportionally impacted, especially in developed nations. Several studies have now shown that the chances of HIV-1 transmission through receptive anal intercourse (RAI) is about 10 times higher compared to vaginal intercourse. Therefore, new strategies are needed to combat rectal transmission of HIV-1. However, the rectal anatomy and physiology, limit dosage form options for drug delivery to this compartment. Suppositories offer several advantages over other rectally administered dosage forms, from a formulation as well as user acceptability perspective. Considering these advantages, this dissertation aims to explore application of the rectal suppository platform to deliver antiretroviral (ARV) drugs as pre-exposure prophylaxis (PrEP) for HIV prevention. In addition, since HIV-1 treatment and products typically utilize more than one ARV, we also explored the potential of developing a suppository product which co-delivered multiple ARV candidates.

Several suppository bases were screened to obtain a desired set of suppository characteristics. From this analysis, two bases (Witepsol H15 and PEG 3350:1000:400 at ratio 60:30:10) were chosen for further evaluation. Additionally, based on factors such as the mechanism of action, physicochemical properties, and drug-resistance profile, tenofovir (TFV), tenofovir alafenamide fumarate (TAF) and elvitegravir (EVG) were selected as the lead ARV candidates. The suppositories were manufactured, characterized and tested for stability as per ICH guidelines. Combination suppositories (TFV and EVG) were manufactured and their pharmacokinetics following a single dose administration was evaluated in a non-human primate (NHP) model. Rectal fluid, tissues (biopsies) and plasma samples were collected to determine the drug exposure achieved using each test suppository dosage form. Furthermore, to evaluate whether

there was any pharmacokinetic advantage gained by replacing tenofovir with its prodrug TAF, combination suppositories (TAF and EVG) were manufactured and evaluated in the same NHP model. The results from both these studies confirmed comparable or in some cases better drug exposure achieved *in vivo* using a suppository dosage form as compared to other dosage forms such as gels and enemas. The studies presented in this dissertation provide a proof of concept that suppositories can be used as potential ARV drug delivery platform for PrEP from HIV.

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ABC	ATP-binding cassette
AIDS	Acquired Immune Deficiency Syndrome
API	Active Pharmaceutical Ingredient
ARV	Antiretroviral
ART	Anti-Retroviral Therapy
ACN	Acetonitrile
ASPIRE	A Study to Prevent Infection with a Ring for Extended Use
ATCC	American Type of Culture Collections
BCS	Biopharmaceutical Classification System
BCRP	Breast Cancer Resistance Protein
BV	Bacterial Vaginosis
CDC	Centers for Disease Control & Prevention
CFU	Colony-Forming Unit
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DTPA	Diethylene Triamine Penta Acetic Acid
DP	Diphosphate
DPV	Dapivirine
DSC	Differential Scanning Calorimetry
ELISA	Enzyme Linked Immunosorbent Assay
EC50	Half Maximal Effective Concentration
FTC	Emtricitabine
GRFT	Griffithsin
GI	Gastrointestinal
GP	Glycoprotein
HAART	Highly Active Anti-Retroviral Therapy
H & E staining	Hematoxylin & Eosin Staining

HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
H_2O_2	Hydrogen Peroxide
Hu-BLT	Human bone marrow liver thymus
IC50	Half Maximal Inhibitory Concentration
IACUC	Institutional Animal Care and Use Committee
IBD	Inflammatory Bowel Disease
INSTI	Integrase Strand Transfer Inhibitor
IDU	Injection Drug Users
IHC	Immunohistochemistry
IVR	Intravaginal Ring
LC-MS	Liquid Chromatography-Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MDR1	Multi Drug Resistance Protein 1
MVC	Maraviroc
MS	Mass Spectrometry
MSM	Men who have Sex with Men
MTN	Microbicide Trials Network
N-9	Nonoxynol-9
NDA	New Drug Application
NIAID	National Institute of Allergy and Infectious Diseases
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NHP	Non-Human Primates
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PK	Pharmacokinetics
PrEP	Pre-Exposure Prophylaxis
qPCR	Quantitative Polymerase Chain Reaction
RAI	Receptive Anal Intercourse
RM	Rectal Microbicide

RH	Relative Humidity
STIs	Sexually Transmitted Infections
SIV	Simian Immunodeficiency Viruses
SHIV	Simian-Human Immunodeficiency Viruses
SLC	Solute Carrier
SPECT/ CT	Single Photon Emission Computed Tomography/Computed Tomography
TP	Triphosphate
TFV	Tenofovir
TAF	Tenofovir Alafenamide Fumarate
TDF	Tenofovir Disoproxil Fumarate
VCF	Vaginal Contraceptive Films
VFS	Vaginal Fluid Simulant
VOICE	Vaginal and Oral Interventions to Control the Epidemic
UV	Ultraviolet
UNAIDS	United Nations Programme on HIV/AIDS
US FDA	United States Food and Drug Administration
WHO	World Health Organization

Preface

I would like to express my sincere gratitude and regards to the School of Pharmacy at University of Pittsburgh. After spending more than six years in the city and getting trained by such professional faculty and pioneers in the different aspects of science at the School of Pharmacy, I have come to realize the reasons behind PITT being regarded as one of the top 10 graduate programs in the country.

I would like to express my sincere gratitude and heartfelt thanks to my adviser and professional mother Dr. Lisa Rohan. Lisa has trained me from a naïve undergraduate to an independent scientist I have become today. Her teaching philosophy, "don't do something you don't understand or just for the sake of doing it" really helped me come a long way from where I was and aided me to grow as a better thinker. Her belief in getting things perfect has made me a very detail-oriented person and scientist. Apart from science, Lisa has taught me to become a better person and inculcated compassion and humbleness in me. Being trained under the tutelage of Dr. Rohan and working in her lab gave me several opportunities to improve several skill sets, scientific and non-scientific. One of the key qualities about Lisa's lab which I will always cherish is the freedom and breadth of areas that a student or trainee is exposed and encouraged to pursue. Lisa's collaboration with scientists throughout the country in other academic institutes, government agencies and companies provided me an enormous wealth of knowledge which helped me succeed in my graduate career. Her lab has expertise from varied backgrounds which opened new doors and enforced me to seek new opportunities in new and unfamiliar areas. This provided me a new direction which helped me securing my next position in Clinical Pharmacology space. She has always wished best for her students and truly appreciates seeing her students excel in their respective careers. For the same reason she has never said no to anything that can help my grow personally and academically.

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Dedication

This PhD is dedicated to my parents, Shriratan and Rita Jhunjhunwala, my brother and his wife, Kamal and Urvashi Jhunjhunwala and my niece Aarna Jhunjhuwala. I am who I am because of my family. Thank you for raising me and making me capable of achieving this.

1.0 Introduction

1.1 The Global AIDS pandemic

Acquired Immunodeficiency Syndrome (AIDS), is a life-threatening disease caused by Human Immunodeficiency Virus (HIV). HIV-1 infections are typically chronic and can only be treated using anti-retroviral therapy (ART) [1]. Although several efforts are currently being undertaken to develop vaccines and interventions to avoid AIDS acquisition, there is no cure for HIV/ AIDS till date. According to the most recent report published by the Joint United Nations Programme on HIV/AIDS (UNAIDS), in 2017, 36.9 million people lived with HIV-1 infection globally [2]. Amongst these, 35.1 million were adults and the remaining 1.8 million were children (<15 years of age). In total, since the beginning of the HIV/ AIDS epidemic, there have been 35.4 million deaths due to AIDS-related illnesses and ~1.9 million people were newly infected in the year 2017 [2]. The most common transmission mechanism resulting in new infections are men who have sex with men (MSM) and injection drug users (IDUs) [3, 4]. Although the number of new HIV-1 infections have significantly reduced (47%) from its peak in 1996 (3.4 million), newer and more effective strategies are required to combat and reduce the rate of new infections, especially for high-risk populations such as MSM. Usually, there is a standard line of treatment for HIV-1 infections based on the severity of the infection and other associated co-morbidities such as tuberculosis and cancer. Although there is no cure for HIV-1, it can be prevented in HIV-1 naive individuals by using products designed for prophylaxis or by treating the HIV-1 positive patients, keeping the viral load under control with the help of highly active anti-retroviral therapy (HAART). The most common treatment regimen used for HIV-1 infection is a single pill (Truvada®) with combination of multiple anti-retrovirals (ARVs) – emtricitabine (FTC) and Tenofovir Disoproxil Fumarate (TDF).

Sexual transmission of HIV-1 has been one of the most common routes of the widespread AIDS pandemic. A recent survey conducted by the Centre of Disease Control and Prevention (CDC) in 2017, confirmed that 38,739 new HIV-1 infections were diagnosed in the United States, about 66% of these new diagnosis (25,748) were caused by gay and bisexual men. Although the

number of new HIV-1 diagnoses have remained stable from 2012 to 2016, the new infections due to men who have sex with men (MSM) has increased from 63% (2010) to 66% (2017). According to Beyrer et al. “minimal to no decline” in the number of new HIV-1 infections among MSM were seen, especially young aged MSM [5]. Tremendous efforts are being undertaken to develop products for pre-exposure prophylaxis (PrEP). The newer generations of ARVs and recent advancements in ART are capable of restricting viral replication and keeping the viral load under control for people living with HIV-1 infections. Adhering to ART and constantly monitoring viral levels can significantly increase the lifespan of HIV-1 positive individuals [6-8]. Currently the FDA has approved only two oral PrEP products, Truvada® (emtricitabine 200mg and tenofovir disoproxil fumarate 300mg) and Descovy (emtricitabine 200 mg and tenofovir alafenamide 25 mg). In addition to oral PrEP, numerous approaches are being pursued for products that directly target the site of viral entry for topical PrEP. Although recent advances in ART has made significant progress in developing treatment interventions to control the viral load, the number of deaths and new infections observed every year indicate the massive burden the AIDS pandemic holds over mankind. This demonstrates that despite the current efforts, safe and effective topical products for HIV-1 prophylaxis are still lacking. One of the robust ways to tackle the overwhelming rectal transmission rate in MSM population is the use of rectal microbicides (RM). RMs are products that can be administered in the rectal compartment pre- or post-sexual intercourse and can significantly reduce the probability of HIV-1 acquisition [9, 10]. The microbicides containing ARV drugs are capable of hindering the viral entry and disrupt HIV-1 replication in the local tissue and prevent spread of infection into the $CD4^+$ T cells present in the vagina or rectum. In addition to MSM, RMs may also help reduce HIV-1 transmission risk in heterosexual couples engaging in receptive anal intercourse (RAI). Therefore, in addition to improving the existing ART regimens, it is crucial to develop new strategies to prevent new infections and restrict the widespread of HIV-1.

1.2 The Human Immunodeficiency Virus (HIV): pathophysiology and ARV targets

HIV is a type of retrovirus which infects by integrating into the host/ human DNA in order to replicate. Retroviruses can be typically classified into two broad categories, simple and complex. HIV is one of the complex retroviruses, and can also be regarded as a lentivirus, which is a genus known to cause chronic and deadly infections followed by long incubation periods [11]. The HIV replication is a complex multi-step process which involves the action of several key enzymes in a chronological order. By inhibiting the function of one or more of these enzymes, the HIV replication can be blocked, which can restrict further viral replication and spread in the host immune system.

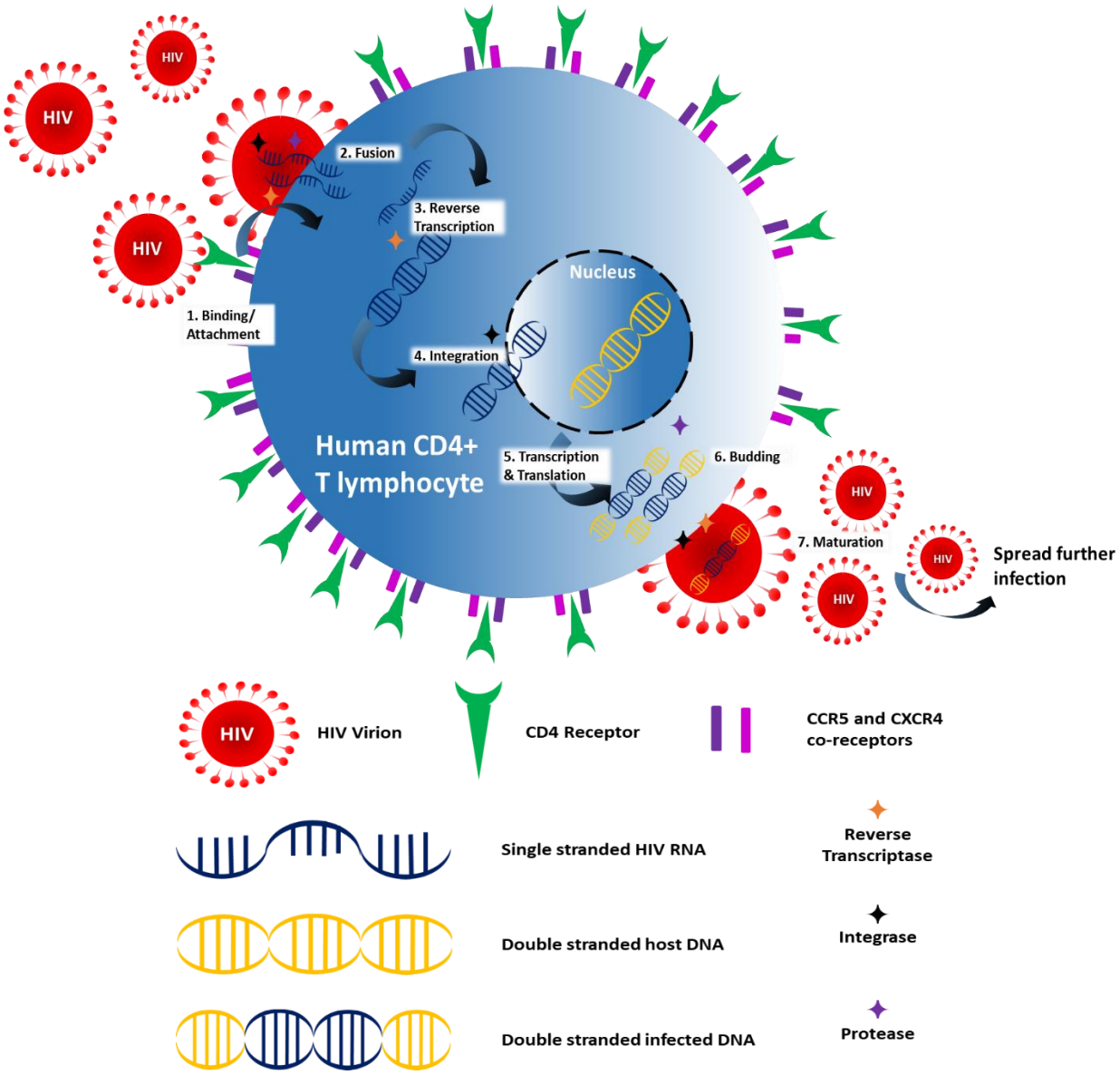


Figure 1 HIV replication lifecycle

(1) Binding/ attachment of the HIV with the human $CD4^+$ T lymphocyte (2) Fusion and release of the HIV viral protein and enzymes into the host cell (3) Reverse transcribing the viral RNA to viral DNA (4) Integrating the viral DNA into the human DNA (5) Transcription and translation to create multiple copies of the infected DNA (6) Budding and assembly of new HIV virions (7) Maturation of the newly formed HIV virions. Adopted from [12, 13]

Once HIV virions enter the host system, the first step of the HIV-1 replication is the attachment and entry of the HIV-1 virion into the host cell (Step 1 of figure 1). The HIV-1 virion envelope consists of two essential glycoproteins (gp) 120 and 41; these gps are programmed to attach to

specific receptors and co-receptors such CCR5 and CXCR4 which are present on the surface of $CD4^+$ T lymphocyte. Attachment of these gps and receptors causes binding and entry of the HIV-1 virion into the $CD4^+$ T lymphocyte. This step can be inhibited by a class of ARV agents called entry or fusion inhibitors. Upon entry, key enzymes and viral RNA strands of the HIV-1 cell are released into the $CD4^+$ T lymphocyte (Step 2 of figure 1) [14]. These components typically consist of the viral RNA and three essential enzymes that bolster the HIV-1 infection namely, reverse transcriptase, integrase and protease. One of these enzymes is called reverse transcriptase. The role of this enzyme is to reverse transcribe the single stranded viral RNA to double stranded viral DNA (Step 3 of figure 1). This reverse transcription can be blocked by a class of ARV agents called non-nucleoside reverse transcriptase inhibitor (NNRTI) or nucleotide reverse transcriptase inhibitor (NtRTI). Once a double stranded viral DNA complex is formed, it has to be integrated into the host DNA. This is followed by transportation of the newly formed viral DNA into the host cell nucleus and integrate it into the host cell genome. This integration is performed by an enzyme called integrase (Step 4 of figure 1). This integration can be restricted by a class of drugs called integrase strand transfer inhibitor (InSTIs). It has been shown that ARVs which act in later stages of replication such as InSTIs are able to better inhibit the HIV-1 replication [15] than those which act at early stages of replication. Once the viral gene is incorporated into the host genetic system, multiple copies of the infected DNA are created by continuous transcription and replication (Step 5 of figure 1). The last step of the HIV-1 replication is the maturation of the newly formed HIV-1 viruses, which is accomplished by an enzyme called protease (Step 6 and 7 of figure 1). These matured copies of the HIV-1 virus initially form a viral reservoir locally at the site of infection and then further proliferate to infect other matured $CD4^+$ T lymphocytes. This formation and maturation of the HIV-1 can be inhibited by a class of ARV agents called protease inhibitors.

1.3 Current research strategies to manage HIV infections

As mentioned in section 1.1, AIDS caused due to the HIV is a global pandemic and a single intervention strategy for AIDS management is insufficient, therefore several different approaches and strategies are available or currently being investigated. Based on the HIV pathophysiology

described in section 1.2, HIV-1 is a complex retrovirus and consists of complicated cellular mechanisms to establish a life-long infection. Primarily, HIV infection can either be treated using different approaches or can be prevented by PrEP (oral, topical and systemic) or potential vaccines that are currently under development. However, due to latest technological advancements and cutting-edge research in the past three decades, several research strategies and targets to potentially cure or eradicate this deadly infection have been identified. The current HIV management interventions which are either available on the market or under clinical development can be classified into three broad categories, (1) Treatment using Highly Active Anti-Retroviral Therapy (HAART), (2) Vaccines and (3) Microbicides for prevention. Other strategies that are being extensively investigated include regulation and silencing of long non-coding RNA (lncRNA), which plays a significant role in HIV replication.

HAART has been very successful and widely utilized to treat patients with AIDS. Moreover, due to increased incidences of ARV resistant virus strains, continuous strategies are being sought to improve already existing effective anti-retroviral therapy (ART). These strategies include exploring novel combinations of ARVs (refer section 1.10), developing more potent and drug resistant ARVs (ex. Dolutegravir versus Raltegravir), and developing long-acting formulations (Cabotegravir (CAB) and Rilpivirine (RPV)) [16] which avoid daily oral regimen to increase treatment adherence. CAB (GSK1265744), a structural analog of dolutegravir is an integrase strand transfer inhibitor which is currently being investigated as an oral tablet as well as long-acting nanosuspension which could potentially be used for treatment as well as prevention of HIV-1 [17]. CAB's long systemic half-life of 40 days post oral administration renders it suitable for achieving sustained protection. In initial clinical studies, it has been shown that the long-acting formulation of CAB was readily absorbed when administered intramuscularly (IM) and subcutaneously (s.c.). Further, repeat-dose PK studies provided clinically relevant plasma concentrations of CAB for extended periods of time, supporting a monthly or bi-monthly dosing regimen [17]. The LATTE-2 study was a randomized, open-label, phase 2b, non-inferiority trial evaluating long-acting IM CAB with RPV [16]. The study population included treatment-naïve HIV-1 infected adults. The study was conducted in three arms, each treatment arm first received oral CAB plus abacavir-lamivudine combination for a 20-week induction period followed by random assignment to one of the three treatment arms at 2:2:1 ratio. The three treatment arms included (1) long-acting CAB plus RPV at 4-week intervals (2) long-acting CAB plus RPV at 8-

week intervals and (3) Continued the oral CAB plus abacavir-lamivudine combination [16]. The viral suppression (HIV-1 RNA <50 copies/ mL) was monitored for the study populations in all the three arms and they were followed up to 96 weeks. A Bayesian approach was adopted to determine if the viral suppression achieved for each long-acting regimen was non-inferior to the oral regimen. According to the 96-week results reported by LATTE-2 study, both the long-acting CAB plus RPV dosing regimens were as effective as daily oral therapy at HIV-1 suppression. Moreover, the evaluated all injectable formulations were well accepted and tolerated [16]. In addition to a potential effective HIV-1 treatment regimen, the long-acting CAB was evaluated as a potential prevention strategy in a pre-clinical non-human primate (NHP) model as well. The long-acting CAB formulation protected NHPs when challenged with simian/human immunodeficiency virus (SHIV) intravaginally [18, 19] as well as intrarectally [20].

Furthermore, vaccines when available will be the most effective strategy against new HIV infections. Unfortunately, so far, no vaccine has been approved. However, significant studies and failures have allowed a few vaccine candidates to reach late stages of clinical development and demonstrated promising results for HIV prevention [21-23]. Recently, a first-in-human trial was conducted to evaluate safety, immunogenicity and mucosal shedding of an oral, replicating adenovirus 26 vector vaccine for HIV-1 [21]. A total of 22 healthy adults were enrolled in the study, amongst them, 18 participants received a single dose of a replication-competent, highly attenuated Ad26 vector that expresses mosaic HIV-1 Env (rcAd26.MOS1.HIV-Env, "rcAd26") in dose escalation of 10^8 to 10^{11} and four participants received the placebo. The participants were monitored for 10 days post vaccination to observe reactogenicity and any vaccine-related adverse events up to 112 days. The results demonstrated only mild to moderate adverse events caused due to vaccines. Moreover, the mucosal shedding results did not indicate any infectious rcAd26 viral particles in the rectal or oropharyngeal secretions from any of the participants. Although the highly attenuated rcAd26.MOS1.HIV-Env vaccine was well tolerated, the poor immunogenic response post single dose suggested the replicative capacity of the vector was too attenuated. In conclusion, considering the immunogenic responses and mucosal shedding results, less attenuated viral vectors are desired in the future live, oral HIV-1 vaccines [21]. Similarly, a mosaic HIV-1 vaccine was evaluated in a multicenter, randomized, double-blind, placebo-controlled, phase 1/2a clinical trial (APPROACH) and in rhesus monkeys (NHP 13-19 study) [23]. The vaccine candidate was tested in few different regimens using IM and demonstrated favorable safety and tolerability in humans.

Moreover, all the regimens were also evaluated in a pre-clinical NHP model [23]. Post-vaccination NHPs were repetitively challenged by heterologous SHIV, and the results demonstrated 67% protection against SHIV infection. In conclusion, the mosaic HIV-1 vaccine was capable of inducing comparable immune response in humans and NHPs. This vaccine is currently being investigated in a phase 2b clinical efficacy trial in Sub-Saharan Africa [23].

Although HAART is highly effective, once a permanent HIV infection is established, infected individuals have to receive life-long ART as ARVs can only help in viral suppression keeping the viral load under control. However, in order to completely cure an infected individual, identifying and eradication of the persistent replication-competent provirus present in latent stages in the memory $CD4^+$ T cells is essential. Therefore, studies are currently undertaken to better understand the underlying mechanism and factors involved in HIV-1 replication. Several studies have recently identified the role of long non-coding ribonucleic acid (lncRNA) in HIV pathogenesis and recognized targets which can be exploited to potentially arrest HIV replication [24-29]. Due to the involvement of lncRNAs in HIV pathophysiology, studies targeting lncRNA to potentially cease viral replication have been reported [30]. Chao et. al. identified a key lncRNA which regulated HIV-1 replication through epigenetic regulation of the HIV-1 promoter [26]. In this study, peripheral blood samples from HIV-1 infected donors were collected and primary monocyte-derived macrophages (MDMs) were cultured. A genome-wide expression analysis of lncRNAs was conducted in MDMs. The results from this analysis recognized a specific lncRNA, HIV-1 enhance lncRNA (HEAL) that was upregulated in HIV-1 infected MDMs, microglia and T-lymphocyte [26]. The authors were able to identify two key mechanisms through which HEAL would form a complex with RNA-binding protein FUS, aiding the HIV replication. To further validate their hypothesis, using RNA interference (RNAi) mediated knockdown and CRISPR-Cas-9 mediated knockout, they were able to prevent HIV-1 reactivation in T cells and microglia after stopping azidothymidine (well-known ARV) treatment, in an *in vitro* setting. In summary, the results from this study provide a potential strategy to eradicate the latent viral reservoirs by targeting the HEAL [26]. Additionally, some other interventions specifically targeting the latent, quiescent, memory CD4+ T cells using host paracaspase MALT1 inhibitor, MI-2 are being explored and currently being investigated [31].

Although vaccine against HIV will significantly reduce new HIV infections, so far there is no vaccine approved. Therefore, other prevention strategies such as microbicides are warranted. In this category, oral Truvada® has proven highly effective and approved for use in human. However, topical microbicides are designed to act as the first line of defense at the site of viral entry, rectum or vagina. Topically applied anti-retrovirals provide significant concentrations at the mucosal sites, which are targets of HIV transmission. A few products have established efficacy and reached late stages of clinical development (refer section 1.6.2). Being a product designed for local delivery, topical microbicides have minimal possibilities of initiating an immunogenic response. Enough ARV concentrations at the local site of action are potentially capable of restricting viral entry in the systemic circulation. Moreover, considering the socioeconomic status of several AIDS dominated developing nations, microbicides can be beneficial as they can be easily accessible.

1.4 Mucosal HIV transmission mechanism

Some of the common modes of HIV-1 transmission are mother-to-child transmission, injection devices used by drug users, blood transfusions and unprotected sexual intercourse. Amongst these modes of transmission, unprotected sexual intercourse (anal/ vaginal) is one of the most common pathways of spreading HIV-1 infection. There is a plethora of information and literature investigating the replication and the HIV-1 life cycle. However, very little is known about the viral transmission through the mucosal sites such as the vagina and rectal compartment. Therefore, some experts in the field investigated this mucosal HIV-1 transmission using non-human primate (NHP) models to identify the key potential targets for ARV agents to act upon and timelines associated with the establishment of acute and chronic HIV-1 infection post initial exposure to HIV-1 [32, 33]. Using NHP models, it was found that there is limited window (30-60 mins) of exposure for HIV-1 to establish initial infection [32]. This is primarily because mucosal sites such as the vagina and rectum are highly perfused and have an abundance of HIV-1 target cells such as the $CD4^+$ T lymphocytes, especially in the superficial layers of the mucosa. These infections are further bolstered as the key cellular components such as the stratified and columnar epithelium are exposed due to the disruption in the epithelial integrity caused due to the sexual intercourse [33].

Post viral attachment, HIV-1 begins to replicate, leading to production of more viruses, establishing an initial viral foci in the mucosal tissue by about 16-72 hours post-exposure. Due to the host's natural immune response, further CD^+ T cells are activated and leads to an influx in the infected areas. Newly produced viruses infect these immune cells and further propagate the infection eventually disseminating in the drainage lymph nodes (24-72 hours), where the infection can be detected [33]. Over the period of next few days to weeks, local expansion of the virus into self-sustaining reservoirs leads to systemic dissemination [32].

To avoid this life-long systemic HIV-1 infection, it is crucial to restrict the viral replication at the beginning of the initial exposure. It is essential for the microbicides to be present at the right time at the right place at optimum drug concentrations. Therefore, microbicides targeting these transmissions at the mucosal site of viral entry (vagina/ rectum) are warranted. There is some evidence in pre-clinical and clinical studies which prove that microbicides are capable of providing protection against HIV-1 acquisition when administered before or in some cases after coitus. Hence, studies presented in this dissertation are focused on developing microbicides that can potentially inhibit HIV-1 transmission.

1.5 Increased risks of acquiring HIV via receptive anal intercourse

According to several surveys conducted by the CDC, the estimated per-act probability of acquiring HIV-1 from an infected source through receptive anal intercourse (RAI) (138/ 10,000 exposures) is about 17 times higher than compared to receptive vaginal intercourse (8/ 10,000 exposures) [34]. From a physiological perspective, the rectal cavity is lined with a single layer of columnar cells compared to multiple layered stratified squamous epithelium of the vagina; making the rectal compartment more susceptible to damage and increasing probability of viral entry [10]. The thickness of the rectal epithelium (~25µm) is about one-tenth compared to the vaginal epithelium (215µm) [35]. Apart from the innate physiology of the rectal compartment, engaging in RAI causes some trauma and damage to the rectal epithelium, eventually breaching the epithelium making entry easier for STDs and HIV-1. Additionally, the rectal compartment is known to have perfused vasculature, which may facilitate the passage of infected seminal fluid to

enter the systemic circulation through the traumatized blood vessels and establish initial infection [35]. Furthermore, the rectal columnar epithelium has a large number of HIV-1 target cells as compared to the vaginal epithelium [10, 36]. Studies have shown evidence of presence of the macrophages and dendritic cells (DCs) in the rectal epithelium which HIV-1 typically binds to. In addition, the rectal lymphoid follicles comprise of specialized M cells, which are known to bind to HIV-1 and spread the infection in lymphoid tissues underneath [36]. Also, unlike the vaginal compartment which has restricted entry after a point, the rectal compartment is comparatively open ended and extends up to the small intestine and beyond, therefore the product designed for rectal administration should be capable of protecting the entire region.

1.5.1 Heterosexual couples engaging in receptive anal intercourse

Historically, it was believed that anal intercourse is primarily associated with homosexual men and not heterosexual couples. But results from several studies and surveys conducted in the recent past state otherwise [37-39]. Some studies have indicated that engaging in anal intercourse is more common in heterosexual men and women compared to homosexual men. A study conducted in 12,571 men and women in the age range 15-44 years, confirmed that about one-third of the studied population engaged in anal intercourse [39]. Moreover, engaging in anal intercourse was correlated with age groups, 10.9%, 29.6%, 35.4% and 34% women; and 11.2%, 32.6%, 38.9% and 41% men within age groups 15-19, 20-24, 25-34 and 35-44 years, respectively. The same study also evaluated the use of condoms during anal intercourse and the results indicated that only 13.3-25.5% women and 22.2-34% men used condoms while engaging in anal intercourse. This indicates the extent of heterosexual individuals engaging in anal intercourse are exposed to the risk of contracting HIV-1 and other related sexually transmitted infections (STIs). There are several similar studies conducted in different parts of the world to evaluate the prevalence of heterosexual couples engaging in anal intercourse [40-43]. The results from different regions might vary, but in totality, anal intercourse amongst heterosexual females and males is not uncommon. From a behavioral perspective, a recent qualitative study evaluated why women engage in anal intercourse [44]. According to results from this study, six key motivating factors were identified as reasons why women would engage in anal intercourse. The reasons were (1) Desire to avoid vaginal sex

(on period, discharge), (2) To please her partner, (3) Women's own desire for anal sex, (4) Quid pro quo Money or drugs exchanged for anal sex, (5) Under the influence of drugs or alcohol, (6) No Consent Coercion Did not know she could refuse [44]. However, some of the reasons demonstrate that in some situations the women engaged in anal intercourse against their consent or to please their male partners. In such cases, utilizing a rectal microbicide product with or without the partners knowledge might benefit the women by reducing the chances of transmitting any STIs.

1.5.2 Men who have sex with men

According to surveys conducted by the Centre of Disease Control & Prevention (CDC), in 2010, 47,500 new HIV-1 infections were reported in United States. Amongst these, about 80% (38,000) infections were accounted by men. Moreover, within the 38,000 newly infected men, about 78% (29,800) accounted for men who have sex with men (MSM) [45]. The same report also categorized the new infections in the US based on gender and other transmission factors like injections drug users (IDUs) etc. Amongst the newly infected male population in the US, MSM contributed (28,500) about 10.5 times more than heterosexual men (2700) and about 26 times more compared to IDUs (850) [45]. These figures indicate the prevalence of MSM over other HIV-1 transmission factors. Although the number of people living with HIV-1 infections have declined over the past two decades, the number of new HIV-1 infections caused due to MSM have either remained stable or in some areas even increased [46]. Due to this rate of rising infections amongst MSM, some articles have regarded this as the new HIV-1 epidemic among MSM [46-48].

1.6 Current pre-exposure prophylaxis (PrEP) strategies

Several strategies are being adopted to develop effective ways to combat HIV-1 infection. One of the most recent and effective ways to restrict the initial HIV-1 acquisition is pre-exposure prophylaxis (PrEP). PrEP involves administration of medication to individuals with high risk of contracting HIV-1 infection before getting exposed to potentially infected individuals. PrEP can be classified into oral (pills by mouth) or topical (microbicides either rectal or vaginal).

Appropriate administration of oral PrEP products can significantly reduce the risk of HIV-1 transmission from person to person.

1.6.1 Oral PrEP

The United States Food and Drug Administration (US FDA) approved the first oral PrEP product in the year 2012. The product was developed and marketed by Gilead Sciences Inc. under the brand name Truvada®. Truvada® consists of a combination of tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) in a single pill. This is designed for individuals with high-risk of contracting HIV-1 infections and is supposed to be administered once daily. Truvada® was granted approval by the US FDA largely based on the results of two randomized clinical trials (Preexposure Prophylaxis Initiative (iPrEx) trial [49] and Partners PrEP Study [50]). The first clinical trial was a random placebo-controlled trial of FTC + TDF conducted with 2499 HIV-seronegative men or transgender women who have sex with men. The results from this clinical trial confirmed 100 HIV seroconversions during the follow up period; out of which 36 subjects in the TDF + FTC group and 64 from the placebo. This demonstrates that the TDF + FTC group was able to achieve a 44% reduction in HIV incidence rate [49]. Similarly, an oral PrEP trial (PARTNERS PrEP study) was conducted in Uganda and Kenya with 4758 heterosexual serodiscordant couples [50]. This study design consisted of three treatment regimens, once daily TDF only, TDF-FTC and matched placebo, and they were followed for up to 36 months. The results from the PARTNERS study demonstrated a relative incidence rate of 67% with TDF only and 75% with TDF-FTC combination when compared with placebo. Moreover, from a safety perspective two out of eight HIV-1 infected subjects (at baseline) developed drug resistance [50].

In spite of the success of oral PrEP, several post-analyses of the above-mentioned clinical trials indicated that adherence to the treatment i.e. taking one pill a day without missing a dose and using alternative strategies for safe sex practices is essential to stay protected. Also, missing a dose might eventually lower TFV levels below the required trough levels leading to sub-optimal drug exposure which can increase chances of drug resistance [49]. Therefore, alternative strategies for “on-demand” purposes were sought. A recent clinical trial was conducted to evaluate if Truvada® can potentially be utilized as on-demand oral PrEP product as well [51]. The Intervention Préventive

de l'Exposition aux Risques avec et pour les Gays (IPERGAY) study group conduced a double-blind, randomized trial to evaluate the safety and efficacy of TDF-FTC among men who have unprotected anal sex with men. In total, 400 participants were enrolled in the study and randomly assigned to two treatment arms, 199 in the TDF-FTC group and 201 in the placebo group. Participants in both arms administered the product before or after sexual activity (median of 15 pills per month) and were followed up for about 9 months. According to the results from this trial, 16 total infections were observed during the follow up period, two in the TDF-FTC group and other 14 were in the placebo group. This demonstrates a relative reduction in HIV-1 incidence of about 86% in the TDF-FTC group [51]. Although the rates of adverse events (AEs) in both groups were similar, the TDF-FTC group indicated higher rates of GI and renal AE compared to placebo group [51]. In summary, even oral products such as TDF-FTC combination oral pill can be used as an on-demand product for HIV PrEP, but along with the potential possibilities of increased AEs. Products administered topically could potentially reduce the incidences of AEs associated with oral PrEP, due to reduced dosing and minimal systemic exposures.

1.6.2 Topical PrEP and its advantages

Microbicides are topical drug delivery systems which can be used as a measure of prophylaxis against sexually transmitted infections (STIs) such as HIV-1. Microbicides are typically administered rectally or vaginally prior to sexual intercourse, as this can significantly reduce the probability of infections. Microbicides can be advantageous compared to other prophylactic interventions because they act as the first line of defense by delivering drugs locally at the site of viral entry. This restricts the viral entry into the body thereby preventing the virus from establishing an initial infection and avoiding the formation of local viral reservoir. This way a systemic irreversible infection can be avoided. More importantly topical microbicides are capable of delivering high doses at the site of entry and potential infections with low systemic exposures, resulting in less toxicity. Usually microbicide products are in the form of rings, gels, suppositories, enemas and films [52]. Some products such as tenofovir (TFV) gel [53], dapivirine (DPV) vaginal rings [54] and gels [55] have reached late stages of clinical development as on-demand products for pre-exposure prophylaxis (PrEP). Additionally, microbicides consisting of active moieties that

can regulate innate factors such as metabolizing enzymes and vaginal/ gut flora-fauna which can be advantageous in providing protection against HIV-1 and other sexually transmitted infections [56]. Microbicides with such strategies are being actively investigated for the development of on-demand products [53, 57].

Few products like TFV gel and DPV vaginal rings have demonstrated efficacy and have reached phase III of clinical development as either on-demand product or just for PrEP [53, 58]. Several such products are currently being actively investigated for an on-demand product as well as for prophylaxis. These products have a capability to deliver the drug locally at the potential site of infection i.e. either the rectum or vagina. This way HIV-1 entry at the local site can be blocked, preventing penetration into the tissue and entry into the systemic circulation. From a drug delivery perspective, factors such as metabolizing enzymes, efflux and influx transporters, innate hormonal levels etc. govern the microbicide's performance *in vivo*. Therefore, it is very crucial to design microbicides with ARV agents which take into the account these factors for optimal dosage form design.

1.7 Current status of rectal microbicide development

Growing evidence in the literature indicates the compelling need of rectal microbicide (RM) products in different parts of the world [59-62]. Conventionally microbicide product development was predominantly focused on vaginal administration. A study conducted by the CDC suggests that the risk of contracting HIV-1 via RAI is 10 times higher as compared to insertive vaginal intercourse and 5 times higher than receptive vaginal intercourse. Owing to the recent increased incidences of HIV-1 infection caused through RAI, strategies to target populations including heterosexual couples engaging in RAI are warranted. The rectal cavity is lined with a single layer of columnar cells compared to multiple layered stratified squamous epithelium of the vagina; making the rectal compartment more susceptible to damages leading to viral entry [10]. Furthermore, the rectal columnar cell epithelium is fairly populated with the HIV-1 target cells compared to the vaginal epithelium; which does not possess the target cells at the surface, rather in the vaginal tissues beneath the cell layers [10, 36]. From a physiological perspective, the rectum

and vaginal compartment demonstrate unique characteristics which are crucial for microbicide product development. Due to differences in their respective surface areas, products for rectal application should cover a much larger surface area compared to vaginal compartment [9].

