

**THE ROLE OF EPITHELIAL INTEGRITY AND METABOLIZING  
ENZYMES IN TOPICAL MICROBICIDE EFFICACY FOR THE  
PREVENTION OF HIV SEXUAL TRANSMISSION**

by

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SEXUAL TRANSMISSION**

Minlu Hu, Ph.D.

University of Pittsburgh, 2016

To further reduce the number of HIV-1 infections, efforts must be made to prevent new acquisition of HIV in healthy population. Although the antiretroviral-based topical (microbicides) and oral pre-exposure prophylaxis (PrEP) products have been evaluated in a number of clinical trials, the results from these studies have been inconsistent. The aim of my dissertation study is to examine whether the modulators of tissue integrity and metabolizing enzymes could affect antiretroviral drug exposure and efficacy in cervicovaginal tissues.

First of all, we demonstrated that the several commonly used excipients which increased paracellular permeability of cervicovaginal tissue did not significantly reduce tenofovir (TFV) activity in HIV-1 prevention in an *ex vivo* explant tissue model. In addition, TEER, morphology, permeability, and MTT-based tissue viability do not necessarily change in parallel with each other and the use of a single measurement cannot accurately reflect the effect of excipients on cervicovaginal tissue integrity. Also, we found that the mRNA of several Phase I and II metabolizing enzyme isoforms, such as CYP1A1, CYP1B1 and UGT1A1, are highly expressed in the human female genital tract. In phosphorylating enzymes study, we found that the majority

of TFV-related phosphorylating enzymes showed significantly decreased mRNA level in T cells as compared to their levels in cervicovaginal tissues. Furthermore, our results indicated that medroxyprogesterone acetate and progesterone treatments changed the activity of phosphorylating enzymes toward different directions in cervicovaginal epithelial cell line (VK2) and a T cell line (PM1). Medroxyprogesterone acetate, progesterone, IL1 $\beta$  and IL8 treatment resulted in altered tenofovir diphosphate level in a vaginal epithelial cell line and a T cell line. Taken together, these studies provide valuable information on the excipients' effect on multiple aspects of cervicovaginal tissue integrity, as well as what biological factors may impact the effectiveness of TFV and its clinical potential. Such information will facilitate the efforts toward optimized vaginal PrEP products for HIV-1 prevention.

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## LIST OF EQUATIONS

Equation 2.1

$$J_{ss} = \frac{Q}{AT} \quad (\text{p } 37)$$

Equation 2.2

$$P_{app} = \frac{dQ}{dt \cdot A \cdot C_d} \times 60 \quad (\text{p } 38)$$

## LIST OF ABBREVIATIONS

**HIV**, human immunodeficiency virus; **AIDS**, acquired immune deficiency syndrome; **PrEP**, pre-exposure prophylaxis; **LFGT**, lower female genital tract; **PK**, pharmacokinetics; **PD**, pharmacodynamics; **NRTI**, nucleoside/nucleotide reverse transcriptase inhibitor; **NNRTI**, non-nucleoside/non-nucleotide reverse transcriptase inhibitor; **TFV**, tenofovir; **TFV-MP**, tenofovir monophosphate; **TFV-DP**, tenofovir diphosphate; **TDF**, tenofovir disoproxil fumarate; **FTC**, emtricitabine; **N-9**, Nonoxynol-9; **DEPC**, diethyl pyrocarbonate; **DNase**, deoxyribonuclease; **cdNA**, complementary DNA; **RT-PCR**, reverse transcriptional polymerase chain reaction; **IHC**, immunohistochemical; **H & E staining**, hematoxylin & eosin staining; **NBF**, neutral buffered formalin; **ANOVA**: Analysis of variance; **PBMCs**, peripheral blood mononuclear cells; **IL**, interleukin; **MPA**, medroxyprogesterone acetate; **P4**, progesterone; **GRAS**, generally-regarded-as-safe;  $P_{app}$ , permeability coefficient; **CPM**, count per minute; **NHP**, non-human primate; **ARV**, antiretroviral; **CYP**, cytochrome P450; **UGT**, uridine 5'-diphospho-glucuronosyltransferase; **API**, active pharmaceutical ingredient; **STI**, sexually transmitted infections; **HSV-2**, herpes simplex virus 2; **H&E stain**, hematoxylin and eosin stain; **DMEM**, Dulbecco's Modification of Eagles Medium, **MTT assay** [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] assay; **PBS**, phosphate buffered saline; **disodium EDTA**, disodium ethyl-enediaminetetraacetate; **BHA**, butylated hydroxyanisole;  $^{14}\text{C}$ -mannitol, D-[1- $^{14}\text{C}$ ]-Mannitol;  $^3\text{H}$ -propranolol, L-[4- $^3\text{H}$ ]-Propranolol; **IACUC**, Institutional Animal Care and Use Committee; **TEER**, transepithelial electrical resistance; human papillomavirus (**HPV**); **FGT**: Female genital tract; **GAPDH**: Glyceraldehyde 3-phosphate; adenylate kinase (**AK2**); adenylate kinase AK3L1 (**AK4**); **NME1** or **NDPKA**, nucleoside diphosphate kinase 1; **NME2** or **NDPKB**, nucleoside diphosphate kinase 2; **CKB**, creatine

kinase, brain; creatine kinase; **CKMT1**, mitochondrial 1; creatine kinase, **CKMT2**, mitochondrial 2; **HAART**, highly active antiretroviral therapy.

## PREFACE

Completing my graduate training would not have been possible without the support and help of many people.

I owe an enormous amount of gratitude to my advisor Dr. Lisa C. Rohan who brought me into her laboratory as a graduate student in 2010. She has been an excellent advisor and providing me in-deep scientific guidance, enormous encouragement and humongous support throughout my graduate study.

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Life is full of ups and downs, but I am lucky enough to have support, comfort, help and love around me.

This work is dedicated to my advisor Dr. Lisa Rohan who brought me into this fantastic field and changed my life.

# 1. INTRODUCTION

## 1.1. Sexual transmission of HIV

### 1.1.1 HIV epidemic

HIV infection remains a global health challenge with an estimated 2.7 million people who are newly infected each year [1]. The untreated infection may lead to Acquired Immune Deficiency Syndrome (AIDS) and cause a weakened immune system, which in turn may ultimately result in opportunistic infections and cancers. Since the first report of AIDS in 1981, millions have died from the disease. Thanks to advances in antiretroviral therapies, to date, patient's life-span has been improved and the death rate decreased by 42% since 2004 [1]. However, despite these advances, there is still no effective vaccine or curative therapy for HIV available. AIDS is still a leading cause of death worldwide and 1.2 million people died of AIDS in 2014. To further reduce AIDS-related deaths as well as the number of HIV infections, efforts must be made to prevent new acquisition of HIV in healthy population, and effective ways to prevent new HIV infections are still desperately needed.

### 1.1.2 HIV mucosal transmission

Among the possible modes of HIV transmission (unprotected sexual intercourse with an infected partner, contact with HIV-infected blood via transfusions and needle sharing, and Mother-to-Child), sexual transmission is the most common in the majority of countries. This is especially true in highly-infected areas in sub-Saharan Africa such as Botswana and Swaziland, where the sexual transmission of HIV accounted for more than 70% of the total new infections[2]. In addition, women continue to be one of the populations at highest risk for HIV

infection [3]. Therefore developing an effective female-controlled biomedical intervention such as a microbicide is a high priority. Microbicides are products that can be applied vaginally or rectally to reduce the transmission of sexually transmitted diseases, including HIV.

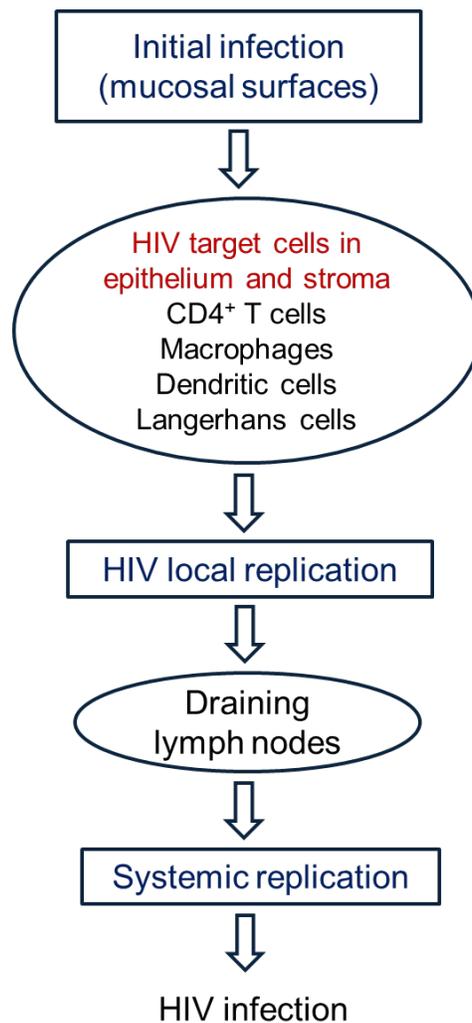
HIV sexual transmission can occur via both vaginal and anal intercourse; and the virus can be found in the body fluids such as semen and vaginal fluid of an infected person. The schematic representation of HIV mucosal transmission is described in Figure 1.1. In women, HIV can pass into systemic circulation by penetrating the lining of the vagina, cervix and uterus[4]. Furthermore the risk of HIV transmission is increased if the woman has an abrasion inside or sore around her vagina [5]; this makes it easier for the virus to reach local immune cells. Anal intercourse carries a higher risk of HIV transmission than vaginal intercourse. This is because the lining of the rectum is columnar cells, which are more delicate than the lining of the vagina (non-keratinized stratified squamous epithelium) and more likely to be damaged during sex [6].

## **1.2. Developing topical microbicides to prevent the sexual transmission of HIV**

### **1.2.1 The promise and challenges in microbicide product development**

To prevent HIV sexual transmission, we need to target early events in HIV infection [7, 8]. Based on the non-human primate (NHP) model, the very early events of local HIV transmission is the most vulnerable step in the HIV replication cycle. As such, this time period offers the best opportunity for preventing viral expansion. Supported by these findings, we now know viral particles need to infect CD4+ host immune cells within the mucosal tissues and establish early infection [9]. It is critical to prevent or significantly reduce this early infection to curtail later replication and dissemination of HIV-infected immune cells. However, if the virus does

successfully establish local expansion and spread into the draining lymph nodes, eradicating the virus from the body becomes much more challenging. Therefore, the goal of therapies aimed at preventing HIV sexual transmission is to eliminate the virus before it successfully expands in the local tissue, including the genital tract and rectal tissue.



**Figure 1.1 Schematic representation of HIV mucosal transmission**

There has been great interest in developing antiretroviral (ARV)-based prevention strategies to reduce HIV sexual transmission [10]. Pre-exposure prophylaxis (PrEP) refers to the process of taking ARVs before HIV exposure to avoid infection. PrEP includes both oral and topical drug products, as well as other routes of administration under development such as intrauterine device, injectable, and implant. In 2012 FDA approved Truvada (emtricitabine, FTC/tenofovir disoproxil fumarate, TDF) as the first oral PrEP product to be used daily to help people at high risk avoid infection with HIV.

Vaginal microbicides are products that can be applied directly to the vagina, a primary mucosal site of HIV infection. Compared to oral administration, vaginal products allow for higher drug concentrations at the site of viral entry, which potentially leads to a greater PrEP efficacy. However, the results from clinical trials to date are not reproducible enough to demonstrate the effectiveness of this prevention strategy [11]. For example, 1% tenofovir (TFV), vaginal gel was able to reduce HIV incidence by 39% in the CAPRISA 004 trial [12]; however, the VOICE and FACTS trials using the same dosing regimen showed no efficacy of the TFV 1% vaginal gel [13-15]. The results of some TFV-based PrEP clinical trials are summarized in Table 1.1[11, 12, 15-24]. Besides the clinical data, a discrepancy between preclinical and clinical results has also been observed for several other microbicide candidates evaluated [25, 26]. For the oral PrEP trials, the iPrEX study which involved daily oral use of Truvada reduced the HIV acquisition by 44% in men who had sex with men (MSM) [17], whereas FEM-PrEP and VOICE trials showed no statistically significant efficacy in HIV-negative women[27, 28]. It has been well accepted that the adherence is one of the keys justifying the difference among trials, which in turn can impact the level of active drug in colorectal and vaginal tissues. Studies of oral TFV have

revealed that TFV and tenofovir diphosphate (TFV-DP) levels are more than 100 times higher in colorectal tissue than in cervical/vaginal tissue after a single oral dose. As such, there is an urgent need to enhance the effectiveness of microbicide products to optimize their clinical potential.

The vaginal and ectocervical mucosal surface serves as the portals for heterosexual transmission of HIV and therefore play a fundamental role in the pathogenesis of the primary infection. The outer layers of the vaginal epithelium consist of epithelial cells, followed by stromal tissue underneath. The cells targeted by HIV, including CD4<sup>+</sup> T cells, macrophages, and dendritic (Langerhans) cells, can reside in both the vaginal epithelium and stroma (Figure 1.2), both of which are vulnerable to HIV penetration and infection[7]. After vaginal intercourse, HIV replication begins slowly in the resident immune cells in the vaginal lumen or tissue for several hours expanding to recruited immune cells and transmission to draining lymphnodes. Therefore, the goal of microbicide delivery is to ensure sufficient drug exposure in the tissue prior to HIV exposure [29]. That is to say, the drug molecules should be able to penetrate the epithelium, and be maintained above the therapeutic level for sufficient time to completely protect target cells from surrounding viral particles.

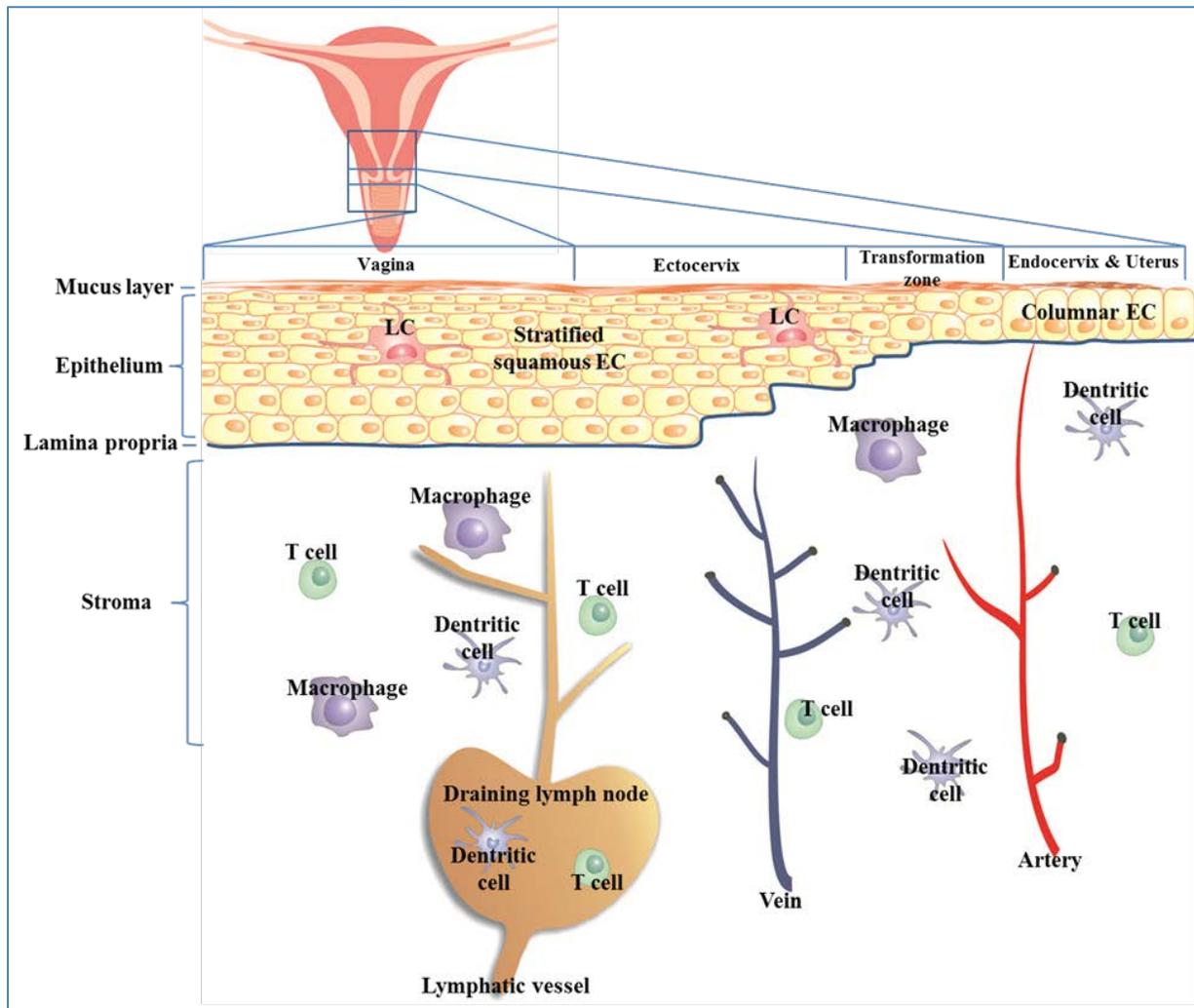
**Table 1.1 Tenofovir-based PrEP Clinical Trials Summary**