Although significant progress has been made in the development of vaginal microbicides, a safe and effective product for rectal application has yet to be developed. A rectal microbicide can be advantageous as it can act as a secondary protective barrier to HIV-1 acquisition even in cases where condoms breaks or slips-off while engaging in receptive anal intercourse [63]. First generation rectal microbicides contained only one active pharmaceutical ingredient (API) but recent advancements have explored multiple APIs in a single product [57, 64, 65]. Single layer of epithelial cells in rectal mucosal membrane makes the rectum more susceptible to HIV-1 infection. Therefore, from a rectal drug delivery perspective, different development strategies are required compared to the multi-layered epithelium of the vagina [63, 66]. Additionally, the gastro-intestinal tract serves as a rich source of HIV-1 target cells. Isolated lymphoid follicles are present throughout the colon and their number increases in the anus further becoming abundant towards the rectum. This makes individuals who engage in RAI extremely susceptible to infections. Drug delivery options to prevent transmission in the rectum include gels, enema, douches [67] and suppositories [68].

1.8 Current vehicles for rectal drug delivery

1.8.1 Gels

Gels have been traditionally used as a topical drug delivery dosage form for the treatment of various disease conditions. Due to the adaptable properties and wide array of options the gels provide, they can be utilized for several applications, such as transdermal, ocular, oral, rectal and vaginal. Therefore, gels with ARV agents have been recently explored as a potential HIV-1 prevention strategy as well [53]. A vaginal gel with 1% TFV was one of the first attempts for pre-exposure prophylaxis of PrEP of HIV-1 [53]. Similarly, other ARV candidate like Maraviroc has

also been investigated using the same vaginal gel formulation [69]. Although the TFV vaginal gels were proven efficacious in the clinics, with the significant increase in number of heterosexual couples engaging in RAI (section 1.5.1), a rectal formulation was warranted. With good safety, acceptability and efficacy data from studies with vaginal applications, the gels were explored for rectal drug delivery as well, especially for heterosexual couples and MSM engaging in RAI. Therefore a few attempts are currently under pursuit to develop dual compartment gels (rectal and vaginal). A recent study evaluated the potential of rectal SHIV transmission post vaginal administration of TFV + FTC gel and found a 4.5-fold reduction in risk of acquiring infection using a macaque model [70].

Although the rectal gel formulation was well tolerated and efficacious, it was associated with few behavioral and acceptability concerns. Factors like the need for an applicator to administer the gel, and messiness caused while administering or leakiness post administration raised adherence and acceptability issues in the clinics. Additionally, depending on the potency of the ARV agents, some APIs would require administration of higher volumes of gels to achieve target concentrations *in vivo*. Moreover, incorporating hydrophobic APIs in water-based gels, without using surfactant might be challenging. Therefore, other drug delivery systems were explored for rectal PrEP to improve user adherence and acceptability.

1.8.2 Enemas & Douches

Enemas are liquid dosage form typically available in a bottle attached with an elongated tube to facilitate ease of administration into the rectum. They are typically used as treatment options for constipation and stimulate bowel movement. Some individuals also use enemas for rectal cleansing as a measure of pre-treatment before surgeries or administration of medications. Moreover, enemas are commonly used by the MSM for rectal cleansing and hygienic purposes before engaging in RAI. As mentioned in section 1.5.2, MSM is one of the highest risk populations for contracting HIV-1 infections. Therefore, several researchers have taken advantage of this already existing route of administration and dosage form for HIV-1 prevention [71-75]. Researchers hypothesized that incorporating ARVs in the enemas might solve two purposes with a single-application, cleanse the rectal compartment as well as provide potentially therapeutically effective levels of ARVs

locally, to restrict viral entry at the site of action. Some progress has already been made using ARV containing enemas in clinical trials, from a safety and adherence perspective [72, 73, 76], and in animal models from an pharmacokinetics (PK) perspective [74]. Initial exploratory studies were conducted in nine men with iso-, hyper- and hypo-osmolar enema solutions to evaluate distributions, safety and acceptability [72]. The results from this trial indicated good acceptability among the three evaluated enema products. In addition, mucosal permeability, tissue penetration and colonic distribution were evaluated using radiolabeled [99mTc-diethylene triamine Penta acetic acid (DTPA)]. The radiolabeled DTPA enabled detailed analysis of distribution and toxicity via single photon emission computed tomography/computed tomography (SPECT/CT). Although no preference was observed amongst the evaluated enema products, comparing all the evaluated attributes, isoosmolar enema seemed superior or similar compared to others [72]. Furthermore, using TFV as a model ARV, Xiao et. al. evaluated iso and hypo-osmolar enema solution at two different dosing levels (high – 5.28mg/mL; low – 1.76mg/mL) [74]. Amongst the evaluated formulations, high-dose hypo-osmolar enema solution demonstrated highest uptake of TFV and TFV- diphosphate (DP) in tissues. Most importantly, the TFV-DP levels obtained post high-dose hypo-osmolar enema dosing was enough to prevent infection in *ex vivo* challenges of rectal tissues post intra-rectal dosing. These results demonstrate that enema could be a potential delivery platform for ARV delivery [74].

1.8.3 Inserts

Inserts are oval shaped polymeric tablets capable of quickly dissolving in the rectal compartment. The inserts are being developed by Conrad & Eastern Virginia Medical School (EVMS). Results from one of the first studies conducted evaluating vaginal inserts was presented at the Conference on Retroviruses and Opportunistic Infections (CROI 2019) in Seattle, Washington [77]. Dobard et. al evaluated TAF/ EVG combination in a vaginal insert using macaque model. A total of 14 pig-tailed macaques were used in this study, wherein, 6 animals received TAF/ EVG vaginal insert followed by $SHIV_{162P3}$ exposure once a week for up to 13 weeks post product insertion. Similarly, 8 animals received matched placebo inserts followed by viral exposure. The results from this study indicated 7 out of 8 macaques who received placebo inserts

were infected and only 1 out of 6 macaques that received TAF/ EVG insert were infected. In totality, TAF/ EVG inserts were able to achieve 92% efficacy against viral exposure [77]. Positive results from animal studies have progressed this product to first-in-humans study, an ongoing phase 1 study evaluating the TAF/ EVG insert [78]. The positive results from this study have encouraged researchers to pursue similar inserts as fast-dissolving tablets for rectal drug delivery as well. A phase I clinical trial conducted by Microbicides Trial Network (MTN – 035) is currently evaluating different dosage forms including inserts, to assess acceptability, tolerability and adherence.

1.8.4 Suppositories

Suppositories are semi-solid dosage forms designed to either melt or dissolve at body temperature and deliver the drug in the rectal compartment. A few studies have investigated the most preferred dosage form amongst gels, enemas, insert and suppositories, and the results indicate mixed preference amongst tested subjects [68, 76]. This is primarily because user preferences might vary, considering the advantages and disadvantages associated with each dosage form. However, suppositories have been on the market for decades and have evolved from naturally occurring cocoa butter and Theobroma oil to semi-synthetic triglycerides. Suppositories have been widely investigated for treatment of constipation, inflammatory bowel disease (IBD), Crohn's disease etc. Broadly, suppositories have several advantages over other dosage forms, such as ease of administration as suppositories do not need an applicator like gels. Additionally, the wide range of suppository bases available on the market makes it versatile providing the opportunity to incorporate a wide range of APIs (hydrophilic, hydrophobic, liquid APIs) which can be challenging with other dosage forms. In addition to that modification of suppository bases, the mono-, di- and tri-glyceride ratios, the melting points of the suppositories can also be altered [79]. This can be significant while formulating suppositories with APIs which are known to reduce its melting point. The addition of ingredients like surfactant can also modify drug release rates [80]. Moreover, suppositories offer greater flexibility in terms of shape and sizes, which offers higher drug loading capacity compared with gels and inserts. Therefore, considering these advantages and

using “another tool in the box” approach, our goal was to develop ARV containing suppository products which can be used for HIV-1 prevention.

However, suppositories offer many strategic advantages compared to other dosage forms including principles such as ease of manufacture, capability of incorporating a wide range of hydrophilic and hydrophobic small molecules and providing a higher drug loading capacity [81]. In some cases, additives like surfactants or absorption enhancers may be included in suppository bases to improve drug absorption from suppositories [82-84]. IOI Oleo Chemical provides witepsol suppository bases of different grades, for instance one of the available grades, ‘S’ consists of a non-ionic ethoxylated emulsifier. Incorporation of this additive makes this grade more suitable for hydrophobic drugs. Similarly, addition of absorption enhancers like sodium laurate (C12) along with taurine in fat-soluble suppository bases can help the hydrophobic APIs partition better than in the aqueous rectal fluid eventually improving drug dissolution. This strategy can prove advantageous for poorly absorbable drugs, improving their overall absorption [84].

1.9 Key performance attributes for suppository bases

Suppositories are currently being explored as a potential platform for anti-retroviral therapy [85]. However, as discussed in section 1.8.4, it is important to understand the behavior of suppositories upon administration in the rectal cavity. The rectal cavity is a dynamic environment with significant intra and inter-person variability. Considering the anatomy of the rectal cavity, factors such as blood supply, surface area, presence of feces etc. can vary from person to person which can alter product performance. On the contrary, product attributes such as pH, solubility, absorption enhancers, dosage form size and weight (enema vs gel vs suppository) can also affect its performance by impacting the physiological parameters such as permeability and absorption. Hence, to better predict the *in vivo* performance of suppositories, it is crucial to investigate their impact on physiological parameters like drug absorption and factors affecting permeability.

Suppositories are broadly classified into two categories, fatty-acid based (fat-soluble) and polyethylene-glycol (PEG) based (water-soluble) suppositories [86]. Both types are generally, nonirritating, melts or dissolves at body temperatures, chemically stable and have some advantages

and disadvantages associated with each type. Amongst these two types of bases, PEG-based suppositories being water soluble, dissolve in the rectal fluid and mucus. Therefore, they might absorb moisture from its surrounding environment, which could cause irritation to rectal mucosa or discomfort post suppository administration [82, 86]. However, by modifying the chain length of the PEG polymers in the suppositories, the dissolution rate, melting point and firmness can be altered to better suit the formulation requirements [85]. On the other hand, fat-soluble suppository bases melt at body temperature giving a smooth and pleasant texture and feel to the user. Moreover, compared to water-soluble PEG polymers, the fat-soluble suppository bases offer greater flexibility to incorporate a wide variety of drugs with different physicochemical properties. Bases such as witepsol-grades (IOI Oleo chemical, GmbH, Hamburg, Germany) and various suppocire-grades (Gattefosse USA, New Jersey, USA) are hard-fats primarily composed of mono-, di- or tri-glycerides in different ratios. These ratios govern the physical nature of the suppository bases; wettability, brittleness and melting point.

1.10 Combination strategies applied to receptive anal intercourse

Several federal and private entities such as the National Institute of Allergy and Infectious Diseases (NIAID), Centers for Disease Control and Prevention (CDC), academic laboratories in universities and the pharmaceutical industry have worked in conjunction to develop anti-retroviral (ARV) small molecules for treatment of HIV-1. The primary goal of HIV-1 therapy with ARVs is to restrict the viral replication in the infected host and keep the viral load under control, eventually leading to better quality of life and increased life-span. Due to the recent developments and increasing number of newly acquired HIV-1 infections in some selected populations [87], several small molecules used for HIV-1 therapy are also being evaluated for their potential application for topical, oral or systemic pre-exposure prophylaxis (PrEP) [88]. Currently the only two FDA approved products for oral PrEP on the market are Truvada® (emtricitabine 200mg and tenofovir disoproxil fumarate 300mg) and Descovy (emtricitabine 200 mg and tenofovir alafenamide 25 mg), both being combinations. As explained in section 1.2, HIV-1 replication is a complicated and multi-step process which occurs rapidly and needs several enzymes and co-factors at every

step in the replication process. Due to the rapid onset of infection, the time to treatment initiation post infection is very crucial and therefore, several studies have been conducted to evaluate an optimum time window for beginning HAART.

However, irrespective of the time of treatment initiation, it is well known that a combination of multiple ARVs works better than a single ARV [89]. Therefore, several combinations of ARV from different classes are already on the market for HIV-1 treatment. Adapting from this strategy, few studies were conducted evaluating daily intake of a single ARV TFV for prophylaxis with the objective of maintaining therapeutically active concentrations of TFV in individuals with high risk behaviors for HIV-1 acquisition. However, adhering to this once a day regimen was crucial to avoid breakthrough HIV-1 infections. Moreover, sub-optimal levels of ARV in the plasma raises of risk of drug resistance. Therefore, adapting the combination strategies from HAART, a few studies evaluated the potential benefits of incorporating combination of more than one ARV for oral PrEP [49, 50]. As mentioned in section 1.6.1, results from these studies led to the approval of Truvada®, the first of its kinds oral PrEP product which was a fixed dose combination of TFV and Emtricitabine (FTC). Learning from the oral PrEP and HAART approach, our goal was to utilize the same principle and apply it to topical PrEP. Especially when topical products like TFV gel have shown efficacy, alone as well as in combination with other ARVs. Few studies have already evaluated combination of ARVs for rectal [90] and vaginal [70, 91] applications for topical PrEP. Early development microbicides comprised of various non-specific agents which modulated different parameters and targeted HIV-1 using multiple strategies. A focused and improvised approach can be adopted by using ARVs which target specific steps of the HIV-1 lifecycle [36]. Herrera *et al*. investigated non-nucleoside reverse transcription inhibitor (NNRTI) and nucleotide transcription inhibitors (NtRTI) alone as well as in combinations using cell-based and tissue models [92]. Tenofovir (TFV), UC-781 and Dapivirine were assessed using TZM-bl cells and colorectal explants; all three APIs exhibited a reduced IC_{50} against HIV-1 in combination with another ARV as compared to single API proving that combinations were better compared to drugs used alone in *in vitro* models [92]. Moreover, Ferir *et al*. evaluated a novel entry inhibitor Griffithsin (GRFT) with different classes of ARVs and observed higher potency with combinations compared to GRFT alone. Moreover, amongst the combinations evaluated, a 3.6-fold increase in antiviral potency of integrase strand transfer inhibitor (InSTI) Elvitegravir (EVG) was observed when tested in combination with GRFT [93].

Moreover, there is evidence in the literature demonstrating the benefits of late-acting ARVs such as INSTIs [15]. Combining these two approaches, a recent study evaluated a combination of TAF and EVG in a long-acting nanoparticle formulation to potentially prevent vaginal transmission of HIV-1 using a humanized bone marrow-liver-thymus (hu-BLT) mice model [94]. In this study, TAF and EVG were incorporated in PLGA-containing nanoparticles and administered to mice vaginally and compared to control group (No drug). Both the groups were challenged with HIV-1 at day 4 and plasma viral load was evaluated on a weekly basis to assess infection. The results from this study demonstrated that all mice in the control group were infected with HIV-1, however 100% of the mice treated with TAF + EVG NPs were uninfected when challenged at day 4 and 60% mice were uninfected at day 14 [94]. Although the TAF + EVG combination was not evaluated against TAF alone or EVG alone, this was a proof of concept study indicating that long-acting NPs demonstrated protection against HIV-1 acquisition in a hu-BLT model with TAF + EVG combination.

1.11 Anti-retroviral prodrug approach

Due to tenofovir's (TFV) poor oral bioavailability, it is typically administered in the form of a fumarate salt of its prodrug TFV disoproxil [95]. As described earlier in section 1.10, tenofovir disoproxil fumarate (TDF) was first approved by the FDA for oral prophylaxis in 2012 as a single pill (Viread®) or in combination with emtricitabine (FTC) (Truvada®). Although TDF has been effective, for prevention, it has to be administered orally at 300mg to achieve therapeutically active concentrations of tenofovir-diphosphate (TFV-DP) intracellularly (figure 2). However, TDF rapidly metabolizes to TFV in the plasma and TFV has demonstrated extended systemic circulation due to its longer half-life [95]. Recent studies have demonstrated renal toxicities and bone-related side effects associated with long-term elevated TFV levels [96-98] (refer section 5.1 and figure 2). Therefore, other alternatives to improve TFV's safety and reduce off-target exposure was sought. A prodrug, tenofovir alafenamide fumarate (TAF) which was being developed by Gilead Sciences Inc., demonstrated favorable PK properties [99]. Based on *in vitro* studies TAF (EC_{50} HIV-1: 0.005 µM) demonstrated about 1000- and 10- fold higher potency than TFV (EC_{50} HIV-1: 5 µM) and

TDF (EC_{50} HIV-1: 0.05 µM) [99]. Moreover, TAF was stable in the biological matrices it was evaluated in, improved intracellular accumulation of the pharmacological active form TFV-DP and several such benefits compared to TDF and TFV [100]. TAF is readily absorbed in the gut followed by the plasma. Upon absorption in the HIV target cell, TAF is hydrolyzed by lysosomal carboxypeptidase cathepsin A (CatA) intracellularly to form a metastable moiety TFV-Alanine (TFV-Ala) (figure 2). Further, TFV-Ala is acid hydrolyzed by phosphonamidase to TFV. TFV is finally metabolized to TFV-monophosphate (TFV-MP) and then its pharmacologically active form TFV-diphosphate (TFV-DP) [99-101]. TFV-DP is responsible for restricting reverse transcription of the HIV-1 virion, described elsewhere [95]. Such advantages of improved efficacy and safety lead to the recent approval of TAF for oral prophylaxis in combination with emtricitabine (FTC) (Descovy®) (refer section 5.1).

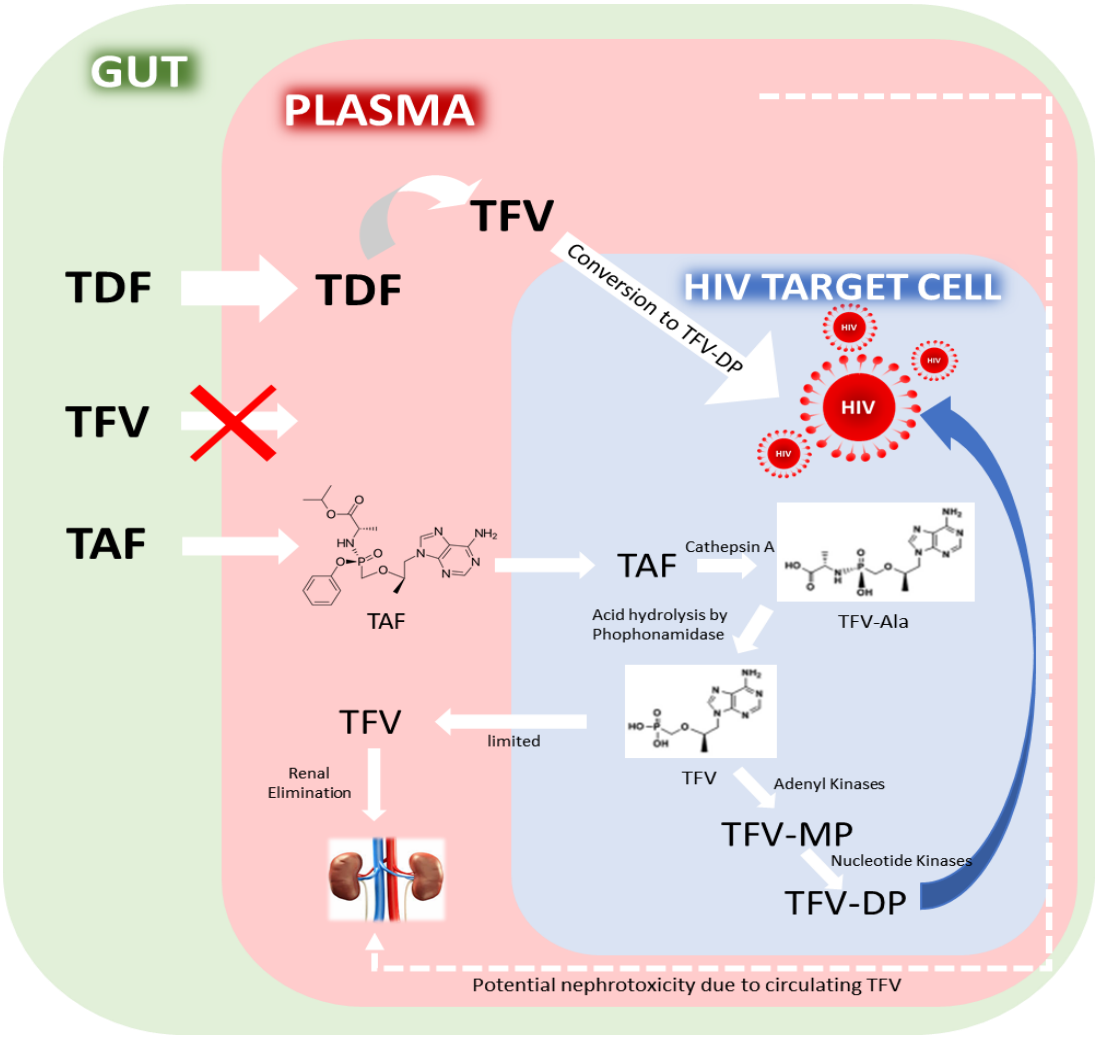


Figure 2 TDF, TFV and TAF *in vivo* metabolism and disposition

Tenofovir Disoproxil Fumarate (TDF), Tenofovir (TFV) and Tenofovir Alafenamide Fumarate (TAF) *in vivo* disposition. TFV cannot be absorbed in the gut, therefore administered as the fumarate salt of its disoproxil fumarate (TDF). TDF is readily absorbed through the gut in plasma and metabolized to TFV. TAF is easily absorbed through the gut and metabolized to a metastable metabolite TFV-Ala by Cathepsin A. It is further metabolized intracellularly to TFV by acid hydrolysis. TFV is then eliminated from the HIV target cell to plasma and then renally eliminated. In the HIV target cell, TFV is then metabolized to the pharmacologically active form TFV-monophosphate (TFV-MP) and TFV- diphosphate (TFV-DP). Reproduced from [99, 101]

1.12 Significance of efflux transporters for anti-retroviral modulation

As described in section 1.10, combinations of ARVs have better outcomes and efficacy compared to single ARV-based therapy is a well-established fact [89]. But the advantages of combinations are associated with other caveats such as the potential of drug-drug interactions (DDIs) [89]. Most HIV-1 treatment regimens currently on the market consist of combination of more than one ARVs belonging to different classes, in a single product. The incorporation of more than one API in a single product usually involves and requires DDI studies based on the number of ARVs. Irrespective of the class of ARV, ARVs are well known for their DDIs with other ARVs as well as ABC and SLC transporters [89, 102, 103]. Therefore, pharmaceutical companies and other organizations developing ARVs have to conduct several DDI studies to design appropriate treatment regimens taking several factors into account.

From a topical drug delivery perspective, there was lack of data establishing the transporter expression in the rectal and vaginal compartments. Therefore, *in vivo* ARV modulation caused due to transporter disposition was unpredictable. But with the recent advancements in the topical microbicide field within the last two decades, several clinical and pre-clinical trials evaluating different dosage forms for vaginal or rectal PrEP were conducted. Some results from these trials demonstrated varying *in vivo* exposures of different ARVs in the mucosal tissue. For instance, about 62 and 73% protection against HIV acquisition was observed in serodiscordant couples with either TDF alone or in combination with emtricitabine (FTC) respectively, in the Partners PrEP trial [104]. But other trials like the FEM-PrEP (daily Truvada®) and one arm (TDF alone) of VOICE failed to protect women from acquiring HIV [105, 106]. Such results directed the scientists to investigate reasons for different outcomes observed in the trials. Some experts in the field initially thought that the half-life of different agents and extent of mucosal penetration could be possible reasons [106]. In addition, expression of efflux and uptake transporters in the rectal [107] and vaginal compartments could also possibly responsible for modulation of these ARVs. Therefore, several studies were conducted to evaluate expression of the transporters at the site of entry (rectum and vagina) [108] and in some cases even further in the female genital tract, the endo- and ectocervix [103]. In a recent study, vaginal, cervical and colorectal tissue samples were collected from 98 unique donors, either from surgical waste specimens or cadaver tissues. The

mRNA and protein expression levels for key efflux (MDR1, MRP2 and MRP4) and uptake (OAT1, OAT3 and OATP1B1) transporters from these tissue specimens were determined using quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC) [108]. According to the results from these studies, both vaginal and colorectal tissue sample expressed all the three uptake transporters at mRNA levels, and MRP4 being the highest amongst the three. However, the uptake transporters were expressed in minority of the tissues, even lower OAT1 in the colorectal tissues [108]. When these results were compared to some observation from the clinical studies, the authors were able to identify some relevance and overlap between the *in vivo* exposures achieved (in separate trials) with the transporter expression in different compartments. Therefore, this and other clinical studies provide evidence that transporters could potentially play a role in ARV disposition [108, 109], making it essential and worthwhile to investigate if an ARV is substrate for key uptake and efflux transporters. Studies was not only limited to human samples, but extended to pre-clinical animal models as well. This was necessary because several pre-clinical animal models such as mice [110] rabbits [111] and monkeys [109] are used for ARV-based microbicide pharmacokinetic assessment.

Similarly, two studies were conducted which predict the expression and functionality of efflux transporters in the mucosal tissues. The first study conducted a single and multiple dose PK study with Maraviroc (MVC) in 12 HIV-negative men by orally administering 300mg MVC twice daily for 8 days [112]. MVC exposures were determined in the saliva, semen and rectal tissue post oral administration. The results from this study concluded that high MVC concentrations were observed in the rectal tissue [112]. MVC being a known P-gp substrate and considering the P-gp expression in the colorectal and vaginal tissues, Nicol et. al hypothesized that MVC accumulation will be higher in the colorectal compartment compared to female genital tract [108]. The second study administered a single oral dose of Truvada® (TDF + FTC) to 15 healthy individuals and determined exposure levels over the next 14 days from the rectal, vaginal and cervical tissues [106]. In addition to TDF and FTC, they also determined the exposure levels of their respective active metabolites, tenofovir diphosphate (TFV-DP) and emtricitabine triphosphate (FTC-TP). The 14-day PK results demonstrated that the TFV and TFV-DP concentrations in the rectal fluid were about 100-fold higher compared to vaginal and cervical tissues. Moreover, overall exposure ($AUC_{1\text{-}14d}$) for TFV in the rectal tissues was 2989 ng.days/g was about 34-fold higher compared to concentration in plasma 91 ng.days/g. This indicates that both TFV and TFV-DP have higher

concentrations in the rectal tissues compared to vaginal tissues and the plasma [106]. TFV being a known MRP2 and MRP4 substrate, considering the efflux transporter expression described above, Nicol et. al hypothesized that transporter expression in the colorectal compartment could be potentially responsible for such drastic exposures in different compartment post oral administration [108]. In summary, the studies described above demonstrate the expression of efflux and uptake transporters in the colorectal and vaginal compartment and signify the need of conducting further pharmacological studies. Especially from a topical drug delivery standpoint, mucosal penetration and achieving sufficient ARV concentration in the local tissue is essential to stay protected.

1.13 Pre-clinical animal models to investigate microbicide candidate

Appropriate selection of animal model for evaluating the microbicide candidates is critical. Historically, non-human primates (NHPs) have been used as the primary animal model for safety and efficacy studies of topical microbicides. NHPs are considered the best suited animal model for microbicide evaluation as they closely mimic the human anatomy and physiology [113, 114]. Physiological factors in the macaques such as the anatomy, microflora, gross cellular morphology, menstrual cycles, mucosal immune responses are very similar to humans [115, 116], hence advantageous for evaluation of either vaginal or rectal applications. A study to establish the similarities in the rectal microbiome and tissues was conducted in healthy humans and macaques [116]. Researchers in this study measured and compared rectal histology and microflora from 80 pig-tailed macaques and 40 reproductive-age women [116]. According to the histology results obtained from macaques, approximately 15 layers of stratified squamous epithelium were observed, which was consistent with the biopsies from humans. Observations of the lamina propria of the tissues indicated loose connective in human as well as macaque histology. Moreover, based on comparisons made with about 10 strains of anaerobic microorganism obtained from the macaque and human rectum, most of them were very similar (in %). However, colonization of some H_2O_2 producing lactobacilli varied from humans to macaques, and beta hemolytic streptococci were totally absent in macaques compared to humans [116].

Amongst the available species of NHPs, three species are the most commonly utilized for microbicide evaluation, rhesus macaques (Macaca mulatta), pig-tailed macaques (Macaca nemestrina) and Cynomolgus macaques (Macaca fasicularis) [113-115, 117]. Several years of research and investigations of each species have led to the selection of specific animal models for testing of particular microbicides, vaccines for prevention and eradication of HIV-1 [117]. Each species has their own practical and scientific features which make them more suitable for testing of specific products. For instance, although cynomolgus macaques are less expensive and smaller compared to rhesus macaques, few studies have indicated that the collection of vaginal biopsies and colposcopy can be challenging due to the smaller vaginal compartment in cynomolgus macaques [114]. Similarly, researchers prefer pig tailed macaques for vaginal studies as they closely mimic the human female genital tract (FGT) and able to give birth year around compared to rhesus macaques, which are seasonal breeders [114].

The similarities in the NHPs and human FGT also provide great flexibility in terms of study designs and the purpose of the microbicide being tested [113, 117, 118]. NHPs are susceptible to the simian immunodeficiency virus (SIV) as well as the simian-human immunodeficiency viruses (SHIV), which were specifically engineered to study different steps and aspects of the viral transmission [114]. Moreover, to better predict HIV-1 transmission in the presence of rectal and vaginal microbicides, several low and high dose inoculations can be conducted. Similarly, a single exposure challenge, versus multiple exposures SIV/ SHIV challenge studies can be conducted to better simulate clinical conditions and determine microbicide efficacy. This is primarily possible because NHP's immune system consists of similar receptors and co-receptors which are required for mucosal viral transmission. These similarities even bolster studies investigating specific steps of the viral transmission such as fusion/ binding and integration [114].

1.14 Research goals and objectives

RAI remains one of the highest risk sexual behavior in the current times. RAI is not only commonly observed within MSM but heterosexual couples as well. Therefore, based on the increased susceptibility associated with unprotected RAI, a microbicide product focused toward

rectal administration is warranted. Although there are few dosage forms currently under pursuit for rectal administrations, suppositories offer more versatility to incorporate various ARV agents with varied physicochemical properties. Suppositories also overcome disadvantages associated with some rectally administered dosage forms, such as the need of an applicator for gels, potentially making it more acceptable to users, increasing adherence and utility. Therefore, we explored the potential of suppository as a platform for safe and effective delivery of ARV agents for HIV-1 PrEP. In addition to developing the suppository platform, learning from the HIV-1 treatment regimen, our goal was also to explore the advantages and possibilities of ARV combinations as topical formulations. Moreover, to address the toxicity issues associated with some ARV agents such as TFV, prodrugs such as TAF (refer section 1.11), which have been proven effective orally, was explored as a potential ARV candidate for topical formulations. Several behavioral surveys and studies investigating sexual practices and product use have indicated concerns with available dosage forms as potential microbicides for HIV-1 prevention. Therefore, from the user's preference perspective, considering "another tool in the box" approach gives the user more options to choose from. To address this research question,

Our goal is to develop a rectal microbicide product using suppository as a platform of PrEP for HIV-1 prevention with combination of anti-retroviral drugs.

This goal will be achieved by completing the following objectives:

Objective 1: To investigate the impact of suppository bases on key performance attributes (Chapter 2)

Objective 2: Conduct studies toward identification of a suitable anti-retroviral candidates for *in vivo* studies (Chapter 3)

Objective 3: Conduct *in vivo* pharmacokinetic studies with Tenofovir and Elvitegravir containing suppositories to assess local and systemic exposures (Chapter 4)

Objective 4: Conduct *in vivo* pharmacokinetic studies with Tenofovir alafenamide fumarate (TAF) and Elvitegravir containing suppositories to evaluate pro-drug benefits using lower dosing levels (Chapter 5)

2.0 Investigate the impact of suppository bases on key performance attributes

2.1 Introduction

The selection of suppository bases is vital and the decision needs close attention as some drugs tend to alter formulation properties, or in some cases can lead to incompatible ingredients in the formulation. Therefore, most manufacturers provide a decision tree to help select the suitable suppository base depending upon the physicochemical characteristics of the drug. In addition, there are several factors associated with selection of suppository bases such as the hydroxyl value, melting point and desired release characteristics. Hydroxyl value of a suppository base is defined as the amount (milligrams) of potassium hydroxide (KOH) required to neutralize the acetic acid. It indicates the number of free hydroxyl (-OH) groups in the suppository base and usually range within 0-100. In addition to the hydroxyl value, another key parameter used to assess suppository base's compatibility with the drug is melting point. Some drugs tend to lower the melting point of the suppositories, which could cause melting of suppositories during transportation or in the user's fingers making it messy and difficult for administration. This will eventually lead to poor patient compliance. In such cases, bases with higher melting point are desired. Apart from various aspects affecting the formulation, factors such as patient compliance and adherence should also be considered while choosing a suppository base because few studies have shown sub-optimal drug exposures due to poor patient adherence to the product [119, 120]. From a user's perspective, a successful microbicide candidate must be user-friendly, causing no discomfort while handling or administering the product.

Use of condoms along with rectal microbicides is not uncommon for couples engaging in RAI while using rectally administered products such as lubricants, enemas, suppositories, gels etc. Historically, some oil-based lubricants are known to damage condoms, therefore water-based lubricants are recommended along with the condom use. To better understand the acceptability and adherence of rectal products in a wide range of study populations, few clinical trials evaluating the concomitant use of condoms with rectal microbicide products have been conducted [9, 121]. Some preliminary experiments from our laboratory indicate that prolonged exposure of condoms

to some suppository bases can lead to weakening and deterioration of the condoms. Therefore, the aim of the studies described in this chapter is to determine the compatibility of two types of the most commonly used condoms with a series of fat-soluble suppository bases with varying hydroxyl values (0 to 50). Additionally, *in vitro* permeability experiments were conducted to assess if the hydroxyl value of suppository bases can impact the drug permeability and absorption through the rectal mucosal epithelium.

Lastly, our collaborators in Atlanta, Georgia at the Centers for Disease Control & Prevention (CDC) conducted a preliminary evaluation of the spreadability of suppositories in the rectal compartment of macaques scheduled for euthanasia. In this study, placebo suppositories of PEG-base and a witepsol base containing a blue dye (Witepsol – Winsor & Newton Cobalt Blue; PEG – Royal Blue icing color) were administered rectally to evaluate the extent of exposure that can be achieved by different types of suppository bases. Results from this preliminary study indicated that PEG-based suppository was able to spread up to 6 cm from the anal margin while the witepsol suppository could spread up to 2 cm only. Therefore, PEG-based suppositories were selected to further evaluate for *in vivo* spreadability and exposure studies. Tenofovir (TFV) was used as the model microbicide drug for this experiment. TFV was selected as it is one of the most widely investigated API as topical microbicide. These studies will give us a thorough understanding of the extent of local *in vivo* exposures that can be achieved using suppository as a dosage form for ARV drug delivery.

2.2 Materials

Witepsol-grade and Suppocire-grade suppository bases were procured from IOI Oleo Chemical GmbH (Hamburg, Germany) and Gattefosse Corporation (Saint-Priest, France), respectively. TROJAN™ ENZ™ lubricated condoms and TROJAN™ Supra™ BARESKIN™ lubricated condoms were purchased from a local distributor (PureRED). Radio-labelled markers (^{3}H propranolol and ^{14}C mannitol) for permeability studies were purchased from Perkin-Elmer or American Radiolabeled Chemicals Inc. (ARC, St. Louis, MO). The electrode tips, electrodes, and tissue mounting sliders were purchased from Physiologic Instruments, San Diego, CA. Hank's

Balanced Salt Solution (HBSS) used in the permeability experiments was purchased from Gibco by life technologies.

2.3 Methods

2.3.1 Condom puncture strength

Our objective was to test the compatibility of fatty bases with different condom materials by measuring the condom puncture strength. Latex and polyurethane are the two most commonly used materials to manufacture condoms. Hence, we selected TROJAN™ ENZ™ (latex) and TROJAN™ Supra™ BARESKIN™ (polyurethane) condoms for this test. The sample preparation procedures were adapted from the standard test for determining compatibility of personal lubricants with natural rubber latex condoms (ASTM 7661-10 (American Society for Testing and Materials)). The schematic of the procedure followed to conduct condom puncture strength is represented in figure 3. Briefly, the condoms were removed from the original packaging, unfolded completely along their length and cut into a rectangular section (n=10/ treatment group). Different suppository bases were melted in separate beakers and several rectangular shaped condoms were completely submerged into the bases. According to the steps adopted from the ASTM 7661-10 method, the condoms were incubated in the suppository base at 40°C for one hour. The condoms were washed with warm tap water to remove residual suppository base from the condom surface. The condoms were gently wiped with paper towels and left to air dry. These condoms were then tested for puncture strength (n=3 tests/ condom) using a texture analyzer (TA-XT. Plus, Hamilton, MA) connected to a data acquisition and analysis software.

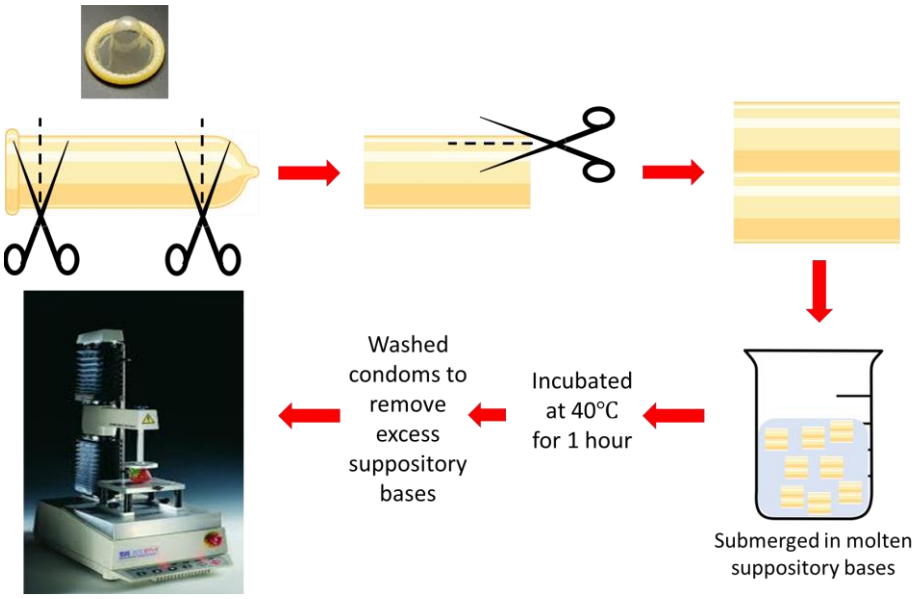


Figure 3 Schematic illustrating stepwise procedure of the condom puncture strength test

Condoms were withdrawn from pouches, cut to obtain a rectangular shaped single layered condom, dipped into the suppository base, incubated at 40°C, washed with warm water to remove access suppository bases and tested using TA.XT Plus Texture Analyzer

A 5kg load cell was attached to the movable arm of the instrument and condoms were placed on a stationary platform using TA-90 holder with exposed condoms through which the TA1/8th probe would puncture the condoms. Due to elasticity of the condoms, an initial increase in force is observed as the material stretches until it is punctured with the probe, creating a hole in the condom. The force (kilogram) required to completely puncture the condoms was recorded as the puncture strength (figure 4). According to the FDA guidelines regarding condom's compatibility with different test items, if the differences in airburst and tensile parameters between the test and control is greater than 20%, it is regarded as not compatible. However, our results were not based on the standard airburst test procedures, and our goal was to determine if there was a relationship between the hydroxyl value of the suppository bases and condom's puncture strength, therefore it was irrelevant if condoms were compatible with different suppository bases or not.

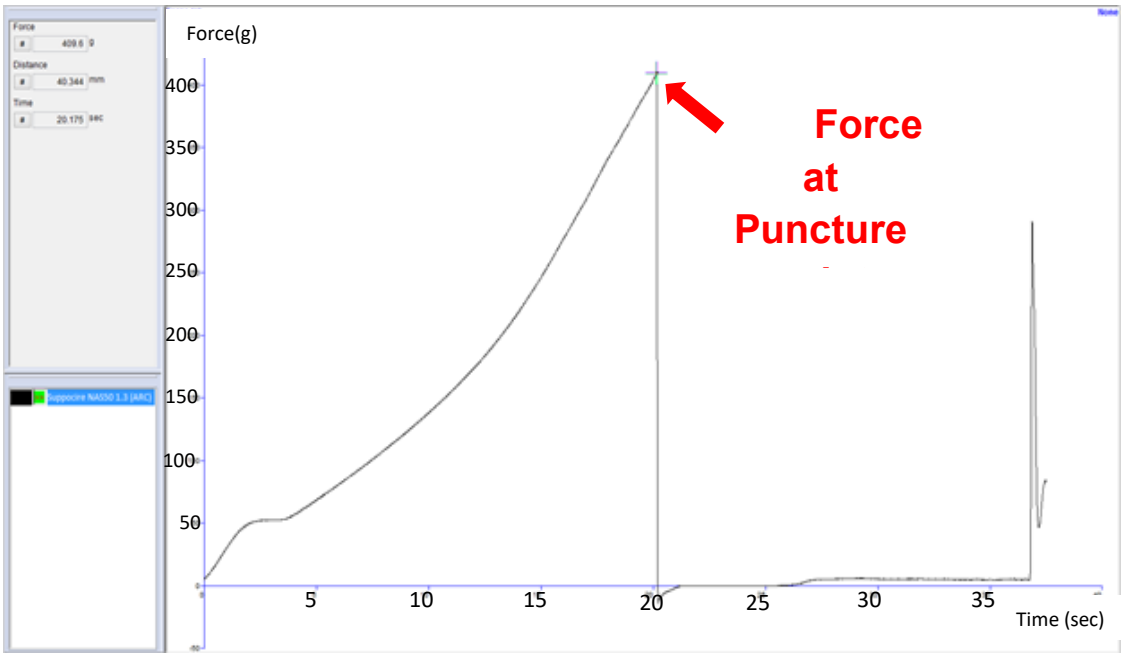


Figure 4 Typical test graph post condoms puncture strength test

Post product exposure each condom was tested thrice, 10 condoms/ treatment group. The initial increase in force due to elasticity of the condom material. The highest force required to puncture a whole in the condom was recorded as the puncture point (represented by the red arrow) X-axis - Time (seconds), Y-axis - Force (g).

2.3.2 In vitro permeability – Ussings chamber

Some evidence suggested that the hydroxyl value of the suppository bases can impact rectal absorption and permeability [122]. Schmitt et. al. conducted a PK study to determine if the HV of suppository bases impacted Carprofen (lipophilic non-steroidal anti-inflammatory) absorption in a dog model [122]. In addition to rectal solution and oral Carprofen, they administered three suppository formulations with hydroxyl value 1 (Massa Estranium 299 – ME299), 25 (Massa Estranium B - MEB) and 45 (Massa Estranium A - MEA) to the dogs and determined basic PK parameters. According to their initial results a C_{max} of 21.2, 27 and 30.1 mg/mL; and $AUC_{0-\infty}$ of 311.1, 357.6 and 335.2 h.mg/L were observed from ME299, MEB and MEA respectively. Although significant differences in exposures were not achieved, upon further deconvolution and calculating cumulative fractions of the dose absorbed post-administration, differences were observed. The most hydrophilic suppository base (MEA) demonstrated a T_{max} and mean absorption

time (MAT) of 1.7 and 2.15 hours respectively, compared to 2.4 and 4.22 hours from ME299 [122]. These results confirmed that Carprofen absorption increases with decrease in HV of the suppository bases. Similar to this study, few other studies conducted so far have used animal models to investigate the effects of suppository bases on rectal epithelium [86, 123]. Therefore, to better understand, further validate these findings and investigate the mechanisms responsible for changes in *in vivo* drug exposure permeability experiments using freshly excised human colonic tissues was conducted.

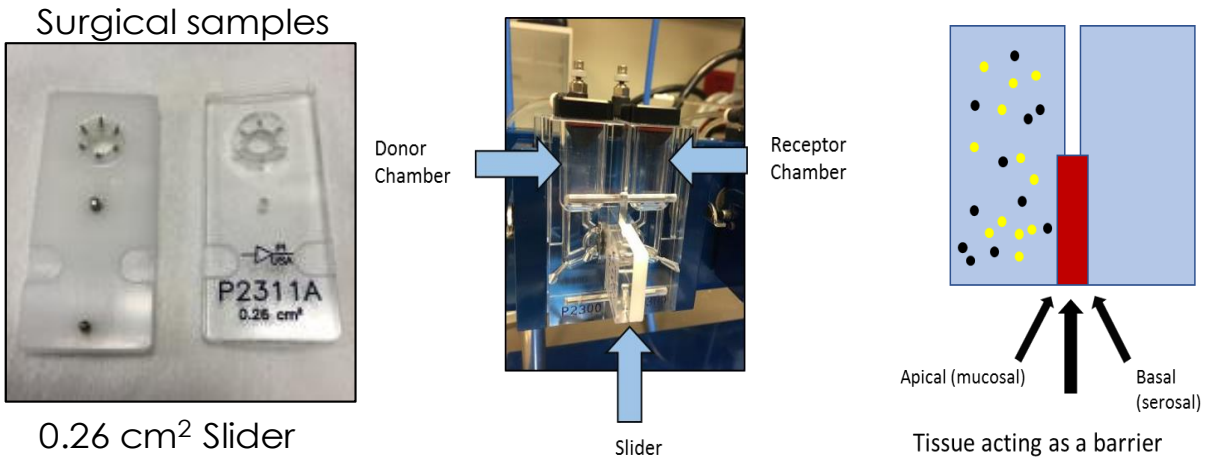


Figure 5 Schematic representing the Ussings chamber methodology and instrument setup

Left to right, slider used to mount the tissues separating the donor and receptor chambers; Image of one of the eight Ussings chamber with donor and receptor chamber separated by the sliders; Schematic representing the orientation of apical and basal layers of the colon tissue

Human colonic tissues were obtained from the tissue procurement facility at the Pitt Biospecimen Core within 2 hours of surgical excision (IRB: 0602024 project#240). Normal human colorectal tissues with pre-set inclusion criteria (no inflammation, no chemotherapy/radiation, 18-79 years age range) were acquired from people undergoing colorectal or gastro-intestinal surgery for non-inflammatory conditions. Post-collection, the excess stromal layer of the tissues was manually trimmed with surgical scissors to obtain a single thin tissue slice representing the apical layer of the colon. Using 10mm biopsy punches, multiple smaller sections were obtained, and their individual thickness was measured using a digital Vernier Caliper. A section was collected as control for pre-exposure evaluations. These biopsies were then individually mounted on the tissue

mounting sliders (area – 0.26 cm^2) and placed in the Ussings Chamber apparatus, apical side facing the donor compartment. The schematic representation of Ussings chamber experimental setup is represented in figure 5. In order to the mimic body temperature the target temperature for the experiment was 37°C but the water bath was set at 40°C to compensate for the heat loss from water bath to the chambers. Previous work in our lab has demonstrated that setting the water bath temperature at 40°C results in 37°C in the chambers. The tissues were also supplied with 95% O_2 and 5% CO_2 to maintain viability throughout the experiment, and the flow was controlled using microvalves. The impact of fat-soluble suppository bases on tissue permeability was tested for suppository bases of low (5) and high (50) hydroxyl value. The donor solution was prepared by mixing 1:1 ratio of molten suppository bases with HBSS containing ^{3}H propranolol (transcellular permeation) and ^{14}C mannitol (paracellular permeation) and placed in a heated water bath at 40°C to avoid solidification and separation of the bases. The donor solution was added to the donor compartment (5 mL) and aliquots were withdrawn from the receptor (HBSS) compartment at pre-determined time points and the amount of radioactivity permeated across the tissue was monitored using liquid scintillation counting (LSC). To ensure tissue integrity over the course of the experiment, trans-epithelial electrical resistance (TEER) was monitored and recorded.

Apparent permeability (P_{app}) was calculated using the equation below, and the P_{app} of control (no treatment) was compared to each, low and high hydroxyl value suppository bases. Any observed statistical differences were considered as an impact on the trans or paracellular permeability, indicating potential interference with drug absorption.

Apparent permeability (P_{app}):

$$P_{app} = \left(\frac{dQ/dt}{C_0 \times A}\right)$$

2.3.3 Tissue processing and staining

Immediately after completion of the permeability experiments, the mounted tissues were withdrawn and placed in labelled embedding cassettes, followed by overnight formalin treatment. The next day, these tissue cassettes were transferred to 70% Ethanol and kept at least for 24 hours

before processing. The tissue processing for Hematoxylin and Eosin (H & E) staining involved several steps of dehydration: 95% ethanol for 1 hour; 100% ethanol for 1 hour (3 times); xylene for 1 hour (2 times); and finally, paraffin (at 50°C) for 3 hours. Post dehydrating the tissues, they were embedded into paraffin blocks using an embedding station (Lecia EG 1160). Using the microtome (Olympus CUT 4060), 5µM sections were cut from the paraffin blocks and transferred to a glass slide. H & E staining was conducted on the tissue sections and section tissues were evaluated visually using a microscope (Zeiss Axioskop 40) to determine if any morphological changes occurred post product exposure.

2.3.4 In vivo pilot studies to evaluate TFV and TFV-DP exposure from PEG-based suppositories in non-human primates

One of the key attributes to evaluate *in vivo* suppository performance is the ability of the product to melt or dissolve, based on the nature of the suppository and spread in the rectal compartment. Such exposure results can give us a better understanding of the *in vivo* performance that can be achieved using suppositories, which can be compared to exposures achieved from other rectally administered dosage forms. Based on the findings in pilot study described in section 2.1 which showed PEG-based suppositories reached deeper in the colon compared to witepsol suppositories, this study was conducted only with PEG-based suppositories (refer section 3.3.1 for manufacturing and characterization of TFV suppositories). The intact colon was isolated and split into three section of 5 cm each (1-5 cm, 6-10 cm and 11-15 cm) from the anal margin. Biopsies and samples were collected from each section and analyzed for drug exposure. The goal of these studies was to determine the extent of TFV and TFV-DP exposures that can be achieved in the rectal compartment (rectal fluid, tissues and plasma) with suppository dosage form using a non-human primate model. TFV was selected as the model-drug for this experiment as it is the most widely studied ARV for topical PrEP using different dosage forms. Since TFV is converted to its active form Tenofovir- diphosphate (TFV-DP) intracellularly, by determining both TFV and TFV-DP levels we expected to gain an in-depth understanding of the extent of drug exposures achieved *in vivo*. Forty milligram TFV was used as the dosing level to match the previous studies conducted

with other rectally delivered products such as gels [124, 125]. The *in vivo* pilot studies described here were conducted by the ARV prophylaxis activity team at the CDC in Atlanta, GA.

In this pilot study, two pig-tailed macaques scheduled to be euthanized were dosed with PEG-based suppositories (PEG 3350:1000:400 at 60:30:10) containing TFV (40 mg). The suppositories were manufactured by us and shipped to the topical ARV prophylaxis labs at CDC. Two hours post-dosing, the macaques were euthanized and their intact rectum and colon tissue (~15 cm) was isolated. This tissue specimen was gently washed with saline to remove remnants of feces and then dissected into smaller 5 cm sections from the anal margin for drug exposure analysis. Multiple biopsies were collected from every section using 3.7-mm biopsy forceps (Radial JawTM 3 Biopsy Forceps, Boston Scientific) and immediately homogenized in 1 mL of ice-cold 80% methanol. TFV and TFV-DP levels from every section using validated LC-MS methods described previously [91, 126]. Rectal lymphocytes were isolated using established enzymatic degradation methods [91, 126] and intracellular TFV-DP levels were determined.

2.3.5 Statistical Analysis

The *in vitro* permeability experiments were conducted on three different tissues obtained from different patients. Several biopsies were obtained from each tissue and split between treatment groups or controls. Tissues for same treatment groups and controls were pooled from three individual experiments and statistical analysis was performed. Statistical analysis was performed using GraphPad Prism version 7.0. The P_{app} was compared using ordinary one-way ANOVA analysis. Dunnett's multiple comparison test was conducted comparing each treatment group (High and low hydroxyl value) with control. P values less than 0.05 were considered statistically significant.

2.4 Results

To select an appropriate fat-soluble suppository base, we conducted preliminary *in vitro* characterization and dissolution studies comparing semi-synthetic and naturally occurring suppository bases from different manufacturers such as witepsol, suppocire and cocoa butter [79]. Amongst these three tested fat-soluble base classes, the suppository bases belonging to witepsol grade demonstrated most visually pleasant and optimum *in vitro* characteristics. Therefore, witepsol was selected as the lead class of suppository bases. The manufacturer provides several grades of witepsol bases such as witepsol E, S, H and W to choose from. Each grade has further subtypes (H35, S58, E76, W31 etc.), primarily differentiated by melting points (31 - 44°C). There are several factors such the drug loading, physicochemical properties (Biopharmaceutical Classification System (BCS) classification) of the API, melting point lowering capacity, presence of surfactants etc. that govern the selection of appropriate suppository base. Based on these factors and a decision tree provided by the manufacturer, witepsol H class was selected as the lead category. Furthermore, we conducted a short survey determining which grades of witepsol bases are most commonly used in marketed products, witepsol H15 used in several products compared to other grades. In addition to the most common suppository base on the market, the results from the condom compatibility studies (section 2.4.1) signifies the utility of suppository bases with lower hydroxyl value, and witepsol H15's hydroxyl value was within 3-5 (Range 3-55). In summary, based on current and previous studies conducted in our lab [79] and the survey mentioned above, we selected witepsol H15 as the lead suppository base representing fatty-acid based suppository bases for *in vivo* PK studies.

Similarly, to select an appropriate water-soluble suppository base, we conducted preliminary studies evaluating three different PEG-based formulations [79]. PEG-based suppositories can be manufactured by combining PEG's of different molecular weights (PEG 400, 1000, 3350, 4000) at various ratios. Three PEG formulations, PEG 8000:400 (at ratio 60:40), PEG 3350:1000 (at ratio 25:75) and PEG 3350:1000:400 (at ratio 60:30:10) were manufactured and characterized. These molecular weights and ratios govern the visual appearance, dissolving time and firmness of the PEG suppositories. Amongst the investigated PEG-based suppositories, based on visual observations and *in vitro* dissolution results we selected PEG 3350:1000:400 (at ratio 60:30:10).

Furthermore, just like fat-soluble suppository bases condom compatibility studies with PEG-based suppositories were also conducted. However, PEG-based suppositories do not melt at 40°C, hence they had to be dissolved in water at 1:1 ratio and then tested for condom compatibility. The condom puncture strength was not affected by PEG-based suppositories. In conclusion, based on current and previous studies, PEG 3350:1000:400 (at ratio 60:30:10) was selected as the lead water-soluble suppository base for further evaluation.

2.4.1 Condom compatibility

The goal of these experiments was to evaluate if commonly used condoms were compatible with suppository bases. Latex and polyurethane condoms were exposed to a series of suppository bases to determine their puncture strength pre- and post- product exposure. Thirteen suppository bases with a wide range of hydroxyl values were chosen for evaluation in this study (table 1). Water and mineral oil were used as negative and positive controls, respectively. The results in table 1 demonstrated a varied impact of suppository bases on mechanical properties of condom with % difference in puncture strength measurement (from baseline) ranging from 12 to 78%. Puncture strength is a test used to measure the resistance of condom against tearing or puncture a hole in the material. This test is utilized as a model to evaluate the overall condom integrity. The observed changes in condom puncture strength were correlated with the hydroxyl value of the suppository bases.

Table 1 Puncture Strength Values Pre and Post Exposure to Suppository Base

Top to bottom – Suppository bases with increasing hydroxyl values. Condoms without any treatment were evaluated as the baseline. % difference was calculated by comparing each treatment group with baseline. Puncture strength for every suppository base represents the force (g) required to puncture the condoms. Evaluated Hydroxyl value range 3 – 45. Results represented as mean of 10 condoms ± standard deviation (SD)

Suppository base grades	Hydroxyl value	Puncture strength Polyurethane condoms (TROJAN™ Supra™ BARESKIN™)	% Difference (from baseline)	Puncture strength Latex condoms	% Difference (from baseline)

				(TROJAN™ ENZ™)	
Baseline (no product)		1907 ± 465	-	744 ± 57	-
Water (-)		2139 ± 318	-	703 ± 87	-6
Mineral Oil (+)		NA	NA	354 ± 50	-52
Suppocire H35	**3**	1264 ± 253	-34	454 ± 44	-39
Suppocire AML	**4**	1319 ± 278	-31	498 ± 43	-33
Suppocire DM	**5**	1349 ± 269	-29	554 ± 38	-26
***Witepsol H5**	**5**	1684 ± 391	-12	563 ± 51	-24
Suppocire NA15	**11**	1170 ± 240	-39	526 ± 33	-29
Suppocire E75	**15**	908 ± 177	-52	482 ± 47	-35
***Witepsol H15**	**15**	1327 ± 97	-30	415 ± 23	-43
Suppocire BS2X	**22**	788 ± 193	-59	459 ± 26	-38
Suppocire NA	**25**	667 ± 81	-65	488 ± 36	-34
Suppocire NAI25	**27**	711 ± 112	-63	491 ± 46	-34
Suppocire NAI50	**42**	429 ± 42	-78	474 ± 35	-36
Suppocire NAS50	**44**	465 ± 65	-76	487 ± 33	-35
Suppocire W35	**45**	438 ± 76	-77	453 ± 34	-39

*Excluded from analysis to demonstrate linearity of damages due to increasing hydroxyl value

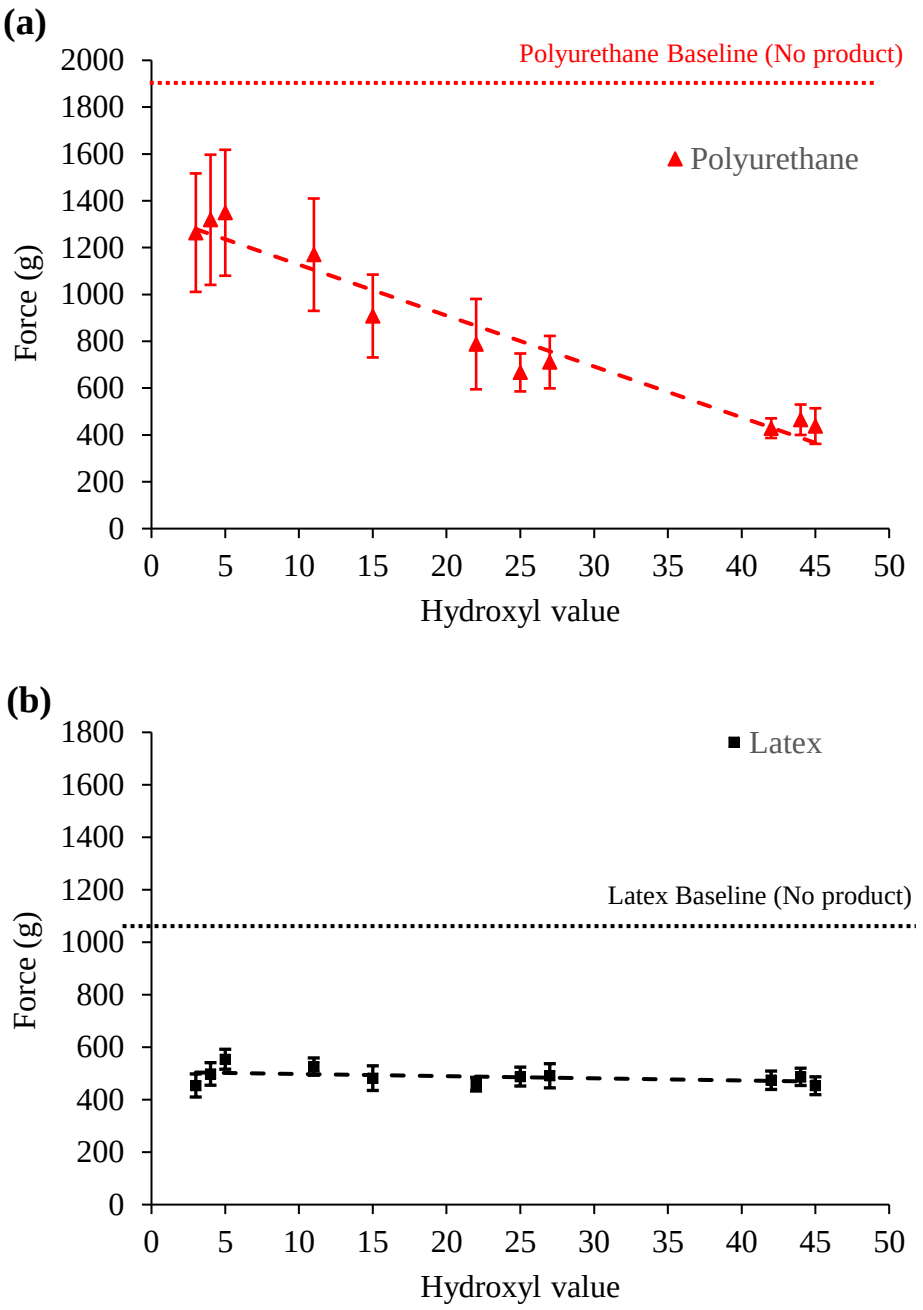


Figure 6 Correlation between hydroxyl value of suppository bases and puncture strength

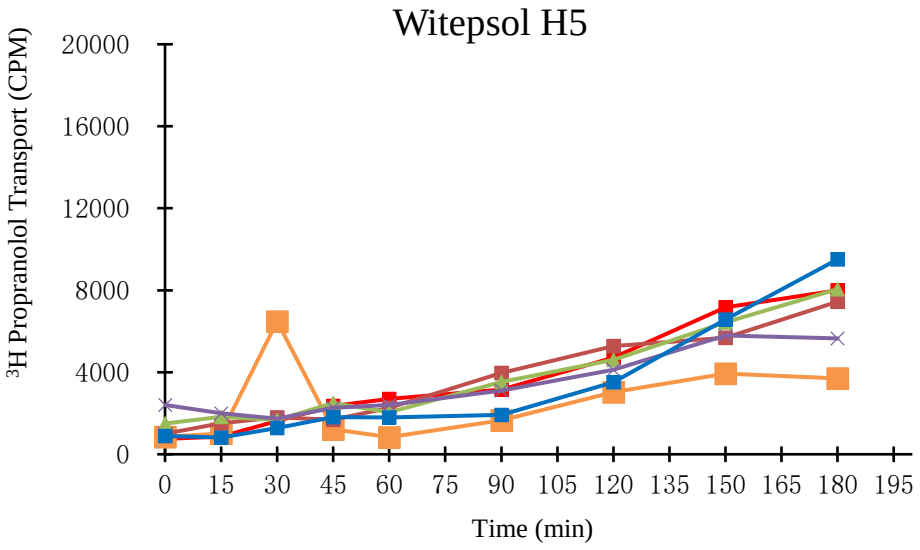
(a) polyurethane condoms represented in red and (b) latex condoms represented in black. (N=10/ hydroxyl value, mean ± SD); The best fit line (dotted line) was displayed using Linear Regression; X-axis – hydroxyl value, Y-axis – Force (g) required to puncture condoms

This relationship is shown graphically in figure 6. A linear relationship (R^2 = 0.9456) was observed between the force required to puncture (rupture) polyurethane condoms and hydroxyl

value of the suppository bases (figure 6a). However, this linear relationship was not observed with latex condoms (R^2 = 0.1859) (figure 6b). Although the latex condoms demonstrated a reduction in puncture strength post treatment with suppository bases, the reduction (damage) observed was not correlated to the changes in hydroxyl value of the suppository bases.

2.4.2 In vitro permeability – Ussings chamber

The aim of conducting *in vitro* permeability studies with human colonic tissue was to determine if the differences in hydroxyl value of suppository bases can impact tissue permeability. The amount of ^{3}H Propranolol and ^{14}C Mannitol permeated through the colonic tissues over time in the presence of two suppository bases was determined. Permeability was measured through quantitation of propranolol or mannitol levels in the receptor compartment using liquid scintillation counting. Differences in permeability were determined through comparison of data from treated or nontreated tissues. Figures 7 (Trans-cellular marker) and 8 (Paracellular marker) show representative data from a single tissue. All studies were repeated for three separate tissues. Figures 7 and 8 compares tissue permeability in presence of witepsol H5 (low), Suppocire NAS50 (high) and control (no treatment).



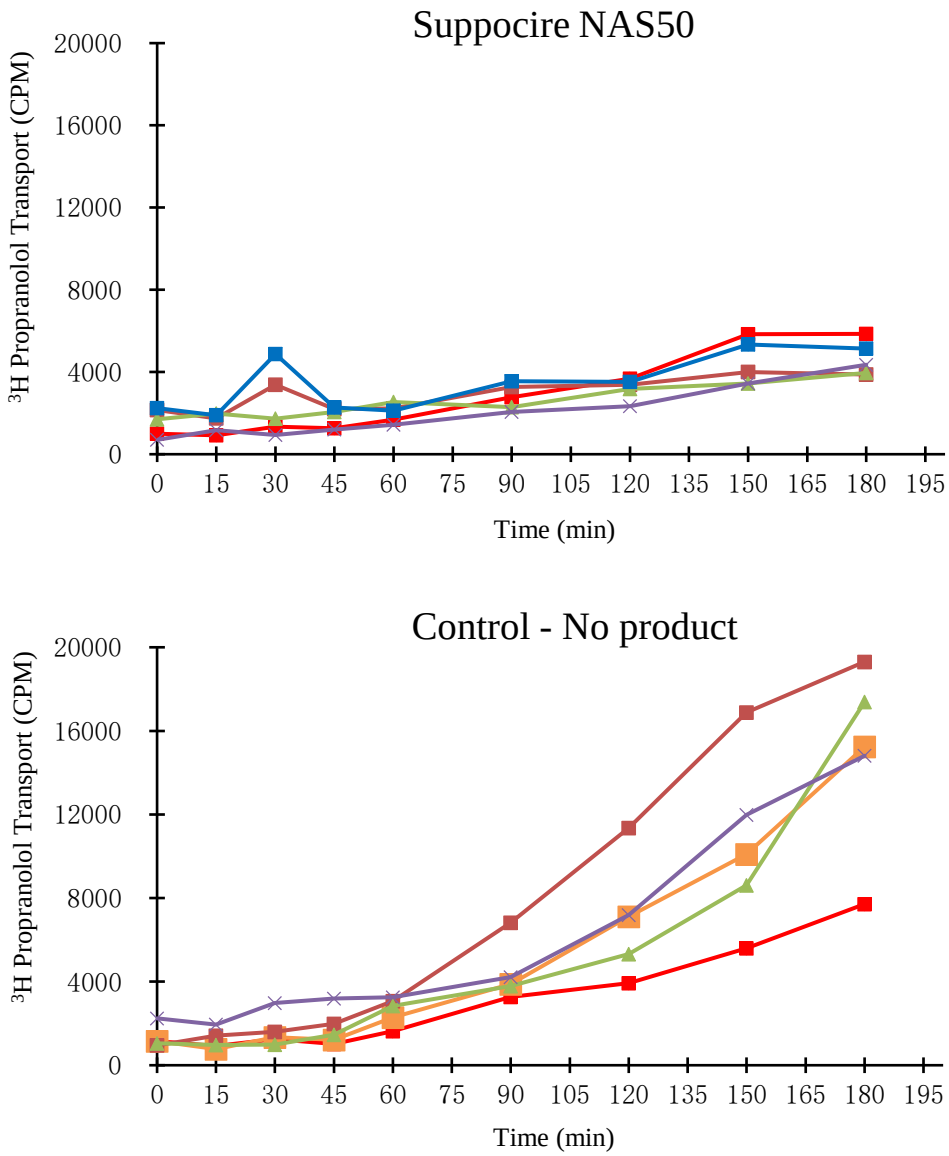
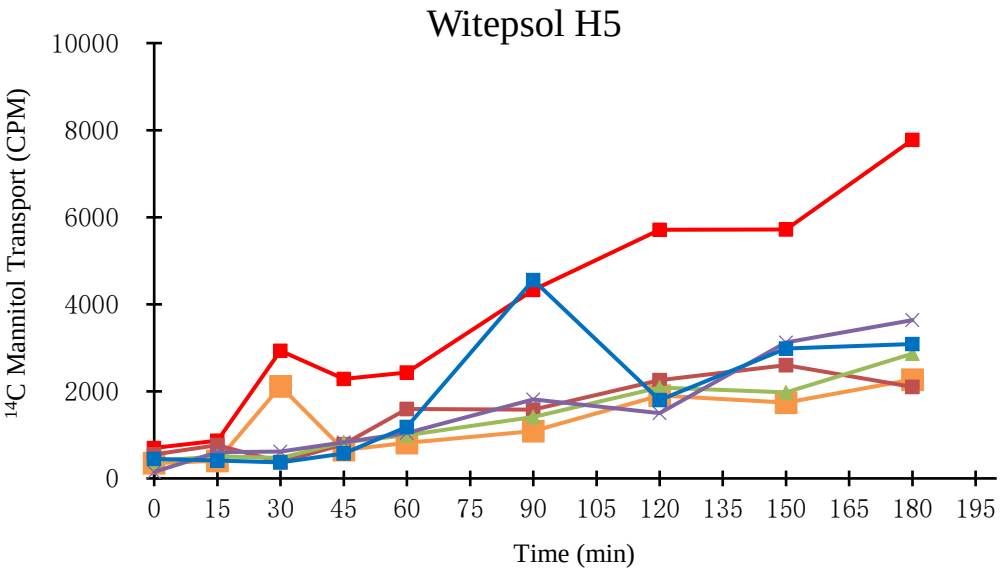
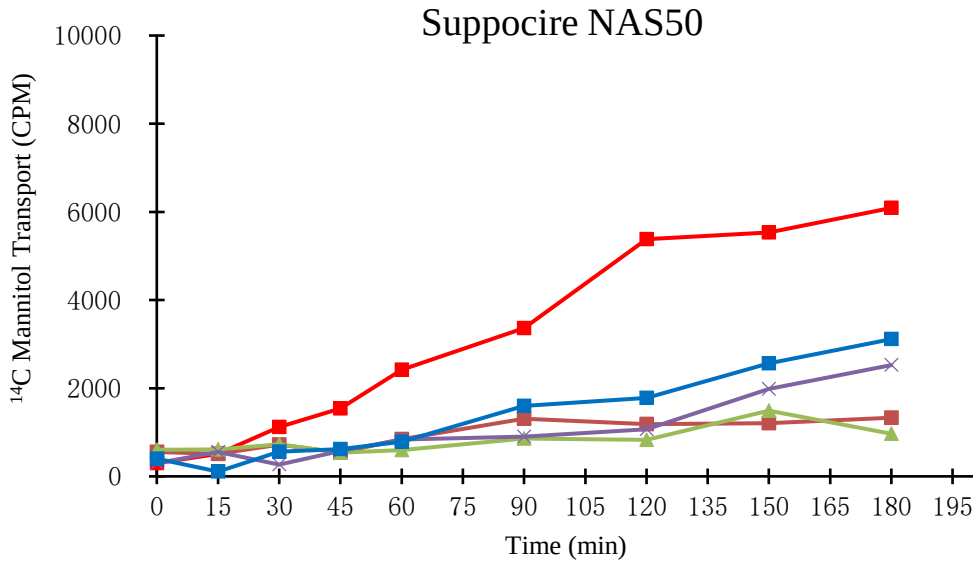


Figure 7 Effect of suppository hydroxyl value on trans-cellular pathways (^{3}H propranolol transport) of rectal absorption

^{3}H propranolol transport in presence of (a) Witepsol H5, (b) Suppocire NAS50 and (c) Control – no product; Each color on every graph represents a single tissue (n=5 for control and Suppocire NAS50, n=6 for Witepsol H5); P_{app} was compared using ordinary one-way ANOVA analysis. Dunnett's multiple comparison test was conducted comparing each treatment group (High and low hydroxyl value) with control. X-axis – Time (min) and Y-axis – ^{3}H Propranolol transport (CPM); $P < 0.05$ were considered statistically significant.


Witepsol H5
^{14}C Mannitol Transport (CPM)
10000
8000
6000
4000
2000
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195
Time (min)


Suppocire NAS50
^{14}C Mannitol Transport (CPM)
10000
8000
6000
4000
2000
0
0 15 30 45 60 75 90 105 120 135 150 165 180 195
Time (min)

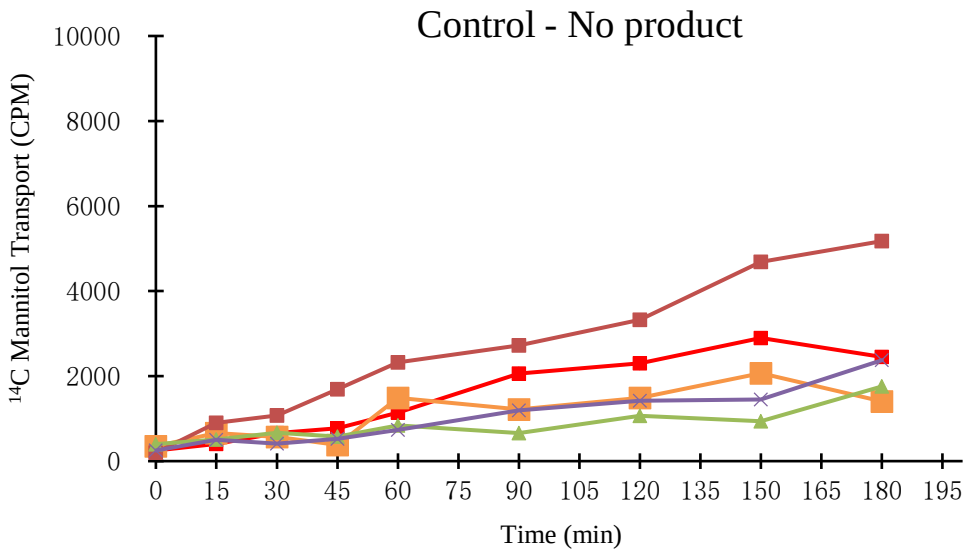


Figure 8 Effect of suppository hydroxyl value on paracellular pathways (^{14}C mannitol transport) of rectal absorption

^{14}C mannitol transport in presence of (a) Witepsol H5, (b) Suppocire NAS50 and (c) Control – no product; Each color on every graph represents a single tissue (n=5 for control and Suppocire NAS50, n=6 for Witepsol H5); P_{app} was compared using ordinary one-way ANOVA analysis. Dunnett's multiple comparison test was conducted comparing each treatment group (High and low hydroxyl value) with control. X axis- Time (mins) and Y-axis ^{14}C Mannitol transport (CPM); P< 0.05 were considered statistically significant.

The first linear phase was observed up to 60 min, followed by the second phase up to 180 min. Several slopes were calculated using different time points and the linear portion from 60-150 minutes was considered for P_{app} calculations. P_{app} for ^{3}H propranolol was 1.49 x 10^{-5}, 1.14 x 10^{-5} and 1.6 x 10^{-5}cm/s for low, high hydroxyl value and control, respectively. Similarly, P_{app} for ^{14}C mannitol were 6.74 x 10^{-6}, 7.85 x 10^{-6} and 8.27 x 10^{-6}cm/s for low, high hydroxyl value and control, respectively. According to ordinary one-way ANOVA and Dunnett's multiple comparison test no significant differences were observed in either transcellular or paracellular route between tissues exposed to the test suppository bases and the control group (no product) (P>0.05 for ^{3}H propranolol and ^{14}C mannitol). Since maximum ^{3}H propranolol permeated through the no product group, we hypothesized that over the testing period, the suppository bases started solidifying creating a physical barrier and leaving minimal-to-no space for any transport through the tissue. However, this cannot necessarily be translated *in vivo*, as we anticipate the suppository bases will spread and

remain in molten state due to the temperature and pressure in the rectal cavity [127]. ^{14}C mannitol transport is not only a paracellular marker, but it can also be treated as a control to ensure the tight junction integrity. Any tear or damage in the tissue would have shown a sharp spike in the mannitol transport, indicating an experimental error, which was not observed in this experiment.

2.4.3 Tissue processing and staining

In order to evaluate if suppository treatment had any impacts of the gross morphology of the colon tissues, H & E staining was conducted. Post *in vitro* permeability experiment completion, the exposed areas of the tissues were collected and fixed for immunohistochemical analysis. The images from H&E staining are presented in Figure 9. A typical normal colon is comprised of single layered epithelium (black arrow), crypts (red arrow), lamina propria, muscularis mucosa and goblet cells (blue arrow) [128] (shown in panel 4(c) and (d) of figure 9). Every horizontal panel of figure 9 are images from a separate treatment group; (1) negative control (no treatment), (2) positive control (Neobee triglyceride), (3) Witepsol H5 and (4) Suppocire NAS50. The analysis was conducted based on degree of damage to some or all the parts of normal colon. Due to limited tissue biopsies from a single patient, only one chamber was used for negative control. The black and red arrows in panel 1(a) and (b) demonstrate intact epithelium and crypts. The epithelium and crypts were completely eroded and absent in panel 2(a) and (b), as neobee triglyceride is known to induce damage to the rectal epithelium. The blue arrow indicates the presence of Goblet cells which are typically present beyond the lamina propria and crypts. However, the epithelium and crypts were visible in panel 2(c) and (d), indicating varied degree of damage in two different chambers. The panel 3(a) and (b) shows intact epithelium and crypts with some damage; and presence of goblet cells in panel 3(c) and (d). Similarly, the panel 4 (a-d) shows the presence of intact epithelium, crypts and goblet cells as well with varied degree of damage in every section. In summary, comparing panel 1(a) and (b) (negative control) with to panels 3 and 4, there is a wide-range of damage associated in both treatment groups. Broadly, the change in hydroxyl value of suppository bases did not impact the gross morphology of the colon tissues three hours post product exposure.