The data was generated from reference [11, 12, 15-24]

Study	Population	Route/Dosing	Efficacy (95 % CI)
<b>CAPRISA 004</b> <i>South Africa</i>	Women (n=889)	vaginal TFV gel (12 hours before/after vaginal intercourse )	39% (6, 60)
<b>VOICE</b> <i>South Africa, Uganda, Zimbabwe</i>	Women (n=5029)	vaginal TFV gel (daily)	Stopped for futility
		oral TDF (daily)	Stopped for futility
		oral FTC/TDF (daily)	no difference as compared to placebo group*
<b>Partners PrEP Study</b> <i>Kenya, Uganda</i>	Serodiscordant couples (n=4747)	oral TDF(daily)	Men, 55% (4, 97)
			Women, 68% (29, 85)
<b>TDF2 Study</b> <i>Botswana</i>	Heterosexual Men (n=656)	oral FTC/TDF(daily)	80.1% (24.6, 96.9)
	Heterosexual Men (n=544)	oral FTC/TDF(daily)	49% (21.7, 80.8)
<b>FEM-PrEP</b> <i>Kenya, South Africa, Tanzania</i>	Women (n=1950)	oral FTC/TDF(daily)	Stopped for futility
<b>FACTS</b> <i>South Africa</i>	Women (n=2029)	vaginal TFV gel (12 hours before/after vaginal intercourse )	no difference between TFV gel (n=6) and placebo gel (n=62) infections

TFV: tenofovir; TDF: tenofovir disoproxil fumarate ; FTC: emtricitabine

\* Similar HIV-1 incidence (cases per 100 person/year, 95% CI) between oral FTC/TDF group (4.7, 3.6–6.1) and placebo group (4.6, 3.5–5.9)[24].



**Figure 1.2 Anatomy of the female genital tract[30]**

LC, Langerhan's cells; EC, epithelial cells

1.2.2 PK/PD relationship of microbicide drugs: the drug tissue level is the key for microbicide efficacy

Most of the microbicides in development utilize anti-retroviral agents as the active ingredient [12, 31-41]. These microbicides specifically protect host cells by disrupting the viral replication cycle [11, 12, 15-24] (Table 1.2), and most of them must penetrate into the cervicovaginal tissue to take effect, as their target cells reside within the tissue. As discussed previously, once HIV penetrates into the vaginal and cervical tissue, the virus can infect resident immune cells before dissemination to local lymphnode, which can take several hours. Therefore, sufficient drug exposure in the tissue is crucial for the efficacy of microbicides. However clinical observations suggest this is not an easy goal to achieve.

**Table 1.2 Mechanism of action of different microbicides candidates**

<b>Mechanism of action</b>	<b>Candidates</b>
Gp120-binding	BMS-806, DS003
Gp41-binding	C52L and T1249
CCR5 inhibitor	PSC-RANTES, CMPD167, Maraviroc
NRTI	Tenofovir, EFdA
NNRTI	MIV-150, TMC120, Dapivirine
Integrase inhibitor	Raltegravir, MK2048

In FACT 001 and other studies, the TFV disposition in cervicovaginal tissue has been analyzed in detail[15]. These studies revealed that a small percentage of vaginally-administered TFV entered tissue, and a tiny portion of the drug in the tissue was converted to the active form TFV

diphosphate [15, 42]. Furthermore, there is large variability in tissue exposure among participants, and the tissue exposure correlated with prevention outcome. In the CAPRISA 004 trial, the HIV incidence rate in women with a vaginal fluid TFV concentration <1000ng/ml (low-exposure group) did not differ from the placebo group. However, the incidence rate was 3 times lower in women with a vaginal fluid TFV concentration >1000ng/ml (high-exposure group) [12]. In the MTN-006 trial, rectal safety, acceptability and pharmacokinetic/pharmacodynamic (PK/PD) of 1% TFV rectal gel were studied. The rectal TFV-DP reached  $C_{max}$  30 min after single rectal exposure and the *in vivo* exposure was significantly correlated with *ex vivo* tissue infectibility suppression (p=0.002) [43]. Other microbicide candidates also exhibit *in vitro-in vivo* correlations. In RMP-01 study, UC781-exposed human colorectal biopsies were cultured *ex vivo* and challenged with HIV [44]. UC781 efficacy was shown to be positively correlated with its concentration in tissue, as reflected by a high EC90:EC50 ratio.

There have been several studies indicating that a higher exposure of microbicide in the vaginal tissue correlates to a lower HIV infection rate [45, 46]. It was suggested that one goal for microbicide delivery in future trials should be achieving the highest tolerable drug concentration in the vagina [45]. There have been efforts in improving drug exposure/concentration in local tissue, such as increasing dose, mucosal-adhesive formulation, sustained-release intravaginal rings, and incorporation of permeation enhancers into microbicide formulation. But there are potential problems associated with these delivering systems such as alterations to the vaginal microbiota and tissue [25, 47-50]. Alternative strategies are needed to increase tissue exposure and efficacy of microbicides. Because the microbicide drug exposure in mucosal tissues positively correlates with HIV prevention efficacy [43, 44, 51], it is necessary to understand the

regulation of the critical determinants of antiretroviral drug concentration in cervicovaginal tissues. This information will benefit the development of novel approaches for microbicide optimization, and will inform the design and interpretation of microbicide PK-PD studies.

### 1.2.3 Sufficient tissue drug concentrations are difficult to achieve

However, due to both behavioral and physiological reasons, it is not easy to achieve sufficient drug concentration in the local tissues even with topical microbicide products. Poor participant adherence observed in the clinical trials of once-daily oral and topical PrEP products is one important factor resulting in the low drug efficacy, but not the only reason[52]. For topically-administered microbicides drug candidates, absorption could play a role. First of all, the mucus layer and the epithelium (made up of tight junctional proteins) of the cervicovaginal and colorectal tracts, consists of primary permeation barriers against drug molecules penetrating into the local tissues, blood, and lymphatic drainage systems which function to extract the drug from the tissue [53]. This is especially true for hydrophilic drugs that have low binding affinity to tissue proteins. Therefore, novel strategies that can increase the tissue penetration of hydrophilic drugs will facilitate the development of topical microbicides. In addition, intracellular enzymes have the potential to metabolize the drug and turn it into its inactive form. For nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) conversion of the drug to its diphosphate/triphosphate form by nucleoside phosphorylating enzymes is necessary for drug efficacy[54]. However, the amount of the parent drug available for diphosphate or triphosphate conversion may be reduced if the NRTIs can be metabolized by other enzyme systems such as cytochrome P450 (CYP) and/or uridine 5'-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT) [55].

As discussed above, microbicides are a promising means to prevent HIV sexual transmission, but the effectiveness of microbicides are inconsistent in the various clinical trials. Therefore, it is crucial to enhance the effectiveness of microbicides to increase their clinical potential. Toward this goal, we need to understand the effect of patient-related and product-related factors on microbicide effectiveness. Several “product-related” and “patient-related” issues have been raised during the studies of microbicides. One issue is the effect of excipients (product-related factor) on cervicovaginal tissue epithelial barrier integrity, which may impact microbicide efficacy. The other issue is the effect of contraceptive hormones and inflammation on the expression and activity of metabolizing enzymes.

#### 1.2.4 Tenofovir (TFV) is a promising microbicide candidate

Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are one of the most critical components of both HIV treatment and pre-exposure prophylaxis (PrEP) [54]. Currently in the US, the initial treatment of HIV infected individuals includes two NRTIs and a third drug from a different class: an integrase strand transfer inhibitor, a non-nucleoside reverse transcriptase inhibitor, or a protease inhibitor with a pharmacokinetic enhancer (cobicistat or ritonavir) [54]. In 2012, the U.S. FDA approved Truvada® (one oral tablet per day) for PrEP in uninfected high risk individuals. Truvada is a combination of FTC and TDF. TDF is the prodrug of TFV which is one of the most widely studied NRTIs in the field of microbicides [56]. It has superior efficacy to other traditional NTRIs such as zidovudine and abacavir, a short- and long-term safety profile, and a low propensity for mitochondrial toxicity make it a favored first-line agent [57, 58].

TFV is a very hydrophilic compound ( $\log D_{7.4} = -3.55$ ) containing two negative charges (two pKa values of 2 and 6.8 respectively) resulting in low intestinal permeability and oral bioavailability, and it is classified as a BCS class III compound [58-60]. To overcome its permeability issues, a prodrug TDF is developed to increase its lipophilicity for oral dosage forms. While vaginally and rectally administered TFV can directly reach the target tissues, orally administered TFV cannot efficiently pass through the gut barrier to reach the genital tissues. Therefore, the prodrug form TDF is used in marketed oral Truvada tablet. TDF is readily absorbed and converted to TFV in the intestinal lumen and plasma by diester hydrolysis [61]. TFV is a nucleotide analogue of deoxyadenosine monophosphate, which requires intracellular phosphorylation to its active diphosphate anabolites. After being taken into cells, TFV is subsequently phosphorylated into the active metabolite, TFV-DP. TFV-DP competes in the cell with the endogenous substrate dATP for incorporation into HIV DNA, and the incorporation of TFV-DP terminates the DNA chain and interrupts HIV synthesis.

TFV has been developed into various dosage forms as both a vaginal and rectal microbicide to combat the transmission of HIV, such as gels (vaginal/rectal), films (vaginal), intravaginal rings (vaginal) and suppository [15, 24, 62-65], but little is known about its metabolism/activation in vaginal or rectal epithelium and immune cells under conditions that are commonly encountered by a potential microbicide user, such as the menstrual cycle, contraceptive use, and tissue inflammation. Since TFV only works after being converted to its active form TFV-DP, the expression and function of TFV-related phosphorylating enzymes (PEs) in tissues and cells relevant to HIV sexual transmission were examined in this dissertation.

### **1.3. Understanding the excipients' effect on cervicovaginal tissue epithelial barrier**

#### **1.3.1 Excipients are crucial components in microbicide formulations**

Pharmaceutical excipients are chemicals that help formulate the active pharmaceutical ingredient (API) into ready-for-use drug products, and play crucial roles in drug formulations. Currently over 1000 materials are used in marketed pharmaceutical products and have various functions such as diluents/fillers, adhesives, disintegrants, lubricants, colors, flavors, coating agents, polishing agents, fragrances, sweetening agents, polymers, and waxes. Traditionally, they are considered as inert ingredients, generally regarded as safe (GRAS) and have no negative effect on human/animal. For vaginal drug delivery systems, excipients help increase the solubility/compatibility/patient acceptability of the API and help maintain the physicochemical stability of the API in the formulated products [66-68]. They are needed for efficient *in vivo* delivery of the API and ensure specific properties of the formulation, such as retention and spreading properties (e.g., viscosity enhancers), stability (e.g., preservatives and humectants), and solubilizing capacity (e.g., solvents/cosolvents, surfactants, and cyclodextrins) [69].

#### **1.3.2 Excipients' impact on the tissue epithelium barrier function**

There have been published reports indicate that some excipients can promote the transport of drugs, such as fatty acid derivatives (e.g. sodium caprylate, sodium caprate, glycerides), surfactants (e.g. polysorbate-80, phosphatidylcholine, sodium salicylate), while others possessed different mechanisms of enhanced drug transport (e.g. EDTA disodium, alcohols) through the skin and the colon [70-74]. The mechanism of these enhancers include: altering the rheological properties of the mucus layer, increasing the thermodynamic properties of the peptide, enhancing

transcellular transport by interacting with phospholipids and/or proteins to increase membrane fluidity and enhancing paracellular transport.

### 1.3.3 The effect of excipients on the tissue epithelial barrier

It may be desired to increase the drug permeability in tissues such as skin and colon. However, we must be cautious when utilizing permeation-enhancing excipients in microbicide formulations, because we don't know whether these excipients are safe to use in the scenario of HIV prevention. The opening of intercellular junctions is not a problem in the delivery of other kinds of drugs, because this opening only causes reversible mild irritation [75]. However, in HIV prevention, excessive opening of the intercellular space might compromise the epithelial integrity. While we might be able to improve drug permeation into tissue, the virus entry may also be facilitated through the intact epithelium, which potentially may result in a net increase in infection risk.

Most of the over-the-counter (OTC) vaginal and rectal products, such as lubricants, typically do not contain pharmacologically active agents that have anti-HIV activity. However, most of those products, if not all, do contain several ingredients or excipients that can contribute to both the physical and chemical properties, such as osmolarity, viscosity, water content, and stability, of the final product. It has been published that some OTC lubricants, such as Astroglide, may have anti-HIV activity resulted by pharmaceutical excipients present in the product [76]. Dezzutti et al tested if the removal of epithelial barrier could increase the HIV-1 infection susceptibility of the explant tissue. They found two-hour-exposure to 0.1% of EDTA increased in HIV-1 replication in the human ectocervical explant tissue as compared to the untreated tissue [77].

In fact, a number of excipients with diverse functions are frequently used in vaginally administered drug products, including the vaginal microbicides tested in preclinical and clinical studies for topical preexposure prophylaxis (PrEP) of sexually transmitted HIV-1 [47, 78-80]. Moench et al. examined several excipients' effect on genital herpes transmission and found that 30% glycerin, undiluted propylene glycol and PEG-8 greatly increased susceptibility to HSV-2 in a mouse model [81]. Gali et al. evaluated the safety of excipients commonly used in vaginal microbicide preparations, including preservatives, cosolvents, surfactants, and cyclodextrins, using HEC-1A cell cultures and cervical explants [74, 82]. They found that most excipients at concentrations near the typical concentration used in vaginal gels compromised cell viability, and the effect was ranked as sodium lauryl sulfate = TPGS 1000 > polysorbate 80 > Cremophor EL (low toxicity). McClelland et al evaluated the effect of vaginal washing on the incidence of HIV-1 infection in 1270 African women between 1993 and 2003. They found that women who partake in vaginal washing, by vaginal douching or washing with water and soap, had higher HIV acquisition risk as compared with women who did not perform vaginal washing. In addition, women who performed vaginal washing with soap had higher risk for HIV-1 infection compared with those who used water alone [83]. Although these findings are still at an early stage, evaluation of safety in the use of pharmaceutical excipients in microbicide product development should be taken into more consideration.

The human vaginal and ectocervical epithelium can be comprised of up to 40 layers of epithelial cells with extensive expression of intercellular tight junctions (TJ). This multi-layered epithelium constitutes a natural barrier to virus [53]. TJ proteins have been reported to limit the penetration

of hydrophilic drugs into mucosal tissues and tissue barriers such as the small intestinal columnar epithelium and blood-brain barrier [84, 85]. In addition, many agents, including hormones and pharmaceutical excipients, have been reported to modulate TJ protein expression and paracellular permeability of the epithelial barriers [86-89]. Several TJ proteins have been reported to be expressed in cervicovaginal epithelial cells [53], and the effect of sex hormones on cervicovaginal TJ have been studied. However the effect of commonly used excipients on TJ proteins and paracellular permeability has not been examined. There is an urgent need to understand this effect since every microbicide product contains multiple excipients in the formulation.

Further, some studies revealed the importance of cervicovaginal tissue barrier function in combating HIV infection [77, 90], so it is necessary to examine the safety of excipients in microbicide products, i.e., whether the excipients could negatively affect tissue integrity, increase tissue vulnerability to HIV infection, or decrease TFV efficacy in HIV prevention.

#### **1.4. Understanding the regulation of metabolizing enzymes for topical microbicide development**

Metabolizing enzymes have been reported to eliminate or activate antiretroviral drugs. The cytochrome P450 (CYP) superfamily is the most important group of Phase I enzymes which catalyze about 90% of the oxidation reactions, such as aromatic hydroxylation, aliphatic hydroxylation, N-oxidation, oxidative desalkylation and epoxide formation[91]. Uridine diphosphate-dependent glucuronosyltransferase (UGT) is one of the key Phase II enzymes which transfer a glucuronide moiety to the molecules. This reaction makes xenobiotic compound more

hydrophilic and is critical for the clearance of most drugs, dietary substances, toxins and endogenous substances [92, 93]. Besides the enzymes that cause elimination of the drugs, there are enzymes that activate antiretroviral drugs in cells as well, such as phosphorylating enzymes activating NRTIs in the cells. Together, these enzymes play an important role in the intracellular pharmacokinetics of antiretroviral drugs. For example, azidothymidine (AZT), an NRTI, was the first U.S. FDA approved drug for HIV treatment. In humans, AZT can be metabolized by CYP and UGT enzymes to 3'-amino-3'-deoxythymidine and zidovudine-5'-glucuronide respectively, and neither of these compounds has antiviral activity [94, 95]. However, AZT can be phosphorylated to AZT-TP to terminate viral DNA chain elongation [95, 96].

The enzyme expression and/or activity can be modulated by hormones, drugs, nutrients, and disease status, as revealed in other tissue types such as liver and lung [97, 98]. However, only sporadic reports on the expression of cervicovaginal metabolizing enzymes exist [99-101], and there is a lack of systematic characterization of enzyme expression in this female genital tract tissues. In addition there is no information about the factors that can regulate cervicovaginal enzyme expression and activity. Therefore, it is necessary to systematically examine the expression of metabolizing enzymes (CYPs, UGTs, phosphorylating enzymes) in human cervicovaginal tissues, identify the modulating factors of these enzymes, and understand how these factors impact the ARVs' concentration.

#### 1.4.1 Understanding the role of CYP and UGT enzymes for topical microbicide research

CYP and UGT enzymes are located in the cell reticulum and affect the residence time of their substrate drugs within the cells. Some of the common human isoforms of CYP and UGT enzymes

are CYPs 1A2, 2C9, 2C19, 2D6, 3A4, UGTs 1A1, 1A4 and UGT2B7[92, 93]. It has been well established that these enzymes control the disposition of administered drugs in various tissues, such as liver, kidney, and brain [102-104]. However, further investigations on cervicovaginal enzymes are hampered by the lack of information on enzyme expression in the ectocervix and vagina of premenopausal women, which are the major sites for microbicide disposition and action. Traditionally, vaginal tissue was thought to lack common drug-metabolizing enzymes, including CYP3A4 [105, 106] . However, more recent reports indicated the presence of some enzymes in human female reproductive tract[107]. In fact, many anti-HIV drugs are substrates for CYPs and UGTs (Table 1.3 [95, 96, 108-127]). Representative marketed anti-HIV drugs from each class are shown. Since the metabolism studies of anti-HIV drugs have been increasingly carried out, it is necessary to explore the CYP and UGT's expression and function in genital tract when developing topical microbicide products that include anti-retroviral as the active pharmaceutical ingredients. The data collected from these studies could help interpret (predict) the drug efficacy and safety, as well as potential drug-drug-interaction.

Biopharmaceutics Classification System (BCS) categorizes drug molecules into 4 classes according to their permeability and solubility: Class 1, high permeability, high solubility; Class 2, high permeability, low solubility; Class 3, low permeability, high solubility; Class 4, low permeability, low solubility [128, 129]. The BCS classification, and the interaction with CYP and UGT enzymes are summarized in Table 1.3.

According to the BCS, Class 1 and Class 2 compounds (with high permeability) are eliminated primarily via metabolism through CYPs, UGTs, etc., whereas Class 3 and Class 4 compounds are primarily eliminated in their unchanged form into the urine and bile [130]. The most new molecular entities are Class 2 compounds. For such compounds developed as topical microbicides that are substrates for phase 1 and Phase 2 metabolizing enzymes, mucosal transporter-enzyme interplay will have an important role in their tissue disposition and elimination.

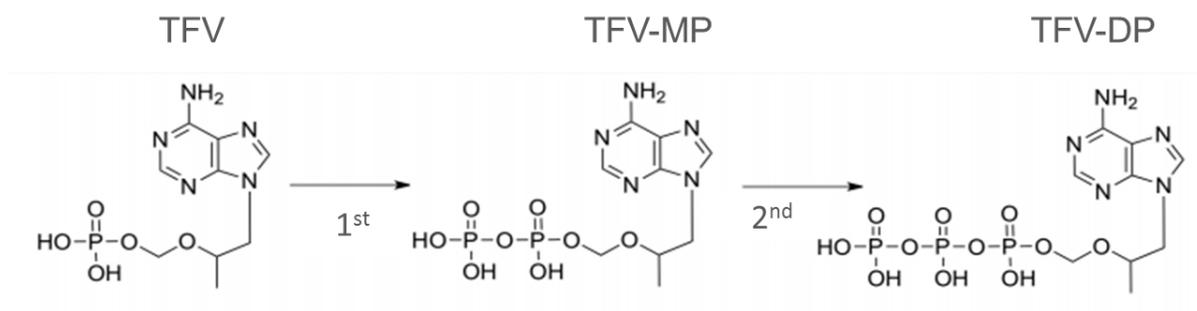
#### 1.4.2 The role of phosphorylating enzymes in tenofovir activation

Kinases and phosphatases are enzymes catalyzing the transfer of phosphate group between phosphate donor and their substrates. Although they both are enzymes that can transfer phosphate, they catalyze opposing reactions. Phosphatases are hydrolases that can remove a phosphate group from a substrate while kinases typically transfer the terminal phosphate group from ATP or GTP to their substrate and results in a phosphate-ester bond in the product. Protein phosphorylation and dephosphorylation are the major posttranslational modifications that regulate the functions of cellular proteins for a wide spectrum of cellular regulation and function by inducing conformational changes or disruption and creation of protein-protein interaction surfaces[131]. It has been reported that protein kinases phosphorylate more than 30% of the cellular proteins [132].

Among various kinase, nucleoside/nucleotide kinases play an crucial role in maintaining equilibrium between the concentrations of different nucleoside triphosphates, cell proliferation, differentiation and development, signal transduction, endocytosis, and gene expression[133]. For example, creatine kinases (CK) catalyze the conversion of creatine and utilizes adenosine

triphosphate (ATP) to adenosine diphosphate and create phosphocreatine which is a cellular transport form of energy between subcellular ATP and ATPases [134]. CK can enhance skeletal, cardiac, and smooth muscle contractility, and therefore reside in tissues and cells where consume ATP rapidly, such as skeletal muscle [135] and brain [136]. It has also been reported that T lymphocytes have high phosphorylation as as compare to relative to dephosphorylation, these cells accumulate dATP and dGTP[137], while B cells are of higher dephosphorylation activity (5'-nucleotidase activity) [138].

Phosphorylating enzymes (PE) activate NRTIs by adding phosphate groups to the compounds; these activated forms are critical for NRTI-based microbicides against HIV transmission [17]. For example, the NRTI TFV is metabolized to its active form, TFV-DP, via phosphorylation within cells, where it can then compete with the endogenous substrate dATP for incorporation into viral DNA[139] (Figure 1.3). The incorporation of TFV-DP terminates the viral DNA chain and stops HIV synthesis. This means, to be effective, TFV requires intracellular phosphorylation to its active form.



**Figure 1.3 Intracellular activation of TFV**

**Table 1.3 Summary of drug metabolizing enzymes and transporters for ARVs**

<b>Class</b>	<b>Anti-HIV drugs</b>	<b>BCS Class</b>	<b>Phase I enzymes (CYPs)</b>	<b>Phase II enzymes (UGTs)</b>	<b>Drug transporters</b>
NRTI	Zidovudine	1	CYP2B, 2C9, 3A, 4A	UGT2B7	BCRP, MRP4 OAT1, OAT2, OAT3, CNT1, CNT3, ENT2
NNRTI	Delavirdine	1	CYP2D6, 3A4		
	Efavirenz	2	CYP2B6	UGT1A1, UGT2B7	
	Nevirapine	2	CYP2B6, 2D6, 3A4		MRP7
Protease Inhibitor	Amprenavir	2	CYP2C9, 3A4, 3A5		P-gp
	Nelfinavir	2	CYP2C9, 2C19, 2D6, 3A4		P-gp
	Atazanavir	2	CYP3A4		P-gp, MRP1, MRP2
	Ritonavir	2	CYP2D6, 3A4		P-gp, MRP1, MRP2
Integrase Inhibitor	Raltegravir	2		UGT1A1	P-gp
	Elvitegravir	2	CYP3A	UGT1A1, UGT 1A3	
	Dolutegravir	2		UGT1A1	
Entry Inhibitor	Maraviroc	3	CYP3A5		P-gp, OATP1B1

\* This table is summarized from references [95, 96, 108-127].

As a nucleotide analogue, TFV has a phosphonate group bonded to the alkyl side-chain, and requires only two phosphorylation steps for activation. Robbins et al. and other groups have reported that adenylate kinase 2 (AK2) probably is the dominant enzyme that catalyzes the second phosphate group to TFV [140, 141]. It was previously reported that the nucleoside diphosphate kinases (NDPKs/NMEs) were the key enzymes that catalyze the last phosphorylation step [142]. However, several other groups have shown that creatine kinase and 3-phosphoglycerate kinase are more likely involved in the final conversion of TFV [143-145]. More recently, Lade et al. reported that different NDPKs may play a role in the conversion of TFV-MP to TFV-DP in a tissue specific manner. For example, pyruvate kinase (PK) appears to be active in PBMCs and vaginal tissue, while creatine kinase appears to be responsible for the formation of TFV-DP in colon tissue [146]. As topical microbicides are designed to be directly applied to vagina or rectum, and oral PrEP product, such as Truvada, can distribute to both vaginal and rectal tissues, it is important to understand the PE's expression on both mucosal sites. However, there are limited data available regarding the expression of PEs in the female genital and colorectal tissue relative to immune cells. Therefore, understanding the expression and function of these phosphorylating enzymes will greatly aid in the study of TFV activation in the female genital tract, including cervicovaginal epithelial cells and immune cells.

#### 1.4.3 Knowledge gaps in current microbicide research regarding phosphorylating enzymes

As discussed above, PEs are critical for TFV activation; however, there are several knowledge gaps in current microbicide research regarding these enzymes and how they impact ARV-based prevention strategies. Since topical microbicides are designed to be applied vaginally or rectally, we face the issue of whether PEs have similar expression and activity in cervicovaginal and

colorectal tissues, as well as in immune cells. In a small PK study conducted at the University of North Carolina, seven healthy women were given a single oral dose of Truvada, and the TFV-DP levels in vaginal and rectal tissues were examined 24 hours after the administration. The investigators found that the concentration of TFV-DP in vaginal tissue was significantly lower than that in the rectal tissue [147]. This variation of TFV-DP levels in different tissues might be attributed to the enzyme variability between the vaginal and rectal compartments.

In another study, Biswas et al. found that several nucleotidases including NT5E, NT5C2, NT5C3L and NT5C showed significantly different expression patterns in human T cells and cervix epithelial cells[148]. Although nucleotidases catalyze the hydrolysis of a nucleotide into a nucleoside and a phosphate and perform the opposite function of PEs, this finding raises the concerns that certain PEs may also have different expression levels in different cell types. Since immune cells are main target cells of HIV, it is necessary to explore the enzyme expression and activity in different cell/tissue types to better understand the ARVs' activation and clearance in the transmission-related tissue and cells.

#### 1.4.4 The potential impact of contraceptive hormones and inflammatory cytokines on PE activity

Hormonal contraceptives (HC), especially progesterone based contraceptives, such as medroxyprogesterone acetate (MPA) injectable contraceptives are commonly used by women in sub-Saharan Africa where the HIV risk is [149, 150]. However, little is known about how HCs could impact PEs activity in cells/tissues [149]. In addition, the association between HCs on HIV-1 acquisition by women is still unclear. There are studies indicating an increased risk of

HIV-1 acquisition and transmission with HC. Heffron et al studied the association between HC use and risk of HIV-1 acquisition by women in South Africa. They found that the rates of HIV-1 acquisition were significant higher in women who used HCs ( $p=0.03$ ) as compared to those who did not [151]. Mitchell et al investigated the effect of MPA on lymphocyte composition in female reproductive tract mucosal tissue. They found that MPA significantly decreased median CD4+ T cell and CD3+ T cell number in vaginal mucosal tissue 12 months after the MPA injection [152]. Coleman et al reported that in an exploratory secondary data analysis of the MTN-001 study, after a daily oral dose of TDF over six weeks, the women using injectable hormonal contraceptives showed significantly decreased TFV-DP levels (~73%) in their PBMC as compared to non-hormone users[100]. However, since this was a secondary data analysis and the study was not designed to address this particular question, the information about the specific type of injectable hormone contraceptives being used by the participants was not collected. Nevertheless, these data substantiated our concerns on the effect of hormonal contraceptives on PEs activity. Considering that the majority of potential microbicide users are likely women of reproductive age and using of some type of HC, it is necessary to investigate whether or not HCs impact PE activity and NRTI conversion [153].

Besides HCs, other biological factors may also impact HIV acquisition and ARVs' activation. Inflammatory cytokines such as interleukin-8 (IL-8) in the macaque genital tract was found to be essential for establishing a vaginal simian immunodeficiency virus (SIV) infection model [154]. In humans, it has been reported the association between increased levels of cytokines observed among those with greatly susceptibility to HIV. Masson et al investigated whether genital inflammation impacts HIV acquisition in young women with high HIV incidence rates in Africa

and found that a genital inflammatory profile contributes to the high risk of HIV acquisition [155]. STIs that cause inflammation, such as herpes simplex virus-2 infection, cervicitis, and vaginosis, are associated with increased susceptibility to HIV-1 infection [156-160]. Mlisana et al reported that 12 pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-8 were significantly higher in women with STIs compared with women who did not have an STI [161]. Among reproductive tract infections, bacterial vaginosis (BV) is the most common cause of vaginitis among reproductive-aged women. The estimates of its prevalence depend on the population studied. In the US, BV prevalence is between 17–19% in family planning clinics and increases to 24–40% in sexually transmitted disease clinics [101]. Increased levels of IL-8 and IL-1 $\beta$  have been found in vaginal fluid obtained from women with BV [162, 163]. For these reasons, we decided to examine the potential impact of contraceptive hormones and inflammatory cytokines on PE expression and TFV conversion.

#### 1.4.5 Considerations in studying metabolizing enzymes for PrEP research

To study the role of PEs in cervicovaginal, colorectal, and immune cells in antiretroviral drug pharmacokinetics, we need to test whether the pharmacokinetic profile of the drugs of interest in transmission-related tissues and cells can be significantly altered by various factors, such as menstrual cycle stages, hormonal contraceptive use, as well as genital tissue inflammation. The potential preclinical models utilized for this purpose could be excised human tissues, *ex vivo* explant cultures, cell cultures and animal tissues. There are several important issues to consider in developing these models.