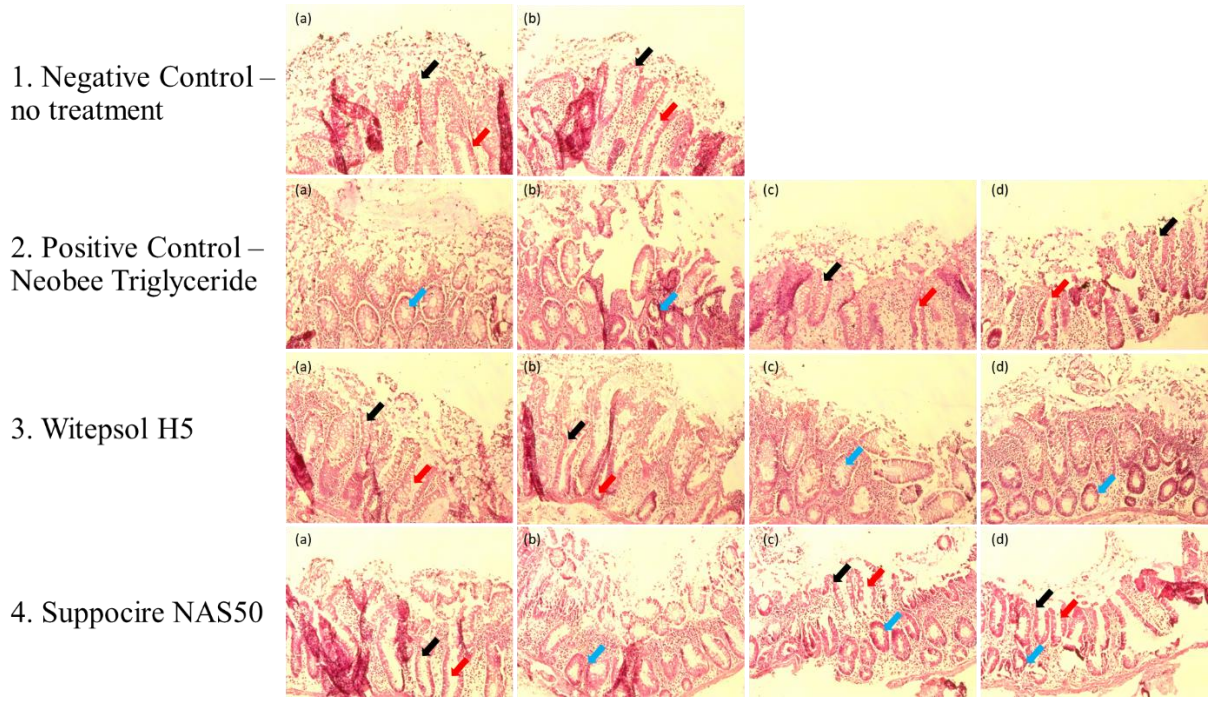


Figure 9 H & E staining images of colonic tissues post treatment

(1) No treatment, (2) Neobee triglyceride, (3) Witepsol H5 and (4) Suppocire NAS50 for every horizontal panel, (a)/ (b) and (c)/ (d) are images from the same slide. Two slides per treatment group were evaluated except no treatment (1). The cellular components are represented using different colored arrows. Epithelium (black arrow), Crypts (red arrow) and goblet cells (blue arrow)

2.4.4 In vivo pilot studies to evaluate TFV and TFV-DP exposure from PEG-based suppositories in non-human primates

Pilot studies were conducted in NHPs scheduled for euthanasia (terminal studies). The NHPs were dosed with PEG-based suppositories containing TFV to evaluate *in vivo* exposure. Detectable TFV and TFV-DP levels were observed in the rectal tissues and rectal lymphocytes respectively, 2 hours post administration as shown in table 2. High TFV and TFV-DP levels in the rectal tissues and lymphocytes suggests enough drug delivery by PEG-based suppositories. At 2 hours, the PEG-based suppositories could achieve 7.6, 2.3 and 3.1 (table 2) times more TFV-DP in rectal lymphocytes compared to TFV/ Maraviroc (MVC) combination rectal gel dosed in macaques previously [69]. Moreover, overall exposure increased from one to two hour, potentially indicating the lag and suppository and drug dissolution post-administration. This indicates that with similar

dosing levels, the PEG-based rectal suppositories can deliver TFV more effectively to the rectal tissue and lymphocytes, the primary site of initial HIV-1 infection.

Table 2 Summary of TFV and TFV-DP levels achieved rectal tissues and lymphocytes post PEG-based suppository dosing in macaque studies scheduled for euthanasia

Two animals were dosed with TFV loaded PEG suppository and euthanized one at one hour and other at two hours post administration. The intact colon was isolated and split into three sections of 5 cm each the anal margin. Biopsies and samples were collected from each section and analyzed for TFV and TFV-DP exposure

	1-5cm, Median	**6-10cm, Median**	**11-15cm, Median**
1hour (n=1)			
TFV, ng/mg	2.80	1.90	2.10
TFV-DP, fmol/mg	567	169	47.5
TFV-DP, fmol/10^6 cells (range – duplicate injections)	1097 (973-1204)	564 (504-600)	479 (399-481)
2hour (n=1)			
TFV, ng/mg	25	9.30	9.60
TFV-DP, fmol/mg	1106	1161	874
TFV-DP, fmol/10^6 cells (range – duplicate injections)	10546 (10580-10546)	8236 (7340-8813)	10693 (9193-10780)

2.5 Discussion and Conclusion

It is well known that some mineral oil-based lubricants deteriorate latex condoms [129]. Hence, concomitant use of oil-based lubricants with latex is not recommended, as this has been previously proven to damage the condom within 60 seconds of exposure. About 90% decrease in the condom's strength was observed using the burst volumes by standard ISO (International Standards Organization) air burst test post exposure. However, concomitant use of suppositories with

condoms and its impact on condom integrity is unknown. Therefore, to better understand this we investigated the impact of fat-soluble suppositories on puncture strength of condoms. Although the air burst test is considered as one of the Standard Test Method for Determining Compatibility of Personal Lubricants with Natural Rubber Latex Condoms (ASTM D7661), we were unable to use this method due to unavailability of suitable equipment. As mentioned earlier, the fat-soluble suppositories are fatty-acid bases composed of different ratios of mono-, di- and tri-glycerides. The ratio of these glycerides governs the hydroxyl value (HV) of the suppository bases. HV is defined as "the number of free hydroxyl groups in the suppository bases". The HV impacts suppository base's properties such as plasticity, viscosity etc. Moreover, the HV also has some surfactant properties, which can improve dissolution wetting etc.

Our results have established a direct relationship between the hydroxyl value of fat-based suppository bases and deterioration of the condoms. Polyurethane is a polymer composed of multiple urethane linkages. Typically, a single urethane link is synthesized by reaction between isocyanates and hydroxyl groups. We predict that the surfactant properties and the additional free reactive hydroxyl groups (in suppository bases with high hydroxyl value value) interact and weaken the urethane linkage, eventually decreasing the force required to puncture the condoms. Therefore, use of suppository bases with high hydroxyl value should be avoided with polyurethane condoms. This is an important piece of information that can be potentially added to the product label, contraindicating prolonged exposures of latex condoms to suppositories with high hydroxyl value. In such cases, polyurethane condoms or condoms made using other materials could be used as possible alternatives. From a rectal microbicide product development perspective, our results give insights about selecting the appropriate suppository bases in future clinical trials involving concomitant use of condoms.

Suppository bases with extreme hydroxyl value were selected. Surprisingly, the results observed in animal studies were not reproduced in our experiments, the changes in hydroxyl value did not correlate with the permeability through the human rectal epithelium. Based on the amounts of ^{14}C mannitol (6.74×10^{-6}, 7.85×10^{-6} and 8.27×10^{-6}cm/s) and ^{3}H propranolol (1.49×10^{-5}, 1.14×10^{-5} and 1.6×10^{-5}cm/s) permeated for low, high hydroxyl value suppository bases, there was no significant difference observed between the control and any of the treatment groups within the experimental timeframe. To our knowledge this is one of the first studies to determine the effect of suppository bases and their hydroxyl value on human rectal permeability. However, the

experimental design had several limitations that makes it difficult to make conclusions. Factors such as procuring fresh colon tissues from human subjects and conducting the experiment within a given time window, obtaining limited tissue sections from a single patient etc. Due to these limitations we were unable to make comparisons from patient to patient and test several suppository bases.

Similar to *in vitro* permeability results, H & E staining images post treatment with suppository bases with different hydroxyl value did not indicate major differences in gross morphology of the colonic epithelium. The basal layer was absent in all tissue sections, and a wide range of damages was observed in different sections of the treated tissues. However, a significant difference in morphology was observed in positive control when compared to treatment groups. Based on visual observations, during the on-going experiment the suppository bases in some chambers started to solidify to some extent, physically blocking the passage though the epithelium. And since O_2 was supplied for stirring of the chambers, the solidification of bases occurred at different degrees over time. This could be one of the explanations to see a wide range of changes in the gross morphology, even varying within the same treatment group (Panel 3(a-d) and panel 4(a-d) in figure 9). As mentioned earlier, based on the gross morphological changes observed, the H & E staining analysis was inconclusive. However, by comparing *in vitro* permeability results with the data in figure 9, any clinical significance in permeability alterations is not anticipated.

The results from our PK studies in macaques demonstrated that PEG-based TFV suppositories were able to achieve detectable TFV and TFV-DP exposures up to 15 cms in the rectum within 2 hours of administration. According to results in a recent PK study in macaques with 1%TFV / 1% Maraviroc (MVC) rectal gel, the gel formulation was able to achieve 43, 9.6 and 246 ng/mg TFV in 5 cm, 10 cm and 15 cm tissue sections from the anal margin [69]. However, our PK results from suppository formulation achieved 25, 9.3 and 9.6 ng/mg TFV in the same anatomical regions, suggesting reduced TFV exposure compared to gels. Despite reduced TFV exposure, the suppositories were able to achieve 1106, 1161, 874 fmol/mg TFV-DP in the tissues and 10546, 8236 and 10693 fmol/ 10^6 cell TFV-DP in rectal lymphocytes, the primary site of HIV-1 infection. Compared to suppositories, the 1%TFV / 1% MCV gel was able to achieve only 415, 429 and 1697 fmol/mg TFV-DP in rectal tissues and 1389, 3588 and 3448 fmol/ 10^6 cells in rectal lymphocytes [69]. These macaques that received the TFV/ MVC combination gel demonstrated 82% efficacy when repeatedly challenged with simian/ human immunodeficiency virus (SHIV).

Therefore, with same dosing levels, if the suppositories could achieve 7.6, 2.3 and 3.1-fold higher TFV-DP compared to 1%TFV/ 1% MVC rectal gel, we anticipate the suppositories to be efficacious. As mentioned earlier, we predict the observed increased exposure with suppositories compared to gels is due to the nature of the dosage form (solid versus liquid). Although gels demonstrate improved dissolution, it is associated with leakiness, which could be the potential reason of observed reduced exposure *in vivo*. Moreover, these PK results indicate that in spite of reduced TFV exposures, drug released from the suppositories is rapidly absorbed into tissues and converted to TFV-DP intracellularly establishing rapid absorption. This could be due to the time required for TFV dissolution from the gel formulation versus instant dissolution from suppositories.

2.6 Acknowledgments

We would like to acknowledge IOI Oleo Chemical GmBH and Gattefoose for providing suppository bases samples. This project used the UPMC Hillman Cancer Center and Tissue and Research Pathology/ Pitt Biospecimen Core shared resource which is supported in part by award P30CA047904. I would like to acknowledge Charles Dobard, PhD, Walid Heneine, PhD, Sunitha Sharma and colleagues at the Centers for Disease Control & Prevention (CDC), Atlanta, GA for designing and conducting PK studies and graciously providing us the PK data. In addition, I would like to acknowledge the analytical team at CDC for sample collections and processing. I would also like to acknowledge the assistance provided by Sabina Spektor from Pittsburgh Science & Technology Academy in conducting the condom compatibility experiments.

3.0 Studies toward identification of a suitable anti-retroviral candidates

3.1 Introduction

Considering the mechanism of action of different classes of ARVs and potential specific targets, studies have been conducted to investigate stage dependent inhibition of HIV-1 infection using single drugs from various classes of ARVs. Combination strategies have also been studied with respect to their potential use in preventing HIV-1 infection. [15, 57, 130, 131]. The authors in one of these studies used a cell culture model to test stage-dependent inhibition of HIV-1 replication [15]. PM1 cells were cultured and infected by the virus, followed by seeding on a 96-well plate. Twenty-four hours post-seeding, these cells were treated with different classes of ARVs at pre-determined concentrations. And twenty-four hours post drug addition, the supernatants were collected and tested for either p24 (ELISA) or viral RNA load (qRT-PCR). Using this cell culture model, the results suggested that later-acting ARVs have the potential to inhibit the viral replication to a greater extent compared to earlier acting classes of ARVs [15]. This study also showed the potential benefit of combining non-nucleoside reverse transcriptase inhibitors (NNRTIs) and integrase strand transfer inhibitors (InSTIs). Moreover, since the goal of the studies described in this chapter is conducting studies to identify suitable ARV candidates which can be incorporated in suppositories as a delivery platform, the most investigated classes of ARVs were selected. Amongst the available classes of ARVs drugs belonging to each class inhibit a specific enzyme within the HIV-1 replication cycle.

In addition to these specific classes of ARVs, our secondary objective was also to investigate the potential benefits of delivering prodrugs topically. Few studies have demonstrated the benefits of achieving similar *in vivo* therapeutic exposures with lower doses of prodrugs [132, 133]. Here, we determined if this phenomenon can be translated when prodrugs are delivered rectally as well. Therefore, in order to determine the benefits of prodrug approach Tenofovir Alafenamide Fumarate (TAF) was selected as the prodrug candidate (Refer figure 2 for TAF activation pathway). TAF is an FDA approved drug already on the market as a combination pill for HIV-1 treatment. TAF is a promising candidate for topical product development due to its good water

solubility and improved efficacy [134]. Hence, to evaluate our primary (NNRTIs + InSTIs) and secondary (prodrug – TAF) objectives, NNRTIs (Tenofovir (TFV), TAF) and InSTIs (Elvitegravir (EVG) and MK-2048) were selected as the two classes of ARVs for the *in vitro* characterization and dissolution studies described in this chapter.

Amongst the ARVs we selected, role of transporter in the modulation of TFV and TAF is reported in the literature [89]. However, insufficient evidence was available for its involvement of transporters in InSTIs transport, hence, we conducted a survey to determine if every available InSTIs was a substrate for efflux transporters (table 3). This survey indicated that Cabotegravir [135], Raltegravir [136, 137], and Dolutegravir [138, 139] have been proven to be P-gp and BCRP substrates. However, amongst the available InSTIs, data in the literature supporting P-gp and BCRP's role in EVG modulation was not available. Only a few studies suggested that efflux transporters could play a potential role in Elvitegravir's (EVG) modulation but did not provide any concrete evidence [140-142]. Hence, we investigated if EVG was a substrate of efflux transporters. Using a well-established trans-well flux assay with MDCKII cell lines, we conducted *in vitro* permeability studies to determine if EVG is a substrate of efflux transporters P-gp and BCRP. This information can be beneficial to predict *in vivo* performance using various topical formulations. For instance, higher doses might be required to circumvent the efflux, assuming EVG is a substrate for these transporters.

Table 3 Efflux transporter substrate profile for current InSTIs

InSTI	P-gp	BCRP	Reference
Cabotegravir	✓	✓	[135]
Dolutegravir	✓	✓	[138, 139]
Raltegravir	✓	✓	[136, 137]
Elvitegravir	?	?	[140-142]

3.2 Materials & Instrumentation

Tenofovir (TFV) was purchased from WuXi Apptec. Elvitegravir (EVG) and Tenofovir Alafenamide Fumarate (TAF) was graciously provided by our collaborators at the Centers for Diseases Control & Prevention (CDC), Atlanta, GA. MK-2048 was provided by Merck & Co. Witepsol H15 suppository base was provided by IOI Oleo Chemical GmbH as free sample. Polyethylene Glycol (PEG) with molecular weights 3350, 1000 and 400 were purchased from Spectrum Chemicals. Disposable plastic suppository shells were purchased from Professional Compounding Centers of America (PCCA). Breaking point of suppositories was measured using TA.XTPlus Texture Analyzer (Texture Technologies Corp., Hamilton, MA/Stable Micro Systems, Godalming, Surrey, UK) and associated TA.XTPlus Texture Analyzer probes. *In vitro* drug dissolution studies were conducted using USP Apparatus Type 1 basket (Distek T2100 series) dissolution apparatus. *In vitro* dissolution and drug content samples were analyzed using High Performance Liquid Chromatography (HPLC). The HPLC system used to develop and validate the assays for every analyte was an Ultimate 3000 (Dionex) equipped with a photodiode array detector. Chromeleon version 6.70 (Chromatography Management System) was used as the data management and analysis software.

Madin-Darby canine kidney wild-type (MDCKII-wt) and MDCKII-Multi-drug resistance protein (MDR1) (P-gp overexpressing) cell lines were purchased from American Type Culture Collection (ATCC). The *in vitro* transwell flux experiments were conducted using Costar™ Transwell™ Clear Polyester Membrane Inserts For 12-Well Plates (12mm diameter, Growth area: 1.12cm^2; Insert vol.: 0.5mL; Pore size: 0.4µm). Cell culture reagents like Dulbecco Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, trypsin, 1x Phosphate buffered saline solution (PBS) were purchased from either Corning Lifesciences or Gibco. The inhibitor Elacridar (GF120918) was purchased from Sigma Aldrich. The T75 flasks, disposable plastic pipettes, reservoirs, filters, disinfectants were purchased from Fischer Scientific. A Carl Zeiss invertoskop 40C microscope was utilized to observe cell growth and required confluency. A LabDoctor Mini Incubated Shaker with non-slip rubber mat was purchased from Midwest Scientific, Valley Park, MO. An EMD Millipore Millicell-ERS2 Volt-Ohm Meter with probe was purchased from Fischer

scientific to measure TEER. A Waters Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS) instrument was used for EVG quantitative analysis from the aliquots.

3.3 Methods

3.3.1 Suppository Manufacturing

Suppositories were manufactured by fusion molding technique. Witepsol suppository pellets were melted using a water bath at 45°C and drug was added in small portions to avoid formation of aggregates. The base-drug mixture was subjected to slow hand-shaking or an overhead mixer depending on the batch size. Once a uniform suspension of the drug in suppository base was achieved, it was slowly poured into the disposable plastic suppository strips (1-1.5grams). PEG suppositories were manufactured by mixing PEG polymers with molecular weights 3350, 1000 and 400 at the ratio 60:30:10. They were weighed and melted such that the temperature of molten mixture was maintained between 65-70°C after addition of each polymer. Further, drug was added in small proportions to avoid aggregate formation. Like witepsol suppositories, once a uniform suspension of the drug in PEG base was achieved, it was slowly poured into the disposable plastic suppository strips and allowed to cool to room temperature.

3.3.2 Drug Content Analysis

Suppositories were manufactured with 40mg/unit for every test drug (TFV, TAF, EVG, MK-2048) and base (Witepsol and PEG). Drug content analysis was performed by extracting the drug from suppositories using the procedures described below, followed by quantitative analysis using High-Performance Liquid Chromatography (HPLC). Drug loaded suppositories were placed in a 20mL volumetric flask and heated using a water bath at 45°C and 65°C for witepsol and PEG suppositories respectively. Once melted the samples were subjected to different extraction procedures based on the analyte as described below.

TFV – A HPLC assay was developed for TFV quantification. The HPLC assay was conducted on an Ultimate 3000 (Dionex) which is equipped with a photodiode array detector (260 nm). Chromeleon version 6.70 (Chromatography Management System) was used as the data management and analysis software. Separations were achieved on a Phenomenex Gemini C18 column (150 X 4.66 mm) at 25°C with a flow rate of 1mL/ min. The mobile phase was 10 mM potassium phosphate buffer + 2 mM tetrabutylammonium sulphate (tBAHS) pH 5.7: MeOH at ratio 80:20. The retention time of TFV was 5.3 ± 0.2 mins and 20 uL injection volume was used. A calibration curve was made with the concentration range 5-200 µg/ml. The limit of detection (LOD) for TFV was 0.1 ug/mL and limit of quantification (LOQ) was 0.32 µg/mL. Each suppository was placed in a 20 mL volumetric flask and melted using water bath. The volume was made up to 20 mL with 20% methanol and 100 uL of 18% sodium hydroxide (NaOH) solution as added. Addition on NaOH solution was necessary as TFV's solubility was pH dependent. TFV was extracted by sonication the samples for at least 10 mins. The solution was allowed to cool to room temperature so that the fat-soluble bases separate from the organic phase. 1 mL aliquots were withdrawn and centrifuged at 10000 rpm for 10 mins. The supernatant was filtered using 0.22µm PTFE syringe filters. Suitable dilutions were made using 20% methanol and analyzed using HPLC.

TAF – A similar HPLC method using the Ultimate 3000 Dionex system equipped with a photodiode array detector (260 nm) was developed for TAF quantification as well. Separations were achieved on a Phenomenex Gemini C18 column (150 X 4.66 mm) at 25°C with a flow rate of 1mL/ min. The mobile phase was 25 mM potassium phosphate + 5 mM tetrabutylammonium bromide (tBAB) pH 6.0: MeOH at ratio 80:20 and acetonitrile. The retention time of TAF was 14 ± 0.2 min and 20 µL injection volume was used. A calibration curve was made with the concentration range 10-200 µg/ml. The LOD for TAF was 0.6 µg/mL and LOQ was 1.25 µg/mL with S/N ratio = 12.43 ± 1.06 and 3.82 ± 0.35 respectively. Each suppository was placed in a 20 mL volumetric flask and melted using a water bath. The volume was made up to 20 mL with 100% methanol. The solution was allowed to cool to room temperature so that the fat-soluble bases separate from the organic phase. TAF was extracted by vigorous vortexing and sonication. 1 mL aliquots were withdrawn and centrifuged at 10000 rpm for 10 mins. After centrifugation, 1:100 dilution was done with 25% acetonitrile. The final sample for analysis will contain methanol, acetonitrile and water at the ratio 10:22:68. These were then filtered using 0.22 µm PTFE syringe filters before injecting into the HPLC system.

EVG – EVG drug content analysis was also conducted on the same Ultimate 3000 Dionex system equipped with a photodiode array detector (275 nm). Separations were achieved on a Phenomenex Synergi 4µ Polar column (150 X 2.00 mm, RP 80A) at 37°C with a flow rate of 0.5mL/ min. The mobile phase was 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile. The retention time of EVG was 7.1 ± 0.2 mins. A calibration curve was made with the concentration range 1-100µg/ml with 5 µL injection volume using 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile at 80:20 as the solvent. Each suppository was placed in a 20mL volumetric flask and melted using a water bath. The volume was made up to 20 mL with acetonitrile and the drug was extracted by vigorously vortexing the samples. The solution was allowed to cool to room temperature so that the fat-soluble bases separate from the organic phase. 1 mL aliquots were withdrawn and centrifuged at 10000 rpm for 10 mins. The supernatant was filtered using 0.22µm PTFE syringe filters. Suitable dilutions were made using 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile at 70:30 and analyzed using HPLC.

MK-2048 – MK-2048 drug content analysis was conducted on the Ultimate 3000 Dionex system equipped with a photodiode array detector (344 nm). Separations were achieved on Xbridge C18 5µm HPLC column (50 X 2.1 mm) at 40°C with a flow rate of 1mL/ min. A gradient phase with mobile phase 0.1% Formic Acid (FA) in water (A) and acetonitrile (B). The retention time of MK-2048 was 5.5 ± 0.2 mins. A calibration curve was made with the concentration range 0.75-50µg/ml with 20 µL injection volume using 30% acetonitrile in 0.1% Formic Acid (FA) in water as the solvent. Each suppository was placed in a 20 mL volumetric flask and melted using a water bath. Melted suppositories were dissolved in 20 mL 100% acetonitrile followed by vortexing for 1-2 minutes for drug extraction until the drug was dissolved. The solution was allowed to cool to room temperature so that the fat-soluble bases separate from the organic phase. 1 mL aliquots were withdrawn and centrifuged at 10000 rpm for 10 mins. Suitable dilutions were made acetonitrile first, followed by 30% acetonitrile in 0.1% Formic Acid (FA) in water. Final samples were filtered using 0.22 µm PTFE syringe filters before injecting into the HPLC system.

3.3.3 Breaking Point of drug loaded suppositories

Breaking point of suppositories was measured using TA.XTPlus Texture Analyzer. The conical edge of the suppositories was trimmed using a blade to obtain flat surfaces on both ends. The suppositories were placed on a flat platform (TA-90) and tested using a flat surface probe (TA-58). The height between suppository and probe was calibrated to 10 mm, and length travelled by the probe after contacting the suppository surface was set to 5 mm to ensure the suppository was broken completely. The standard 5 kg load cell was replaced with the 30 kg load cell as in some cases more than 5 kg force was required to crush the suppository. Tests were conducted with drug loaded suppositories and placebo suppository as a control.

3.3.4 In vitro dissolution of suppositories

A USP type 1 apparatus was used for the dissolution studies. Based on USP guidelines the distance between the inner surface of the vessel and the basket was manually adjusted to 25 ± 2mm. Sink conditions were maintained and 900 mL 0.5% SDS was used as the dissolution medium. Two ml aliquots were collected at 15, 30, 45, 60, 75, 90, 105 and 120 minutes and replaced with fresh medium. These samples were filtered using a syringe and 0.22µm PTFE syringe filters, diluted and analyzed using HPLC. HPLC analysis was conducted as described in section 3.3.2.

3.3.5 In vitro trans-well flux assay

In vitro trans-well flux permeability studies were conducted using MDCKII – wt and MDCKII-MDR1 (P-gp overexpressing) cell lines, a well-established model to investigate P-gp substrates. Both cell lines were withdrawn from cryostorage, thawed and cultured in T75 flasks until 80% confluency was achieved. Required confluency was determined visually using a microscope. Once confluent, the cells were seeded on to Costar™ Transwell™ inserts at 500K cells/ well seeding density. The monolayer integrity was measured by monitoring and recording TEER using EMD

Millipore Probe every other day until required confluency was achieved. The threshold to conduct experiment was set to 200Ω for MDCKII-wt and 400 Ω for MDCKII-MDR1. This trans-well flux assay was validated with a known P-gp substrate and inhibitors, ^{3}H Digoxin and Elacridar (GF120918), respectively. Based on results from validation experiments and literature, 5µM Elacridar (GF120918) was identified as the optimum inhibitory concentration. A 2 µM EVG stock solution was prepared (DMSO < 0.1%) in HBSS. Directionality experiments were conducted by calculating B→ A and A→ B flux of EVG in MDCKII-wt and MDCKII-MDR1 cells. Two stock solutions of 2 µM EVG were prepared, one with 5 µM GF120918 and one without. To avoid competitive binding with the P-gp transporters, the cell monolayer was incubated with 5 µM GF120918 solution for 30 mins prior to beginning of the experiment. Further, the solutions were replaced with stock solutions in the donor chamber and fresh HBSS in the receptor chamber. 100µL aliquots were collected at 0, 30, 60, 90 and 120 minutes and replaced with fresh HBSS. The drug content was analyzed from the aliquots using UPLC. The trans-well plate was subjected to constant stirring at 37°C. Apparent permeability (P_{app}) was calculated based on the formula mentioned in section 2.3.2. Efflux ratio (ER) was calculated by dividing the P_{app} of B→ A by A→ B flux of EVG for the MDCKII-wt and MDCKII-MDR1 cells.

3.3.6 Statistical analysis

The *in vitro* characterization of all the different suppositories were statistically analyzed using one-way ANOVA with Tukey's multiple comparison post-hoc test at 30 min as maximum drug was released and it was the first *in vivo* sampling time point. Multiple comparisons were conducted using placebo suppositories as control group. The efflux transporter experiment was conducted three times, each experiment was conducted in triplicate wells. The data from three experiments was pooled together and statistical analyses was conducted. One-way ANOVA was conducted comparing each treatment group, Apical (A) to Basal (B), B to A, with and without inhibitors for the MDCKII-wt cells and MDCKII – MDR1 overexpressing cells as well. Post-hoc comparisons were conducted using Tukey multiple comparisons test. P values less than or equal to 0.05 were considered statistically significant.

3.4 Results

3.4.1 Weight variation & appearance of drug loaded suppositories

It is essential to understand if addition of ARVs impact the appearances or weights of the suppositories. Therefore, suppositories (batches of 20 units) with each drug were manufactured and weighed separately. Based on visual inspection, incorporation of the selected drugs did not impact the appearance except the MK-2048 in PEG base suppositories. Incorporation of MK-2048 in the PEG suppository mixture imparted a yellow coloration to the mixture. Based on previous studies conducted in our lab (unpublished data) with other dosage forms, we had anticipated this change in coloration, so we modified the pH to acidic which dissipated the yellow coloration. The weight of all four drug-loaded suppositories was within 2% relative standard deviation (RSD) (table 4). However, they were significantly different from each other and when each API suppository was compared with placebo (one -way ANOVA with multiple comparisons using Dunnett's multiple comparison test; $P < 0.05$).

Figure 10 Witepsol H15 suppository batches with TFV, EVG and MK-2048 (left to right)

Table 4 Summary of physicochemical characteristics of drug-loaded suppositories

Single entity Polyethylene Glycol (PEG) (top) and Witepsol (bottom) suppositories with EVG, MK-2048, TFV and TAF (40mg each) were manufactured and characterized for weight variation, drug content uniformity, appearance/ feel and breaking point. Data represented as mean ± standard deviation (SD)

PEG 3350/1000/400					
Test	**Elvitegravir**	**MK-2048**	**Tenofovir**	**TAF**	**Placebo**
Weight Variation (g, mean ± SD, n=20)	1.45 ± 0.02	1.46 ± 0.01	1.46 ± 0.02	1.48 ± 0.02	1.49 ± 0.01
Drug content (mg/suppository ± SD, n=3)	35.7 ± 3.12	35.2 ± 1.44	26.7 ± 0.63	37.9 ± 2.36	NA
Appearance/ Feel	White/ slightly sticky to touch	White/ slightly sticky to touch	Unpleasant odor	White/ slightly sticky to touch	White/ slightly sticky to touch
Breaking point (kg, mean ± SD, n=3)	4.36 ± 0.96	3.76 ± 0.56	3.70 ± 0.7	2.36 ± 0.07	1.31 ± 0.14

Witepsol H15					
Test	**Elvitegravir**	**MK-2048**	**Tenofovir**	**TAF**	**Placebo**
Weight Variation (g, avg ± SD, n=20)	1.19 ± 0.02	1.19 ± 0.01	1.19 ± 0.01	1.20 ± 0.02	1.23 ± 0.01
Drug content (mg/suppository ± SD, n=3)	37.3 ± 3.57	41.3 ± 1.29	25.2 ± 3.78	36.3 ± 1.97	NA
Appearance/ Feel	Smooth - drug gathered on tip	Smooth texture	Smooth - drug gathered on tip	Smooth - drug gathered on tip	Smooth texture
Breaking point (kg, mean ± SD, n=3)	5.33 ± 0.59	5.64 ± 0.52	5.86 ± 0.22	>6	5.07± 0.98

3.4.2 Drug content uniformity

Drug content analysis was conducted to ensure uniform drug distribution amongst units in a batch. Therefore, drug extraction was performed on three randomly picked suppositories out of the batch of 20 units followed by the drug quantification using HPLC. Forty milligrams per unit was selected as the target dosing levels based on several pre-clinical studies conducted with the same drugs using different dosage forms. The results from drug content uniformity is represented in table 4. The RSD of drug content in all batches for both suppository types was under 5%, which confirms the drug is uniformly distributed in suppositories.

3.4.3 Breaking point

The primary purpose of this test is to determine if manufactured suppositories (water-soluble or fat-soluble bases) can withstand the pressure encountered during handling and transportation. Suppositories can be broadly classified either as elastic or brittle in nature [143]. We conducted the breaking point test for suppositories using the TA.XT Plus Texture Analyzer. We developed an in-house method to conduct this test. Suppositories were placed vertically on a flat surface and a flat-headed probe applied pressure from the top. The force required to crush the suppository unit was considered as the breaking point. During the test it was observed that PEG suppositories were crushed slowly, forming a collapsed mass instead of breaking or splitting into pieces like the witepsol suppositories. This suggests that PEG base suppositories were more elastic whereas the witepsol suppositories were brittle. Moreover, the witepsol drug loaded suppositories were not significantly different with each other as well as when compared to placebo control. However, the PEG suppositories were significantly different when compared with each other and with the placebo, accept TAF suppositories (one -way ANOVA with multiple comparisons using Dunnett's multiple comparison test; $P< 0.05$). Although there is no gold standard available for the breaking point strength, some researchers have suggested breaking point above 1.8 kgs to be optimal [143]. Breaking point of witepsol suppositories ranged between 2-4.5kgs and PEG-based suppositories 5 ->6kgs. Therefore, both witepsol and PEG suppositories can be regarded as acceptable.

3.4.4 *In vitro* dissolution

Our goal was to compare dissolution of hydrophilic and hydrophobic drugs from the same suppository base; hence a common dissolution medium was used. 0.5% SDS was used as the dissolution media to maintain sink conditions, considering the hydrophobic drugs and to ensure quality control for the suppository batches. The witepsol H15 suppository bases primarily consist of fat-soluble glycerides and considering the lipophilic nature of MK-2048 and EVG, only ~20% MK-2048 and EVG released within the first two hours of experiment (figure 11a). Based on the visual observation, the witepsol suppositories quickly melted and formed a cake like mass in the mesh basket, slowly releasing the drugs. Because of the increased affinity between lipophilic drugs and suppository bases, slow drug release was observed. On the contrary, both the hydrophilic drugs (TFV and TAF) were completely released within the first 30 minutes due to better partitioning into the aqueous dissolution media. But these distinctly different drug release profiles were not observed in PEG-based suppositories. Irrespective drug's nature, 100% drugs were released within the first the 30 mins of the dissolution studies, except MK-2048 (figure 11b). We suspect the pH adjustment made while manufacturing the PEG-based MK-2048 suppositories might have modified the PEG polymeric bonds chemically, resulting in a slower dissolution. However, 100% MK-2048 was released within 60 minutes. Using one-way ANOVA the drug dissolution from witepsol suppositories was statistically significant. Based on tukey's multiple comparison test, all the APIs were significantly different except EVG vs MK-2048 ($p=0.9614$) and TFV vs TAF ($p=0.8863$). Similarly, for PEG suppositories the drug dissolution was statistically significant using one-way ANOVA. Based on tukey's multiple comparison test, all the APIs were significantly different except EVG vs TFV ($p=0.7511$), EVG vs TAF ($p=0.7239$) and TFV vs TAF ($p=>0.9999$).

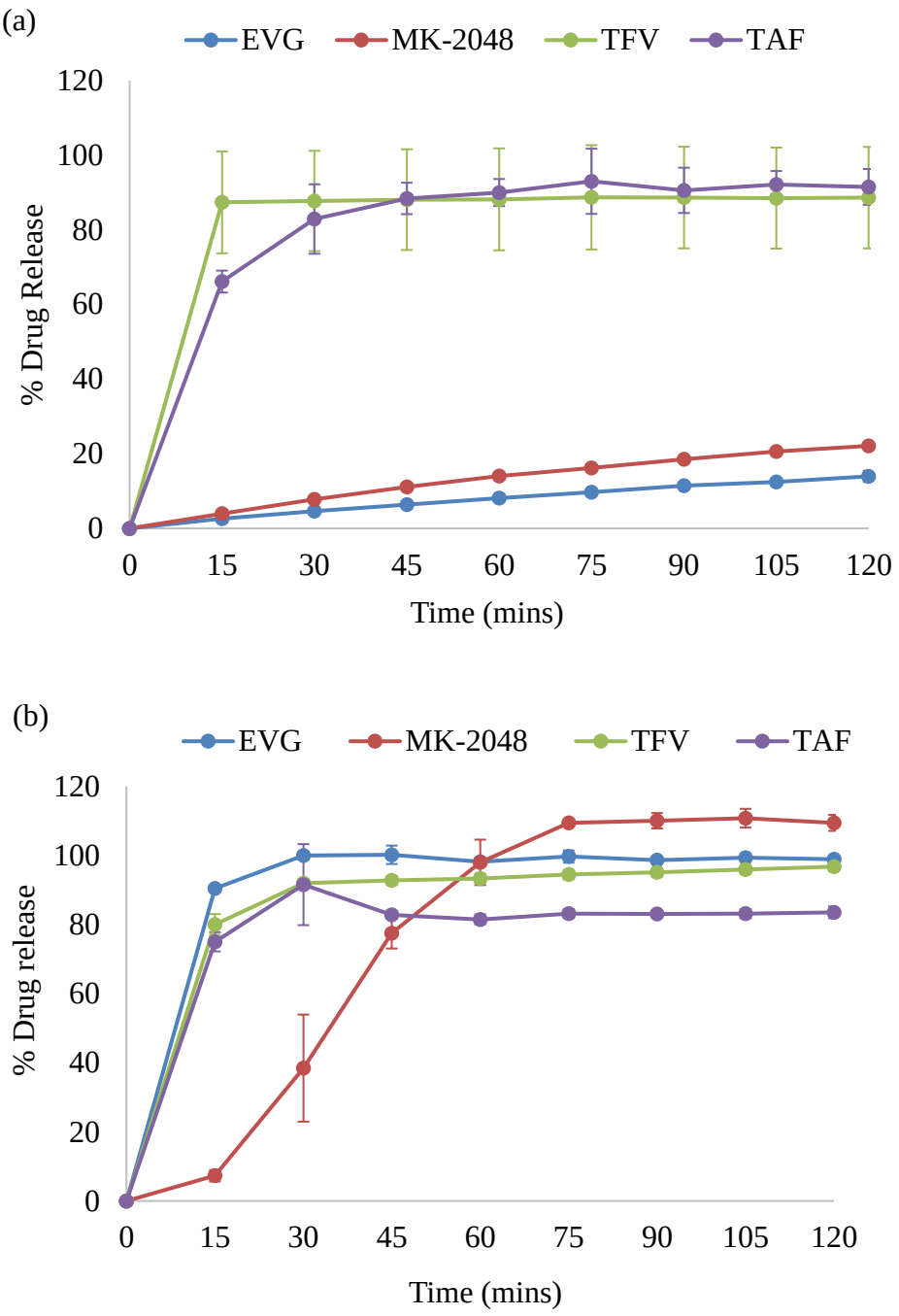
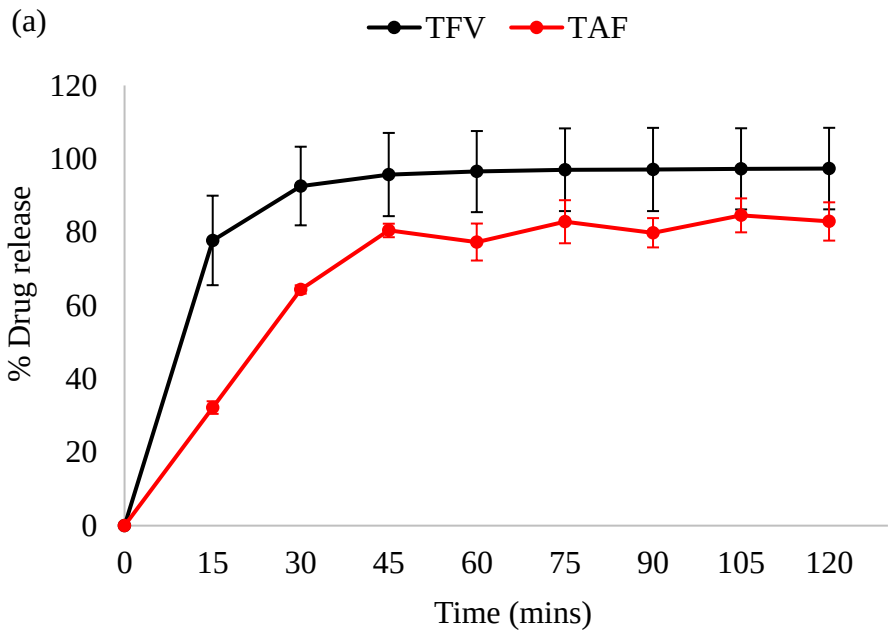


Figure 11 *In vitro* drug dissolution of EVG, MK-2048, TFV, and TAF

In vitro dissolution was conducted using USP apparatus Type 1 with single entity suppositories (40mg) (a) Witepsol H15 and (b) PEG 3350:1000:400 at ratio (60:30:10). 0.5% SDS was used as the dissolution media; X-axis – Time (mins) and Y-axis - % drug release; one-way ANOVA with tukey's multiple comparison (N=3 suppository units/ API, mean ± SD)

The role of surfactant (SDS) in the dissolution media was to achieve sink conditions for the hydrophobic drugs. However, amongst the evaluated APIs, TFV and TAF were hydrophilic in nature. Addition of SDS in the dissolution media for APIs with good solubility would enhance the drug dissolution, making it difficult to observe the differences in drug dissolution profiles (if any). Hence to clearly predict *in vitro* dissolution rates for the two hydrophilic drugs, the dissolution studies for TFV and TAF suppositories were performed in 1xPBS (figure 12). Although they are both hydrophilic drugs, the logP of TFV and TAF is -1.5 and 1.6 respectively. More than 90% TFV was released within 30 mins, but only about 65% TAF was released in that time (figure 12a). However, this difference in release rates was not observed in PEG-based suppositories (figure 12b). Based on visual observation, the PEG-based suppositories completely dissolved in 1xPBS within 5-10 mins of beginning the experiment, hence released almost 100% within 15 mins of dissolution. Additionally, based on unpaired t-test TFV release was significantly different then TAF from witepsol suppositories but non-significant from PEG suppositories (p=0.7983).