Currently utilized preclinical models for microbicide testing (safety and efficacy) can be categorized into *in vitro*, *ex vivo*, and *in vivo* models. The *in vitro* models include cancerous cell lines Hec-1-A, HeLa, Caski, Caco-2, as well as End1/E6E7, Ect1/E6E7 and VK2/E6E7 [18, 20, 21]. End1/E6E7, Ect1/E6E7 and VK2/E6E7 cell lines are derived from normal cervicovaginal epithelium but immortalized by stable-transfected HPV proteins E6 and E7. The *ex vivo* models include ectocervical, vaginal and colorectal tissue explants [11, 22, 23]. The *in vivo* models include mouse, rabbit, sheep, bovine, and macaque [31-33, 164-167]. These preclinical models have been used to evaluate both the safety and efficacy of microbicides such as vaginal irritation, drug distribution, tissue absorption, and drug EC<sub>50</sub>, etc.

Since there is no established model for studying the metabolizing enzyme function in cervicovaginal/colorectal tissue; model characterization and validation work is needed. This can be initiated from examining enzyme expression and localization in the transmission-related tissues and cells of the preclinical models. A comparison of enzyme characteristics between the models and intact human tissues could also enhance our understanding on model validity in microbicides development.

Currently, human PK studies of microbicides are mainly conducted by collecting biopsied tissue (vaginal, cervical and/or rectal) and by analyzing the drug tissue concentration. However, ARV concentration in tissue may not directly reflect their levels in the HIV target cells. A recent study conducted in our lab using excised human ectocervical tissue showed that dapivirine (an NNRTI), released from a vaginal film, accumulated near the basal layers of cervicovaginal epithelium throughout the upper part of the stroma, while the amount of drug reaching the deep stroma was

very low [168]. This observation led us to consider the design of microbicide pharmacokinetic/pharmacodynamics (PK/PD) studies and the interpretation of the results, especially during early-phase clinical trials. In these trials, the effectiveness of microbicide products is examined by the *ex vivo* challenge assay, where cervical and vaginal tissue biopsies are taken from product-exposed participants, then the tissues are exposed to HIV and cultured *ex vivo* for several days followed by determination of HIV replication. Since the biopsies are small pieces of tissue and usually superficial samplings, the drug concentrations measured in these samples mainly reflect the epithelial layers and upper part of the stroma. They do not necessarily reflect drug concentration in the deeper part of the stroma. In the case of dapivirine, the PK/PD results obtained using tissue biopsies tend to overestimate the drug's capability of penetrating the tissue and preventing HIV infection *in vivo*[169]. Since dapivirine mainly cumulated in the epithelium, the drug level in biopsy samples might be much higher than its level in deeper tissue like stroma. As the immune cells are the main targets for HIV, to understand what factors could potentially impact microbicides efficacy, it is important to study the PEs functions in both immune cells and epithelial cells.

### **1.5. Hypothesis and specific aims**

As discussed above, microbicides are a promising means to prevent HIV sexual transmission, but the effectiveness of microbicides, especially TFV-containing microbicides, is not robust and reproducible. We need to understand specific factors that may affect microbicide efficacy. Toward this goal, we need to understand the effect of patient-related and product-related factors on microbicide effectiveness. Several issues have been raised during the studies of microbicides. One issue is the effect of excipients (product-related factor) on cervicovaginal tissue epithelial

barrier. Pharmaceutical excipients present in topical microbicides formulations can potentially irritate mucosal epithelium which would lead to compromised barrier function through affect the cellular junction protein, and/or immune reaction that could increase the susceptibility of HIV infection. These excipients also impact drug metabolism in genital tract and rectal tissues. The other issue is the effect of contraceptive hormones and inflammation (product-related and patient-related factors) on the expression and activity of metabolizing enzymes. Excipients are widely used in microbicide formulations and some excipients have been reported to alter the tight junction proteins (epithelial barrier) [170, 171], so it is necessary to examine the safety of excipients in microbicide products. In addition, enzymatic conversion is an important step in PrEP drug activation or deactivation. The enzymes that may affect certain classes of ARV drug metabolism and effectiveness include CYPs, UGTs, and PEs. Furthermore, it is necessary to examine the effect of PrEP relevant factors (menstrual cycle, exogenous hormones, and inflammation) on enzyme expression and activity.

To address these research questions, we hypothesize that cervicovaginal tissue integrity and metabolizing enzymes are critical determinants of tissue exposure and efficacy of antiretroviral drugs in HIV-1 prevention. Furthermore, modulators of tissue integrity and metabolizing enzymes affect antiretroviral drug levels and efficacy in cervicovaginal tissues.

Aim 1. Examine the effect of commonly used excipients on the paracellular and transcellular permeability in human ectocervical epithelium. Radiolabeled mannitol and propranolol will be used for paracellular and transcellular permeability assessment. The TEER assay, morphology examination, and MTT assay will also be utilized to examine the safety of the excipients. These

data will facilitate the understanding of cervicovaginal tissue integrity regulation, and will inform the development of novel strategies that aim to enhance drug penetration through modulating tissue integrity.

Aim 2. Examine the expression and regulation of metabolizing enzymes in cervicovaginal tissues of human and animal models, as well as human cell cultures used in microbicide product testing. The expression of CYPs, UGTs and phosphorylating enzymes in human and macaque cervicovaginal tissues (endocervix, ectocervix, and vagina) will be systematically examined. In addition, the role of highly expressed nuclear receptors in the regulation of the metabolizing enzymes will be examined in cell models. The results obtained in this aim will provide useful information on the presence of metabolizing enzymes in cervicovaginal tissues.

Aim 3. Examine the effect of contraceptive use and inflammation on the expression and activity of the highly expressed PE enzymes will be studied using cervicovaginal cell lines and immune cell cultures. The effect of the identified enzyme-regulating factors on antiretroviral drug efficacy in HIV-1 prevention will also be studied in immune cell cultures. The results will reveal the effect of commonly encountered factors on metabolizing enzyme expression and activity. These data will contribute to the development of novel strategies that aim to increase drug exposure through modulating metabolizing enzymes.

## **2. THE EFFECT OF COMMONLY USED EXCIPIENTS ON THE PARACELLULAR AND INTRACELLULAR PERMEABILITY OF HUMAN ECTOCERVICAL EPITHELIUM.**

### **2.1. Introduction**

Pharmaceutical excipients are chemicals that help formulate the active pharmaceutical ingredient (API) into ready-for-use drug products. According to their role in the pharmaceutical product, excipients can be categorized as chelators, antioxidants, solvents/cosolvents, preservatives, etc. They can help increase the solubility/compatibility/patient acceptability of the API and help maintain the physicochemical stability of the API in the formulated products [66-68, 172]. A number of excipients with diverse functions are frequently used in vaginally administered drug products, including the vaginal microbicides tested in preclinical and clinical studies for topical preexposure prophylaxis (PrEP) of sexually transmitted HIV-1 [47, 78-80].

In the design and development of vaginal formulations, the effect of formulation ingredients on the integrity of the cervicovaginal epithelium should be evaluated. This is especially true in the formulation of vaginal microbicides which are intended for use during sex. The ectocervical and vaginal epithelium is composed of the multi-layer stratified squamous epithelial cells with TJ at the basal epithelial layer [53]. The intact epithelium functions as a natural barrier against sexually transmitted infections (STIs) such as HIV-1 [173, 174]. If a formulation excipient denudes epithelial layers or opens intercellular junctions, it would compromise the epithelial integrity and potentially increase the risk of viral penetration and infection [50]. The importance of epithelial integrity in HIV prevention was exemplified by the failures of two phase III trials testing the nonoxynol-9 (N-9) vaginal gel (COL-1492) and the 6% cellulose sulfate (CS) gel. In these trials, N-9 and CS were tested as microbicide candidates for HIV-1 prevention, but were

found to increase HIV infection [48, 49, 175]. A key reason for this unsatisfactory outcome was the disruptive effect of these two agents on cervicovaginal epithelial layers [25, 47-50]. Therefore, when formulating vaginally administered drug products for the prevention of STIs, the epithelial safety of the formulation ingredients must be evaluated.

Despite extensive utilization of excipients in vaginal drug products for PrEP, there is limited understanding regarding the safety of vaginal product excipients in the context of HIV-1 prevention. In clinical trials, “universal HEC gel” has been used in the placebo arm, which contains several pharmaceutical excipients. The safety of many vehicles and single excipients were either unknown or only grossly evaluated. Currently, robust and reproducible efficacy has not been observed in the TFV vaginal gel clinical trials for the prevention of sexually transmitted HIV-1. Safety concerns have been raised regarding the formulation components in addition to APIs [176]. There is a growing body of evidence suggesting that excipients may interact with human cervicovaginal epithelium. Some hyperosmolar over-the-counter (OTC) lubricant gels have been found to reduce the tissue viability and result in epithelial fracture/sloughing in an ectocervical explant model [77]. Several other tissue explant and mouse studies reported that treatment with excipients that are frequently used in vaginal products could alter the cervicovaginal tissue integrity and even increase the rate of vaginal HSV-2 transmission [74, 81, 177]. McClelland et al evaluated the effect of vaginal washing on the incidence of HIV-1 infection in 1270 African women between 1993 and 2003. They found that women who partake in vaginal washing, by vaginal douching or washing with water and soap, had higher HIV acquisition risk as compared with women who did not perform vaginal washing. In addition, women who performed vaginal washing with soap had higher risk for HIV-1 infection compared

with those who used water alone [83]. However, the models utilized in these studies were cervicovaginal cell lines, explants, or rodents. The effect of these treatments on intact human cervicovaginal tissue remains not well-known. Clearly, the safety profile of product components in addition to the API needs to be better understood to inform the product development, clinical trial design and data interpretation. Toward this goal, it is necessary to examine the effect of various extensively-used excipients on cervicovaginal tissue in a clinically relevant model, and to determine if excipients influence active product efficacy.

In the current study, we aimed to examine the effect of commonly used excipients on the paracellular and intracellular permeability of human ectocervical epithelium. Meanwhile, the TEER assay, morphology examination, and MTT assay will be utilized to examine the safety of the excipients. The results obtained in this aim will provide useful information on the effectiveness and safety of commonly used excipients as TJ modulators in human ectocervical tissue. These data will facilitate the understanding of cervicovaginal TJ regulation, and will inform the development of novel strategies that aim to enhance drug penetration through modulating TJ proteins. Overall, the results obtained in this chapter will test our hypothesis on cervicovaginal tissue integrity, and fulfill the research objectives proposed in Aim 1 (Chapter 1 Hypothesis and Aims section).

## **2.2. Materials and methods**

### **2.2.1. Materials and instruments**

The excipients tested in this study are summarized in Table 2.1 D-[1-14C]-mannitol (14C-mannitol) and L-[4-3H]-Propranolol (3H-propranolol) were obtained from Perkin-Elmer

Life Sciences (Boston, MA). Disodium EDTA, benzoic acid, citric acid, BHA, propylene glycol, sorbic acid, glycerin, methylparaben, propylparaben, and N-9 were obtained from Spectrum Chemical Manufacturing Corp (New Brunswick, NJ). Tenofovir (TFV) drug substance was obtained from International Partnership for Microbicides. All other chemicals and solvents were obtained from Fisher Scientific, Inc. (Fair Lawn, NJ). All the excipients and permeability markers were dissolved in phosphate buffered saline (PBS, 1X), pH 7.4, (46-013-CM, Mediatech, Inc. Manassas, VA) at the concentrations denoted in Table 2.1. Other instruments used in the study were: Ussing Chamber system VCC MC6 Multichannel Voltage/current Clamp with DM MC6 Single Channel Electrode Input Module and Dummy Membrane (Physiologic Instruments, Inc., San Diego, CA). Beckman pH meter (Model 300, Fisher, Pittsburgh, PA), Beckman Coulter Futura Flat Bulk Combination pH electrode (BK511066, Fisher, Pittsburgh, PA).

### 2.2.2. Placebo and TFV vaginal gel

The universal placebo formulation was described by Tien et al previously [178] and was listed in Table 2.1 The gel pH was adjusted to 4.4 with sodium hydroxide. TFV gel is composed of 1% TFV incorporated into a formulation containing a gelling agent (hydroxyethyl cellulose, HEC), glycerin, EDTA, citric acid, and the preservatives methyl and propyl parabens.

**Table 2.1 Formula of the “universal” placebo vaginal gel**

<b>Component</b>	<b>% (w/w)</b>
Sodium chloride, USP	0.85
Hydroxyethyl Cellulose NF (250HX)	2.70
Sorbic Acid, USP	0.10
Sodium Hydroxide 1.0N	0.35
Purified water, USP	96.0
Hydrochloric acid 1.0N	As needed
Sodium Hydroxide 1.0N	As needed

2.2.3. Ethics statements

All human ectocervical tissues used in this study were obtained through the University of Pittsburgh Health Sciences Tissue Bank as per approved IRB protocol PRO09110431. All tissues were de-identified and collected through an Honest Broker.

2.2.4. Human ectocervical tissue

Human ectocervical tissue was obtained from a total of 87 patients (30 to 55 years old) undergoing hysterectomy for indications that were unrelated to the present study. Tissues were held at 4°C in Dulbecco’s Modification of Eagles Medium (DMEM) (Mediatech, Inc. Herndon, VA) during transfer from the surgical room to the lab, which occurred within 5 hours of surgical excision. The tissues were used immediately upon procurement or snap-frozen and stored at -80°C until use. The frozen MeOH was used to freeze tissue as previously described [179]. Briefly, crushed dry ice was added to methanol (1:1 w/v) to form a slurry. The tissue was placed in a re-sealable specimen storage bag (Fisher Scientific) and immersed in the slurry for 1-2 minutes until frozen. Tissue was then stored at -80°C until use. To thaw, the specimen storage

bag containing tissue was placed in a 37°C water bath for 5-10 minutes until thawed. All ectocervical tissues used in this study were classified as histologically normal based on H&E staining results. In addition, our preliminary studies demonstrated that frozen and fresh ectocervical tissues did not differ in basal levels of TEER, epithelial morphology, and mannitol permeability. The epithelial layer was isolated using a Thomas-Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ) and epithelial thickness was measured by a micrometer prior to the studies as described previously [179]. Briefly, the thickness of two microscope slides was determined first. Then, the tissue slice was sandwiched between the two slides and the total thickness was measured. The value obtained for the slides was subtracted from the value of the slides plus the tissue to give the tissue thickness. Snap-frozen tissue with methanol and dry ice: fresh tissue was placed in a resealable zipper closer bag (Bitran specimen storage bag; Fisher Scientific) and immersed in the methanol with dry ice for a few seconds to freeze.

#### 2.2.5. Electrophysiological measurements

TEER was measured using an Ussing Chamber system with sliders (P2311A, circular diameter= 5.7 mm, area = 0.26 cm<sup>2</sup>, Physiologic Instruments, Inc., San Diego, CA) for mounting of tissues. The excised ectocervical epithelia were mounted in the Ussing chamber and equilibrated for 60 minutes with PBS filled in both hemichambers (apical and basolateral). TEER monitoring was initiated by replacing the PBS in the apical chamber with excipient solutions. Hemichambers were bubbled continuously with environmental air to provide oxygenation and stirring of the solutions. The mounted tissue samples were exposed periodically to a 1 mV bipolar pulse of 100-ms duration. The resultant short circuit current ( $I_{sc}$ ) was recorded in a real-time manner throughout the 6-hour incubation period, with a multichannel voltage clamp through a pair of

Ag/AgCl electrodes immersed in 3% (w/v) agarose gel containing 3.0 M of potassium chloride. Data were acquired and processed at 0.1 to 1 Hz (Acquire and Analyze Rev II software, version 3.1; Physiologic Instruments). TEER ( $\Omega \cdot \text{cm}^2$ ) was determined by using the current deflections with Ohm's law. The TEER at the 6-hour time point was compared to the initial value (shown as Time 0) to indicate the effect of excipient incubation on TEER.

#### 2.2.6. Histological evaluations

Histological evaluation was conducted on all tissue specimens from the permeability and TEER experiments as previously described [47, 180]. Briefly, tissue pieces were obtained before (denoted as pre-tissue) and after the excipient solution exposure, fixed in acid alcohol or formalin for 12-24 hours followed by 70% ethanol, 95% ethanol for one hour, 100% ethanol for one hour, xylene for two hours, paraffin for one hour and for three times, and embedded in paraffin using the Leica EG 1160 embedding station. Tissues were then sectioned at 5  $\mu\text{m}$  with the Olympus CUT 4060 microtome and placed on slides for further hematoxylin and eosin (H&E) staining procedures where hematoxylin stains the nuclei of cells purple and eosin stains the other structures of the tissue section. Pictures were taken using a Zeiss (Genna, Germany) Axioskop 40 microscope with AxioVision Software.

#### 2.2.7. Permeability studies

The permeability studies were performed using a Franz Cell system as previously described [179] with minor modifications. The 7mm Franz-cells (PermeGear Inc., Hellerstown, PA) were water-jacketed and the temperature was maintained at  $37 \pm 0.5^\circ\text{C}$  throughout the experiment. The receptor chamber was filled with 5.0 mL 1X PBS (pH 7.4) solution and continuously stirred. The

excised epithelia (fresh or frozen) were placed in the Franz Cell with the epithelial side facing the donor solution, which yielded a diffusion area of 0.385 cm<sup>2</sup>. The tissue was equilibrated with PBS (pH 7.4) in the donor compartment for 15 minutes prior to the study. The study was initiated by replacing the equilibrating PBS with 450 μL of <sup>14</sup>C-mannitol (12.5 μCi·mL<sup>-1</sup>) or <sup>3</sup>H-propranolol (1.0 μCi·mL<sup>-1</sup>), with or without excipients. After initial experimental setup, a 50 μL sample was taken from the donor compartment to measure the baseline exposure level. Aliquots of 200 μL were removed from the receptor compartment at 15, 30, 45, 60, 120, 180, 240, 300, 360 min after initiation, and replaced with fresh PBS (pH 7.4) to maintain sink conditions. Radioactivity was measured using a Wallac 1409 DSA Liquid Scintillation Counter (Perkin Elmer Life Sciences, Inc., Boston, MA).

#### 2.2.8. Calculation of Flux and Permeability

The cumulative radioactivity in the receptor compartment, as a percentage of the initial radioactivity in the donor compartment, was plotted over time to reflect the permeation profile of <sup>14</sup>C-mannitol through the epithelial layers. The calculations of steady-state flux and apparent permeability ( $P_{app}$ ) were based on Fick's First Law of Diffusion. Steady-state flux ( $J_{SS}$ ) was calculated as:

$$J_{SS} = \frac{Q}{AT} \quad (\text{Equation 2.1})$$

where  $Q$  is the quantity of substance crossing membrane [counts per minute (cpm)],  $A$  was the surface area (cm<sup>2</sup>) of the tissue exposed to the solution, and  $t$  is the time of exposure (min). The units for  $J_{SS}$  were: cpm cm<sup>-2</sup> min<sup>-1</sup> [181].

Apparent permeability coefficient,  $P_{app}$ , was calculated as:

$$P_{app} = \frac{dQ}{dt \cdot A \cdot C_d} \times 60 \quad (\text{Equation 2.2})$$

Where  $dQ/dt$  was the slope of the curve of  $Q$  (amount in cpm) versus  $t$  (time in minutes),  $A$  was the surface area ( $\text{cm}^2$ ) of the tissue exposed to the solution, and  $C_d$  is the initial radioactivity of the  $^{14}\text{C}$ -mannitol in the donor compartment ( $\text{cpm} \cdot \text{mL}^{-1}$ ). The units for  $P_{app}$  were: ( $\text{cm} \cdot \text{s}^{-1}$ ).

To account for tissue thickness variation between experiments,  $DK$  was also plotted for treatment.  $DK$  was obtained using the equation  $DK = Ph$ , where  $D$  is diffusivity ( $\text{cm}^2/\text{s}$ ),  $K$  is the partition coefficient,  $h$  is tissue thickness ( $\text{cm}$ ). Since  $K$  is a dimensionless value, the rank order of the diffusivity of the products is the same as the tissue permeation coefficients above.

#### 2.2.9. Tissue viability analysis

The MTT assay for epithelial viability was performed using a previously reported procedure with modification [182]. In brief, the freshly excised tissues used in the permeability experiments were collected after the final sampling time point, and incubated with 0.2 mL of 0.5 mg/mL MTT dissolved in PBS for 3-4 hours at  $37^\circ\text{C}$ . The MTT solution was then discarded and the tissues were weighed. The formazan formed in the cells was extracted overnight in 2 mL of n-propanol, and the absorbance was measured at 540 nm with readings at 620 nm serving as blank, in a regular UV-spectrophotometer. In this study, tissue viability was calculated as the  $\text{Abs} \cdot \text{mg}^{-1}$  compared to the control tissues incubated with PBS in the permeability experiment. No frozen tissue was used in MTT assay.

#### 2.2.10. Ex vivo efficacy study

The normal human ectocervical tissues were used in this study. Polarized explant cultures were set up as previously described [47, 182]. Briefly, the explant was placed with the luminal side up in a transwell system. The edges around the explant were sealed with Matrigel™ (BD Biosciences, San Jose, CA). The explants were maintained with the luminal surface at the air-liquid interface. The lamina propria was immersed in medium for ectocervical explants. Cultures were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere. 2× TFV or TFV/excipient solution were prepared in complete culture medium, and 100 µl of 2× TFV or TFV/excipient solution was added to the apical side of the explants followed by adding 100 µL of 5 × 10<sup>4</sup> tissue culture 50% infective dose (TCID<sub>50</sub>) of HIV-1<sub>BaL</sub>. The final concentration of TFV was 500µM and the excipients concentrations were listed in Table 2.2 Twenty-four hours after application, the explants were washed and fresh culture medium was applied to the basolateral compartment. Every 3 to 4 days over a 3-week period, supernatant was collected and stored at -80°C for further analysis. Fresh medium was replaced in the basolateral compartment. At the day 21, the supernatant was tested for HIV-1 replication using the p24gag enzyme-linked immunosorbent assay (ELISA, Perkin Elmer Life and Analytical Sciences, Inc., Waltham, MA, USA).

#### 2.2.11. Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD). Statistical analyses were conducted by using Student's *t* test and *p* < 0.05 was considered statistically significant. The differences between multiple groups, at various time points was performed using the one way analysis of variance (ANOVA). All error bars represent standard deviations.

### **2.3. Results**

### 2.3.1. Comparing fresh tissue vs snap freezing tissue

Because the fresh human cervical tissues are difficult to obtain, we have to use the frozen tissue in the following studies as well, so we evaluated the mannitol permeability for both fresh and frozen tissues. Given the fact that no statistically significant difference ( $p>0.05$ ) in mannitol permeability through fresh and frozen tissues (Table 2.2), in the following permeability studies we used both fresh and frozen tissue.

**Table 2.2  $^{14}\text{C}$ -mannitol Apparent Permeability ( $P_{\text{app}}$ ) through Human Ectocervical Tissue Evaluated for Fresh and Frozen Tissues**

	<b>Frozen tissue (n=6)</b>	<b>Fresh tissue (n=4)</b>	<b>p value</b>
<b><math>J_{\text{ss}}</math> (cpm /cm<sup>2</sup>/min)</b>	57.23 ± 48.17	19.71 ± 11.93	0.09
<b><math>P_{\text{app}}</math> (cm/sec) *10<sup>6</sup></b>	0.352 ± 0.190	0.492 ± 0.296	0.19
<b>DK (cm<sup>2</sup>/sec)</b>	0.020 ± 0.011	0.035 ± 0.025	0.10
<b>t (lag) (sec)</b>	39594 ± 29231	31976 ± 24436	0.34

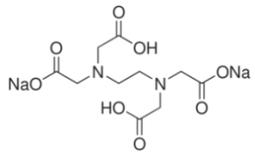
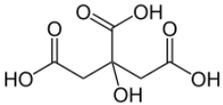
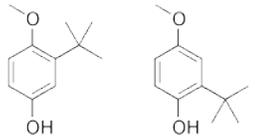
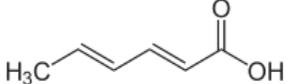
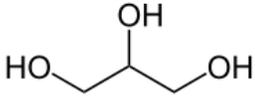
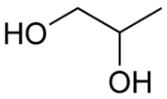
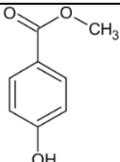
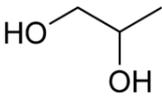
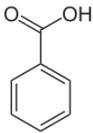
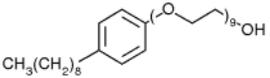
Tissues were obtained from premenopausal women undergoing hysterectomy for benign conditions, with an age range of 30 – 55 years old.

### 2.3.2. Selection of excipients and determination of experimental conditions

The excipients examined in this study were: disodium EDTA, citric acid, sorbic acid, benzoic acid, BHA, glycerin, propylene glycol, methylparaben and propylparaben (Table 2.3). These excipients have been widely used in vaginally administered products. For example, glycerin,

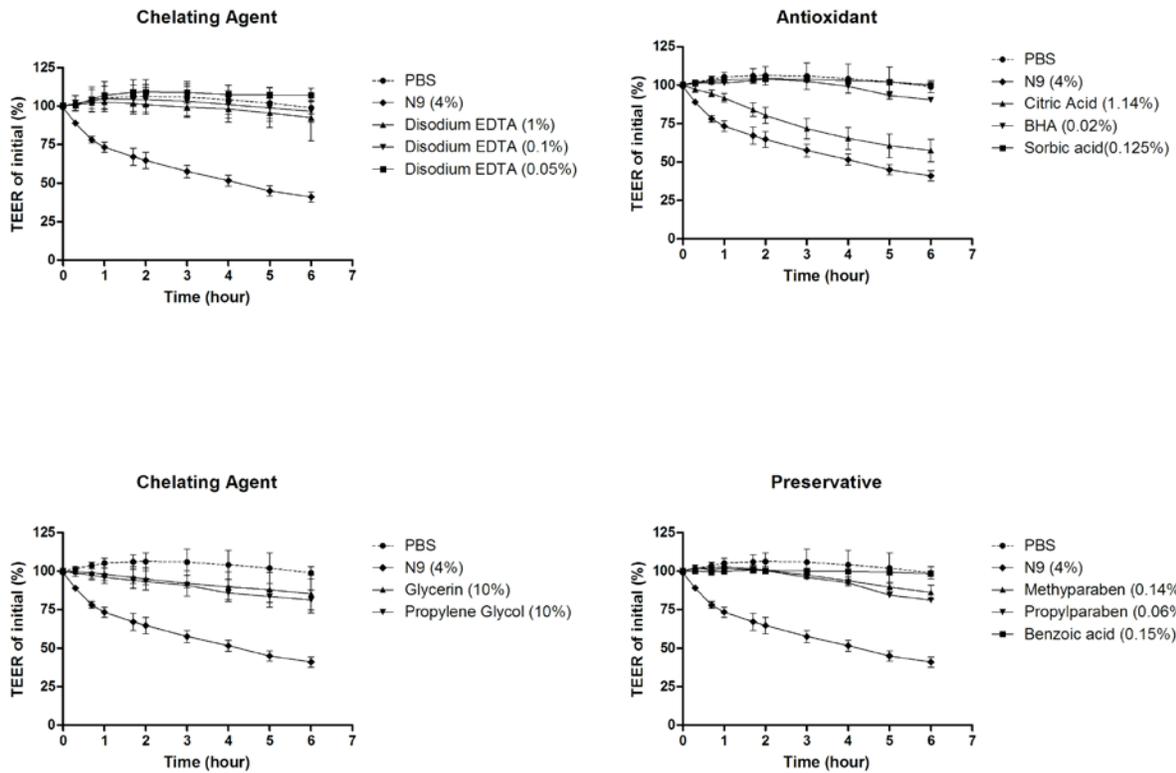
propylene glycol, sorbic acid, methylparaben, and propylparaben were used in the 1% TFV gel [47] and 0.05% dapivirine gel [183] evaluated in clinical trials such as CAPRISA 004, VOICE, FACTS 001, IPM003 and IPM005B trial [80, 184, 185]. Although identified as generally-regarded-as-safe (GRAS) by the Food and Drug Administration (FDA), several of these excipients were reported to affect the epithelial barrier in human cell culture or animal models. Excipients chosen for evaluation represent a variety of functional categories and diverse chemical structures. The excipient concentration used in this study was determined based on the following considerations: disodium EDTA is widely reported to decrease the integrity of intestinal cell monolayer (Caco-2) and excised intestinal epithelium, so three concentrations (0.05, 0.1, and 1%) were chosen to cover the relevant levels of use. For all other excipients a single concentration was studied. The concentration chosen was based on the common level of use in vaginal products. Due to the dilution by cervicovaginal fluid and other body fluids, the concentration of excipients in cervicovaginal fluid is usually lower than that in administered drug product, and the results obtained in this study could indicate the effect of those excipients at highest possible *in vivo* concentrations. The incubation time for all experiments was 6 hours.

**Table 2.3 Excipients used in the study**

Class	Excipient	Commonly used	
		Concentration range (%)	Chemical structure
Chelator	Disodium Ethylenediaminetetraacetic acid (Disodium EDTA)	0.01-0.1	
	Citric acid	0.1-2.0	
	Butylated Hydroxyanisole (BHA)	0.005-0.02	
Antioxidant	Sorbic acid	0.05-0.2	
	Glycerin/ Glycerol	5-15	
Solvent/Cosolvent	Propylene Glycol	5-80	
	Methylparaben	0.1-0.18	
Preservative	Propylparaben	0.02-0.1	
	Benzoic Acid	0.1-0.2	
Positive control	Nonoxynol-9 (N9)	-	

### 2.3.3. The effect of excipients on cervicovaginal epithelium TEER

The effect of excipients on TEER was monitored in a real-time manner using an Ussing Chamber instrument. As shown in Table 2.4 and Figure 2.1, the negative control PBS-treated tissues showed stable TEER throughout the 6-hour assay period, while the positive control N-9 (4%) resulted in an approximate 40% decrease in TEER after 3 h incubation, and a 59% decrease after 6 h incubation. The difference in TEER between PBS and N-9 groups appeared as early as 20 minutes post exposure. These results were consistent with previous clinical and preclinical observations with PBS and N-9, confirming the ability to distinguish between benign and disruptive treatments using this experimental setup. For tested excipients, disodium EDTA (0.05%, 0.1%, 1%), sorbic acid (0.125%), and benzoic acid (0.15%) did not show any effect on TEER of ectocervical epithelium throughout the 6-hour assay period when compared to PBS. Methylparaben (0.14%), propylparaben (0.06%), glycerin (10%), propylene glycol (10%), and BHA (0.02%) exposure resulted in a slightly decreased TEER value. This decrease was negligible at 3 h, and was between 10-20% at the 6 h time point. On the other hand, exposure to citric acid (1.14%) resulted in a significant decrease in TEER (25% at 3 h and 43% at 6 h).