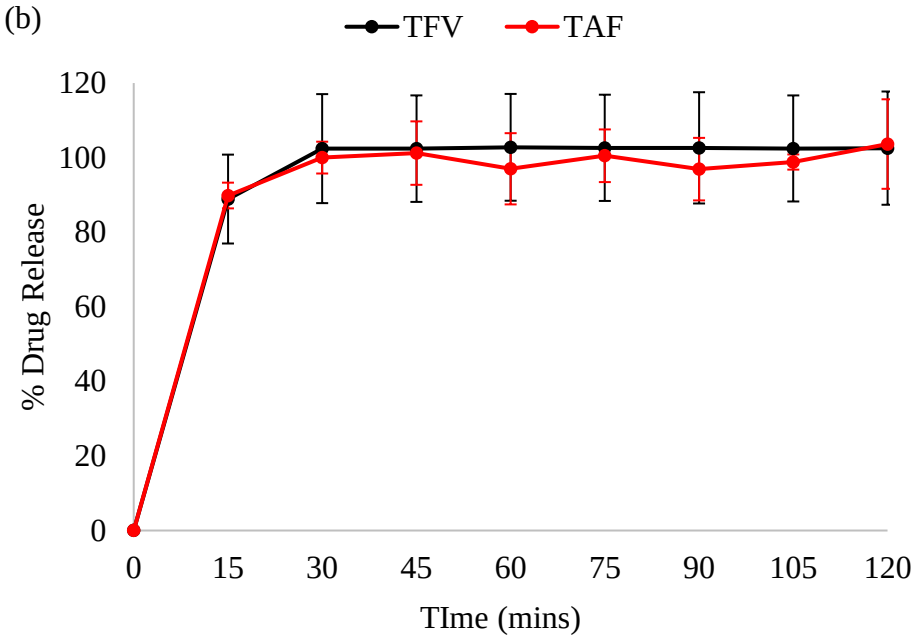


Figure 12 *In vitro* drug dissolution of TFV, and TAF

In vitro dissolution was conducted using USP apparatus Type 1 with single entity suppositories (40mg) (a) Witepsol H15 and (b) PEG 3350:1000:400 at ratio (60:30:10). 1 x PBS was used as the dissolution media; X-axis – Time (mins) and Y-axis - % drug release; unpaired t-test at 30 mins (N=3 suppository units/ API, mean ± SD)

3.4.5 Determining if Elvitegravir is a substrate for efflux transporter P-glycoprotein (P-gp)

P-glycoprotein belongs to the ATP binding cassette (ABCB1) transporter family. As discussed in section 1.12, there is evidence of efflux transporters being expressed in the colorectal region. Moreover, ARVs are well-known for multiple DDIs and usually administered as combinations of more than one ARV in a single product. Hence, it is essential to investigate if the ARVs being developed as a potential topical product are substrates for key efflux transporters. If the ARVs are substrates and not administered in sufficient amounts to saturate the transporter function, they could potentially affect the ARV exposures *in vivo*. Figure 13 demonstrates the results from trans-well flux assays conducted to determine if EVG was a P-gp substrate. Panel A demonstrates that the EVG apparent permeability (P_{app}) in MDCKII-wt cell line and is primarily driven from B→A

side (3.74 x 10^{-6} cm/sec) compared to A→B side (1.22 x 10^{-6} cm/sec), increased the efflux ratio (ER) to 3.06. With addition of 5µM GF120918, the P_{app} was reduced to 2.02 x 10^{-6} cm/sec and 1.30 x 10^{-6} cm/sec for B→A and A→B side, respectively with an efflux ratio of 1.55. This results in a 49% inhibition in MDCKII-wt cell line. Similarly, panel B illustrates an EVG P_{app} in P-gp (ABCB1) overexpressing MDCKII-MDR1 cells, wherein the B→A (5.39 x 10^{-6} cm/sec) side compared to A→B side (0.57 x 10^{-6} cm/sec) increasing the efflux ratio to 9.3. Upon addition of GF120918, the P_{app} was inhibited to 2.5 x 10^{-6} cm/sec and 0.79 x 10^{-6} cm/sec respectively, reduced the efflux ratio to 3.15. A 66% inhibition was achieved in MDCK-MDR1 cells which is above the suggested value of 50% inhibition to be considered as a P-gp (ABCB1) substrate [144]. However, GF120918 is a non-specific P-gp inhibitor, and is known to inhibit another efflux transporter called Breach Cancer Resistance Protein (BCRP) expressed by ABCG2. Therefore, the 49% and 66% inhibition observed in MDCKII-wt and MDCKII-MDR1 cells could be due to inhibition of both the efflux transporters P-gp and BCRP. In summary, these cell culture experiments confirm that EVG is a P-gp (ABCB1) and BCRP (ABCG2) substrate. However, further studies are required to confirm the specific efflux transporter.

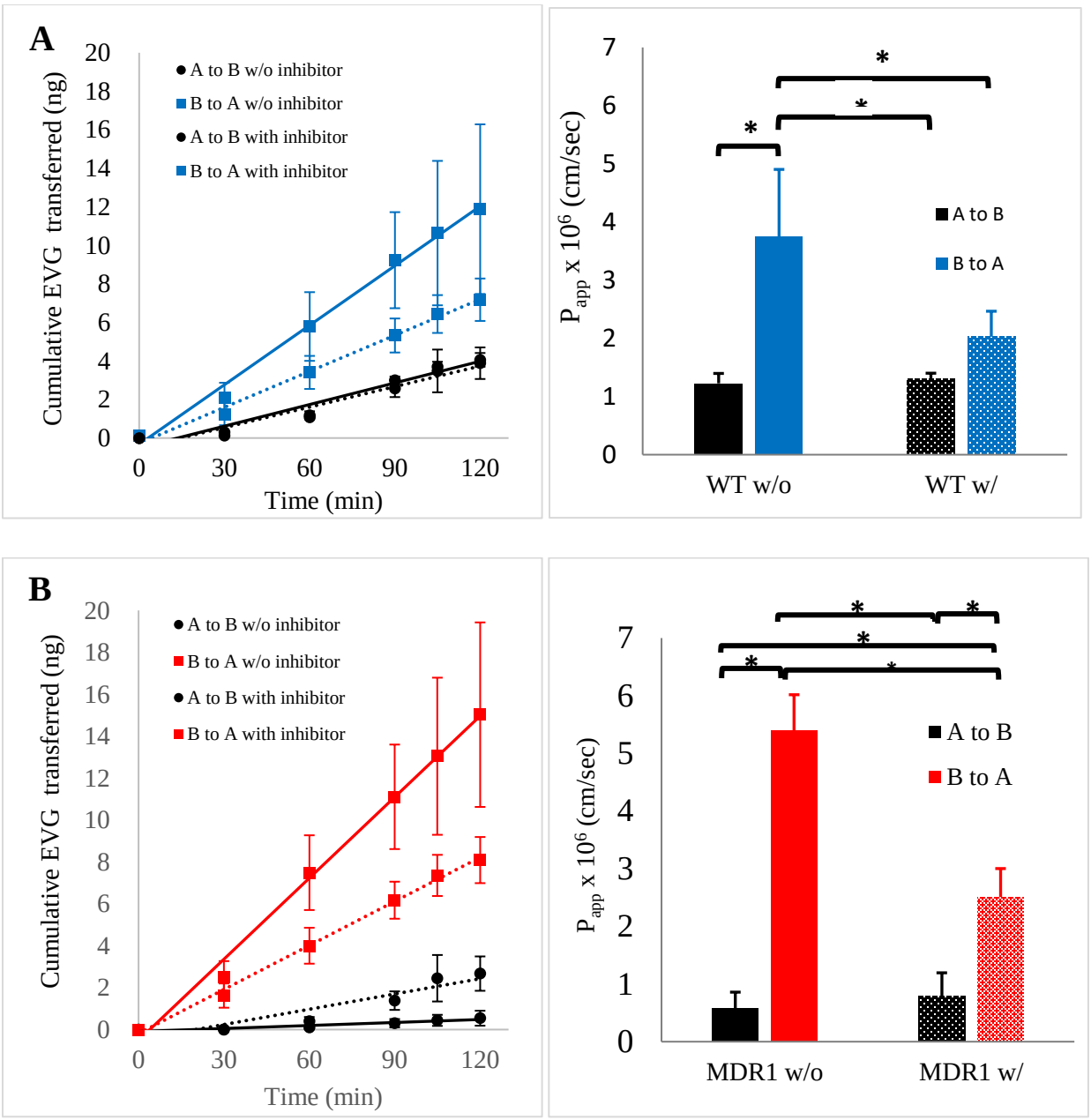


Figure 13 EVG transport directionality (Panel A left) and EVG Apparent permeability (Panel A right) in MDCKII-wt; EVG transport directionality (Panel B left) and EVG Apparent permeability (Panel B right) in MDCKII-MDR1

Elvitegravir (EVG) concentration was 2µM and GF1209018 concentration was 5µM; Data represented as mean of three experiments ± standard deviation (SD) (N=3 wells/ group); Apparent permeability (P_{app}) was calculated using equation in section 2.3.2. One-way ANOVA with post-hoc Tukey's test ($P \leq 0.05$)

3.5 Discussion and conclusion

The primary objective of the experiments presented in this aim was to conduct studies that would help identify suitable ARV drug candidates. Suppositories with these candidates would be manufactured, characterized and evaluated *in vivo* using macaque models. Our criteria for selection of ARVs was to choose the most investigated class, so that we could use data from the literature and other relevant studies to make assessments about PK exposure and efficacy achieved by the suppository as a dosage form, hence NNRTIs were selected. Secondly, as explained in section 3.1, from a mechanism of action perspective, several researchers have indicated the benefits of late-active ARVs have proved to be beneficial as compared to ARVs acting in earlier stages (NNRTIs, entry inhibitors etc.) of HIV-1 replication [15, 145]. Dobard et. al has demonstrated that InSTIs come into action at later stages of the HIV-1 replication cycle, therefore this class of ARVs could potentially provide protection for longer duration, in some cases even post-exposure [145]. Using a rectal gel containing 1% L-870812, two out of three macaques were protected from acquiring SHIV infection, when administered 30 mins before exposure. Additionally, a 1% raltegravir containing rectal gel protected five out of 6 macaques from acquiring SHIV infection when applied 3 hours post exposure. These results clearly prove the advantages of using InSTIs for PrEP. ARVs from both these classes are already on the market as combination pills for HIV-1 treatment. So, there is minimal risk of any potential drug-drug interactions. Also, combining these two classes of ARVs could potentially provide synergistic effects [15].

According to the initial characterization results, although the suppository weights were within 2% RSD, both witepsol and PEG drug loaded suppositories were significantly different from each other as well as when compared to placebo control (one -way ANOVA with multiple comparisons using Dunnett's multiple comparison test; $P< 0.05$). However, incorporation of either hydrophilic (TFV, TAF) or hydrophobic (EVG) ARVs did not have any significant impact on the appearance of the suppositories, except MK-2048. These observed results were anticipated as suppositories bases offers the possibility of incorporating a wide range of drugs with various physiological properties. Additionally, the size of suppositories used in our studies can typically incorporate 100 mg drug or more, and we loaded with 40mg only. The overall weights and appearances were not

affected (exception MK-2048) with different ARVs. Upon further investigation, the yellow color change was identified due to the protonation of MK-2048 caused by pH change in the molten PEG suppository mixture. Therefore, the color change was reversed by addition of NaOH solution. Although there are no guidelines specifically for drug content uniformity in suppositories, general guidance's for drug product suggests the range should be within 90-110% of the label claim. All the suppository batches were within this range accept PEG-based suppositories with EVG, MK-2048, TFV and witepsol TFV suppositories. We hypothesized that this could primarily be due to an ineffective drug extraction procedure. TFV demonstrates a pH dependent solubility profile, therefore, even with an organic solvent, complete drug extraction could not be achieved. Upon addition of 100µL 5N NaOH solution, all the undissolved TFV easily dissolved. This specific pH adjustment step was later incorporated into the TFV extraction procedure for future studies. Although, small batches of 20 units were manufactured, uniform drug distribution was achieved in all batches (<5% SD). Some studies have indicated that changes in particle size can have an impact on drug distribution in suppositories [80], however this will only be primarily applicable for batches with larger quantities.

The rationale in evaluating breaking point of a suppository is to ensure the product can withstand the potential pressure exerted while handling and transportation. Hence a specific test or range of value is not predefined. Additionally, any incompatibilities between the suppository bases and APIs could potentially affect the suppository integrity, altering its breaking point. Various investigators have used different techniques (either manual or automatic) to determine the breaking point of suppositories [143, 146]. However, the underlying principle behind each test remains the same, the suppository is placed on a flat surface either vertical or horizontal and with the help of a probe, increasing weights are applied and the weight at which the suppository breaks is referred as the breaking point. In some cases maintaining constant travel distance upon contact, the force required to break the suppository is referred as the breaking strength [80]. Based on the same principle, we designed an automated breaking point test as described in section 3.3.3. Overall, the incorporation of hydrophilic and hydrophobic drugs did not have an impact on the breaking point of the witepsol suppositories, but PEG suppositories were significantly different when compared to placebo control (one -way ANOVA with multiple comparisons using Dunnett's multiple comparison test; $P < 0.05$). The witepsol suppositories were more brittle and harder compared to PEG-based suppositories which were softer and elastic in nature. The breaking point

for witepsol H15 suppositories from our studies (5-6kgs) were comparable to the Sulpride SD suppository formulation (5.5kgs) using the same base. Similarly, the PEG-based suppositories in our studies (2-4kgs) were also comparable to the Sulpride SD suppositories (1.8 - 2.2kgs), however the PEG polymers used in both bases were with different molecular weights and ratios. The molecular weights of PEG polymers impact the chain length which in turn affects the firmness and dissolution of the PEG-based suppositories (section 2.1).

Witepsol H15 is one of the most commonly available and widely investigated hard-fat suppository base [84, 143, 147]. The glyceride content in witepsol H15 makes it adaptable to a series of drugs with various physicochemical properties. Similarly, although not very widely preferred, PEG-bases are also evaluated as suppositories [80, 123, 146], with different molecular weights and ratios. There are several approaches available which can be utilized to modify the dissolution rates by incorporating other ingredients [80, 84, 123, 146, 147] or modify the drugs [143], eventually either sustain or accelerate the drug release. For instance, addition of starch hydrolysates in both lipophilic and hydrophilic suppository bases have the potential of altering drug dissolution rates [146]. Increasing amounts of starch hydrolysates, caused a rapid drug dissolution from cocoa butter suppositories, whereas drug dissolution was delayed from PEG-based suppositories. Similarly, addition of solids fats with high melting point to fatty-acid base like witepsol H15 have the potential to prolong rectal absorption without affecting the exposures [147]. This approach could be beneficial if longer duration of therapeutic window is desired or reduce dosing frequencies. Our dissolution results from witepsol H15 using the selected ARVs clearly demonstrate the difference in drug release rates is dependent on the physicochemical properties of the drugs. Due to the lipophilicity of EVG and MK-2048, a delayed release was observed with witepsol H15 as the primary ingredient. Whereas due to the hydrophilic nature of TFV and TAF, they had more affinity to the aqueous dissolution medium, perhaps the interactions were exaggerated due to the presence of surfactant (SDS). This distinctly different drug dissolution rates can potentially benefit us considering an on-demand product for HIV-1 prevention. As discussed earlier, although the dissolution media is not biologically relevant, if similar dissolution rates were achieved *in vivo*, the NNRTI could act as the first line of defense against HIV-1 virions upon entry and the delayed InSTI release could potentially act as a secondary defense mechanism for resistant mutant viruses.

As mentioned in sections 1.12 and 3.4.5, investigating DDIs for ARVs is crucial from a topical drug delivery perspective. Upon initial investigation, we determined that data supporting EVG's efflux transport modulation was not available (table 3 summarizes the efflux transporter profile of InSTIs). The *in vitro* permeability results presented in figure 13 prove that EVG is a P-gp (ABCB1) and BCRP (ABCG2) substrate. GF120918 is well-known inhibitor for P-gp as well as BCRP transporters, therefore the inhibitions observed was not P-gp specific. The results confirm that EVG is a substrate for P-gp as well as BCRP, but in order to determine if EVG is a substrate for BCRP, similar studies with specific inhibitions like Verapamil (known specific P-gp inhibitor) are warranted. Although EVG is a highly potent InSTI but has to be administered at higher doses orally, boosted with Cobicistat as it is readily metabolized by CYP enzyme. However, according to the product label, no clinically meaningful DDIs were found when EVG was co-administered with known P-gp inhibitors or inducers [148]. But lower doses might be required to achieve local therapeutic exposures when administered topically. This is valuable information to have while interpreting *in vivo* PK data and evaluating exposures of different ARVs

In spite of high potency, EVG belongs to Biopharmaceutical Classification System (BCS) class II making it a highly permeable drug. According to the product label published by Gilead Sciences, Inc. EVG's tissue distribution (tissue to plasma concentration) post oral administration is greater than 1 for liver and GI tract [148]. But specific information for colorectal tissue distribution is not available. However, upon [^{14}C] EVG administration, the radioactivity in the tissues largely declined parallelly with the plasma, with almost complete elimination by 96 hours [149]. Therefore, since EVG was almost completely eliminated within 96-hour window, we do not anticipate tissue retention and any systemic toxicity post rectal administration. Similarly, another recent study evaluated PK and tissue distribution of EVG and TAF using long-acting nanoparticle administered subcutaneously (s.c.) [150]. They evaluated tissue distribution of free drugs as well as in nanoparticles for all major organs including the vagina and colon as well. According to their results, the half-life ($t_{1/2}$) of free-EVG in plasma, colon and vaginal tissue was 10.8, 12.5 and 30.8 hours [150]. In terms of tissue distribution, since the $t_{1/2}$ of plasma and colon tissue was very close, this further establishes that EVG tissue retention post rectal administration should not be concerning. However, this might not be true for vaginal administration as the $t_{1/2}$ was increase about 3 times in the vagina compared to plasma.

Based on the results presented in chapter 2 (section 2.5), witepsol H15 was selected as the base of choice for fat-soluble suppository bases and PEG 3350:1000:400 (at ratio 60:30:10) was selected as the base of choice for water-soluble suppository bases. Similarly, based on results presented in chapter 3, two hydrophilic (TFV and TAF) and two hydrophobic (EVG and MK-2048) APIs were selected as the lead ARV candidates. During the course of investigation and selection, the clinical development of MK-2048 for treatment of HIV-1 was put on hold due to poor PK results. Therefore, amongst the selected ARVs, MK-2048 was withdrawn from further *in vivo* studies. In summary, witepsol H15 and PEG 3350:1000:400 suppositories loaded with either TFV and EVG or TAF and EVG will be manufactured and tested *in vivo* using a non-human primate (NHP) model. The NHP animal model is widely used in microbicide research and well-established model for safety and efficacy evaluation.

3.6 Acknowledgments

We would like to acknowledge Phillip Graebing for helping with the analytical method development and validations. We would also like to acknowledge Dr. Phillip Empey for providing his scientific inputs and expertise for the trans well flux assay experiments conducted in the efflux transporter studies.

4.0 Conduct in vivo pharmacokinetic studies with tenofovir and elvitegravir containing suppositories to assess local and systemic exposure

4.1 Introduction

A recent behavioral study conducted by Carballo-Dieguez *et al.* assessed the use of placebo rectal gel versus rectal suppositories in sexually active HIV-seronegative men engaging in RAI [68]. This study was primarily conducted to determine men's preference for suitable RM vehicle amongst available dosage forms [68]. The results indicated that the studied population preferred rectal gels over the suppositories, however the authors highlighted that development of suppositories should not be completely disregarded due to a few limitations of their study protocol and products tested [68]. Limitations such as the weight, size and choice of suppository base evaluated in their studies might not be appropriate and might have an impact of subjects preferring one dosage form over the other. We predict this could be a potential factor responsible for preference of gel over suppositories. Hence, one of our objectives was to overcome such limitations pertaining to suppositories and explore the potential application of a suppository as potential RM. Apart from a suitable dosage form, user-friendliness is an important factor to be considered while investigating RMs. For instance, in spite of a safe and effective rectal gel product, some users reported gel would tend to leak and become messy due to the semi-solid nature. This affected their regular sexual behaviors, eventually leading to poor user adherence and compliance. Hence, a few strategies are currently being pursued to develop consumer driven and user-friendly dosage forms which do not impact or interfere with a couple's routine sexual behaviors.

EVG was first approved by the FDA in 2012 for HIV-1 treatment in ARV-treatment-experienced adults and has recently demonstrated the potential to be a valuable microbicide candidate [145]. It was primarily developed to overcome the drug resistance caused by previous InSTIs such as Raltegravir. Several studies have suggested the advantages of InSTI's, which are known to target the later stages of HIV-1 replication cycle [15, 151]; the late-acting ARV phenomenon can be exploited and used in the favor of designing topical microbicides with a

combination of InSTI and other ARVs [151]. Shimura *et. al.* evaluated the antiviral activity and drug-resistance profile of EVG and proved that EVG can effectively inhibit HIV-1 replication in various multiple-drug resistant clinical isolates [149].

In terms of physicochemical properties, as TFV belongs to Biopharmaceutical Classification System (BCS) class III and EVG belongs to BCS class II, TFV can easily solubilize in the rectal fluid acting as the first line of defense. Whereas EVG's higher permeability can potentially be advantageous and provide prolonged tissue retention. Especially because EVG tissue distribution (plasma to tissue concentration) is greater than 1 for the liver and GI tract [148] (refer section 3.5). Moreover, TFV being a NRTI restricts the HIV replication cycle in the earlier stages compared to InSTIs which inhibit comparatively later stages of the HIV replication cycle, perhaps providing protection for longer durations. Hence, we hypothesize that a single product with combination of TFV and EVG will lead to prolonged therapeutically active drug levels due to different physicochemical nature of the APIs and likely improve protection due to their diverse mechanism of action. We conducted a proof of concept study to demonstrate that suppositories can be a potential RM product for HIV-1 prevention. In this chapter we explored the utility of a suppository, with a hydrophobic and a hydrophilic API co-delivered in a single product, using a water soluble (Polyethylene Glycol based) and fat-soluble (Witepsol) rectal suppository formulations. Based on the factors discussed in chapter 3, a NNRTI TFV was used as the hydrophilic candidate; and an InSTI Elvitegravir (EVG), which has been previously proved as a late acting anti-retroviral (ARV) agent, was used as the hydrophobic candidate. The dosing levels for TFV were selected based on clinical trials conducted earlier. PEG and witepsol suppositories with 40 mg TFV and 40 mg EVG were manufactured and characterized for their physical and chemical attributes. An *in vivo* crossover pharmacokinetic study was conducted in non-human primates (NHPs) comparing both suppository formulations to evaluate local and systemic exposures post product administration.

4.2 Materials

Tenofovir (TFV) was procured from CONRAD, Arlington, VA. Elvitegravir (EVG) was provided by Gilead Sciences, Foster City, CA. The fat soluble base witepsol was received as a free

sample from IOI Oleo GmbH, Suppocire bases from Gattefosse. Cocoa butter and three Polyethylene-Glycol (PEG) polymers of different molecular weights 3350, 1000, 400 were purchased from Spectrum Chemical. The plastic disposable suppository molds were purchased from Professional Compounding Centers of America (PCCA).

4.3 Methods

4.3.1 Manufacturing of TFV and EVG loaded suppositories

TFV and EVG loaded suppositories were manufactured by fusion molding technique using disposable plastic suppository molds. Witepsol Suppositories bases were melted in a water bath and drugs were added in small portions to avoid formation of aggregates. The base-drug mixture was subjected to slow hand-shaking or an overhead mixer depending on the batch size. Once a uniform suspension of the drugs in suppository base was achieved, it was slowly poured into the disposable plastic suppository molds (1-1.5grams). PEG suppositories were manufactured by mixing PEG polymers with different molecular weights. They were weighed and melted in increasing order of melting points, such that the temperature of molten mixture was maintained between 65-70°C after addition of each polymer. Further, TFV and EVG were added in small proportions to avoid aggregate formation. This was followed by pouring the drug-loaded molten mass into disposable plastic suppository molds.

4.3.2 In vitro characterization of Suppositories

Visual inspection was conducted to examine the color, surface and texture of the suppositories. Each unit was visually inspected for cracks and pits possibly caused by air entrapment.

TFV Drug content: A previously developed and validated Ultra Performance Liquid Chromatography (UPLC) assay was modified to quantify TFV from suppositories and assess the

drug content uniformity. A Waters Acquity UPLC equipped with a UV detector and EMPOWER data-processing software was used. Separations were achieved on an ACQUITY UPLC BEH C18 column (1.7µm, 2.1 X 50mm; Waters) fitted with a guard column (1.7µm, 2.1 X 50mm; Vanguard) at 20°C. The mobile phase was a mixture of 90% 10mM K_2HPO_4 + 5mM t-Butylammonium bisulfate (tBAHS) adjusted to pH 5.7 using 10% phosphoric acid and 10% methanol. The retention time for TFV was 2.8 ± 0.3 minutes. A calibration curve was prepared in the concentration range 1-200µg/ml. Each suppository was placed in a 20 mL volumetric flask and melted at 65°C for PEG based suppositories and 45°C for fat soluble bases using water bath. The volume was made up to 20 mL with 10% methanol and the drug was extracted by vigorously vortexing the samples. The mixture was allowed to cool to room temperature so that the fat-soluble bases separate from the organic phase. 1mL aliquots were withdrawn and centrifuged at 10K rpm for 10 mins. The supernatant was filtered using 0.22µm PTFE syringe filters. Suitable dilutions were made and analyzed using HPLC.

EVG Drug content: The High-Performance Liquid Chromatography (HPLC) system used to develop and validate the assay for EVG was an Ultimate 3000 (Dionex) which is equipped with a photodiode array detector (275 nm). Chromeleon version 6.70 (Chromatography Management System) was used as the data management and analysis software. Separations were achieved on a Synergi 4µ Polar column (150 X 2.00 mm, RP 80A; Phenomenex) at 37°C with a flow rate of 0.5mL/ min. The mobile phase was 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile. The retention time of EVG was 7.1 ± 0.2 mins. A calibration curve was made with the concentration range 1-100µg/ml. Each suppository was placed in a 20mL volumetric flask and melted at 65°C for PEG based suppositories and 45°C for fat soluble based using water bath. The volume was made up to 20 mL with acetonitrile and the drug was extracted by vigorously vortexing the samples. The solution was allowed to cool to room temperature so that the fat-soluble bases separate from the organic phase. 1 mL aliquots were withdrawn and centrifuged at 10000 rpm for 10 mins. The supernatant was filtered using 0.22µm PTFE syringe filters. Suitable dilutions were made using 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile at 70:30 and analyzed using HPLC. Aliquots were withdrawn and filtered using 0.22µm PTFE syringe filters for HPLC analysis.

Weight variation was determined by weighing individual unit using an analytical balance. The deviation of each unit from the average of the batch was calculated and the acceptance criteria were set up as 5% relative standard deviation (RSD).

Hardness testing was measured using Texture Analyzer TA.XT.plus, which was operated using "Exponent" software. Due to the conical shape of the suppository, it was trimmed to obtain flat surfaces on both sides. The suppositories were placed on a flat platform (TA-90) and tested using a flat surface probe (TA-58). The height between suppository and probe was calibrated to 10 mm, and length travelled by the probe after contacting the suppository surface was set to 5 mm to ensure the suppository was broken completely. The force required to break the suppository (highest peak on the graph) was considered as the breaking point.

Melting point of the suppositories was measured by a Differential Scanning Calorimeter (DSC) purchased from Mettler Toledo. Approximately 2 to 8 mg of suppository sample was weighed in an aluminum crucible and sealed using a sealer. The heat flowing through this crucible was compared to an empty crucible as a reference and change in heat flow was measured. The highest point of this graph was considered the melting point of the sample.

Disintegration time (DT) for suppositories was determined using Electrolab Disintegration tester apparatus (Model no: ED 2L, Version 1.1). 700mL of 1X PBS at pH 7.4 was used as the medium and was heated at 37 ± 0.5° C. The time point at which the PEG based suppositories completely dissolved or the fat soluble bases completely melted was recorded as the DT.

***In vitro* release** was conducted in a Distek T2100 series dissolution apparatus. We used the USP Apparatus Type 1 basket for this experiment. The distance between the inner surface of the vessel and the basket was manually adjusted to 25 ± 2 mm. Sink conditions were maintained by using 500 ml 5% SDS as the dissolution medium. Two mL aliquots were collected at 15, 30, 45, 60, 75, 90, 105, 120, 150 and 180 minutes and replaced with fresh medium. These samples were filtered using a syringe and 0.22 μm PTFE syringe filters, diluted and analyzed using HPLC. Drug content was determined from these samples using the above mentioned HPLC method. Drug dissolution profiles were calculated using Microsoft Excel.

4.3.3 Formulation stability

Long term stability (5°C for 1 year) and accelerated stability (25°C/ 60%RH for 6 months) of the TFV and EVG combination suppository is currently being monitored. In order to replicate the marketed product, we individually sealed the suppositories using a manual heat sealer (lab-scale). The stability was conducted in the disposable plastic suppository shells to replicate the marketed formulations.

4.3.4 In vivo single dose pharmacokinetic studies in non-human primate (NHP) model – study design

These studies were conducted by the ARV prophylaxis activity team at the CDC in Atlanta, GA. TFV and EVG combination rectal suppositories were tested in a non-human primate (NHP) model described previously [69]. A cross-over study design using 6 rhesus macaques in 2 groups was utilized to test the developed products (figure 14). Witepsol H15 and PEG 3350:1000:400 (60:30:10) based suppositories with combination of 40 mg each of TFV and EVG were evaluated, with a 1-week washout period between product administrations. The dose of these APIs was selected based on previous pre-clinical and clinical studies. Suppositories were inserted 1 cm in the rectal cavity maintaining the same dosing site as before. To minimize any interference in drug absorption due to fecal matter, each animal received a rectal wash with sterile saline (3-4 mL) and stool softeners were included in their diet to effectively remove feces. Plasma, biopsy and rectal fluid samples were collected at 2, 5 and 24 hours to evaluate systemic and local exposure, respectively. The sample collection time points were selected based on the protocols defined in the Institutional Animal Care and Use Committee (IACUC) guidelines. Briefly, macaques had to be anesthetized for every sample collection and there were limitations for anesthetizing a macaque within a 24-hour time window, therefore the time points were selected accordingly. Biopsy samples were collected using 3.7-mm biopsy forceps (Radial JawTM 3 Biopsy Forceps, Boston Scientific). Sample collection was never repeated for more than one time point from the same animal after product administration. For instance, an animal was administered with a dose in the first week and sample was collected at two hours only, this animal would go through a one-week

washout period post sample withdrawal. The same animal was administered with another dose in second week and sample was collected at five hours only, followed by a one-week washout period. In summary, independent sampling was conducted from all animals for both treatment groups at every time point.

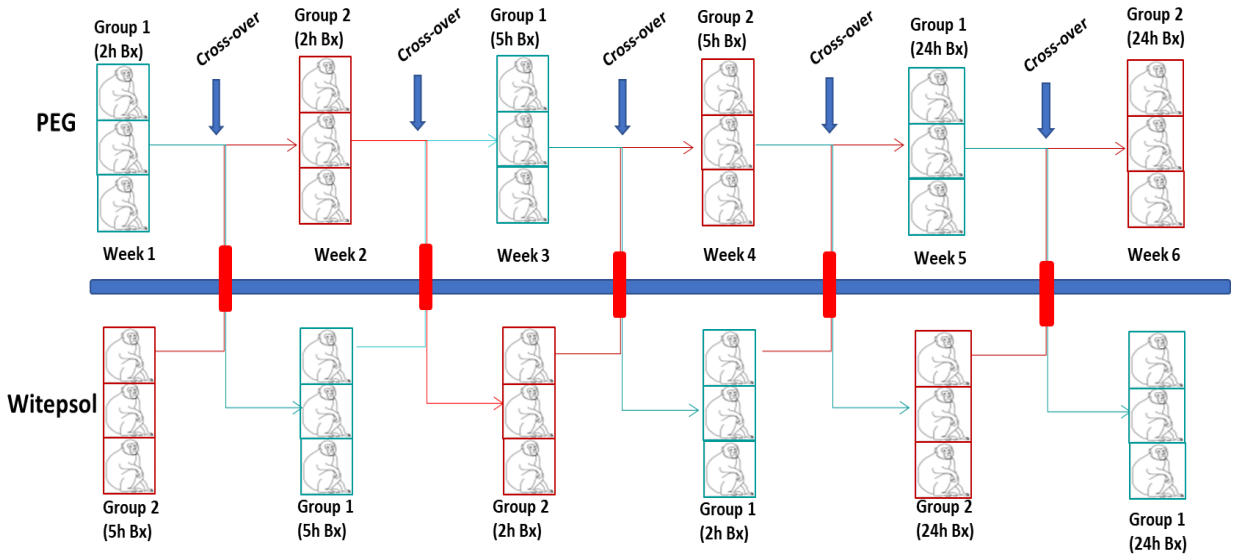


Figure 14 Single dose pharmacokinetic study design in non-human primates

Witepsol and PEG suppositories with 40mg TFV + 40mg EVG were evaluated in the cross-over single dose PK study. Independent sampling was conducted from every animal with a minimum one-week washout period after every sample withdrawal. N=3 / treatment group, crossed over every week. (Bx – Biopsies)

4.3.5 In vivo sample preparation and analysis

All the sample preparation and analyses were conducted by the ARV prophylaxis activity team and associated analytical team at the CDC in Atlanta, GA. EVG and TFV concentrations in the blood plasma and biopsies were measured using separate analytical methods. Irrespective of the analyte being determined, biopsies were collected using 3.7-mm biopsy forceps (Radial JawTM 3 Biopsy Forceps, Boston Scientific). Once the biopsy samples were procured, they were transported on ice to the appropriate lab and personal. Each biopsy sample was then placed in a 50 mL conical tube with 1 mL 80% methanol. The biopsies were minced using sterile surgical scissors and stored in -80°C overnight. Next day, the minced samples were either centrifuged and supernatant was

removed or filtered through 0.45µM attached to a 5cc syringe. The resultant solution was transferred and saved in a 2 mL screw-cap vial at -80°C for future analysis. TFV and TFV-DP levels from biopsies and plasma were determined using previously developed and validated Liquid Chromatography – Mass Spectrometry (LC-MS) method [152].

4.3.6 In vivo pharmacokinetic data and statistical analysis

The *in vitro* dissolution results and stability data is represented as mean ± standard deviation (SD), however the *in vivo* PK data represented as median ± range. GraphPad PRISM 7.0 was used to process and analyze the data. Statistical differences were calculated within suppository bases (PEG versus witepsol) as well as across time points using two-way ANOVA. P values ≤ 0.05 were considered statistically significant. These analyses were conducted for every biological matrix i.e. rectal fluid, biopsies and plasma. Sidak's multiple comparison post-hoc tests were conducted for *in vivo* PK data wherever required.

4.4 Results

4.4.1 Suppositories in vitro characterization

Three PEG-based suppository formulations and three fat soluble base suppository formulations from different manufacturers were prepared (table 5). The tested suppository bases were selected using the decision tree provided by the manufacturers and compatibility with physicochemical properties of the APIs. The dosing levels were calculated based on some preliminary data from the TFV gels used in clinical trials. Forty mg of both APIs were incorporated in every suppository base mentioned above. Each suppository formulation was characterized for weight variation, breaking point, disintegration time, melting point, *in vitro* dissolution and drug content uniformity.

The force required to break the suppository (breaking point) was used to evaluate the hardness of various suppository formulations. Amongst the three tested fat-soluble suppositories, the

hardness was in the following order: Witepsol H15> Suppocire A> Cocoa butter (table 5). Cocoa butter is a naturally occurring material and has the lowest melting point and different chemical composition compared to witepsol H15 and suppocire A, hence tend to break easily compared to the other synthetic suppository bases. The suppocire A and witepsol H15 primarily consist of glycerides and are specifically designed to melt at body temperature for rectal and vaginal administration, but also withstand pressure during handling and transportation. Amongst the three tested PEG suppository bases, the order of hardness was PEG 8000/400> PEG 3350/1000> PEG 3350/1000/400 (table 5). However, due to the soft nature of the PEG suppositories, they lost their shape when compressed by the texture analyzer probe instead of breaking in 2 halves. The maximum force required at 5 mm to compress the suppository was recorded as the breaking point.

Table 5 Suppositories characterization results

Three PEG-based suppository formulations and three fat soluble base suppository formulations from different manufacturers were manufactured and characterized for hardness, disintegration time and melting point. Data represented as mean ± standard deviation (SD)

Suppository Bases	Hardness (kg, mean ± SD, n=4)	Disintegration time ranges (mins)	Melting Point (°C, n=2)
Suppocire A	2.83 ± 0.28	7-8	37.1
Witepsol H15	5.08 ± 0.98	6-8	34.7
Cocoa Butter	0.38 ± 0.29	3-4	33.1
PEG 8000/400 (60:40)	2.57 ±0.21	12-15	56.3
PEG 3350/1000 (25:75)	2.05 ± 0.20	8-9	36.3
PEG 3350/1000/400 (60:30:10)	1.31 ± 0.14	7-8	56.4

The Differential Scanning Calorimetry (DSC) results indicated minimal differences between placebo and drug loaded suppositories, indicating that the co-existence of both APIs does not affect

the melting point of the suppository. Two PEG based formulations showed melting point higher than body temperature. However, this should not interfere with the performance of suppositories because the PEG polymers dissolve in the rectal cavity and not melt at body temperatures. Although the melting points of the witepsol H15 suppositories were 34.77°C and 34.73°C, higher melting points can be achieved by selecting other grades of witepsol.

Another characterization parameter used to evaluate the suppository dissolution is the disintegration time i.e. the time taken by a suppository to completely dissolve at body temperature. Considering the pH of rectal fluid, 1X PBS at pH 7.4 was selected to conduct this test. As mentioned above, the fat-soluble suppository bases are designed to melt at body temperature and they disintegrate relatively quickly, due to the elevated temperatures. Since the PEG-based suppositories dissolve and not melt, in order dissolution to occur the PEG-based suppositories will have to be continuously dipped in buffer for extended periods of time. However, the constant oscillations caused by the disintegration apparatus created a movement wherein suppositories were in and out of the buffer media rather than continuously staying dipped. These oscillations reduced contact time between suppositories and PBS, hence taking relatively longer for PEG-based suppositories to disintegrate. PEG suppositories took the longest time to dissolve in the order PEG 8000/400 > PEG 3350/1000 > PEG 3350/1000/400 (table 5).

All suppository formulations were tested for amount of drug released over a period of 3 hours with pre-defined time intervals. A surfactant (5% Sodium dodecyl sulfate) was used for *in vitro* drug dissolution studies to maintain the sink conditions for hydrophobic EVG. TFV release from all fat-soluble bases was faster and higher as compared to EVG (figure 15). However, the extent of TFV release from different bases at 30 min was significantly different ($p<0.0001$) with 82%, 41% and 18% TFV release from witepsol H15, suppocire A and cocoa butter, respectively. On the other hand, only 44%, 16% and 4% EVG ($p<0.0001$) was released from the above-mentioned fat-soluble bases, respectively at 30 minutes (figure 15), indicating about 2-3 fold reduced EVG release compared to TFV. 30 minutes was selected as the time point of comparison as the suppositories tend to dissolve or melt quickly upon administration. These differences in drug release of TFV and EVG can be explained by the lipophilicity of both APIs. EVG, being a more lipophilic drug, had a higher tendency to bind with the fat-soluble suppository bases compared to TFV.

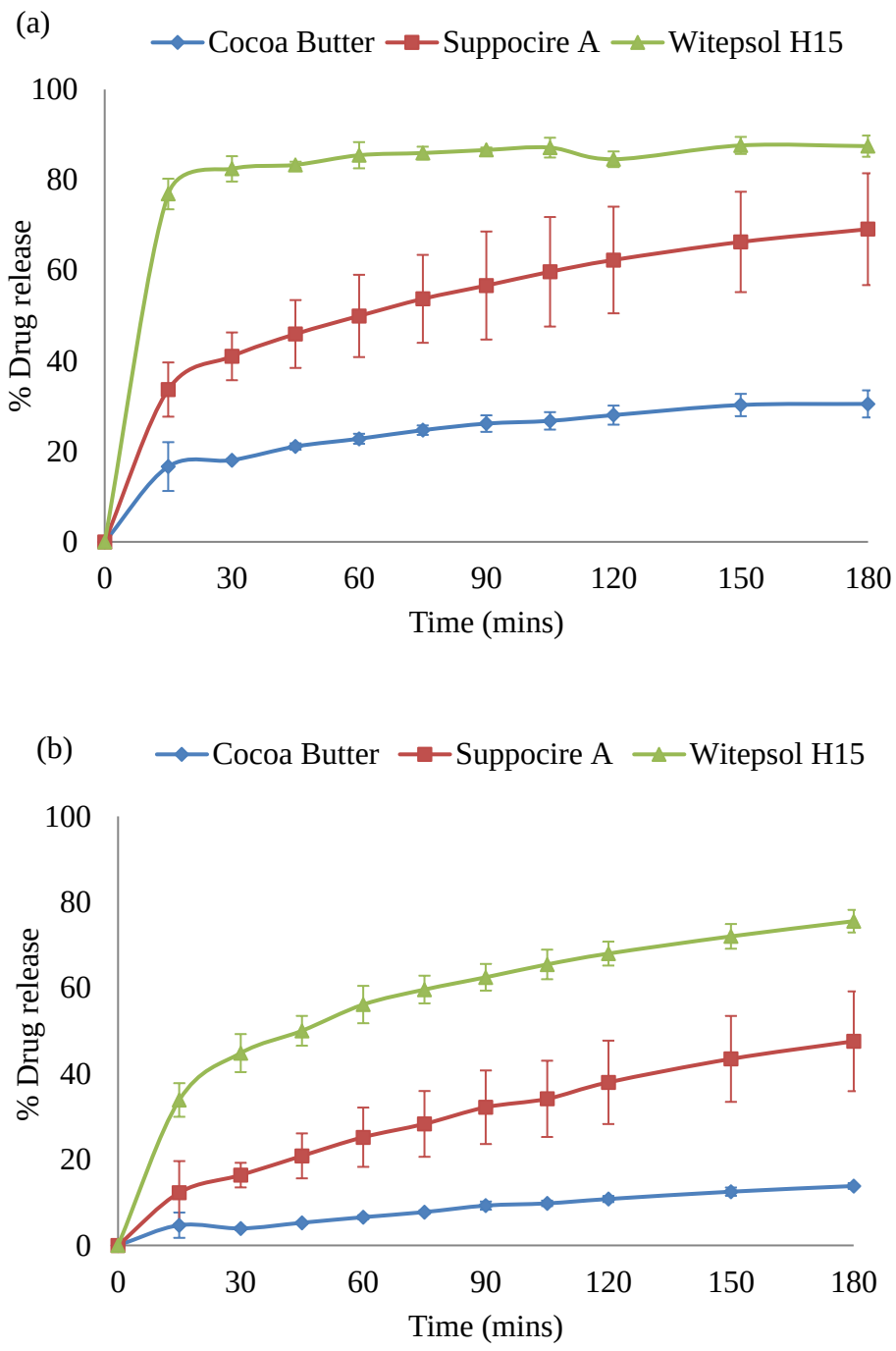
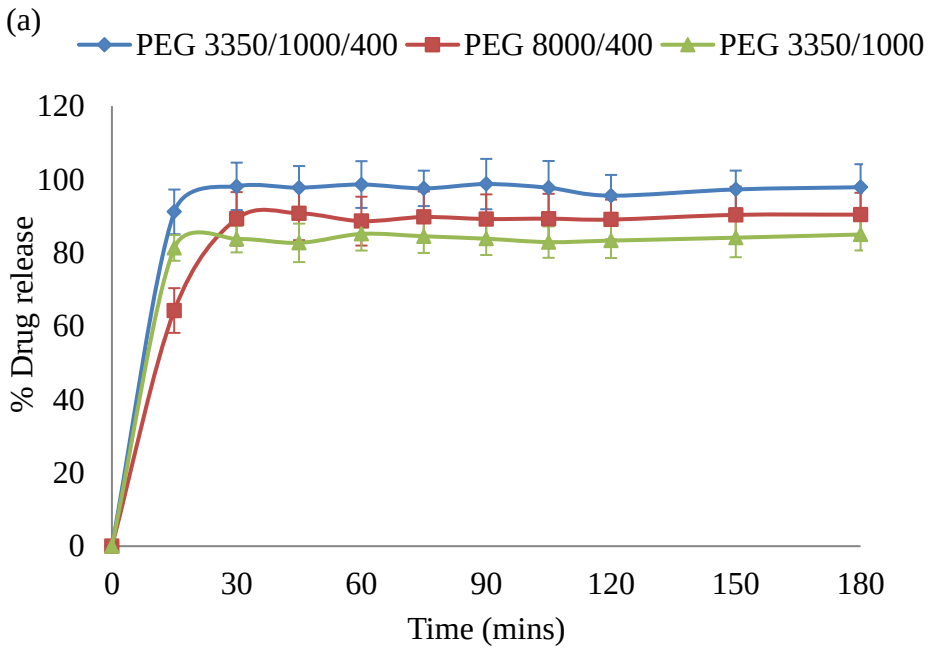


Figure 15 *In vitro* dissolution of (a) Tenofovir and (b) Elvitegravir from Cocoa Butter, Suppocire A and witepsol H15 suppositories in 5% SDS

In vitro dissolution was conducted using USP apparatus Type 1 for combination suppositories (40mg TFV + EVG). 5% SDS was used as the dissolution media; X-axis – Time (mins) and Y-axis - % drug release (n=3/ formulation, mean ± SD, one-way ANOVA)

The PEG based suppositories were completely dissolved in the dissolution media within the first 15 mins of the test, indicating the burst release occurred within the first fifteen minutes (figure 16). Due to this immediate suppository dissolution, no significant differences (one-way ANOVA) were observed for TFV ($p=0.5441$) as well as EVG ($p=0.1723$) from different PEG based suppositories (figure 16). Although no significant differences were observed amongst the three tested PEG formulation (one-way ANOVA), PEG 3350/1000/400 at ratio 60:30:10 showed the greatest and quickest drug release. In addition, compared to other two PEG formulations, the PEG 3350/1000/400 was most visually pleasant and with lowest hardness, potentially providing increased patient compliance.



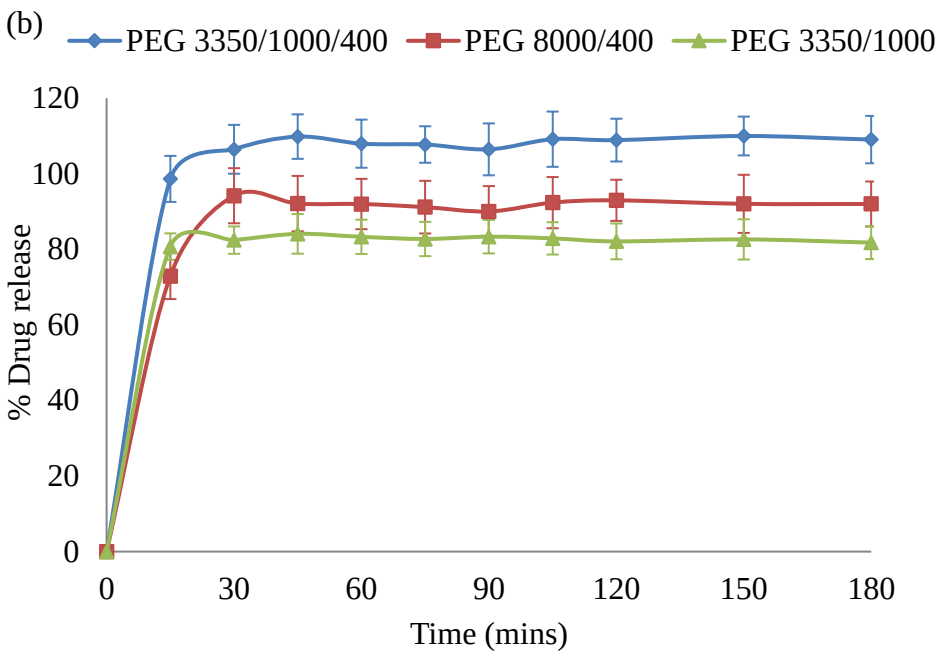


Figure 16 *In vitro* dissolution of (a) Tenofovir and (b) Elvitegravir from PEG 3350:1000:400 (60:30:10), PEG 8000/400 (60:40) and PEG 3350/1000 (25:75) suppositories

In vitro dissolution was conducted using USP apparatus Type 1 for combination suppositories (40mg TFV + EVG). 5% SDS was used as the dissolution media; X-axis – Time (mins) and Y-axis - % drug release (n=3/ formulation, mean ± SD, one-way ANOVA)

4.4.2 Stability assessment

The results of long-term and accelerated stability studies for the TFV and EVG combination suppositories are presented in tables 6 (Witepsol H15) and 7 (PEG). No change was observed from witepsol H15 suppositories up to two months at long-term as well as accelerated conditions. However, the PEG suppositories started melting at accelerated conditions within the first 2 weeks of stability studies making the suppositories sticky. This could have potentially been due to incorporation of the TFV and EVG in the PEG base, lowering its original melting point. The breaking point of the suppositories was evaluated as per methods described in section 4.3.2. The breaking point of witepsol suppositories after 2 months at both stability conditions were comparable to time zero. However, the breaking point of PEG suppositories decreased at two-months (1.6kg) compared to time zero (5.3kg), and this was probably due to the melting of the

PEG suppositories at accelerated conditions. The melting point of witepsol suppositories was evaluated using the method described in section 4.3.2. The melting point at two months was higher compared to time zero and 1 month. As the melting point apparatus is a very coarse method of evaluating the time required for suppositories to melt, this could potentially be due to the variability in visual observation capacity of the evaluator. Since the PEG suppositories dissolve in water and not melt, elevated temperatures don't impact the melting of PEG suppositories, therefore only witepsol suppositories was evaluated. TFV and EVG drug content was determined using methods described in section 4.3.2. The suppositories were randomly selected from the batch for every test, and we predict that the ones selected at time zero happened to be of higher drug content compared to rest of the batch. Also, the drug content results for up to two months suggest a wide drug distribution amongst the units within the batch. We predict this was due to ineffective mixing and manual filling during suppository manufacturing. Therefore, we do not anticipate this issue using automated suppository manufacturing equipment. Although TFV and EVG drug loading was same, based on two months drug content results, TFV indicated better drug distribution compared to EVG. TFV drug content was lowered at two weeks compared to initial drug content, we anticipate the random suppositories selected for analysis had lowered drug content, hence can be regarded as outliers. However, close monitoring will be required while analyzing drug content for the remaining time point to confirm if this was an outlier.

Table 6 Long-term and accelerated stability study results for TFV + EVG witepsol H15 suppositories (Mean ±SD, n=3 units/ test/ time point)

		Long-term Conditions (Mean ± SD, n=3)				
Tests		**Time 0**	**T 1 Month**	**T 2 Month**	**T 3 Month**	**T6 Month**
Appearance		White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque
Breaking point (kg)		9.23 ± 2.22	8.55 ± 1.12	7.77 ± 0.8	8.07 ± 0.9	9.46 ± 0.3
Melting Point (mins)		9.43 ± 1.6	9.46± 3.48	20.17 ± 0.29	11.09 ± 2.67	8.76 ± 0.43
Drug Content (%)	**EVG**	46.13 ± 1.1	43.74 ± 0.7	40.93 ± 0.20	38.80 ± 1.41	36.04 ± 1.83
	TFV	40.77 ± 0.89	39.89 ± 0.98	37.94 ± 1.10	39.06 ± 0.59	35.63 ± 0.59

		Accelerated Conditions (Mean ± SD, n=3)					
Tests		**Time 0**	**T 2 weeks**	**T 1 Month**	**T 2 Month**	**T 3 Month**	**T 6 Month**
Appearance		Same as above	White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque
Breaking point (kg)			-	8.42 ± 0.61	7.83 ± 0.66	7.77 ± 1.4	7.36 ± 1.24
Melting Point (mins)			-	8.31 ± 1.06	17.73 ± 1.62	11.50 ± 0.48	12.51 ± 0.52
Drug Content (%)	**EVG**		39.33 ± 1.18	47.04 ± 1.39	43.18 ± 0.71	39.61 ± 1.05	35.39 ± 1.46
	TFV		23.88 ± 3.22	37.78 ± 1.41	38.28 ± 1.56	36.64 ± 3.32	37.87 ± 0.88

Table 7 Long-term and accelerated stability study results for TFV + EVG PEG suppositories (Mean ±SD, n=3 units/ test/ time point)

		Long-term Conditions (Mean ± SD, n=3)				
Tests		**Time 0**	**T 1 Month**	**T 2 Month**	**T 3 Month**	**T6 Month**
Appearance		White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque
Breaking point (kg)		5.30 ± 0.49	5.07 ± 0.81	3.23 ± 0.50	5.54 ± 2.48	4.81 ± 0.72
Melting Point (mins)		-	-	-	-	-
Drug Content (%)	**EVG**	51.35 ± 1.90	47.87 ± 0.19	42.24 ± 0.31	41.60 ± 1.77	37.66 ± 0.67
	TFV	39.93 ± 0.21	41.02 ± 1.06	40.75 ± 0.22	39.58 ± 3.26	38.02 ± 0.71

		Accelerated Conditions (Mean ± SD, n=3)					
Tests		**Time 0**	**T 2 weeks**	**T 1 Month**	**T 2 Month**	**T 3 Month**	**T 6 Month**
Appearance		Same as above	Sticky due to melting	Sticky due to melting	Sticky due to melting	Sticky due to melting	Sticky due to melting
Breaking point (kg)			-	2.46 ± 0.97	1.6 ± 0.78	1.06 ± 0.67	1.36 ± 0.19
Melting Point (mins)			-	-	-	-	-
Drug Content (%)	**EVG**		43.26 ± 1.62	47.95 ± 0.19	40.67 ± 1.24	38.88 ± 0.59	36.86 ± 0.21
	TFV		41.66 ± 2.31	40.08 ± 0.10	40.14 ± 0.33	37.94 ± 4.39	37.94 ± 1.06

4.4.3 In vivo pharmacokinetic studies in non-human primates (NHPs)

Rectal fluid PK: The drug concentrations achieved in rectal fluid demonstrated no significant difference (ordinary two-way ANOVA) between the witepsol and PEG suppository bases for TFV (p= 0.9771, 0.7026 and >0.9999 for 2, 5 and 24 hours) as well as EVG (p= 0.7518, 0.9698 and >0.9999 for 2, 5 and 24 hours) (figure 17a and 17b). Moreover, comparing the drug release profiles within each suppository base, no significant difference was observed between TFV and EVG as well (TFV LOD - 3ng/mL; EVG LOD - 1.5ng/mL). However, witepsol suppositories demonstrated lower variability amongst replicates compared to PEG suppositories. Results also showed a time-dependent decrease in drug concentrations over 24 hours indicating either absorption or elimination of the APIs. For TFV, ~ 35-fold reduction was observed over 24 hours from the witepsol suppositories, but ~108-fold reduction in PEG based suppositories, possibly indicating rapid TFV dissolution from PEG suppositories. However, EVG reduction was comparable in both suppository bases with 62- and 50-fold reduction from witepsol and PEG suppositories over 24 hours, respectively. Although its uncertain if the achieved exposures in the rectal fluid can provide protection, but TFV levels were few folds above the reported IC_{50} (516 ng/mL) levels at two and five hours. Similarly, achieved EVG exposures in the rectal fluid was few folds higher than the reported IC_{50} (45 ng/mL) value at all three evaluated time points (red dotted line on figures 17a and 17b.

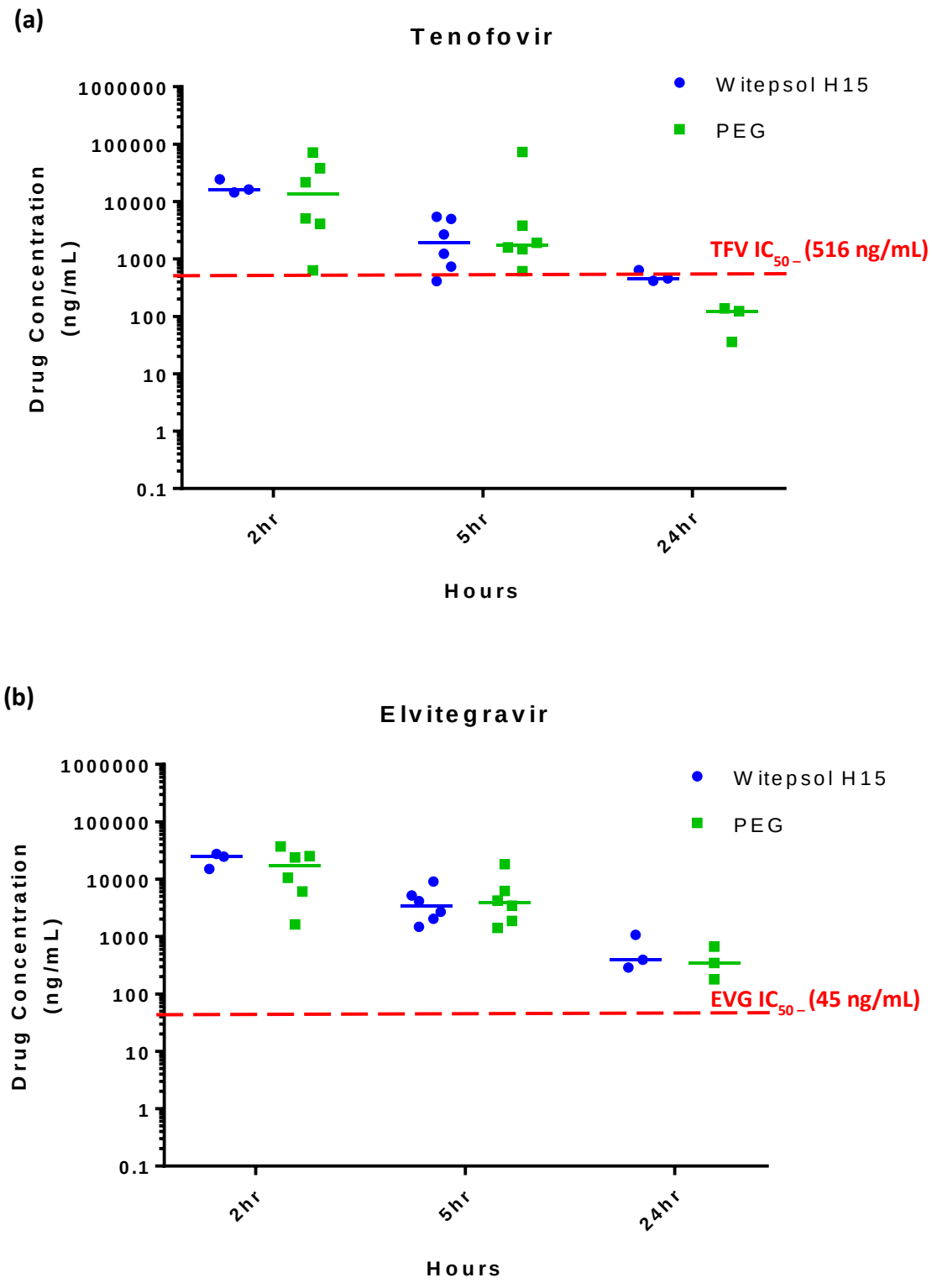
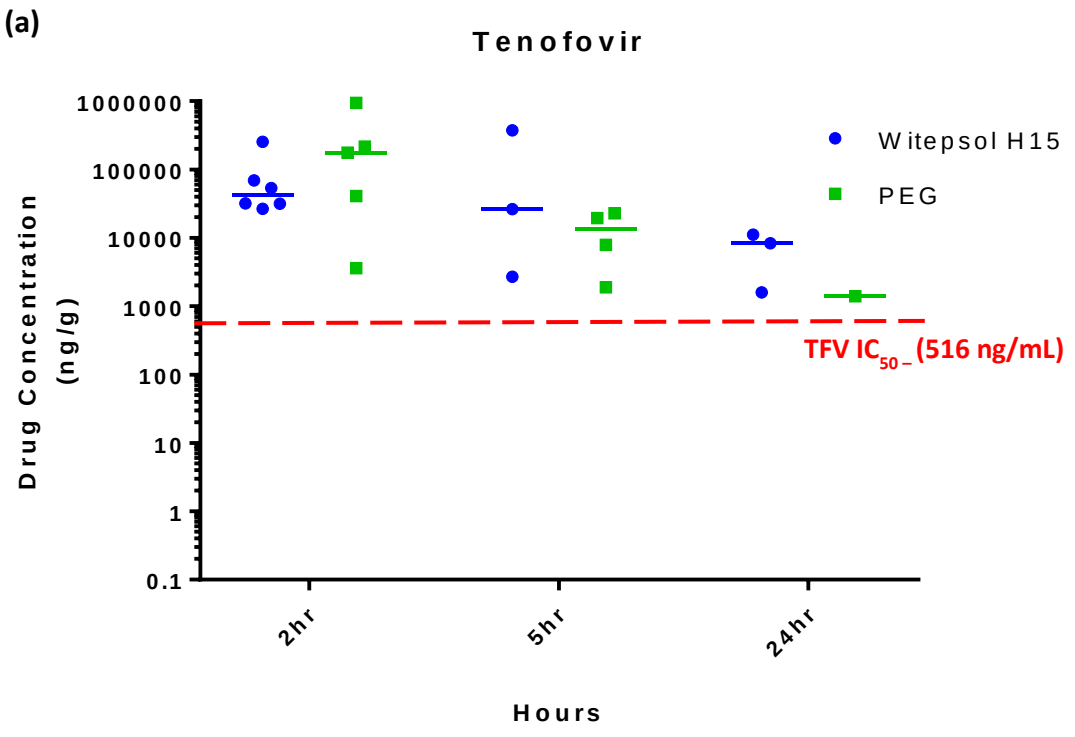


Figure 17 *In vivo* pharmacokinetic profile in rectal fluid following rectal administration of TFV + EVG PEG and witepsol suppository in macaques

(a)Tenofovir and (b) Elvitegravir (n=6; median ± range); Each data point represents an independent measure from an animal; The red dotted line represents the TFV and EVG IC_{50}; X-axis – time (hours) and Y-axis drug concentrations (ng/mL); Two-way ANOVA with Sidak's multiple comparison post-hoc test; P< 0.05 were considered statistically significant

Rectal Biopsies PK: Despite decreasing drug concentrations in the rectal fluid over time, overall both TFV and EVG maintained detectable drug levels during the study period (figures 18a and 17b). This indicates that although the APIs are being eliminated or metabolized from the rectal fluid, but still being absorbed in the tissues. Although it is unclear if the achieved levels are within the therapeutic window to provide protection, both TFV and EVG exposures were above the reported IC_{50} values. At 2 hours, PEG suppositories showed ~3-fold higher drug levels in biopsies as compared to witepsol suppositories for TFV (109ng/mL vs 31.7ng/mL) (figure 18a) and EVG (46.5 vs 15.65ng/mL) (figure 18b). This was not observed at 5 and 24 hours. Moreover, using two-way ANOVA, both TFV and EVG release was non-significant across time points and evaluated bases.



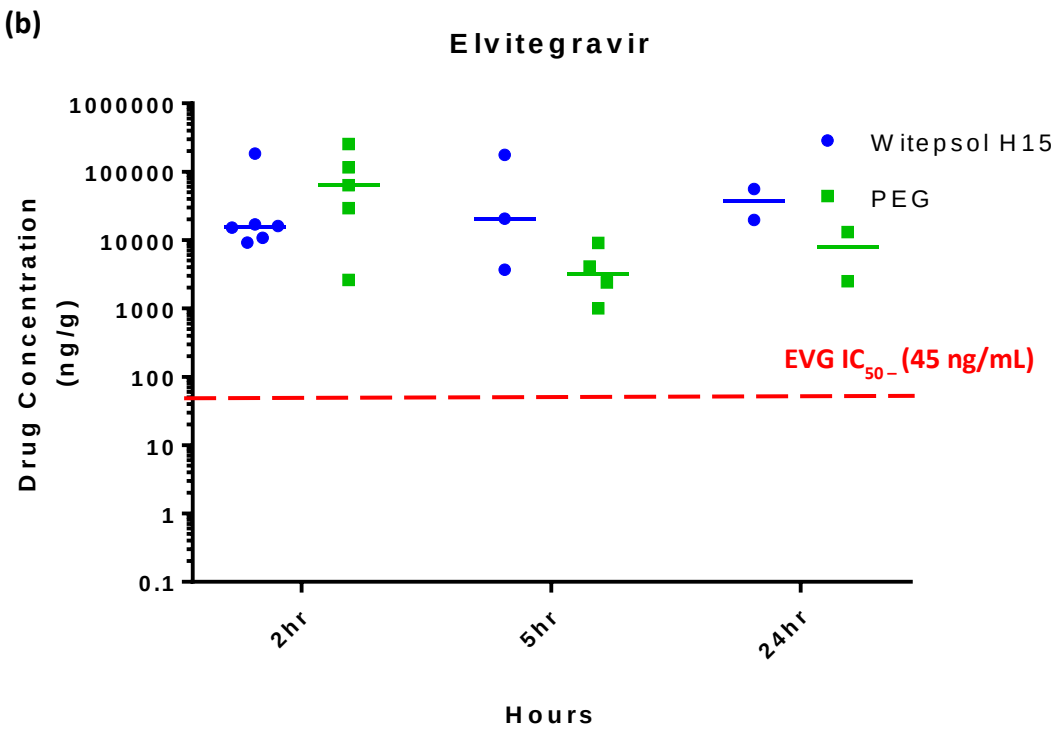
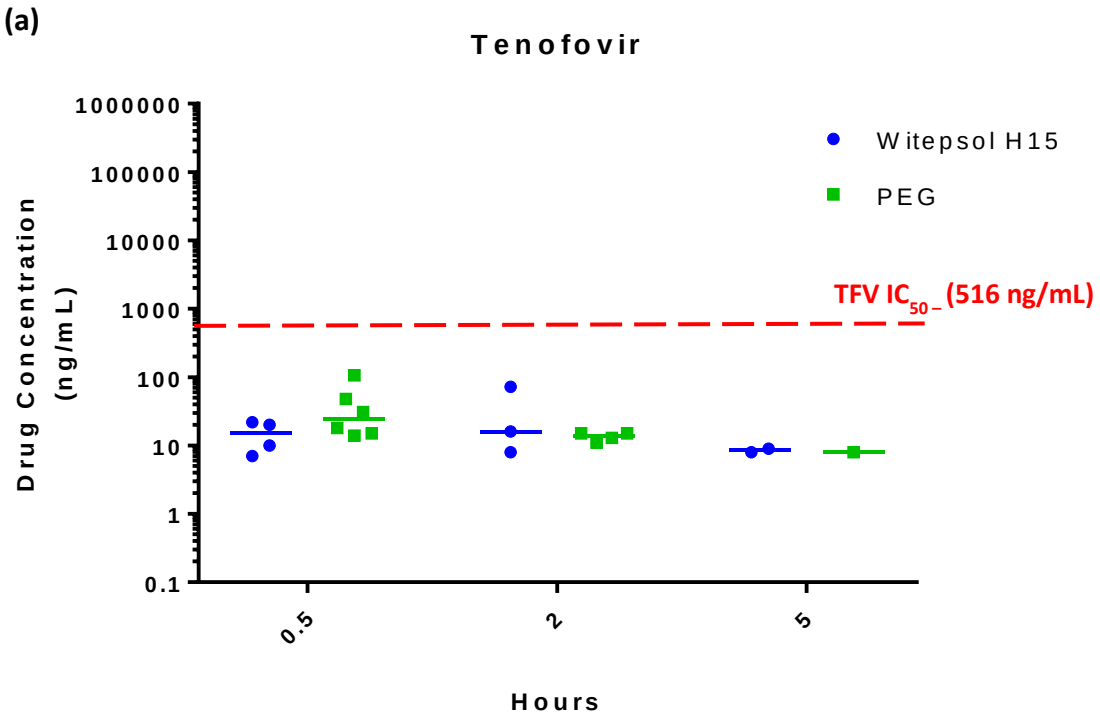


Figure 18 *In vivo* pharmacokinetic profile in biopsies following rectal administration of TFV + EVG PEG and witepsol suppository in macaques

(a)Tenofovir and (b) Elvitegravir (n=6; median ± range); Each data point represents an independent measure from an animal. The red dotted line represents the TFV and EVG IC_{50}; X-axis – time (hours) and Y-axis drug concentrations (ng/mL); Two-way ANOVA; P< 0.05 were considered statistically significant

Plasma PK: PEG suppositories demonstrated ~1.6 fold (25ng/mL) higher levels of TFV as compared to witepsol suppositories (15ng/mL) at 30 mins (figure 19a) However, no significant difference was observed at 2 and 5 hours in these suppositories. In terms of exposure amongst replicate animals (total 6), 6, 4 and 1 animals showed detectable drug levels with PEG suppositories, but only 4, 3 and 2 animals had detectable drug levels with witepsol suppositories at 0.5, 2 and 5 hours respectively (figures 19a and 19b). Overall, least TFV and EVG exposures were achieved in plasma compared to biopsies and rectal fluid. Since the goal of topical delivery using suppositories was for local applications only, systemic concentrations were irrelevant. However, detectable concentrations within the first 30 mins of product administration signifies the rapid delivery and signifies the advantages of first-pass metabolism via the rectal drug delivery

route. Moreover, using two-way ANOVA, both TFV and EVG release was non-significant across time points and evaluated bases from plasma.



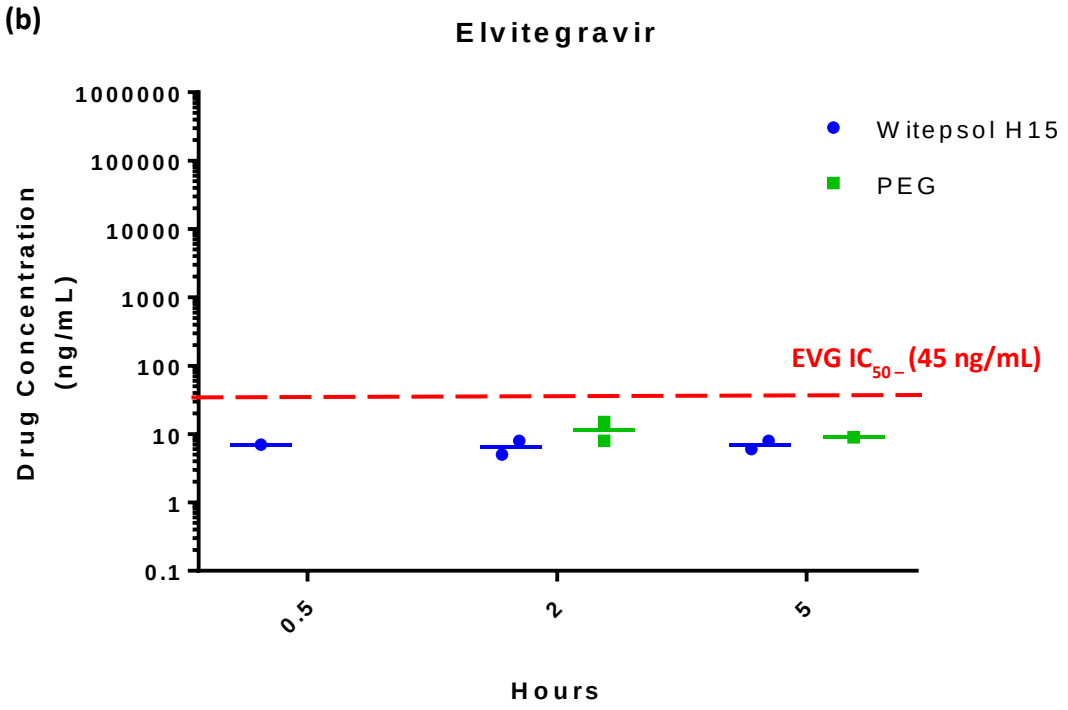


Figure 19 *In vivo* pharmacokinetic profile in plasma following rectal administration of TFV + EVG PEG and witepsol suppository in macaques

(a) Tenofovir and (b) Elvitegravir (n=6; median ± range); Each data point represents an independent measure from an animal. The red dotted line represents the TFV and EVG IC_{50}; X-axis – time (hours) and Y-axis drug concentrations (ng/mL); Two-way ANOVA; $P< 0.05$ were considered statistically significant

Table 8 TFV and EVG Pharmacokinetic summary in macaques following rectal administration of 1% EVG/1% TFV PEG suppository and 1% EVG/1% TFV witepsol suppository

	Tenofovir			Elvitegravir		
Variable	C_{max}, (median ± SE)	T_{max}	AUC_{0-24}, (median ± SE)	C_{max}, (median ± SE)	T_{max}	AUC_{0-24}, (median ± SE)
Rectal Fluid	(µg/mL)	(Hours)	(µg h/mL)	(µg/mL)	(Hours)	(µg h/mL)
PEG suppository	13.3 ± 13.6	2	188 ± 283	17.2 ± 6.7	2	94.8 ± 63.6
Witepsol Suppository	16.3 ± 2.6	2	60.6 ± 22.3	24.5 ± 3.2	2	84.2 ± 28.7
Biopsy						
PEG suppository	0.177 ± 0.192	2	0.571 ± 0.584	0.064 ± 0.050	2	0.259 ± 0.168
Witepsol Suppository	0.043 ± 0.044	2	1.659 ± 2.002	0.020 ± 0.048	5	1.159 ± 0.953
Plasma	(ng/mL)	(Hours)	(ng h/mL)	(ng/mL)	(Hours)	(ng h/mL)
PEG suppository	25 ± 18	0.5	71 ± 27	12 ± 2	2	31 ± 7
Witepsol Suppository	16 ± 17	2	96 ± 59	7 ± 1	-	30 ± 4

For EVG, overall there was minimal to no systemic exposure. Undetectable EVG levels were observed at 0.5 hours and only 3 animals out of 12 animals showed detectable EVG levels at 2 and 5 hours, from PEG suppositories. Similarly, detectable drug levels were observed in only 1, 2 and 2 animals out of 6 animals at 0.5, 2 and 5 hours from witepsol suppositories. These inconsistent results indicate the dynamic nature of the rectal compartment and how difference in factors like rectal fluid volume, buffering capacity and presence of fecal matter can influence drug dissolution and absorption [153]. In summary, detectable TFV and EVG levels in rectal fluid were maintained

for as long as 24 hours post suppository administration, possibly beneficial in providing protection over longer periods. This was demonstrated not only in the rectal fluid but tissues as well; evaluated by analyzing drug content from rectal biopsies, being the primary site of viral entry and transmission.

4.5 Discussion

The potential utility of suppository platform for PrEP was explored, considering its advantages over other rectal dosage forms as well as provide more options to improve patient acceptability and compliance. To our knowledge this is one of the first attempts made towards developing suppositories as potential rectal microbicide product targeting HIV-1 prevention. Suppository dosage forms offer various advantages over other rectally administered dosage forms. Due to the availability of a variety of suppository bases, suppositories can accommodate a wide range of APIs (hydrophilic/ lipophilic/ solid/ liquid). Additionally, suppository dosage form can be advantageous in several ways; unlike gels it does not need an applicator for administration and don't tend to leak or become messy. Although the overall results from a recent behavioral study indicated that men preferred gels over suppositories [68], the authors highlighted a few abnormalities which could potentially govern these results. The weight of suppositories in that study was approximately 8 grams which is 2-3 times larger and heavier than the normal adult suppositories (1.5-3.5g). Also, the suppositories in this study were manufactured using poly-ethylene glycol (PEG) bases. PEG-based suppositories are less common in dispensing pharmacies due to a few disadvantages associated with them. PEG polymers have the tendency to absorb moisture from the environment, hence post-administration it can cause irritation and discomfort to the user [68]. Considering the patient adherence and acceptability, we selected a more realistic and clinically relevant suppository molds. Hence, we conducted our *in vitro* and *in vivo* studies in NHPs using ~2 gram suppository molds. To compare physiochemical attributes *in vivo* behavior and evaluate the choice of suppository bases, we manufactured and studied a panel of both fat- and water-soluble bases

Three water-soluble and three fat-soluble suppository bases were formulated to select an appropriate formulation and consider its impact on drug release and characterization parameters.

For fat-soluble suppository bases, witepsol H15 demonstrated the maximum hardness followed by suppocire A and then cocoa butter. The breaking point of a suppository helps to predict the amount of pressure it can handle while packaging and transporting. Further, it acts as a quality control parameter to assess batch-to-batch variability. Moreover, compared to naturally occurring cocoa butter, witepsol H15 suppository bases offer more flexibility to incorporate a wide range of APIs. The mono-, bi- and tri-glyceride ratios in these suppository bases can be manipulated to provide more flexibility in formulation design. Also, witepsol H15 demonstrated the quickest and maximum drug release *in vitro* from the suppositories compared to suppocire A and cocoa butter. Considering above mentioned factors witepsol H15 was selected for *in vivo* evaluation of fat-soluble suppositories. However, such dramatic differences were not observed within the tested PEG suppository bases. Especially the *in vitro* drug release results indicated that PEG suppositories dissolved within 15 minutes and showed maximum drug release, irrespective of formulation. Hence based on physical appearances and smoothness/ grittiness, PEG 3350/1000/400 (60:30:10) was selected as the favorable PEG-based suppository for *in vivo* evaluation.