**Figure 2.1 Effect of excipients on transepithelial electrical resistance (TEER) in the human ectocervical epithelium**

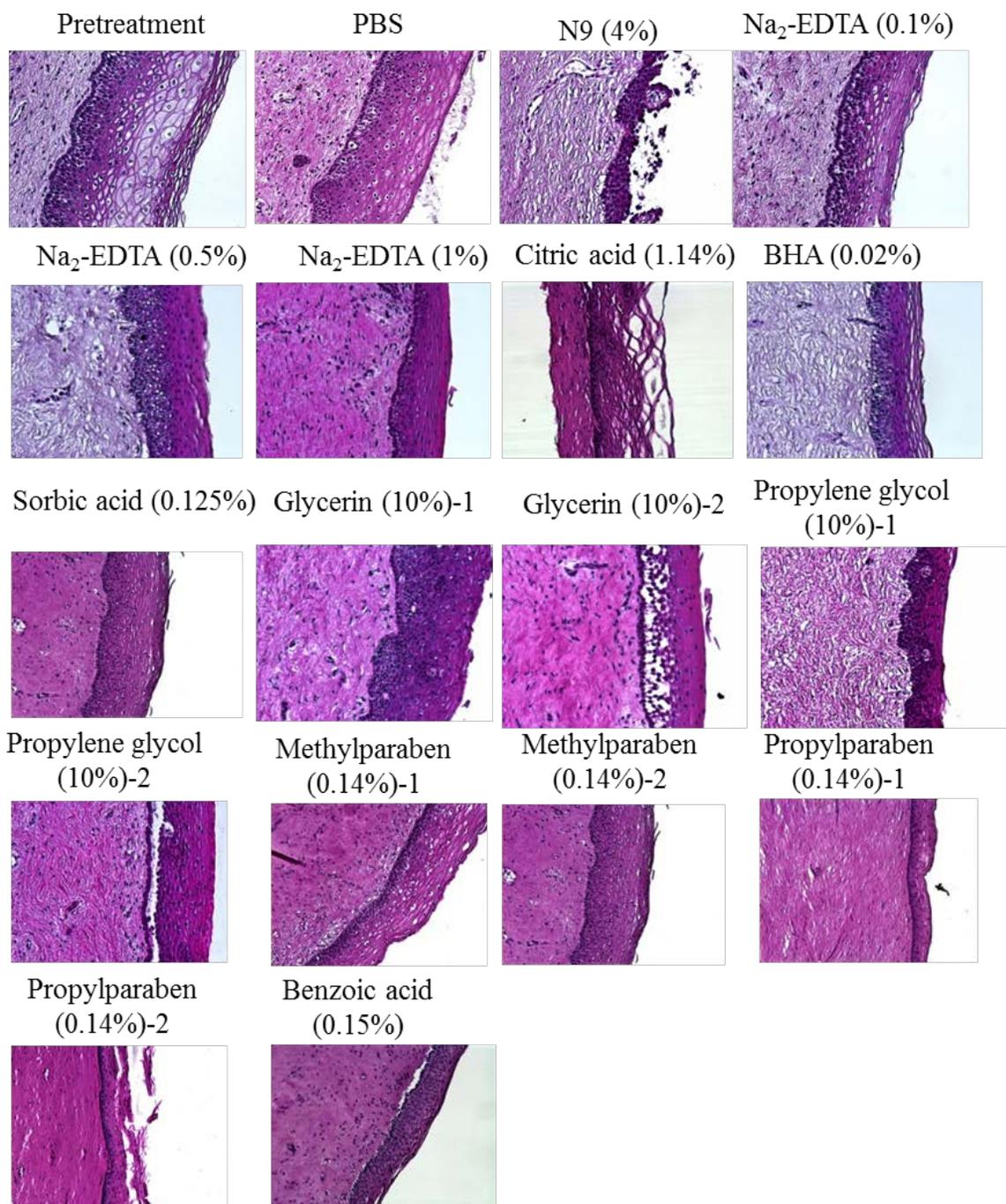
Epithelium was excised from human ectocervical tissue and equilibrated with PBS solution for 1h prior to the TEER measurement. The TEER was monitored at real time during exposure to PBS (control) or different excipient PBS solution for 6 hours using Ussing Chamber. TEER ( $\Omega \cdot \text{cm}^2$ ) was determined by using the current deflections with Ohm's law. The TEER at the 6-hour time point was compared to the initial value (shown as Time 0) to indicate the effect of excipient incubation on TEER. The results shown are the means  $\pm$  s.d. for at least three independent determinations (3 tissues from patients).

#### 2.3.4. The effect of excipients on epithelium

The effect of the excipients on the epithelium was examined using H&E staining of ectocervical tissue. It was anticipated that an intact multi-layer epithelium would be seen for those tissues with unchanged TEER, while detachment of uppermost epithelial layers, or total denudation of

the epithelium from lamina propria would be seen for tissues with significantly decreased TEER. The morphology of PBS and N-9 treated tissues correlated well with their TEER value obtained (Table 2.1 and Figure 2.2). PBS treated tissues maintained an intact stratified squamous epithelium which was comprised up to 40 epithelial layers; no swelling or necrosis of the epithelial cells could be observed. In contrast, the N-9 treated tissues lost multiple superficial layers, which was a result of extensive disruption of epithelial cells and the intercellular tight junctional proteins, and demonstrated a 69% decrease in the TEER by 6 hours (Table 2.4).

Among the tested excipients, citric acid exposure resulted in obvious damage to the ectocervical epithelium. This epithelial damage coincided with an observed 45% decrease in TEER. Other tested excipients, such as EDTA, sorbic acid, and benzoic acid did not significantly affect the tissue TEER during 6 hours exposure at tested concentration. The effects of BHA (0.02%), methylparaben (0.14%), propylparaben (0.06%), glycerin (10%), and propylene glycol (10%) on tissue morphology were heterogeneous. Exposure to these excipients resulted in inconsistent effects on the epithelium with some tissues unchanged from the untreated control while others showing epithelial fracture. This intra-group variability was probably due to inter-individual variability in tissue sensitivity to toxic stimuli. Tissues treated with disodium EDTA (0.1%), sorbic acid (0.125%), and benzoic acid (0.15%), showed unchanged morphology after 6 hours incubation, which was comparable to the PBS group.



**Figure 2.2 The effect of excipients on tissue morphology**

Human cervicovaginal epithelia used in the TEER and/or permeability studies were subjected to the H&E staining. All tissue samples were incubated with excipient solution, PBS, or N-9, for 6 hours at 37°C under 5% of carbon dioxide (CO<sub>2</sub>). For each excipient, at least three treated tissue samples were used for the staining and analysis. For excipients that displayed homogeneous effect on tissues (disodium EDTA at all concentrations, citric acid, BHA, benzoic acid), only one

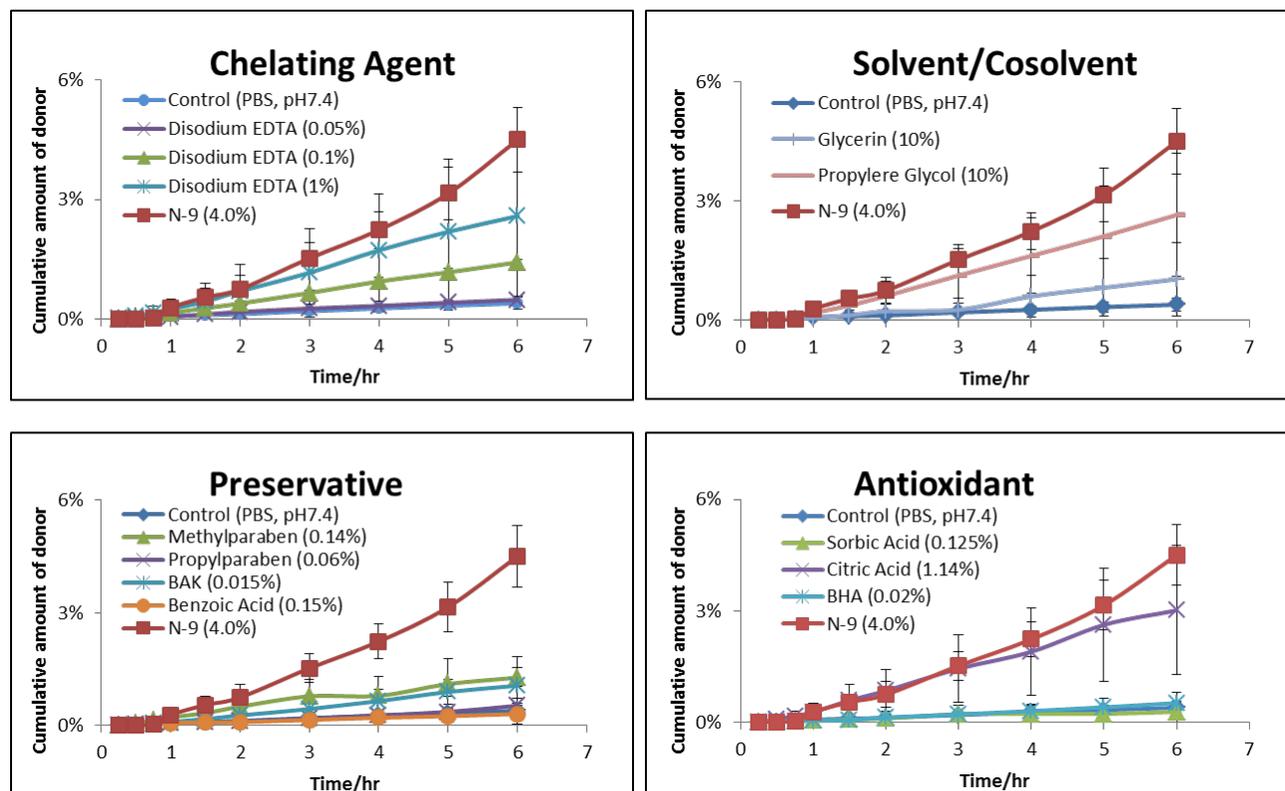
representative picture was shown. For the excipients that displayed heterogeneous effects (sorbic acid, glycerin, propylene glycol, methylparaben, and propylparaben), both the intact morphology and disrupted morphology were shown. Magnification, 20 $\times$ .

#### 2.3.5. The effect of excipients on paracellular permeability

The effect of excipients on paracellular permeability was measured using  $^3\text{H}$ -mannitol. The purpose of this experiment was to confirm TEER observations obtained. For the excipients that decreased tissue TEER and damaged epithelial layers, an increase in the apparent permeability coefficient ( $P_{\text{app-man}}$ ) would be expected; for those excipients that did not affect TEER and tissue morphology, e.g. disodium EDTA (0.05, 0.1, 1%), the  $P_{\text{app-man}}$  would be expected to remain unchanged. As shown in Table 2.4 and Figure 2.3, compared to the PBS treated tissues, N-9 (4%) caused an 11-fold increase in  $P_{\text{app-man}}$ . The slope of the flux curve increased with time, reflecting the gradual disruption of cell layers by the N-9 and corresponding time-dependent increase in epithelial permeability.

Among all the tested excipients, citric acid (1.14%) resulted in the largest increase in  $P_{\text{app-man}}$  (7.6-fold compared to PBS treatment with  $p < 0.01$ , Figure 2.3). This finding is consistent with a dramatic decrease and a significant disruption of cervicovaginal epithelium in TEER, after 6-hour exposure (Figure 2.3). The 1% and 0.1% disodium EDTA significantly increased  $P_{\text{app-man}}$  by 5.0 and 1.5 folds compared to PBS treatment, which appeared paradoxical to the observation that EDTA had no effect on TEER and epithelial morphology (Table 2.4). The effect of disodium EDTA on  $P_{\text{app-man}}$  appeared to be concentration-dependent (Figure 2.3). Propylene glycol, glycerin, and methylparaben caused a moderate-to-large increase in the  $P_{\text{app-man}}$ , consistent with but not proportionate to the change in TEER and morphology they induced. BHA

and propylparaben slightly increased  $P_{app}$ -man, consistent with moderately decreased epithelial TEER and slightly altered epithelial morphology.



**Figure 2.3 The effect of excipients on  $^{14}\text{C}$ -Mannitol transport across human ectocervical epithelium**

The 7mm Franz-cells were water-jacketed and the temperature was maintained at  $37\pm 0.5^\circ\text{C}$  throughout the experiment. The receptor chamber was filled with 5.0 mL of PBS solution and continuously stirred. The excised epithelium was placed between the donor and receptor chamber with the epithelial side facing the donor solution. The study was initiated by replacing the equilibrating PBS with 450  $\mu\text{L}$  of  $^{14}\text{C}$ -mannitol ( $12.5 \mu\text{Ci}\cdot\text{mL}^{-1}$ ) or  $^3\text{H}$ -propranolol ( $1.0 \mu\text{Ci}\cdot\text{mL}^{-1}$ ), with or without excipients. Aliquots of 200  $\mu\text{L}$  were removed from the receptor compartment at 15, 30, 45, 60, 120, 180, 240, 300, 360 min after initiation, and replaced with fresh PBS (pH 7.4) to maintain sink conditions. The time course of  $^{14}\text{C}$ -Mannitol permeation was shown as the amount appearing in the acceptor chamber normalized to the initial amount applied to the donor chamber. Each data point represented the mean  $\pm$  s.d. of at least three independent tissues.

**Table 2.4 Summary of test results**

<b>Excipient (tested concentration, w/v)</b>	<b>TEER (% of initial value)<sup>a</sup></b>	<b>Tissue morphology<sup>b</sup></b>	<b>Fold change in <math>P_{app}</math>-man<sup>c</sup></b>	<b>Viability (%)<sup>d</sup></b>
Disodium EDTA (0.05%)	107.2±4.3	+	1.2	NA
Disodium EDTA (0.1%)	96.7±7.0	+	1.5	96.0±26.7
Disodium EDTA (1%)	92.5±15.0	+	5.0 *	82.5±49.4
Citric acid (1.14%)	57.5±7.3 **	-	7.6 **	10.5±13.3 **
BHA (0.02%)	90.4±0.1 *	+/-	1.2	80.6±45.5
Sorbic acid (0.125%)	100.1±0.6	+	0.7	44.8±21.7 *
Glycerin/Glycerol (10%)	85.2±12.3	+/-	2.5	86.5±21.0 *
Propylene glycol (10%)	81.3±6.4 *	+/-	6.6 *	84.5±32.0
Methylparaben (0.14%)	86.2±4.6 *	+/-	3.9 **	102.8±16.2
Propylparaben (0.06%)	81.2±1.3 **	+/-	1.2	79.2±27.5
Benzoic acid (0.15%)	98.5±1.5	+	0.7	52.6±39.1
N-9 (4%)	41.0±6.1 **	-	11.0 **	59.0±5.4 **

- a. The TEER value was calculated by normalizing the TEER at the 6-hour time point to the initial TEER of the same tissue sample at time zero. Data represent the mean ± SD of at least three independent experiments.
- b. The tissue morphology was arbitrarily classified as intact or disrupted. +, all the examined tissue samples showed intact epithelial layers; -, all the examined tissue samples showed disrupted epithelial layers; +/-, heterogeneous results among the examined tissue samples were observed.
- c. The fold change in  $P_{app}$ -man was calculated by normalizing the Papp-man with excipients to the Papp-man with PBS. Data represent the mean ± SD of at least three independent experiments.
- d. The viability % was calculated by normalizing the MTT value obtained with excipients to the MTT value obtained with PBS (100%). Data represent the mean ± SD of at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$

**Table 2.5 Calculation of permeability parameters of <sup>14</sup>C-mannitol and <sup>3</sup>H-propranolol<sup>a</sup>**