The selected bases were further tested *in vivo* to evaluate PK parameters in an animal model. The observed demonstrated that rectal fluid levels showed that drug levels were maintained in the rectal fluid up to 24 hours irrespective of the formulation or API properties. Despite time-dependent decrease in drug levels over 24 hours in the rectal fluid, detectable and consistent levels were maintained in the rectal tissue after 24 hours. Detectable levels in the rectal tissue RE very noteworthy, because tissues act as the first line of defense against HIV-1 acquisition upon initial infection. Moreover, detectable drug levels were observed in plasma as early as 30 minutes post suppository administration indicating rapid dissolution and drug absorption from the suppositories. The rapid systemic exposure is indicative that drugs administered rectally in suppositories can circumvent first pass metabolism, however the extent of exposure can be influenced due to innate factors like volume and buffering capacity of rectal fluid and interference caused due to presence of feces.

Dobard *et. al.* conducted a similar study with a combination of TFV and MVC, but in a rectal-specific gel formulation and demonstrated about 82% efficacy against SHIV infections in macaques [69]. Our TFV/ EVG combination suppositories (same dosing levels as gels) achieved

total exposure (AUC) of 188 µg h/mL (PEG) and 60.6 µg h/mL (Witepsol H15) (table 8) in rectal fluids as compared to 29300 µg h/mL with TFV gel. Despite about 2 $\log_{10}$ lower TFV exposure in rectal fluid from the suppositories, the plasma exposure achieved (71 & 96 ng h/mL) was comparable with their rectal-specific gel (121 ng h/mL). More importantly, TFV-DP levels (active drug moiety) achieved with witepsol suppositories at 2 hours (773 fmol/mg) and 24 hours (1697 fmol/mg) was approximately 2 and 4 times higher (unpublished data) than the TFV-DP levels achieved post gel administration (415 and 446 fmol/mg), respectively. With 82% efficacy achieved with TFV and MVC rectal-specific gel products, we can establish that the suppositories can provide same or better protection compared to gels. However, minimal EVG systemic exposure with considerable exposures in the rectal fluid and tissues were achieved. These results indicate the suppositories can prove advantageous and useful for delivering hydrophilic and hydrophobic drugs.

Due to the potent nature of INSTIs and their innate property of inhibiting later stages of HIV-1 replication, they can be potentially utilized as effective topical HIV-1 prevention strategy. This property can also be beneficial in providing longer duration of protection against HIV-1 acquisition [15, 149]. This is one of the first attempts towards developing topical formulations with InSTI; EVG. These results give us some preliminary evidence of formulation stability, *in vitro* characteristics and *in vivo* pharmacokinetics of EVG in suppositories, however in-depth toxicity and efficacy studies are essential to confirm these findings.

In summary, the findings from our studies clearly establish that witepsol and PEG-based rectal suppositories demonstrate similar, and in some cases better local and systemic exposure compared to previously proven effective rectal gels in NHPs. Based on TFV and EVG exposures achieved in rectal fluid and tissues using suppositories and comparing with protective levels reported in the literature, suppositories could provide protection against HIV-1 acquisition. However, several dose-response relationship studies and efficacy trial are required to confirm these results. Extended exposures up to 24 hours suggest possibility of longer duration of protection, which can be further validated by conducting efficacy studies in NHPs. In summary, the advantages associated with rectal suppositories over other dosage forms and ARVs exposures achieved in this study proves suppositories to be a viable rectal microbicide candidate and warrants further development for HIV-1 prevention.

4.6 Acknowledgments

I would like to acknowledge Charles Dobard, PhD, Walid Heneine, PhD, Natalia Makarova, PhD, Sunitha Sharma and colleagues at the Centers for Disease Control & Prevention (CDC), Atlanta, GA for designing and conducting PK studies and graciously providing us with the PK data. In addition, I would like to acknowledge the analytical team, Choung Dinh and Amy Martin at CDC for sample collections and processing.

5.0 In vivo Pharmacokinetic Studies with Tenofovir Alafenamide Fumarate (TAF) and Elvitegravir (EVG) containing Suppositories to Evaluate Benefits of Pro-drug at Lower Doses

5.1 Introduction

Tenofovir (TFV) was first approved by the US FDA in 2001 for HIV-1 treatment. It is one of the first-line agents to combat early HIV-1 infection and is placed on the World Health Organization's List of essential medicines. Since then, TFV has been one of the most widely utilized and investigated ARVs, not only for treatment of HIV-1 but for prevention purposes as well. However, over the years TFV administered orally in the form of Tenofovir Disoproxil Fumarate (TDF) has been reported to demonstrate side-effects such as renal toxicities and bone mineral density issues [96, 97]. Initially, post-approval acute nephrotoxicity was documented in patients on TFV-based regimen, but only some indications of chronic kidney damage (CKD) were observed [98]. Although there are several other factors and comorbidities such as co-prescription of other ARVs, preexisting renal conditions, associated diabetes etc., but CKD associated specifically to TFV was highly debated in the field with contradictory results [97, 98, 154, 155]. Recent findings have established the long-term kidney injuries associated with TFV treatment [97]. Some studies have investigated the factors responsible for decline in kidney functions caused due to TFV and TDF treatment. For instance, TFV-induced toxicity was determined using renal proximal tubular cells and potential involvement of mitochondria was investigated. The conclusions from this study suggested that mitochondrial toxicity was associated with intracellular accumulation of TFV in the proximal tubule cells, altering cell proliferation and viability, eventually leading to cell death [155]. Due to several contradictory results from various *in vitro* models and retrospective case studies, solutions to this nephrotoxicity was sought. Considering all the incidences, risk factors and prognosis associated with TFV-induced nephrotoxicity, some approaches to prevent this were proposed [156]. According to results from this survey, addition of

other active agents or antioxidants could prevent some of the damages associated with long-term TFV treatment.

Similarly, to prevent TFV-associated side-effects, a TFV prodrug, tenofovir alafenamide fumarate (TAF) was developed by Gilead in 2016 as a single tablet regimen for HIV-1 treatment (Brand name – Vemlidy®). TAF has demonstrated significantly higher antiviral potency and reduced side-effects profile when compared to parent molecule TFV. These benefits led to its approval as fixed-dose combinations (FDC) in several treatment products approved from 2016-2018. Several studies were conducted during this period to evaluate the benefits of starting with TAF (as a combination) regimens as well as switching from TFV-based combination regimen to TAF-based combination regimen [157-160]. This was not only limited to HIV-1 treatment, but for hepatitis B virus infection as well [159, 161]. Advantages of reduced TAF dosing versus TDF are primarily three-fold, improved potency, efficacy and safety profiles [99]. A recent trial conducted a dose ranging 10-day monotherapy to determine antiviral activity, safety and PK/PD of TAF versus TDF. Thirty-one HIV-1 positive adults received one of the three doses, 8, 25 or 40 mg TAF versus 300 mg TDF. The results from this trial confirmed that 8 mg TAF was able to demonstrate similar decreases in HIV-1 viral load as 300 mg TDF [162]. In terms of efficacy, reduced TAF dosing is not only effective in HIV-1 infected patient population but other specialized types of populations as well. TAF efficacy in patient populations such as treatment naive adults and adolescents, treatment experienced adults following switch, and renally impaired patients has been proven in clinical trials previously [99]. Advantages of efficacy due to reduced TAF dosing is not only limited to monotherapy but several FDCs as well [163-165]. From a safety perspective, a recent retrospective study conducted on 126 HIV-infected patients in Netherlands investigated the benefits of switching from TDF to TAF [157]. Serum parathyroid hormone (PTH) and alkaline phosphatase (ALP) was utilized as the marker for bone loss. Consistent elevation of serum PTH and ALP levels is typically associated with cortical bone loss and high fracture rates [166]. The results from this study indicated a significant decline in serum PTH and ALP levels, after switching from TDF to TAF-based combination therapy. They concluded that the significant decline in serum PTH levels might be primarily responsible for improved bone-related safety associated with TAF. These findings also supported their rationale that the observed results of changes in serum PTH levels ARE corelated to TDF and TAF doses. [157]. TAF is not only beneficial for bone-related side-effects but also has an improved renal safety profile [167, 168]. Similarly, few studies

have demonstrated that administration of reduced doses of TAF is able to achieve similar or in some cases higher intracellular Tenofovir -diphosphate (TFV-DP) levels; the active moiety, when compared to TFV and TDF [167]. This not only improves efficacy but also reduces dose-related toxicity by TFV.

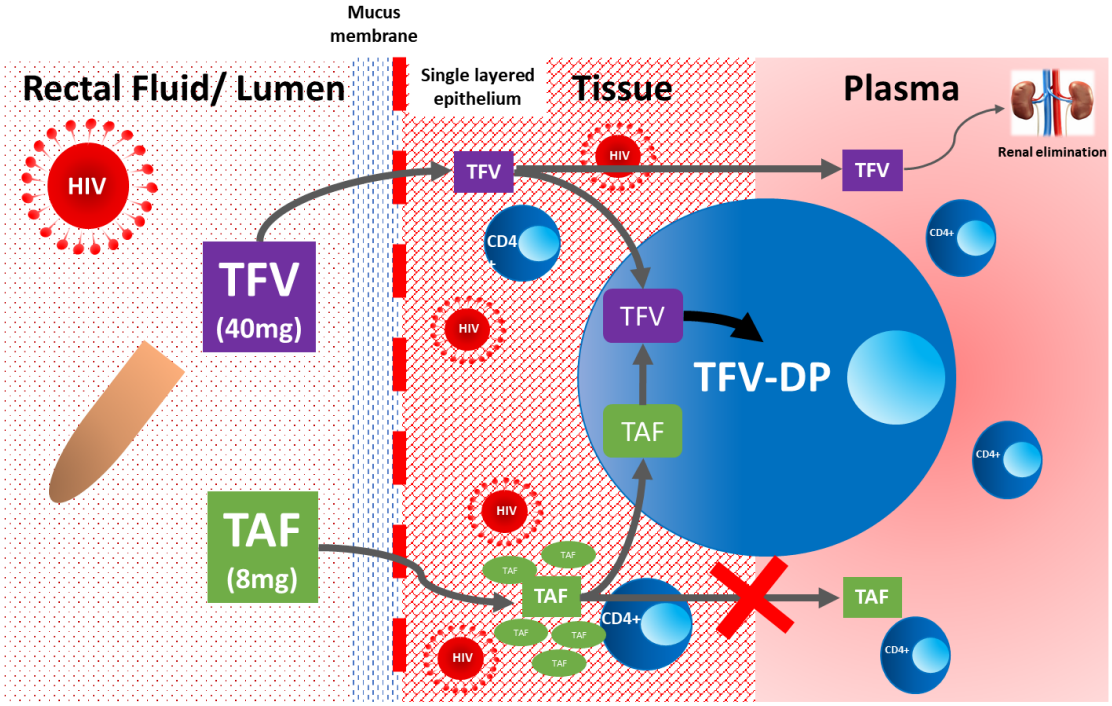


Figure 20 Schematic representing TFV and TAF *in vivo* disposition

Left to Right: Rectal Fluid, biopsy and plasma. Both TFV and TAF permeate through the rectal epithelium at different rates and penetrate the HIV-1 target cell ($CD4^+$ T lymphocyte), present in tissues as well as plasma. Due to TFV's longer half-life, it stays longer in the plasma and demonstrates possibilities of nephrotoxicity. Compared to TFV, TAF has shorter half-life.

In addition to improved potency, efficacy and safety, TAF is metabolized and activated intracellularly using a distinctive mechanism [99, 101]. TAF consists of isopropylalaninyl monoamidite phenyl monoester group which masks two negative charges on the TFV molecule [100, 101]. These charges on parent TFV would limit its permeability, eventually lowering its absorption. Addition of these ester group to parent TFV, improved its lipophilicity, bolstering absorption. Upon penetration into the cells TAF is first metabolized by cathepsin A (CatA) (PMBCs) or carboxyl esterase (Ces1) (liver) leading to a metastable metabolite (figure 20) [101].

This is further hydrolyzed to TFV-Ala conjugate which is then metabolized to parent TFV intracellularly by acidic hydrolysis. The intracellular kinases present in the $CD4^+$ T lymphocytes further phosphorylate TFV to TFV-monophosphate (MP) and finally the pharmacologically active moiety TFV- diphosphate (DP) [99, 100]. However, due to TAF's increased lipophilicity (logP: 1.2) [169] compared to TFV (logP: -1.6) [170], TAF shows improved absorption and half-life, especially in tissues. We anticipate this phenomenon can be taken advantage from HIV-1 prevention perspective. A few studies have evaluated TAF in combination with InSTIs like EVG, in the form of long-acting nanoparticles for prevention of HIV-1 vaginal transmission [94, 150]. As described in sections 4.1 and 1.10, a combination of NNRTI and InSTIs can also prove advantageous from mechanistic perspective. Additionally, the high lipophilicity of EVG might be beneficial for immediate local bioavailability and maintain therapeutic levels for longer duration of time providing longer protection against HIV-1 acquisition (figure 20). Based on the *in vivo* PK data observed in section 4.4.3, TFV and EVG administered as a rectal suppository could achieve desired therapeutic concentrations in the rectal compartment. The plasma PK data (section 4.4.3) demonstrated detectable and consistent TFV and EVG levels up to 5 hours in the macaques.

The primary goal of the studies described in this chapter was to use the previously developed suppository platform (chapter 4) for ARV delivery and evaluate the benefits of prodrugs at reduced doses delivered topically. The benefits and advantages of reduced TAF doses (10mg) compared to TDF (300mg) administered orally for HIV-1 treatment have already been established, which lead to the approval of TAF as an effective HIV-1 treatment. We wanted to determine if these benefits of achieving similar or better intracellular TFV-DP exposures observed in oral treatment, can be achieved with topical delivery of TAF using suppositories. With 40mg TFV dosing in macaques using suppositories (chapter 4), the results demonstrated local as well as systemic TFV exposure. However, our objective was to achieve local concentrations and minimize systemic exposure. Especially because some studies have suggested that sub-therapeutic systemic exposures of ARVs might have potential of developing drug-resistance [33, 171]. Although dose-range finding studies have been conducted in macaques, these studies were conducted using inserts for vaginal administration [172]. Therefore, in addition to determining the benefits of reduced prodrugs dosing, we also wanted to establish if the dose-independent exposure achieved in the vagina can be translated to the rectal compartment as well. To our knowledge this is one of the first studies investigating TAF and EVG dosing for rectal administration.

5.2 Materials

Elvitegravir (EVG) and Tenofovir alafenamide fumarate (TAF) were provided by the Centre of Disease Control & Prevention, Atlanta, Georgia. Polyethylene Glycol (PEG) 3350, 1000 and 400 were purchased from Spectrum chemicals, New Brunswick, NJ, USA. The plastic disposable suppository strips were purchased from Professional Compounding Centers of America (PCCA). Tetra-butyl ammonium bromide, potassium mono- and di-phosphate salts used in the HPLC assays were purchased from Fisher scientific, New Jersey, USA or J.T.Baker, New Jersey, USA.

5.3 Methods

5.3.1 Manufacturing of (TAF and EVG) combination suppositories

Suppositories were manufactured using standard fusion molding method. Based on some studies previously conducted in our lab (unpublished data) PEG suppository formulation with molecular weights (MWs) 3350:1000:400 at ratios 60:30:10 was selected as the formulation for *in vivo* evaluation. Briefly, PEG polymers with descending MWs were weighed and melted using a water bath. This was followed by addition of the APIs (EVG followed by TAF) with constant mixing with an overhead stirrer or magnetic stirrer (to obtain 8 mg EVG and 8 mg TAF/ suppository unit based on batch size). This molten mass was then poured into the plastic suppository shells up to the shoulder. It was cooled down naturally to RT until the suppositories are completely solidified. Suppositories shells were then peeled off, and the excess suppository base was manually trimmed up to shoulders.

5.3.2 In vitro characterization of suppositories

In order to assess the quality of the suppositories and ensure appropriate product characteristics, suppositories were removed from the molds and subjected to visual inspection,

weight variation, hardness and *in vitro* dissolution as per methods described in section 4.3.2. Drug content analysis for EVG and TAF from *in vitro* dissolution samples were conducted using two different HPLC methods. Elvitegravir content was analyzed using the method described in section 4.3.2. However, for the *in vitro* dissolution test, 1% SDS was used as the dissolution media instead of previously described 5% SDS, because 1% SDS was enough to maintain sink conditions for reduced (8 mg) dosing levels.

Drug Content (TAF): The PEG-based suppositories were melted in a 20 mL volumetric flask at 65°C using a water bath. They were dissolved in 100% methanol followed by vigorous shaking to ensure complete drug extraction. These samples were diluted within a suitable range using 25% Acetonitrile, centrifuged and filtered using 0.22µM PTFE syringe filters. The final diluted and filtered solutions were analyzed using HPLC. A Dionex Ultimate 3000 HPLC system with UV or PDA detector was used for TAF analysis. Analysis was conducted on a Phenomenex Gemini C18 150 x 4.6mm column and TAF was retained at 17 mins. 25mM potassium phosphate + 5mM tetra-ammonium butyl bromide (at pH 6): methanol (80:20) and acetonitrile was used as the mobile phase using gradient method.

Drug Content (EVG): Each suppository was placed in a 20mL volumetric flask and melted at 65°C for PEG based suppositories and 45°C for fat soluble based using water bath. The volume was made up to 20 mL with acetonitrile and the drug was extracted by vigorously vortexing the samples. The solution was allowed to cool to room temperature so that the fat-soluble bases separate from the organic phase. 1 mL aliquots were withdrawn and centrifuged at 10000 rpm for 10 mins. The supernatant was filtered using 0.22µm PTFE syringe filters. Suitable dilutions were made using 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in acetonitrile at 70:30 and analyzed using HPLC. Aliquots were withdrawn and filtered using 0.22µm PTFE syringe filters for HPLC analysis. The High-Performance Liquid Chromatography (HPLC) system used to develop and validate the assay for EVG was an Ultimate 3000 (Dionex) which is equipped with a photodiode array detector (275 nm). Chromeleon version 6.70 (Chromatography Management System) was used as the data management and analysis software. Separations were achieved on a Synergi 4µ Polar column (150 X 2.00 mm, RP 80A; Phenomenex) at 37°C with a flow rate of 0.5mL/ min. The mobile phase was 0.2% Formic Acid (FA) in water and 0.2% Formic Acid in

acetonitrile. The retention time of EVG was 7.1 ± 0.2 mins. A calibration curve was made with the concentration range 1-100µg/ml.

5.3.3 Stability assessment

Once a drug product is developed, it is imperative to evaluate the stability as per ICH guidelines. Therefore, the stability for PEG-based TAF/ EVG combination suppositories was performed for 3 months at long-term (5°C) and accelerated (25°C/ 60% RH) stability conditions as per ICH guidelines. Due to limited availability of the APIs, the suppositories were tested at 1, 2 and 3 months at accelerated conditions and only 3 months at long-term conditions. At each time point appearance, breaking point, and drug content uniformity were performed.

5.3.4 Single dose in vivo pharmacokinetics – study design

Based on preliminary results from previous *in vivo* suppository studies (section 4.4.3), the PEG-based formulation was selected for *in vivo* macaque studies. These studies were conducted by the ARV prophylaxis activity team at the CDC in Atlanta, GA. This study was conducted in 6 animals and all animals were subjected to a cleansing protocol before product administration (figure 21). Each animal received a rectal wash with sterile saline (3-4 mL) and stool softeners were included in their diet to effectively remove feces and minimize interference in rectal absorption. Suppositories were inserted ~1cm in the rectum and plasma, biopsy and rectal fluid samples were collected at 2, 5 and 24 hours to evaluate systemic and local exposure, respectively. The sample collection time points were selected based on the protocols defined in the Institutional Animal Care and Use Committee (IACUC) guidelines. Briefly, macaques had to be anesthetized for every sample collection and there were limitations for anesthetizing a macaque within a 24-hour time window, therefore the time points were selected accordingly. Biopsy samples were collected using 3.7-mm biopsy forceps (Radial JawTM 3 Biopsy Forceps, Boston Scientific). All samples were analyzed for TAF, TFV, TFV-DP and EVG concentrations using validated LC-MS assays. Post sample collection, the animals were subjected to a 1-week washout period before the next dose administration.

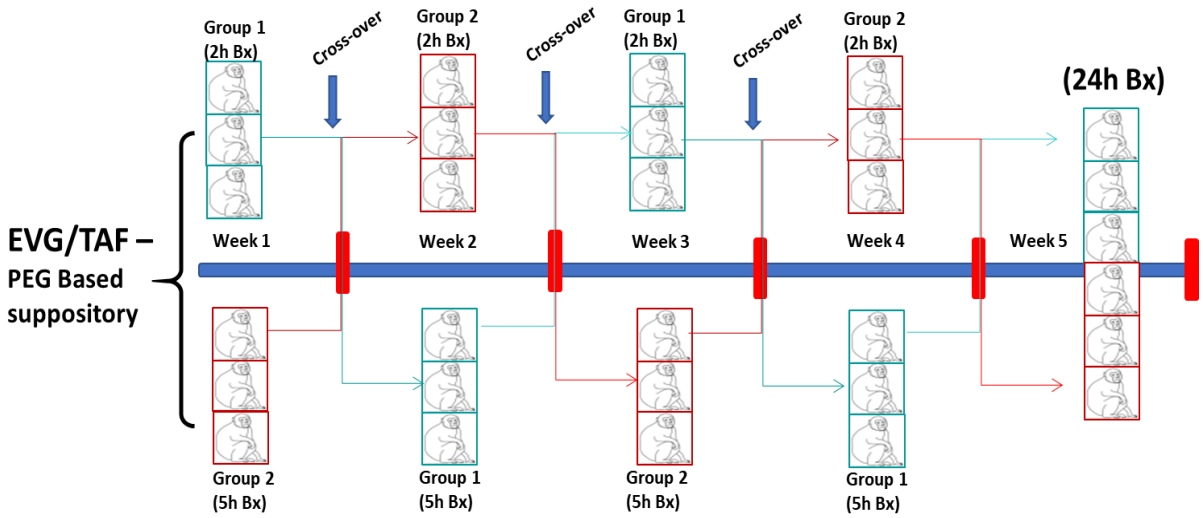


Figure 21 *In vivo* non-human primate single dose cross-over PK study design

PEG suppositories with 8mg TAF + 8mg EVG were evaluated in the cross-over single dose PK study. Independent sampling was conducted from every animal with a minimum one-week washout period after every sample withdrawal. (n=3/ treatment group).

5.3.5 In vivo sample preparation and analysis

Sample preparation: All the sample preparation and analyses were conducted by the ARV prophylaxis activity team and associated analytical team at the CDC in Atlanta, GA. Once the biopsy samples were procured, they were transported on ice to the appropriate lab and personal. Each biopsy sample was then placed in a 50 mL conical tube with 1 mL 80% methanol. The biopsies were minced using sterile surgical scissors and stored in -80°C overnight. Next day, the minced samples were either centrifuged and supernatant was removed or filtered through 0.45µM attached to a 5cc syringe. The resultant fluid was then transferred, labeled in a 2 mL screw-cap vial and utilized for analysis.

Extraction method and sample analysis: EVG and TAF were measured by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Sciex, Foster City, CA, Shimadzu Scientific, Columbus, MD). **Plasma:** A cocktail of 500 µL (490uL of MeOH and 10 uL of internal standard (1000ng/ml), isotopically [d6]-labeled Elvitegravir (Cat #E509002) and isotopically [13C]- labeled Tenofovir (Cat #MC1102) (Toronto Research Chemicals Inc, Toronto,

Canada and Moravek, Brea, CA), were added to 100 µL of plasma to precipitate proteins. After a brief centrifugation and removal of protein precipitates, the supernatant was evaporated to near dryness and re-suspended in 150 µL of 0.2% formic acid in water (mobile phase). **Biopsy:** Biopsy samples were treated in like manner except that they were sonicated for 30 minutes. After a brief centrifugation, 400 µL of supernatant were removed and evaporated to near dryness and re-suspended in 150 µL of 0.2% formic acid in water (mobile phase). **Swab (rectal fluid):** Similarly, a cocktail of 500 µL of methanol containing internal standard were added to the swab samples. After a brief centrifugation, 400 µL of supernatant were removed and evaporated to near dryness and re-suspended in 150 µL of 0.2% formic acid in water (mobile phase). Ten µL of the final solution was injected onto a Unison UK-C18 column (100 x 1 mm, Imtakt, Portland, OR) connected to an HPLC-MS/MS system. An aqueous-acetonitrile mobile-phase gradient was used to elute the drugs from the column and into the analyzer. The gradient began with 100% aqueous up to 0.1 min and then transitioned to 2% acetonitrile at 0.15 min up to 0.3 min, the acetonitrile was increased to 98% over next 0.7 min and held for another 1.5 min, and then the gradient was back to 2% acetonitrile over next 0.5 min and held there till the end. Mass transitions for TAF and EVG of 476.7/270.2, 476.7/176.2, 448.2/344.1, and 448.2/143.1 (m/z), respectively, were monitored in positive mode. The drug concentrations were estimated from a standard curve with a range of 0.5-2000 ng/mL with interday reproducibility of 8.3 %CV. The lower limit of quantification of this assay was 1 and 10 ng/mL for biopsy/swab and plasma, respectively. Since the standards, QCs and unknown samples were in the same matrix, the extraction efficiency was assumed to be same between all samples.

5.3.6 In vivo pharmacokinetic data and statistical analysis

The *in vitro* dissolution results and stability data is represented as mean ± standard deviation (SD), however the *in vivo* PK data represented as median ± range. GraphPad PRISM 7.0 was used to process and analyze the data. Statistical differences were calculated using two-way ANOVA. P values ≤ 0.05 were considered statistically significant. These analyses were conducted for every biological matrix i.e. rectal fluid, biopsies and plasma. Sidak's multiple comparison post-hoc tests were conducted for *in vivo* PK data.

5.3.7 Comparative analysis of in vivo PK data – 40mg TFV with 8mg TAF

Based on our primary hypothesis, the results presented in this chapter establish that the advantages of reduced TAF dosing compared to TFV can be translated to topical drug delivery using a suppository as well. To further validate these findings, we compared the intracellular TFV-DP levels achieved from current studies (chapter 5) and studies presented in chapter 4.

5.4 Results

5.4.1 Suppositories in vitro characterization

A batch of 116 suppository units was manufactured and weighed individually. The weight variation for this batch was within 5% relative standard deviation (1.49g ± 0.018, Mean ± SD). The breaking point was determined to be within 4-6 kgs. Appearance was white, opaque and sticky. PEG-based suppositories are water soluble and therefore dissolve in the rectal cavity. Typically PEG polymers used in our formulation have high melting points, above the body temperature. Hence the melting point of TAF/ EVG suppositories were not indicative of any *in vivo* predictions, therefore the melting point was not determined. Due to the lipophilic nature of EVG, addition of surfactant (1% SDS) was necessary in *in vitro* dissolution test to maintain sink conditions. Based on visual observations, the suppository completely dissolved in the basket within first 15 mins of start of the experiment, completely releasing TAF and EVG within the first 30 mins of dissolution testing (figure 22).

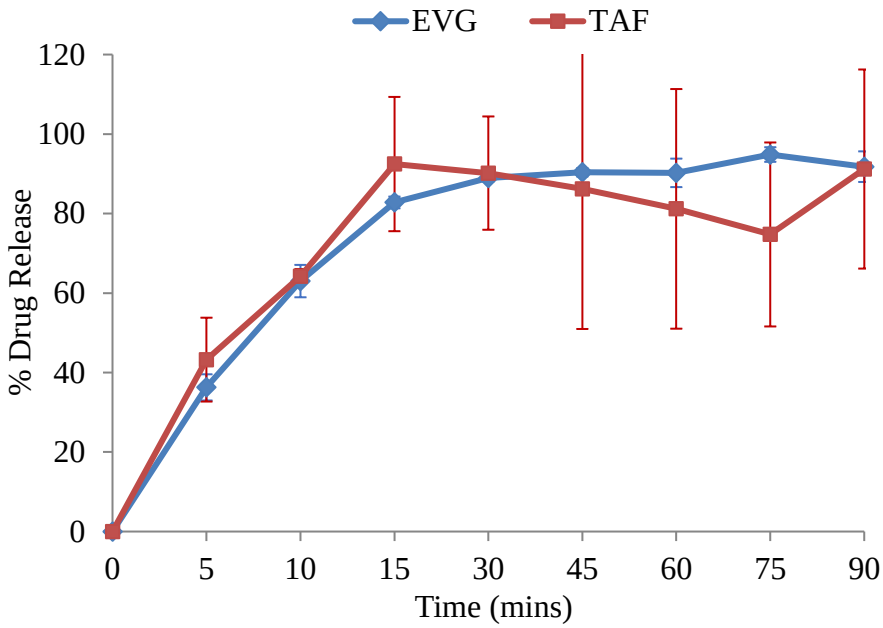


Figure 22 *In vitro* drug release of EVG and TAF from PEG-based suppository

In vitro dissolution was conducted using USP apparatus Type 1 for combination suppositories (8mg TAF + EVG). 1% SDS was used as the dissolution media; X-axis – Time (mins) and Y-axis - % drug release (n=3, mean ± SD, one-way ANOVA)

5.4.2 Stability

The results from the pilot stability studies for TAF and EVG suppositories at accelerated (25°C, 65% RH) and long-term (5°C) conditions are presented in tables 9 and 10, respectively. No change in appearance was observed over 3 months at both tested conditions. While conducting hardness test, some suppositories slipped from its position upon contact by the testing probe. These tests were excluded from the results (marked with *). As described in section 5.3.2., TAF and EVG were analyzed from the suppository using different analytical techniques. The drug content analysis from the stability batch confirmed 87.5% TAF (~7 mg) and EVG (~7 mg) of the target concentration per suppository unit. Therefore, 7 mg was considered as the target value for the stability studies over the investigated period. We predict that lower drug loading was observed because more than calculated units were obtained upon manufacturing from one batch, so API was distributed amongst more units than expected. EVG drug content was maintained between 85-

115% of the target concentration over 3 months at both testing conditions. Similarly, TAF levels were maintained within the 90-110% range of the target value except at two months. This could have just been because of poor distribution or an outlier suppository within the initially manufactured batch, as expected drug content was within the range at three months.

Table 9 Accelerated stability study (Mean ± SD, n=3)

		Accelerated Studies (25°C, 65% RH)				
Tests		**Time 0**	**T 2 weeks**	**T 1 Month**	**T 2 Month**	**T 3 Month**
Appearance		White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque	White/ Opaque
Breaking point		4.48 ± 0.38	5.02 ± 0.82	4.52 ± 0.50	5.37*	6.04 ± 0.07*
Drug Content (% of target)	**EVG**	101	103	97.6	91.3	101
	TAF	98.6	97.8	93.8	85	91.3

Table 10 Long-term stability study (Mean ± SD, n=3)

		Long-term Studies (5°C)			
Tests		**Time 0**	**T 1 Month**	**T 2 Month**	**T 3 Month**
Appearance		White/ Opaque	NA	NA	White/ Opaque
Breaking point		4.48 ± 0.38	NA	NA	5.72 ± 0.54*
Drug Content (% of target)	**EVG**	101	NA	NA	105
	TAF	98.6	NA	NA	99.8

5.4.3 In vivo non-human primate (NHP) pharmacokinetic assessment

Rectal Fluid PK: All prodrug levels, TAF, TFV and TFV-DP levels were evaluated to systematically understand the prodrug approach and determine the prodrug-to-active metabolite

conversion rate in the rectal compartment. TAF was detectable only up to 5 hours in the rectal fluid, however, TFV as well as TFV-DP both levels were detectable up to 24 hours (figures 23 and 24). Detectable EVG levels were observed up to 24 hours, however the C_{max} for all 3 APIs, TAF, TFV (metabolized from TAF) and EVG was reached within 2 hours of suppository administration (0.01×10^4, 1.2×10^4 and 1.0×10^4 ng/mL, respectively) (figure 23), indicating a rapid absorption post product administration; possibly even before 2 hours. Despite the hydrophilic and hydrophobic natures of TAF and EVG respectively, almost identical drug release kinetics were observed *in vivo* from the PEG suppositories. Using ordinary two-way ANOVA, TFV, TAF and EVG release was found non-significant, however they were significantly different over time.

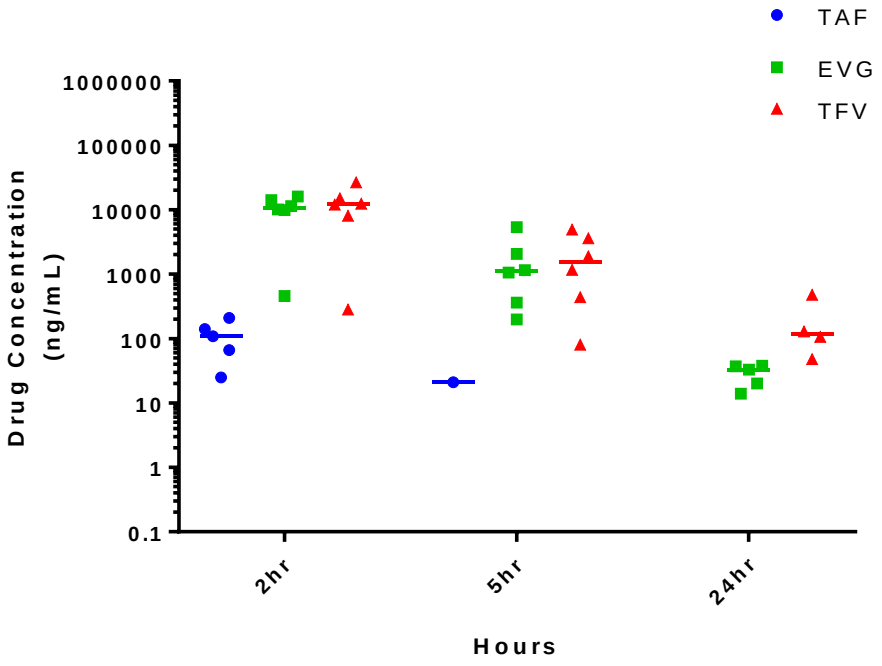


Figure 23 Time course of TFV, TAF and EVG in macaques following rectal administration of 8mg EVG/ 8mg TAF PEG suppository

Each data point represents an independent measure from an animal; n=6; Data represented as median ± range; X-axis – time (hours) and Y-axis drug concentrations (ng/mL); Two-way ANOVA; P< 0.05 were considered statistically significant (TAF LOQ- 1ng/mL; EVG LOQ – 1 ng/mL)

Rectal tissue PK: TAF was detectable in the rectal biopsies at 2-hours post suppository administration (figure 24) because first sample was at 2 hours only. However, TFV was detectable

in the rectal biopsies for up to 5 hours, clearly indicating rapid hydrolysis of TAF to TFV in the rectal tissue. Considering the TFV and TFV-DP levels observed in the rectal fluid, it suggests that although TAF is rapidly hydrolyzed to TFV, the TFV to TFV-DP phosphorylation is prolonged for at least 24 hours (figure 25), perhaps even longer. Using ordinary two-way ANOVA, TFV, TAF and EVG release was found non-significant in the biopsies, however they were significantly different over time.

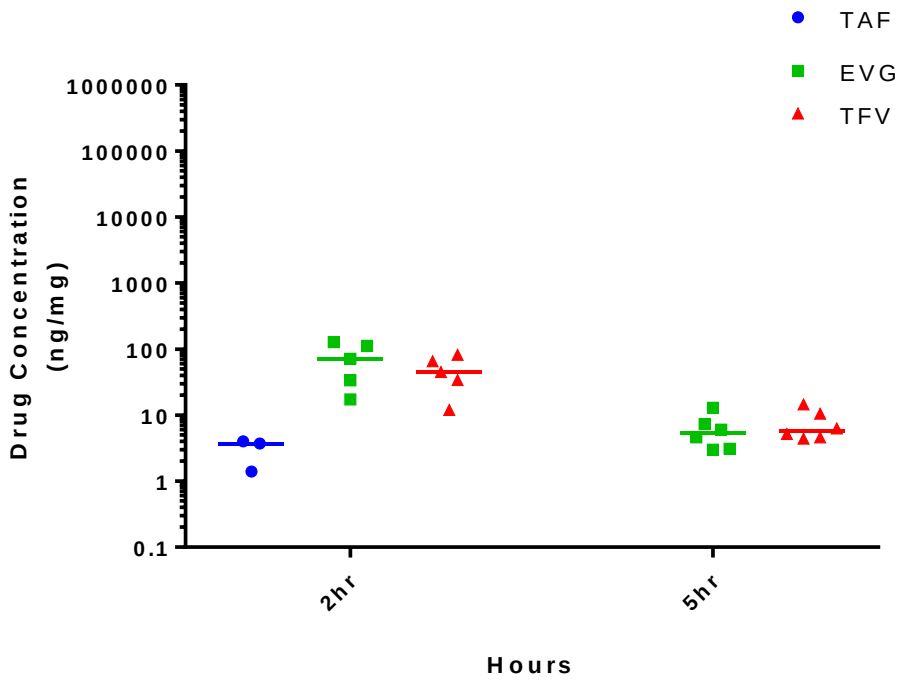


Figure 24 Time course of TFV, TAF and EVG in macaques following rectal administration of 8mg EVG/ 8mg TAF PEG suppository

Each data point represents an independent measure from an animal; n=6; Data represented as median ± range; X-axis – time (hours) and Y-axis drug concentrations (ng/mg); Two-way ANOVA; P< 0.05 were considered statistically significant (TAF LOQ- 1ng/mL; EVG LOQ – 1 ng/mL)

Plasma PK: The lower dosing with the PEG suppositories did not show any TAF and EVG systemic exposure. From a prevention perspective, our goal was local exposures only, so the lower doses might be beneficial to achieve sufficient local exposures only.

Table 11 Pharmacokinetic parameters of TAF and EVG in macaques following rectal administration of 8mg EVG/ 8mg TAF PEG suppository (TFV LOD - 3ng/mL; EVG LOD - 1.5ng/mL)

PEG suppository	Rectal Fluid			Tissue (Biopsy)		
Variable	C_{max}, ng/mL, (median ± SE)	T_{max}, hours	AUC_{0-24}, ng h/mL	C_{max}, ng/mg, (median ± SE)	T_{max}, hours	AUC_{0-24}, ng h/mL
TAF (8mg)						
	0.01×10^4	2	-	1 ± 1	2	-
TFV (from TAF)						
	$1.2 \times 10^4 \pm 0.43 \times 10^4$	2	4.2×10^4	6 ± 2	2	82.9
EVG (8mg)						
	$1.0 \times 10^4 \pm 0.27 \times 10^4$	2	3.4×10^4	5 ± 2	2	117.8

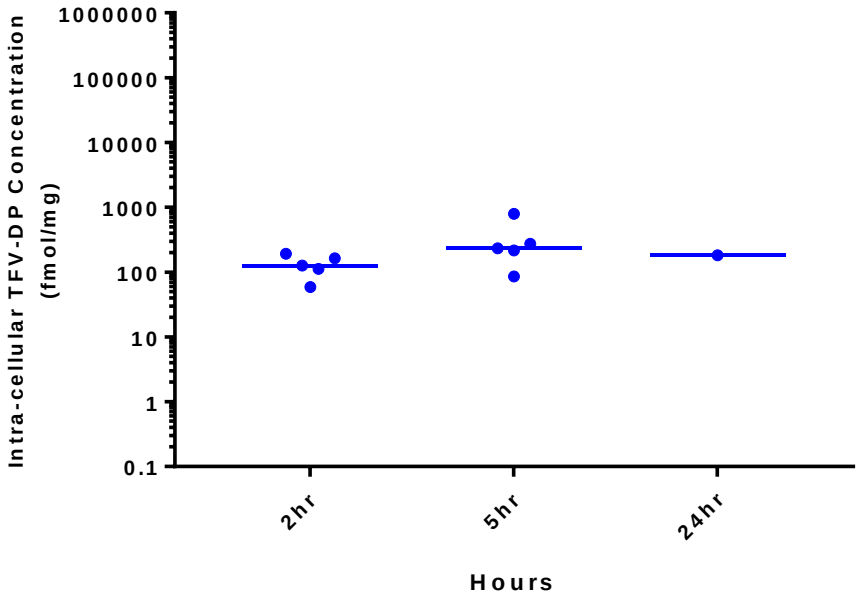


Figure 25 Time course of intracellular TFV-DP in macaques post rectal administration of TAF + EVG PEG suppository

Each data point represents an independent measure from an animal; n=6; Data represented as median ± range; X-axis – time (hours) and Y-axis drug concentrations (fmol/mg)

5.4.4 Comparative pharmacokinetic assessment – 40mg TFV with 8mg TAF

Using Tukey's multiple comparison test, the intracellular TFV-DP levels were significantly different at 2 hours only, no significant differences were observed at 5 and 24 hours (figure 26). However, the overall $AUC_{0\text{-}24}$ from 8mg TAF (PEG suppository) dosing achieved (4.2×10^4 ng*h/mL) about 1.4 and ~4 fold less exposure to 40 mg TFV witepsol suppository (6.0×10^4 ng*h/mL) and 40 mg TFV-PEG suppository (18.7×10^4 ng*h/mL), respectively. In summary, ~8.3 fold dose reduction (TAF molar ratio) is capable of achieving similar (1.4 fold witepsol suppository) or higher (~4 fold PEG suppository) TFV-DP exposures when administered rectally. Moreover, in spite of achieving similar or higher TFV-DP exposures *in vivo*, there was no systemic exposure in the macaques. Considering local target of action, our goal was to achieve drugs exposures in the rectal fluid and tissues, so exposure in the systemic circulation was favorable. This further confirms the secondary hypothesis of achieving local TFV exposures, without systemic exposures with reduce TAF dosing (figure 20). In addition to measuring exposures achieved with different dosing, parent-to-metabolite intracellular conversion ratios could possibly tell us the conversion rate which is valuable information for future PK studies. However, since TAF was detectable in rectal biopsies for 2 hours only, enough data was not available to calculate these intracellular ratios.

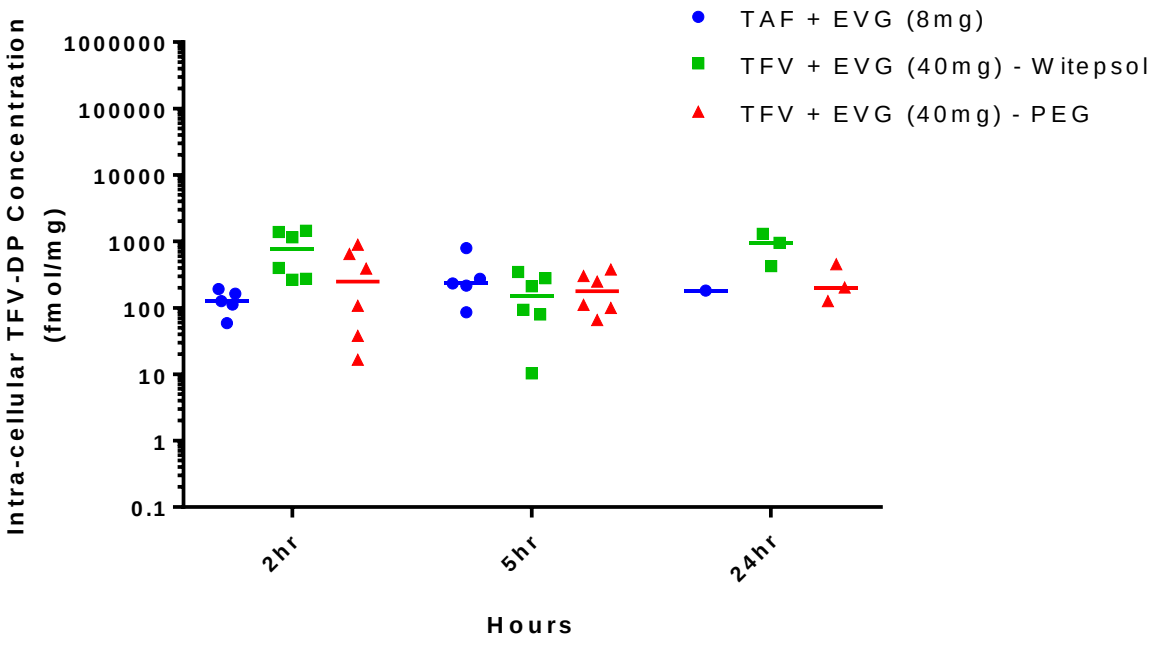


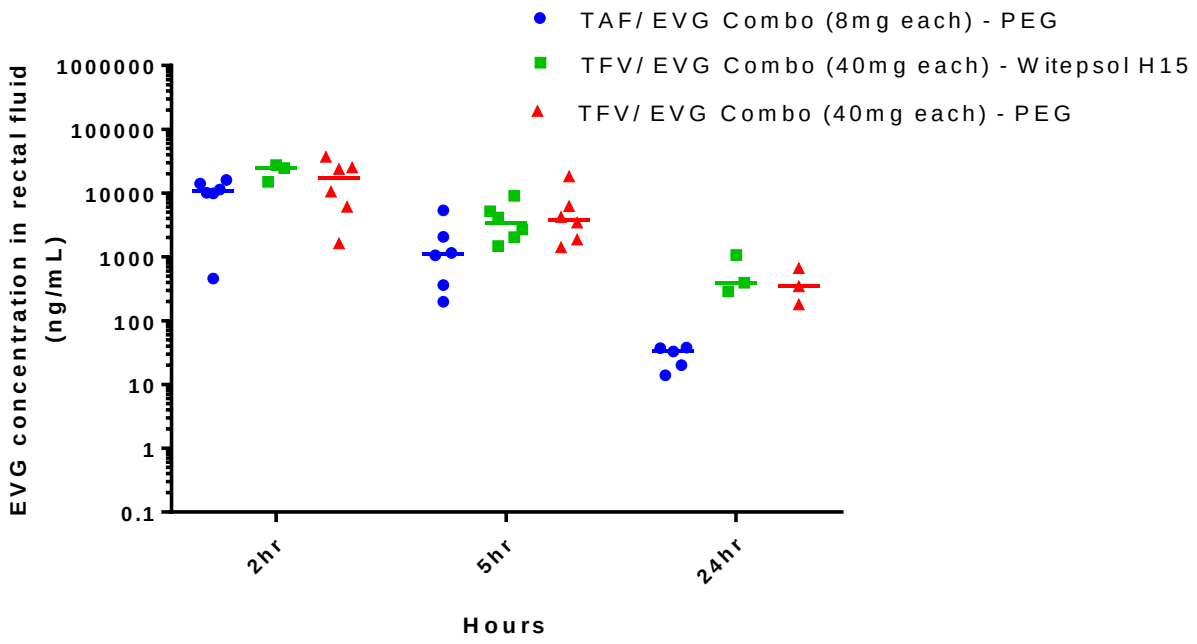
Figure 26 Time course of intracellular TFV-DP levels post rectal administration of suppositories with different doses

Data comparing 40mg TFV (PEG and witepsol) versus 8mg TAF (PEG) suppository. Each data point represents an independent measure from an animal; n=6; Data represented as median ± range; X-axis – time (hours) and Y-axis- TFV-DP concentrations (fmol/mg); Two-way ANOVA with tukey's multiple comparison post-hoc test; $P < 0.05$ were considered statistically significant

Similar to TFV and TFV-DP exposures, we also compared EVG exposures achieved in rectal fluid and biopsies with 8mg EVG (chapter 5) and 40mg EVG (chapter 4) (figure 27). In spite of a 5-fold difference in dose, no significant difference was observed in EVG exposure (AUC_{0-24}) in the rectal fluid between 8mg PEG (3.4×10^4 ng*h/mL), 40mg witepsol H15 (8.4×10^4 ng*h/mL) and 40mg PEG suppositories (9.4×10^4 ng*h/mL) (except 2 hours 8mg PEG vs 40mg witepsol suppository) (Two-way ANOVA) (figure 27a). This indicates that EVG absorption from suppositories is not dose dependent as a 5-fold increase in dose, was able to only achieve a ~2.5-fold higher exposure in the rectal fluid. However, this correlation was not translated in EVG exposures observed in the rectal biopsies. With reduced (8 mg EVG) dosing, detectable EVG levels were observed in the biopsies for up to 5 hours only, compared to 24 hours with 40 mg EVG. Furthermore, a 9.9-fold (1159 ng*h/mL) and 2.2-fold (259 ng*h/mL) increase in AUC_{0-24}

of EVG was observed in 40 mg witepsol H15 and 40 mg PEG suppositories respectively, compared to 8 mg PEG (117 ng*h/mL) suppositories (figure 27b). The therapeutic efficacy of the observed exposures in the rectal fluid and tissues can only be confirmed by conducting efficacy studies in NHPs. The dose independent kinetics observed in the NHPs could possibly be explained due to the modulation by the efflux transporters (described in section 3.4.5) or metabolism due to the CYP enzymes present in the rectal tissue [173, 174]. Especially EVG being a substrate for the efflux transporter P-gp, the absorption could be restricted due to P-gp expression in the rectum [175, 176].

(a)



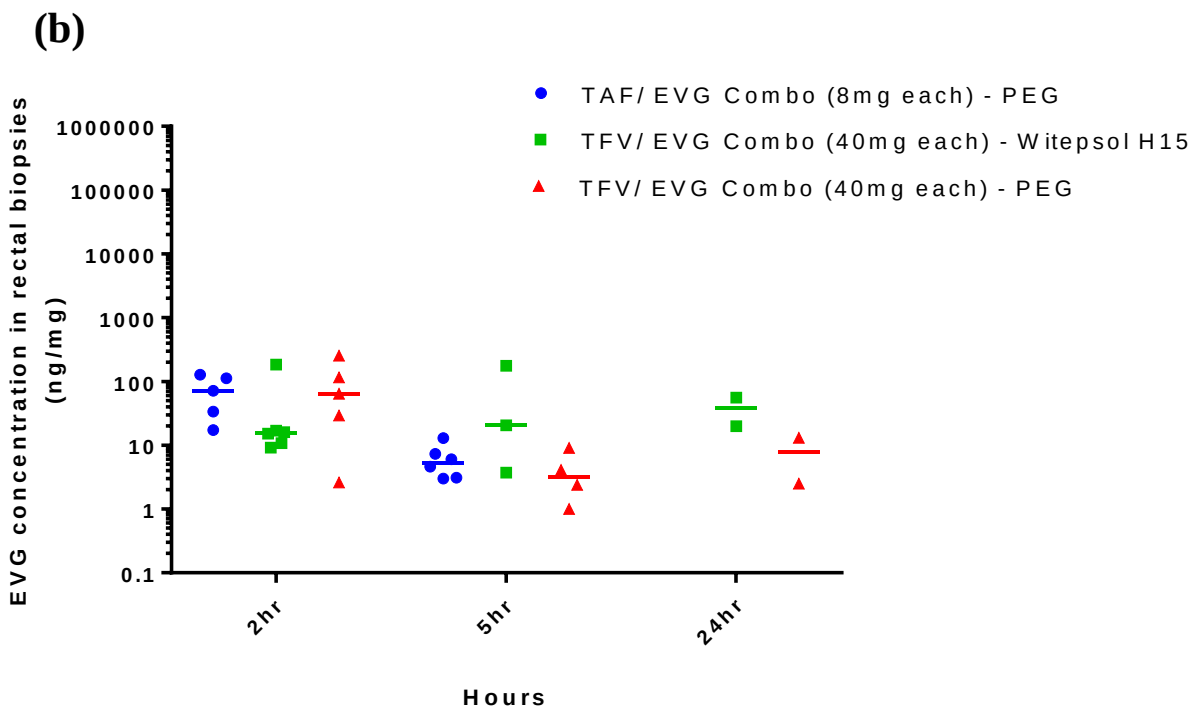


Figure 27 Time course of EVG in macaques following rectal administration of TAF + EVG PEG suppository at different doses

Data comparing 40mg EVG (PEG and witepsol) versus 8mg EVG (PEG) suppository in (a) Rectal fluid, (b) Biopsy. Each data point represents an independent measure from an animal; n=6; Data represented as median ± range; X-axis – time (hours) and Y-axis- EVG concentrations (ng/mL for (a) and ng/mg for (b)); Two-way ANOVA; $P < 0.05$ were considered statistically significant

5.5 Discussion

Using the PEG-based suppository utilized in chapter 4 (PEG 3350:1000:400 at ratio 60:30:10), we manufactured and characterized TAF and EVG suppositories and evaluated *in vivo* pharmacokinetics using the macaque model described in section 4.3.4. Two studies which are currently on-going, are developing TAF-based topical formulations to prevent vaginal transmission of HIV-1 [94, 150]. A recent study conducted a dose-range determination for TAF and EVG for vaginal inserts in macaque model [172]. Combinations of three different dosing levels 8/10, 16/ 20 and 24/ 40mg (EVG/ TAF) were evaluated in pigtailed macaques as vaginal inserts. The PK profiles obtained from this study were similar for all doses and suggested minimal dose

dependence on exposure [172]. The results from this study confirms that the EVG *in vivo* exposure achieved was not dependent on the dose administered, i.e. the incremental increases in doses do not translate to incremental increases in exposures. Although the study was designed for vaginal administration of inserts, however being one of the first studies utilizing TAF and EVG combination in a topical formulation, we used the results from their studies as a point of reference for dose selection in our experiments. This seemed possible because some studies have attempted to pursue vaginal dosing or formulations in the rectal compartment [70], for exploratory purposes. As the TAF and EVG PK profiles mentioned above were not dose-dependent, our goal was to begin with the lowest doses and titer up. Moreover, since we had PK data from previous experiments (chapter 4) with 40/ 40 mg EVG and TFV, we selected 5-fold lower doses 8/ 8mg (EVG/ TAF).

As described in chapter 5.1, TAF and EVG combinations are readily available in FDCs for HIV-1 treatment, therefore incompatibility due to co-existence of TAF/ EVG in a single formulation was not expected. Incorporation of TAF and EVG in the PEG-based formulation did not impact the physicochemical properties of the suppository product. Based on the stability results, the combination PEG-based suppository was stable for up to 3 months in the evaluated (accelerated and long-term) conditions. This further established the TAF and EVG can be formulated in single stable formulation. Moreover, the results from the *in vitro* dissolution studies demonstrated a biphasic drug release, comparable to TFV and EVG in figure 16. The reduction in dose from 40mg to 8mg and substitution of TFV to TAF did not impact the drug release from the PEG-based suppositories. Although both the APIs were partially soluble and dispersed, due to complete dissolution of the suppository instantly, no differences were observed between TAF and EVG release profiles. PEG being a water-soluble polymer, the initial rapid drug release from the suppositories could potentially be due to the addition of a surfactant, perhaps it improved the solubility potential in the dissolution media. The *in vitro* dissolution studies were conducted to ensure quality control (QC) for consistent product attributes from batch-to-batch.

The *in vivo* study in macaques (figure 23) confirmed detectable TAF levels up to 5 hours in the rectal fluid, but TFV up 24 hours, indicating rapid conversion of TAF to TFV in the rectal fluid (figure 20). In addition to the rectal fluid, detectable TFV levels were observed in the biopsies up to 5 hours as well (figure 24), proving rapid absorption of TAF in the rectal tissue. As described

in figure 20, due to improved lipophilicity of TAF, it can easily diffuse through the lipid bilayer and intracellular enzyme rapidly convert TAF to TFV and then TFV-DP via hydrolysis and kinases respectively. Therefore, although TAF is rapidly converted to TFV-DP and is undetectable after 5 hours, the intermediate TFV can still be detected in the rectal tissue up to 24 hours (figure 24). Intracellular conversion of TFV in the tissues and the long intracellular half-life of TFV-DP is responsible for prolonged TFV-DP exposures up to 24 hours (figure 25) [172], with reduced (8 mg) TAF dosing.

Apart from the decreased dosing, we predict that the intra-animal variability and variation in extent of fecal contamination could be responsible for differences in exposures. But the difference in exposures achieved with two different EVG doses indicates that higher doses can be incorporated for extended protections, without systemic exposures. From an on-demand perspective, for shorter duration of protections, 8mg EVG dosing might be enough, if proven therapeutically active.

Overall, the results presented in this chapter confirm our primary hypothesis, that reduced doses of prodrug TAF can achieve equivalent intracellular TFV-DP levels compared to higher TFV doses. It also reiterates that suppositories are a viable platform for ARV drug delivery, with combinations as well. Although several studies have been conducted with TAF in different dosage forms such as rectal enema [75, 94, 150], it is difficult to make comparisons due to differences in animal models (mice versus macaques).

5.6 Acknowledgments

I would like to acknowledge Charles Dobard, PhD, Walid Heneine, PhD, Natalia Makarova, PhD, Sunitha Sharma and colleagues at the Centers for Disease Control & Prevention (CDC), Atlanta, GA for designing and conducting PK studies and graciously providing us with the PK data. In addition, I would like to acknowledge the analytical team, Choung Dinh and Amy Martin at CDC for sample collections and processing.

6.0 Summary of major findings, contributions and future directions

6.1 Introduction

Several studies have demonstrated RAI as the highest risk sexual behavior for HIV-1 acquisition amongst the different modes of HIV-1 transmission [10, 34, 35, 46]. With the recent spike in newly acquired HIV-1 infections amongst MSM and heterosexual couples engaging in RAI, there is an urgent need for safe and effective rectal microbicides [71, 76, 79]. Although a few strategies are currently underway and have shown substantial evidence of being a potentially effective microbicide product, but there is lack of a safe, effective and user compliant product. One of the first dosage forms selected and proven effective in NHP model against HIV-1 acquisition was a rectal specific gel formulation [90]. However, upon evaluation in the clinics, there were reports of product associated problems such as leakiness and messiness. The gel formulation also required an applicator for administration, this would eventually lead to reduced patient compliance and acceptance of the product. These aspects associated with the rectal gel were not favorable to the users before engaging in sexual intercourse, therefore other drug delivery systems were evaluated. Rectal enema solution was explored as one of the rectal drug delivery options for HIV-1 prevention [72, 74]. The microbicide field had high expectations with enemas, because some surveys have suggested that MSM use enemas solution to cleanse the rectal compartment before engaging in RAI for hygienic purposes. Researchers incorporated ARV agents in these enema solutions so that the rectal microbicide does not alter a couple's regular sexual practices. Significant progress has been made with the enema solutions loaded with ARV agents, especially because the liquid nature of the enema solution allowed it to travel deep within the colon, providing extended and enhanced exposure and tissue permeability [72-74]. However, some studies have suggested that administration of large volumes of enemas solution would be required to achieve therapeutic drug concentrations and an applicator is still needed to administer the product. Hence despite safe and effective rectal drug delivery systems, a more user acceptable dosage form was sought.

The choice of drug delivery systems is very limited due to the rectal compartment's dynamic and sensitive nature. Amongst the available dosage forms, the product and its attributes have to be selected carefully. Several factors such as the absorption and permeability of drugs from dosage forms, compatibility with condoms, the appearance and texture have to be favorable to the rectal compartment and the user. This is essential because any incompatibilities or irritation to the rectal epithelium could potentially trigger bowel movement affecting the administered product. So, our goal has been to utilize the existing drug delivery platforms and develop improved products for rectal drug delivery. With advantages associated with rectal suppositories, such as reduced leaky and messiness, avoiding the need of an applicator, this dissertation focuses on exploring the already existing and established rectal dosage form, suppositories as a potential platform for rectal drug delivery of ARV agents. To our knowledge, this is one of the first extensive studies conducted with suppositories loaded with ARV agents specifically targeted towards HIV-1 prevention. Traditional suppository bases are very limited and typically geared towards geriatric or pediatric population who are unable to swallow medications orally. Apart from specific population, suppositories are traditionally used for agents which require a quick onset of action. This is possible because of the physiology of the rectal compartment which avoids first pass metabolism allowing a rapid onset of action. The rectal compartment consists of a very small volume of neutral rectal fluid which has minimal buffering capacity. Moreover, it consists of fenestrated blood vessels which allows rapid absorption into the systemic circulation. Therefore, we need to take advantage of the rectal physiology and develop dosage forms to successfully deliver therapeutic levels of ARV agents. However, this vascular nature of the rectum can act as a double-edged sword. Although rectally administered products can have rapid onset of action, due to bypassing first-pass metabolism, the presence of bowel feces and speckled vasculature from person-to-person can significantly impact drug absorption, leading to wide-ranging *in vivo* exposure. In some cases, this might lead to sub-optimal systemic exposures of ARVs, increasing the chances of drug resistance as well [49].

To develop a successful rectal microbicide product, the first objective in this dissertation was geared towards determining key factors to be considered while selecting suitable suppository bases (chapter 2) followed by conducting studies to select the appropriate bases amongst the available ones on the market. Witepsol H15 and PEG 3350:1000:400 (at ratio 6:30:10) were selected as fat-soluble and water-soluble suppositories, respectively. Based on the physicochemical properties

and mechanism of action, a series of ARVs were selected, suppositories containing each ARV was manufactured with the selected suppository bases and *in vitro* characterization (chapter 3) were conducted to identify lead formulations for future *in vivo* studies. Since ARV agents delivered in rectal dosage forms have previously been proven efficacious, we explored the potential of a combination product with more than one ARV agent in a single product. Furthermore, because of the versatile nature of suppositories, we wanted to test the potential of co-delivering a hydrophobic and hydrophilic API simultaneously. At first TFV and EVG were selected as the ARV candidates for *in vivo* studies. Combination suppositories containing TFV and EVG were manufactured, characterized for qualitative and quantitative properties and tested for stability as per ICH guidelines. In order to determine *in vivo* exposures that can be achieved using the TFV and EVG combination suppositories, single-dose pharmacokinetic were evaluated in a well-established NHP model (chapter 4). Samples were withdrawn from the rectal fluid, tissues and plasma to determine local and systemic exposures achieved using suppositories. Additionally, basic PK parameters such as C_{max}, T_{max} and AUC_{0-24} were calculated to determine the differences in exposures achieved using two different formulation. The *in vivo* exposure results observed from suppositories were compared with results obtained from other dosage forms (literature). Favorable PK results from these studies encouraged us to further test the prodrug approach using same suppositories. In order to test the benefits of prodrugs administered rectally, PEG based suppositories with reduced doses of TAF and EVG were manufactured, characterized and tested for stability as per ICH guidelines (chapter 5). Similar dosing and study design were adopted as described in chapter 4. Once the samples were obtained from the rectal fluid, tissues and plasma, basic PK parameters were calculated. The exposures achieved with parent drug (TFV) and prodrug (TAF) were measured. In summary, the results from the studies conducted in this dissertation is a proof of concept, confirming the use of suppositories as a platform for rectal drug delivery of ARV agents.

6.2 Summary of major findings

Suppositories have been traditionally used for treatment of several disorders such as constipation, hormonal therapy, epilepsy etc. The selection of suppository bases is crucial and is

dependent on various factors which might have been overlooked in the past. The studies presented in this dissertation give some in-depth insights into factors that should be considered while selecting an appropriate suppository base. For instance, although use of oil-based lubricants were contraindicated with latex condoms, there was no evidence of concomitant use of suppositories with condoms (section 2.5). The results described in section 2.4.1 establish an important correlation between hydroxyl value of fat-soluble suppository bases and condoms. As per our knowledge, this is one of the first studies conducted evaluating concomitant use of suppositories with condoms. This significant finding can potentially be used in the product labels or at least taken into consideration for future microbicide development. Although this information is useful from a product development perspective, results from section 2.4.2 demonstrate that this phenomenon does not impact *in vitro* permeability. According to permeability studies conducted with excised human colon tissues, the changes in hydroxyl value did not impact trans and paracellular permeation pathways. This was further confirmed by H & E staining results presented in section 2.4.3. Therefore, we anticipate that the hydroxyl value of the fat-soluble suppositories might not have a significant impact on rectal absorption and permeation of drugs. However, further investigation with more elaborate selection of suppository bases with varying parameters such as, iodine value, peroxide value etc. might be warranted to validate these initial findings.

In addition to the selection of suppository bases, we also investigated if the choice of APIs can impact the overall suppository formulation characteristics. We selected two hydrophilic and two hydrophobic ARV APIs and conducted a series of *in vitro* characterizations. The physicochemical characteristics (table 4) confirms that suppositories loaded with ARV agents can be formulated and that the ARV properties (hydrophobicity) do not impact the overall product quality (except MK-2048). The *in vitro* dissolution results from these studies demonstrated that the physicochemical properties of the API can alter the drug dissolution rates from fat-soluble suppository bases such as witepsol. This was evident when only about 20% hydrophobic APIs (EVG, MK-2048) were released after 2 hours and almost 100% hydrophilic APIs (TFV, TAF) were released within the first 30 mins. This significant difference was not observed with the PEG-based suppositories. Therefore, dissolution rates from fat-soluble suppository bases are dependent on nature of the API, which gives a wider range to modify drug dissolution rates based on the duration or onset of action required. Although the properties of the ARV agent did not impact the overall quality of suppositories, based on some stability studies (chapter 4) conducted, the PEG

based formulation indicated signs of melting at 25°C/ 65% RH. Also based on some results reported in the literature, PEG-based suppositories tend to absorb moisture from the environment, so they may cause discomfort to the users. Therefore, fat-soluble suppositories might be a favorable alternative over PEG-based suppositories with TFV and EVG.

We conducted pilot studies in NHPs to measure *in vivo* exposures that can be achieved with suppositories, being one of the first attempts to test ARV loaded suppositories in animals, prior to any elaborate PK studies. The results from the NHP studies confirmed that TFV administered in PEG-based suppositories were able to achieve exposure up to 15 cm in the colorectal compartment. Comparing the suppository PK data with other rectally administered dosage forms such as gels and enemas, suppositories were able to reach deep within the colorectal compartment, in spite of the semi-solid nature of the formulation. This indicates that suppositories might be a better alternative to gels and enemas for users as they overcome some disadvantages associated with gels and creams such as messiness, leakiness and need for an applicator. Moreover, although our dose was limited to 40 mg in the NHP studies, suppositories are capable of delivering up to 100mg doses as well (unpublished data).

ARVs are well-known for DDIs with innate transporters and metabolizing enzymes, therefore, elaborate DDI studies are typically conducted with newer ARVs. This phenomenon is especially concerning from rectal drug delivery perspective, as some evidence indicated expression of efflux transporters in the colorectal region. However, it is uncertain if they can significantly impact ARV absorption in the rectum. Considering these factors, it was essential to investigate if the ARVs presented in this dissertation are substrates for efflux transporters. Based on our literature survey, data suggested every InSTI was a substrate for efflux transporters, but data supporting EVG was unavailable. Therefore, we conducted experiments to determine if EVG was a P-gp substrate. The trans well flux assay results described in chapter 3 confirmed that EVG is a P-gp substrate. This knowledge can be applied to future topical microbicide product development for dose selection in future clinical studies. In spite of being a P-gp substrate, EVG when administered rectally as suppository formulation, the need of a booster might be potentially eliminated.

In addition to developing a combination rectal microbicide product using suppository as a platform, one of the goals was to assess drug dissolution rates by comparing two APIs with extreme solubilities (TFV and EVG) and by comparing the dissolution of these APIs using two different

kinds of suppository bases. We analyzed the *in vivo* PK data using two different approaches, one comparing the TFV release with EVG (figure 17) and second comparing witepsol versus PEG-based suppository bases. Surprisingly, despite the differences in solubilities of TFV and EVG, no significant differences were observed *in vivo* in the release kinetics at any time points. Similarly, no significant differences were observed between witepsol H15 and PEG 3350:1000:400 as well. Therefore, based on our studies we predict the *in vivo* drug dissolution is possibly independent of the nature of the APIs or the suppository base used. Instead it can vary depending on other innate factors such as presence or absence of fecal matter, volume of rectal fluid, vasculature (fenestrated blood capillaries) present in the rectal compartment etc.

From a mechanism of action perspective, in spite of declining drug exposure over time *in vivo*, the presence of considerable concentrations of TFV and EVG in the rectal fluid indicates perhaps longer duration of action. Especially considering EVGs lipophilicity, it may potentially remain bound to the fat-soluble suppositories bases and stay in the rectal compartment longer than 24 hours. As mentioned earlier, this phenomenon can be beneficial as InSTIs are known to act at later stages of the HIV-1 replication cycle. Moreover, TFV's hydrophilicity can be advantageous as we predict that it will be easily with the rectal fluid. Therefore, it will be capable of arresting replication early in the HIV lifecycle, whereas EVG's hydrophobicity can potentially allow it to penetrate and stay longer in the lipid bilayers of the rectal epithelium and tissues. An ideal product should be able to disintegrate, and dissolve quickly post administration, have longer duration of action, good solubility and permeability in the rectal fluid and tissues, good potency so that the drug loading can be reduced to reduce possibilities of ARV-related toxicities. Based on the observed PK results the suppositories developed in this dissertation were able to demonstrate all the above-mentioned properties of an ideal formulation. Therefore, similar to studies that have co-formulated NNRTIs and InSTIs, our TFV and EVG combination suppository formulation can prove advantageous from a mechanism of action as well as formulation perspective.

6.3 Limitations and shortcomings

The major limitations that were identified from the studies presented in this dissertation are listed below:

One of the limitations in chapter 2 was that the *in vitro* spreadability studies were conducted in PEG-based suppositories only. Although the results confirmed TFV and TFV-DP levels up to 2 hours post administration and confirmed exposure up to 15 cm in the colorectal compartment; investigating witepsol suppositories might give a better understanding of *in vivo* dissolution and predict exposures using different suppository bases. This can be beneficial to understand especially because both suppository bases utilize different mechanism of drug dissolution. Due to the water-soluble nature of PEG polymers, PEG-based suppositories dissolve in the rectal fluid, whereas, fat-soluble suppository bases melt at body temperatures. The dissolution and melting of suppository bases could influence their *in vivo* spreading capacity, eventually altering drug release kinetics. Therefore, investigating both suppository bases would have given a better understanding of their respective behaviors *in vivo*.

Another limitation was the use of non-biologically relevant dissolution media (0.5 % SDS) for the *in vitro* dissolution studies in chapter 3. Using biologically relevant dissolution media such as rectal simulant fluid could have potentially allowed us to make better *in vitro-in vivo* correlations (IVIVC) between the *in vitro* dissolution results and *in vivo* results from the macaque PK studies. However, it was not possible to maintain sink conditions for all APIs in biologically relevant dissolution media. Our goal was to investigate if the physicochemical properties (hydrophilic/ hydrophobic) of the APIs influence the dissolution rates from the suppositories, therefore it was crucial to maintain consistency in the dissolution media. Moreover, only four ARVs were evaluated in chapter 3, to get an in-depth understanding of differences observed in the *in vitro* dissolution rates between a broad range of ARVs with different hydrophobicity's could have been incorporated in these studies.

In chapters 4 and 5, the sampling time points for the *in vivo* macaque PK studies were limited. Well-designed PK studies in animals require sample collection for at least 5 times the half-lives of the APIs to ensure complete elimination. In cases where extensive sample collection is not

possible, at minimum 6-8 sampling time points are essential to clearly observe the absorption and elimination phases PK profile. The PK results indicated T_{max} of 2 hours for every formulation and ARV, but with additional sampling time points, more accurate C_{max} and T_{max} could have been captured. The PK results indicated no-to-minimal differences in drug release profiles from both suppository bases (Witepsol/ PEG) as well as both APIs (TFV/ EVG). With additional sampling time points, differences within suppository bases or ARVs (if any) could have been potentially identified. For instance, fat-soluble suppositories release the drug by melting at body temperatures whereas the water-soluble PEG based suppositories dissolve in the rectal fluid. Based on our *in vitro* characterization results and observations, witepsol suppositories melts within 10-20 mins *in vitro* forming oil globules. However, since water-soluble PEG suppositories dissolve, this observation couldn't be evaluated using any *in vitro* tests. But with additional sampling time pints, these precise differences and *in vivo* performance of the two types of suppositories could have been captured. In addition, the data could have been used for conducting *in vitro in vivo* correlations (IVIVC) for mechanistic understanding of suppository dissolution. Furthermore, collecting additional sampling time points beyond 24 hours could have helped identify the potential duration of action and accurately observe the elimination of TFV and EVG. In addition, collection of additional time points is crucial to better understand the elimination PK tail to assess the duration of efficacy the product can provide as well as indicate any potential of drug resistance. This could be concerning as a longer PK tail with sub-therapeutic *in vivo* levels can increase the chance of drug resistance. However, sample collection from NHPs requires anesthetizing them, and according to IACUC guidelines, NHPs can be anesthetized only 4-5 times in a 24 hours window, limiting number of samples collected.

Although the PK results presented in chapters 4 and 5 indicated similar or better TFV-DP and EVG *in vivo* exposures in NHPs using suppositories, this was based on comparisons made with PK results reported in the literature. Even if suppositories demonstrate favorable PK studies using similar dosing strategy as other dosage forms (gels/ enemas etc.), efficacy studies with HIV-1 challenges are essential to firmly establish if suppositories can provide protection against HIV-1 acquisition.

One of the key limitations with suppositories in general as a dosage form is that it melts at elevated temperatures. Therefore, although most suppositories are required to be stored at room

temperature, they might not be ideal product for regions with elevated temperatures perennially such as some countries in Africa. Such places might require additional care for the product such as refrigeration etc. which could drive the product cost to be higher.

6.4 Significance to the field

The anatomy and physiology of the rectal compartment restricts the drug delivery to only a limited number of dosage forms. Active research for ARV delivery using other drug delivery systems such gels, enemas and inserts are already underway [72-74, 79, 172]. In this dissertation work, an existing traditional drug delivery system (suppository) was explored to develop a product which can be potentially used for pre-exposure prophylaxis (PrEP) against HIV-1 acquisition using ARVs. According to our knowledge, the work presented in this dissertation is one of the first extensive studies evaluating suppository platform for ARV drug delivery.

Several studies conducted in this dissertation pave way for the selection of optimal suppository product for a Phase I clinical trial. Results obtained from the condom compatibility studies, *in vitro* permeability studies, aesthetic appearance etc. all together lead us to narrow down to the most suitable suppository product for future clinical trials. The Microbicide Trial Network – 035 (MTN – 035) is currently recruiting about 210 MSM and transgender women who engage in RAI. The goal of this study is to assess acceptability and preferences amongst three rectally administered placebo products; enema, insert and a suppository.

Historically a few studies were conducted comparing various rectal drug delivery systems as placebos to assess user acceptability, safety and tolerability. Most of these studies indicated suppositories was not the first preference by the users [68]. However, there were several factors that were overlooked while selecting the right suppository product, which could have impacted the results of these studies. Taking these factors such as suppository base, shape and size, we believe the studies conducted in this dissertation can lead to be better product which is more acceptable to the users.

Overall, from a product acceptability perspective, suppositories provide the users more options compared to traditional enemas and suppositories. Apart from acceptability, some products might themselves be more practically viable compared to others. For example, suppositories might not be the first preference in geographical areas with perennial high temperatures as they might melt while storage or administering. In such cases other dosage forms which are currently under pursuit might prove a favorable option. However, semi-synthetic suppository bases offer products with a wide range of melting pints to choose from. Therefore, suppository formulations can be altered to better suit the climatic areas in various geographical conditions.

Moreover, by comparing similar studies from the literature (gels/ enemas) and our *in vivo* exposures achieved using the combination of TFV and EVG, the suppositories were able to achieve similar exposures, in some cases beyond therapeutic levels. This not only substantiates suppositories as a viable ARV drug delivery platform, it also provides evidence of using more than one ARV agent in a single product. In fact, studies presented in this dissertation encourages the field to develop more topical products with more than one ARV agent for improved efficacy and potentially provide protection for longer durations of time.

6.5 Future directions

Although the results described in this dissertation demonstrated comparable PK exposures to already proven 1% TFV gel with rectal suppositories in the NHP model, are the observed levels enough to provide protection against HIV-1 acquisition, still needs further studies. Therefore, one of the immediate future directions is to conduct *in vivo* toxicity and efficacy studies determine if the suppositories developed in this dissertation are capable of providing protection against HIV-1 acquisition.

Before conducting elaborate PK studies with NHPs, the ARV-based microbicide products (single or combinations) could be perhaps evaluated first using the *ex vivo* explant challenge model [177]. This model provides the advantages of having preliminary efficacy data without conducting elaborate PK studies in animals. Typically, this model evaluated the efficacy of microbicide

products by evaluating HIV-1 infections with or without the product and comparing against the respective microbicide product's placebo control.

Furthermore, the studies presented in this dissertation utilized suppositories with TFV and EVG combination, future studies should explore the possibilities of other combinations of ARV agents. The general HIV treatment regimen (HAART) utilizes candidates from several classes of ARVs, similar strategies can be explored for topical drug delivery as well. This can be beneficial especially because suppositories offer greater drug loading capacity than other rectally administered dosage forms, therefore more than two ARV agents from same or different classes can be co-delivered in a single product. The combination phenomenon can be further explored by calculating the combination index (CI) of different combinations from different classes of drugs to determine if any specific combination provides synergism or additive effects. A few studies have been conducted to evaluate if combinations work better than monotherapy [15], but data proving synergism or additive effects with multiple ARVs is still unknown. However, conducting efficacy studies by challenging NHPs with HIV-1 post product exposure is essential before exploring other combinations of ARV agents.

Although the *in vivo* TFV, TFV-DP and EVG exposures achieved with the suppositories matched or exceeded observed levels in the literature with other rectally administered products, the maximum tolerable dose (MTD) is still unknown. Therefore, single ascending dose (SAD) studies for rectally administered product starting with clinically relevant doses should be conducted to determine MTD. This is crucial information as this will establish the maximum dose that can be administered without causing toxicities or adverse effects. Additionally, such data can help establish minimum dosing interval for users who intend to use such products multiple times in a given time period. Irrespective of the dosage forms, establishing the No-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) values for a given API delivered topically can be beneficial for dose selection and future product development.

The products developed in this dissertation were for topical PrEP for on-demand purpose, therefore expected to dissolve and release the APIs rapidly. However, the same suppository platform can be explored for sustained drug delivery by incorporating nanoparticles in the suppository as well. Such a formulation would have the potential to act as a dual-purpose formulation product. For instance, ARVs which act sooner in the HIV replication cycle such as

fusion and reverse transcriptase inhibitors could be incorporated in the suppository vehicle for immediate protection. Whereas integrase and protease inhibitors that can inhibit comparatively later stages of the HIV replication can be incorporated in the nanoparticles for sustained release, perhaps providing protection for longer durations. Apart from mechanism of action perspective, from a formulation standpoint, hydrophobic molecules exhibiting poor drug dissolution can also be incorporated in the nanoparticle for better absorption improving the *in vivo* exposures.

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