Excipient/control	Flux-man (cpm·cm <sup>-2</sup> ·s <sup>-1</sup> )	$P_{app}$ -man × 10 <sup>6</sup> (cm·s <sup>-1</sup> )	Flux-prop (cpm·cm <sup>-2</sup> ·s <sup>-1</sup> )	$P_{app}$ -prop × 10 <sup>6</sup> (cm·s <sup>-1</sup> )
PBS	22.5±23.3	0.5±0.2	70.8±25.0	3.9±1.8
Disodium EDTA (0.05%)	14.1±3.9	0.6±0.1		
Disodium EDTA (0.1%)	17.9±4.3	0.8±0.2		
Disodium EDTA (1.0%)	41.6±41.4	2.6±1.5 *	91.8±37.3	4.8±2.7
Citric acid (1.14%)	104.9±41.5 **	3.9±2.3 **		
BHA (0.02%)	113.9±104.9	0.6±0.4		
Sorbic acid (0.125%)	17.1±17.3	0.4±0.4		
Glycerin/Glycerol (10%)	30.7±28.4	1.3±1.1	113.9±54.3	5.1±3.2
Propyleneg (10%)	76.5±44.0	3.4±2.0 *	94.6±65.8	4.0±3.0
Methylparaben (0.14%)	83.4±45.2 *	2.0±0.3 **	45.0±9.0	2.4±0.3
Propylparaben (0.06%)	47.4±44.7	0.6±0.6		
Benzoic acid (0.15%)	54.9±7.4 *	0.4±0.1		
N-9 (4.0%)	111.2±14.0 **	5.6±0.8 **		

a. The effect on <sup>14</sup>C-mannitol permeability was studied for every excipient while the effect on <sup>3</sup>H-propranolol permeability was studied only for those excipients that caused opposite changes in TEER and <sup>14</sup>C-mannitol permeability. Flux-man: flux of <sup>14</sup>C-mannitol;  $P_{app}$ -man, apparent permeability coefficient of <sup>14</sup>C-mannitol. Flux-prop: flux of <sup>3</sup>H-propranolol;  $P_{app}$ -prop, apparent permeability coefficient of <sup>3</sup>H-propranolol. Data represent the mean ± SD of at least three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$

### 2.3.6. The effect of select excipients on transcellular permeability

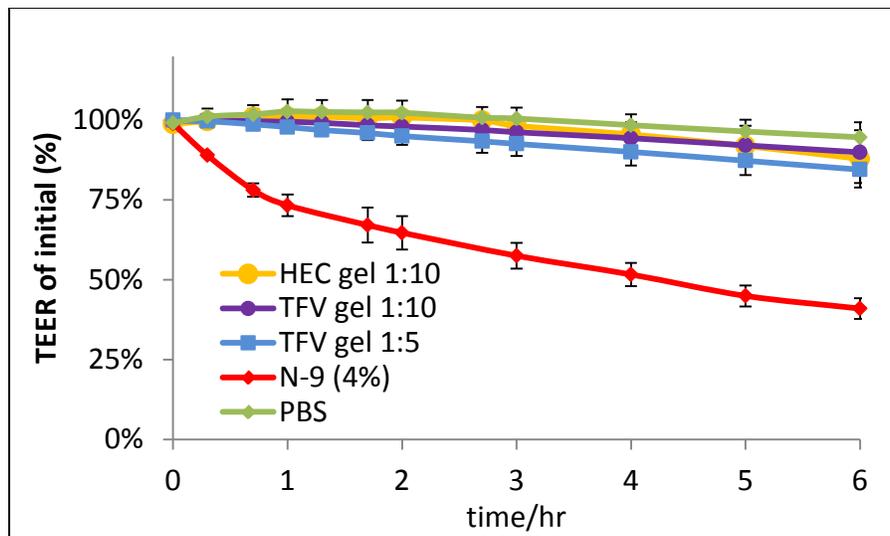
Since disodium EDTA, glycerin, propylene glycol, and methylparaben increased the paracellular permeability, the effect of these four excipients on transcellular permeability was also examined using the transcellular probe  $^3\text{H}$ -propranolol. None of the tested excipients significantly altered propranolol permeability coefficient ( $P_{\text{app-prop}}$ ) at the concentrations tested (Table 2.5).

### 2.3.7. The effect of excipients on epithelial cell viability

The MTT based colorimetric assay was used to measure the effect of excipients on epithelial viability. In live cells, the MTT reagent is metabolized by mitochondrial reductase into colored formazan products. Therefore, a significant decrease in MTT value indicates inhibition of cellular metabolic function. The purpose of this assay in the present study was to examine the effect of excipients on tissue viability and correlate these findings with those of TEER, morphology and permeability studies. If the excipient disrupted the epithelium, the MTT value of treated tissue would be expected to be lower when compared to the control PBS treated tissues (Table 2.4). The MTT value of the N-9 treated tissues was  $59 \pm 5.4\%$  ( $p < 0.001$ ) of the PBS-treated tissues. Among the excipients, exposure to citric acid (1.14%) resulted in a dramatic 90% ( $p < 0.001$ ) decrease in MTT value. This decrease is consistent with the marked TEER and histological changes observed with citric acid exposure. Sorbic acid also induced a significant decrease in the MTT value. Conversely, tissue exposure to disodium EDTA at 0.05%, 0.1%, and 1%, BHA (0.02%), methylparaben (0.14%), propylparaben (0.06%), glycerin (10%), or propylene glycol (10%) did not result in decreases in MTT.

### 2.3.8. The effect of topical microbicide gels on cervicovaginal epithelium TEER

The effect of vaginal TFV (1%) or universal placebo HEC gel on human ectocervical tissue TEER was monitored in a real-time manner using an Ussing Chamber instrument. The TFV gel formulation was the same as that was used for several microbicide clinical studies, such as CAPRISA004 and VOICE. The negative control PBS-treated tissues showed stable TEER throughout the 6-hour assay period, while the positive control N-9 (4%) resulted in dramatically decrease in TEER after 6 h incubation (Figure 2.4). The diluted (1 to 10 and 1 to 5 diluted in PBS) or HEC gel (1 to 10 diluted in PBS) solution did not show any effects on TEER of ectocervical epithelium throughout the 6-hour assay period.

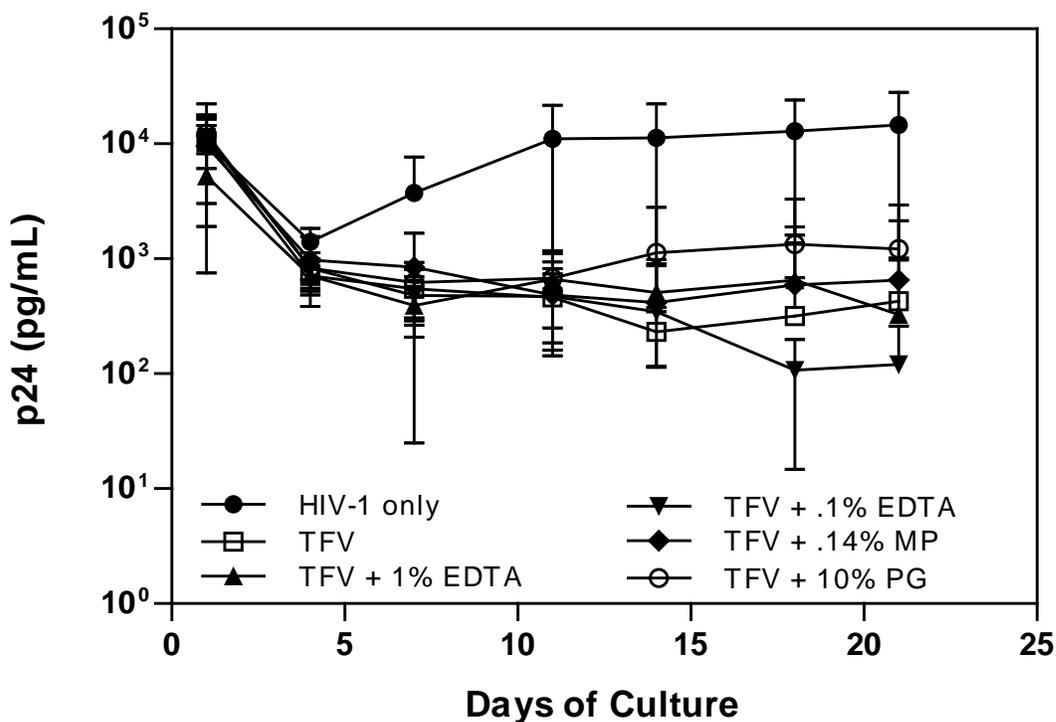


**Figure 2.4 The effect of TFV gel (1%) and HEC gel on transepithelial electrical resistance (TEER)**

The TEER was monitored at various time points during the 6 hours exposure to PBS (negative control), N-9 (positive control), and different TFV (1%) and HEC gel diluted in PBS. The TFV and universal placebo HEC gels were diluted with PBS (1 to 5 or 1 to 10 dilution for TFV gel, and 1 to 10 dilution for HEC gel). Each data point represents the mean  $\pm$  SD of tissue samples from at least three patients.

### 2.3.9. The impact of excipients on microbicide potency

The effect of excipients on tissue integrity was further evaluated in an HIV challenge model using excised human ectocervical tissue cultured *ex vivo*. In the untreated control explants (HIV-1 only), viral replication peaked to  $10^4$  pg/mL on Day 11 and maintained at this level for the rest of the test period (Figure 2.5). The TFV-treated (0.5mM) explants showed decreased p24 in the culture supernatant indicative of this antiretroviral drug in suppressing HIV replication. The p24 curves of the explants treated with TFV and the excipients (EDTA, MP, and PG) closely resembled the curve of TFV-treated only explants, with a few exceptions. No significant difference were found between TFV only and TFV with excipients group. While the explants treated with TFV and PG displayed higher p24 levels than the TFV-treated explants, all excipient-treated explants were less than 10-fold different to the HIV-1 only explants. Overall, there is a clear separation in p24 curve pattern between the positive control group and the groups treated with excipients.



**Figure 2.5 The effect of the excipients on the ex vivo efficacy of TFV using human ectocervical tissue explant cultures**

Polarized explant cultures were set up with the luminal side up in a transwell system. The edges around the explant were sealed with Matrigel™ (BD Biosciences, San Jose, CA). The lamina propria was immersed in medium for ectocervical explants. Cultures were maintained at 37°C in a 5% CO<sub>2</sub> atmosphere. Tissues were exposed to TFV (0.5mM) alone or with different excipient solutions and HIV-1 overnight, followed by washing and 21 days of culture. At study end, the supernatant was tested for HIV-1 replication using the p24gag ELISA. Each point represents mean ± SD of tissue samples from at least three patients. MP, methylparaben; PG, propylene glycol.

#### **2.4. Discussion and conclusions**

Although in vivo penetration studies remain important for any new drug product development, well-defined and controlled *ex vivo* studies are probably more appropriate for the initial

investigations of compounds diffusion/penetration through mucosal tissues before use in human clinical studies. Several diffusion apparatus have been investigated for studying in vitro and ex vivo tissue permeability, such as the static Franz cells, the Ussing chamber, as well as flow-through diffusion cells [186, 187]. These apparatus contain a donor and an acceptor compartment, between which a membrane/tissue can be placed. Artificial membranes, cell layers and human/animal tissues, such as cultured Caco-2 monolayers, excised human and animal intestinal tissue, are frequently used for permeability/diffusion determination of the APIs [188-191]. Compared to the Franz Cell, the Ussing chamber may contain an additional amplifier and data acquisition system, which provides continuous integrity and viability data of the tissue by measuring electrophysiological parameters[192]. Ussing chamber system has been used to study the passive permeability of various drugs across excised rabbit nasal epithelium and mucosa[193], human and rat intestine [190, 194], human scleral tissues[195], as well as Caco-2 monolayer[196]. It also has been used as an ex vivo model to study the role of various ion exchangers, such as sodium-calcium and sodium-hydrogen exchangers in the homeostasis of retinal pigment epithelium in various animal and human models [197, 198].

This study utilized intact human ectocervical tissue directly excised from patients, which overcame the limitation of previous studies. Previously, Grammen et al. and Moench et al. evaluated excipients' effect on cervicovaginal tissue using in vitro cell lines and a mouse model[81, 177]. However, the baseline thickness of the epithelial layers, the types of epithelial cell present in the tissue, as well as the type and abundance of tight junction proteins are quite different between intact human cervicovaginal tissue and these preclinical models[164, 199]. These differences affect the interpretation of previous findings, and limit their applicability

towards optimizing clinically-applied vaginal PrEP products. In our opinion, although these models are valuable tools for early-stage toxicity screening, the surgically excised human cervicovaginal tissue represents a more clinically relevant model for safety evaluation of excipients and formulated products.

In the current study only excised human tissues was used. This is because of among available cell lines, to our best knowledge, there is no cell line that can reconstitute the tight junctional proteins of cervicovaginal tissues. The primary culture of cervicovaginal epithelial cells might be a suitable cell model, but it is practically difficult to use primary due to the limited availability of fresh human cervicovaginal tissues. Among the animal models, the macaque vaginal tissues have the most similarity in anatomy to humans, but harvesting tissue is problematic due to the need to sacrifice the animals. The cervicovaginal tissues of other animal species (mouse, rabbit) have different types of tight junctions and different permeation characteristics compared to human tissue [164, 199]. Therefore, only the excised human tissues were used to study the effect of excipients in the study.

A significant challenge in developing an ex vivo model for permeability studies is the acquisition of fresh human cervical and vaginal specimens. Recently, several hospital established “tissue bank” systems where samples can be frozen and stored for use at a later date. We previously evaluated the different freezing methods effect on the permeability of tritiated water on human vaginal and cervical tissues and found that freezing of human ectocervical and vaginal tissue followed by subsequent storage at  $-80^{\circ}\text{C}$  did not affect  $P_{\text{app}}$  [179]. Thus, in the current study, we used the same freezing method for tissues that could not be processing immediately. Meanwhile,

we also compared the mannitol permeability across fresh and frozen tissue samples (Table 2.2), and again, no significant difference was observed on several permeability parameters between fresh and frozen human specimens. No frozen tissue was used in MTT assay.

Using our model, we have demonstrated that GRAS excipients could alter various aspects of cervicovaginal tissue integrity, including TEER, epithelial morphology, permeability, and viability. In addition, these effects are dependent on excipient concentration and duration of treatment. TFV gel composes of glycerin, EDTA, citric acid, methylparaben and propylparaben. At the concentrations tested in this study, disodium EDTA below 0.1%, sorbic acid and benzoic acid did not compromise tissue TEER, morphology, and permeability. EDTA above 1% did not decrease TEER but significantly increased tissue permeability. In addition, BHA, glycerin, propylene glycol, methylparaben, propylparaben, and citric acid displayed time-dependent negative effects on more than one aspect of tissue integrity.

Further evaluation of the effect of EDTA, propylene glycol, and methylparaben using the *ex vivo* cultured human ectocervical tissues demonstrated that although these excipients altered TEER, morphology, paracellular permeability and viability of excised human tissue to different degrees, they did not exert significant impact on tissue susceptibility to HIV infection when used in conjunction with an active API. This was evidenced by the clear separation in the time-dependent p24 curves between excipients-treated tissues and positive control-treated tissues (Figure 2.5). Taken together, these observations suggest that when evaluating the safety of excipients used in vaginal formulations for the purpose of HIV prevention, the commonly-used *in vitro* testing methods (TEER, histological staining, paracellular permeability, viability) have

limitations and the value of a single assay should not be overstated. A comprehensive analysis of the results from all these assays is more likely to reflect clinically relevant evaluation of excipient's safety. The observations reported in this study also suggest that the tested excipients except citric acid are safe to use for vaginal product development at tested concentrations. However, 1% of citric acid has been used in TFV vaginal gel in several microbicides clinical trials without seeing dramatic side effects. This might be due to the diluted effect by genital secretion and semen, or the shorter exposure time. Also, the side effect might be too light to be observed.

The results from this study have implications for future PrEP studies. Despite widespread use of the excipients tested in this study, there has been reported that some commonly used excipients could alter the permeability of mannitol, thus raising the concern that these excipients could open the intercellular gates to facilitate HIV entry. Gali et al further demonstrated that EDTA and glycerin could increase vaginal tissue susceptibility to viral infection (HSV-2), in a mouse model. We have demonstrated using human tissues that these excipients appear to be safe at clinically relevant concentrations, although they may change the TEER, permeability, viability to various extents. The apparent discrepancies between our results and other studies may be attributable to the difference in models (human tissue vs. cell line or animals) and test conditions (e.g. concentration of excipient). Our conclusion on the safety of most tested excipients is supported by the observation that almost no severe side effects have been reported from clinical use of the vaginal products containing the tested excipients.

Exposure is a critical factor in determination of excipient's safety. At the concentration tested in our study, citric acid is found to harm tissue integrity in every aspect (TEER, morphology, permeability, viability). However, citric acid has been formulated into various vaginal products and no significant toxicity has been observed. In fact the TFV gels tested in our study also contains citric acid, and its clinical use has demonstrated no safety concerns after vaginal use. This discrepancy is presumably caused by limited preclinical safety testing of clinically used vaginal products. The safety evaluation is often conducted in the population with regular product use. Under such conditions, the excipients exert their functions in the formulation (e.g. citric acid as antioxidant), thus the amount of biologically reactive excipients available to the tissue might be less than their total amounts contained in the product. In addition, the excipients are usually gradually released from the product, and a significant amount of excipients might be entrapped in the cervicovaginal mucus. Moreover, the vaginal retention of most administered dosage forms, such as gel, film, tablet, and suppository, is short due to vaginal discharge. Therefore, under regular clinical use, tissue exposure to the product-released excipients might be lower than the concentrations tested in our experimental setting. However, the lessons learned from microbicide trials highlight the necessity of thoroughly evaluating the safety of product components under all conditions that may be encountered by PrEP product users [176, 200].

A variety of product- and patient-related factors could result in higher-than-expected exposure to excipients. The excipient concentration in the product is frequently changed for better product properties. In the context of PrEP, the demographic at highest vaginal infection risk is sex workers and those who have multiple sex partners. These people use the product much more frequently than the rest of the population and their tissue exposure to excipients may be much

higher than the majority of people who use the product less frequently. Indeed, the COL-1492 trial revealed that the sex workers used the N-9 gel >3.5 times per day, and the vaginal toxicity and increased HIV-1 acquisition that were associated with this particular subgroup were not observed among those who used the gel only once per day. In addition to dosing frequency, other patient-related factors such as reduced viscosity of cervicovaginal fluid, could also result in elevated exposure of excipients. In this study, the 1% tenofovir vaginal gel was also found to have no effect on TEER and tissue morphology in our tissue model, but a number of excipients contained in this product affected multiple aspects of tissue integrity when they were administered to the tissue as solutions. These effects, in our opinion, reflect the potential toxicity risk under the conditions of high level product exposure. In these “enhanced exposure” scenarios, it is possible that even the excipients that are shown to be safe in our study could become harmful when their concentrations are markedly increased. Hence, our results can help PrEP researchers with future development and labeling of product formulations, and provides clues for interpreting the suboptimal clinical trial efficacy results.

This study revealed that excipients could result in inconsistent changes in different aspects of cervicovaginal tissue integrity. For example, the excipients-induced increase in paracellular permeability did not necessarily coincide with a decrease in TEER. Also, disodium EDTA did not change TEER at any concentrations tested; however, it caused a concentration-dependent increase in the mannitol permeability, which consist with Dezzutti group’s finding that exposure to EDTA solution increased HIV-1 replication in the human ectocervical explant tissue as compared to the untreated tissue[77]. In addition, the glycerin, propylene glycol, and methylparaben significantly increased mannitol permeability, which was disproportional to the

moderate decrease in TEER. These observations were unexpected, since the decrease in TEER was often accompanied by an increase in permeability in epithelial cell monolayer [201-203], due to the opening of the intercellular space by the co-incubated excipients. In our study, the excised human ectocervical tissue consists of multiple layers of epithelial cells, therefore the impact of excipient on the mannitol permeability across these tissues may not be necessarily same with epithelial monolayer. The transcellular permeability across plasma membrane ( $^3\text{H}$ -propranolol as the probe) was not affected by excipient treatment (Table 2.5), suggesting the un-paralleled change in TEER and paracellular permeability was not due to the compensation from altered transcellular permeability.

Dezzutti et al found that two-hour-exposure to 0.1% of EDTA increased in HIV-1 replication in the human ectocervical explant tissue [77], however in our study, EDTA did not dramatically impact tissue TEER, viability and morphology. Previous studies using kidney cells and cultures suggested that paracellular permeability to electrical currents and non-charged solutes may be regulated by different sets of tight junctional proteins [204-208]. If this is the case, given that multiple types of tight junctional proteins are expressed in human cervicovaginal mucosa [53, 209-211], it is possible that EDTA treatment affected certain types of tight junction proteins that control small molecule movement, but did not affect those that control electrical current flux. Such a change in tight junction, in our opinion, is not comparable to the physical breaches which facilitate HIV-1 entry into submucosal compartment [212-214]. This is determined by the difference in size and entry mechanism between HIV-1 and mannitol. Both cell-free and cell-associated HIV-1 are large, densely charged particles (>100 nm in diameter), and their entry process depends on the interactions between virus and host cells [215, 216]. On the contrary,

mannitol is a small, neutral molecule with a few nm diameter; its tissue penetration does not require interaction with tissue cells, and should be much easier than that of HIV-1 particles. Therefore, the excipients-induced increase in paracellular permeability does not necessarily predict increased risk of HIV-1 entry. From this perspective, the permeability assay alone cannot accurately reflect the comprehensive effect of treatments on the tissue defense barrier against pathogens. Rather, it should be used in combination with other methods (e.g. TEER, histology) for tissue integrity assessments.

Although several excipients altered mannitol permeability through the human ectocervical epithelia at tested concentrations, no obvious changes were found on tissue morphology or TEER. This was also confirmed in the gel TEER studies in which neither vaginal TFV gel nor HEC gel impacted ectocervical epithelium TEER over the tested time period. The TFV (1%) and HEC gel tested in the study was the same formulation that was used for several microbicide clinical trials such as CAPRISA004. The *ex vivo* efficacy study was performed to test if the altered permeability would impact tissue infectivity or ARVs anti-HIV effectiveness. Rohan et al studied the impact of TFV vaginal gel on human ectocervical explant epithelium and found the 24-hours-exposure to diluted TFV gel solution (1:5 diluted in culture medium) showing epithelial sloughing[185]. However, in the current study, diluted TFV gel solution did not cause a dramatical decrease of tissue TEER. This might be due to the different exposure time period (24hr vs 6hr). The results show that the excipients at tested levels did not affect TFV activity and indicate that there is not a definite correlation between the altered paracellular permeability and ARVs efficacy. Based on our results, several fold increase in mannitol permeability appeared insufficient to significantly facilitate the entry of HIV particles.

Carias et al studied the interaction of HIV with the mucosal barriers of the female reproductive tract and found that EDTA (10 mM) could increase virus penetration in human cervical explants [217]. Several experimental differences between the Carias et al study and this study can explain this seemingly contradictory result. The cultured tissues in the Carias et al study are different from the human tissue culture used in our study in terms of the susceptibility to HIV infection. In Carias's paper, nonpolarized explants were used and were exposed to EDTA for 2 hours, followed by exposure to HIV-Bal virus for 4 hours. However, in our study, polarized explants were used and were exposed to HIV virus for 24 hours. In the setting of non-polarized culture (Carias et al.), both epithelium and stroma were exposed to the treatment and virus. In the polarized explant culture (our study), only epithelium was exposed with treatment and virus, which in our opinion, mimicked the *in vivo* scenario of HIV sexual transmission. Also, the EDTA concentration used in Carias et al. study (10 mM) is different from the concentration used in our study, and may not be relevant to the *in vivo* EDTA concentration after the use of the marketed products. In addition, an increase in virus penetration does not necessarily equate to increased infection in the tissue, and it is not certain if this increase in virus penetration is severe enough to cause a clinical impact. In our study, we examined the viral infection marker (p24 concentration), which is not the same as virus penetration. Based on the above discussion, our p24 assay should be a more clinically relevant approach to evaluate the impact of excipient treatments on tissue susceptibility to HIV.

Due to the limited availability of human tissues, both fresh and frozen human ectocervical tissues were used in this study. Our laboratory has previously evaluated the effect of the freeze-thaw process on tritiated water permeability through human cervicovaginal tissue, and no statistically

significant differences in permeability values between fresh and frozen-thawed tissues were observed. We have also compared the  $P_{\text{app-man}}$  between fresh and frozen-thawed tissues used in this study and the result is shown in Table 2.2. The mannitol permeability values were not significantly different ( $p = 0.188$ ) between the two kinds of tissues, indicating that all tissues used in this study had similar baseline permeability.

In addition to the mannitol permeability, the MTT-based cell viability values of several excipients also showed un-paralleled change as compared to TEER/morphology/permeability results. In this study, benzoic acid (0.15%) and sorbic acid (0.125%) caused unexpectedly significant MTT decreases (Table 2.4), in contrast to their TEER, histology, and mannitol permeability data, all of which appeared unchanged after incubation with these two excipients. The effect of these excipients on MTT value was maybe due to their reported ability to interfere with mitochondrial activity instead of destructive cytotoxicity. The MTT assay is known to be insensitive on the cytotoxicity of tested agents [218-220], which is related to the intrinsic limitation of this assay. The agents that do not kill cells but inhibit metabolism may cause the MTT value to decrease as if the treatment could damage the tissue. On the other hand, the cytotoxic effect could be masked if the tested agent could cause moderate cell death but metabolically activate the remaining live cells. In this scenario the net MTT value may appear unchanged. Therefore, the MTT assay was reliable in reflecting epithelial integrity only when the epithelium was severely damaged, after which most live cells were lost. Indeed our data showed that MTT results correlated well with TEER and histology findings only when the excipient treatment greatly destroyed the epithelial cells probably due to changes in lamina propria more than in epithelium. Therefore, this assay can be used to validate the devastating observations

from TEER or histology measurements, but cannot be used as the sole approach to examine epithelial integrity.

## **Conclusion**

In summary, this study has tested the effects of these generally-regarded-as-safe excipients on cervicovaginal tissue integrity, viability and morphology, which have provided implications for future development of vaginal PrEP products. Our study has revealed that a number of excipients could alter certain aspects of cervicovaginal tissue integrity, in a concentration- and/or time-dependent manner. The excipients which increased paracellular permeability of cervicovaginal tissue did not significantly reduce TFV activity in HIV prevention. These results have demonstrated that most tested excipients are generally safe to use in vaginal product formulations for the sake of HIV prevention. However, caution should be implemented in the use of those excipients that could increase permeability, because there might be situations in which the excipient concentration is much higher than the concentrations tested in our study. In this case the negative impact of those excipients on tissue integrity may be much more obvious. Future investigations are warranted to identify factors that can lead to increased tissue exposure to excipients, to reduce the toxicity risk associated with excipients and optimize clinical product use. In addition, our study has demonstrated that TEER, morphology, permeability, and MTT-based tissue viability do not necessarily change in parallel with each other. The use of a single measurement cannot accurately reflect the effect of excipients on cervicovaginal tissue, and it appears necessary to use a combination of multiple approaches to gain a comprehensive understanding. Taken together, the studies presented in this chapter have provided valuable information on the excipients' effect on multiple aspects of cervicovaginal tissue integrity. This

information will facilitate the efforts toward optimized vaginal PrEP products for HIV-1 prevention.

### **Acknowledgments**

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### **3. MRNA EXPRESSION AND LOCALIZATION OF CYP AND UGT ENZYMES IN HUMAN AND ANIMAL TISSUES RELEVANT TO TOPICAL MICROBICIDE**

#### **3.1. Introduction**

Antiretroviral (ARV) drug levels in mucosal tissues and resident immune cells positively correlates with its efficacy against HIV, and one of our goals for future microbicide development is to achieve maximally tolerable drug concentration in the female lower genital tract [221]. The major enzymes involved in antiretroviral drug metabolism include Phase I cytochrome P450s (CYPs) and Phase II UDP-glucuronosyltransferases (UGTs). These two types of enzymes are located on the microsomal membrane and could affect the intracellular concentration of substrate drugs and their metabolites. CYP in human and mammal animals are generally membrane-associated proteins and principally located in the smooth endoplasmatic reticulum (ER) of parenchymal liver cells. However, it has been reported the expression of CYPs in other extrahepatic tissues, such as intestine, brain, kidney and lung [45, 222]. In addition of metabolizing xenobiotics, CYPs are crucial for normal physiology functions in mediating synthesis and metabolism of bile acids, fatty acids, cholesterol, vitamin D and steroid hormones [223-226]. CYPs catalyze various reactions such as aromatic hydroxylations, N-, O- and S-dealkylations, oxidative deaminations and dehalogenations [227-230]. Like CYPs, the study of expression and activity of the UGTs toward xenobiotics, bile acids, and bilirubin have primarily focused on hepatic, intestinal and renal tissues [93, 231-233].

Although it has been established that these enzymes control the absorption and disposition of administered drugs in various tissues, such as liver, kidney, and brain, there is a lack of

characterization of CYPs and UGTs in cervicovaginal tissues in human or animal models used for microbicide research. Since CYPs and UGTs are potentially present in the cervicovaginal tissues and may impact ARVs' local concentration, it is important to understand the expression and function of these enzymes in cervicovaginal tissues. The current study aimed to fill this knowledge gap and examine the expression pattern of Phase I and II metabolizing enzymes in the female lower genital tract (FLGT) of human and animal models. First, the mRNA expression of 9 CYP enzymes and 10 UGT enzymes was examined using conventional RT-PCR, in the FLGT tissues of human and mouse. Since the CYP enzymes are believed to have much greater impact on ARV drug PK compared to UGT enzymes, the expression of CYP1A1 and 1B1, two CYP enzymes that were found positive in human and mouse tissues, were further studied using real-time RT-PCR and immunohistochemical (IHC) staining, in the FLGT tissues of human and macaque, a more biologically relevant model compared to mouse in microbicide research. Finally, the expression of CYP1A1 and 1B1 was compared between pre- and postmenopausal human ectocervix, as a preliminary examination of the effect of menopause on enzyme expression in FLGT.

To our knowledge, this is the first evaluation of CYPs and UGTs in the FLGT. The data generated from animal models are relevant since macaque and mouse are widely used animal models in microbicide research. The mouse model is used by many researchers at the initial stage of microbicide screening, especially in the screening of toxic effects of microbicide products. The pigtailed macaque has been considered as the most biologically relevant animal model in microbicide research. The morphology and physiology of macaque female reproductive and colorectal tracts are very similar to those of corresponding human tissues [199, 234, 235]. For

most functional genes, including the CYP and UGT enzymes, the DNA and protein sequences are highly conserved between human and macaque. The pigtailed macaque has been utilized for the testing of vaginally and rectally administered drug products [199, 234, 235]. Therefore, the enzyme expression in the FLGT of these animal models in comparison to human is important information for the selection of models and interpretation of study results generated from these models. The data presented in this chapter will help us better understand the pharmacokinetic data obtained from clinical trials as well as animal studies, especially when the test drug is a substrate of the metabolizing enzymes studied in this chapter.

### **3.2. Materials and methods**

#### **3.2.1. Acquisition of human, pigtailed macaque and mouse tissues**

Human ectocervical tissues were obtained from premenopausal women (n=7) and postmenopausal women (n=6) undergoing hysterectomy for benign conditions. Human vaginal tissues were collected from 5 women with prolapse. Human liver tissues were obtained from donors (age: 15-75) without hepatic malignancies. Human sigmoid colon tissue were obtained from women (age: 20-79) undergoing surgery for non-inflammatory conditions. The colon tissues used were histologically examined without malignancies. All human ectocervical tissues used in this study were obtained through the University of Pittsburgh Health Sciences Tissue Bank as per approved IRB protocol PRO09110431 and were de-identified and collected through an Honest Broker. The epithelial layer of ectocervical tissue was isolated using a Thomas-Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ) and epithelial thickness was measured prior to the studies as described elsewhere [179].

The three pigtailed macaques used in this study were 12.6, 18.7 and 17.6 years old, and were of reproductively active. The macaques were maintained in Washington National Primate Research Center at the University of Washington, which was supported by the Office of Research Infrastructure Programs of the National Institutes of Health under Grant Number P51OD01042, and in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The macaque tissues (uterus, endocervix, ectocervix, vagina, colorectum, liver) were acquired through the Tissue Distribution Program, which was approved by the Institutional Animal Care and Use Committee.

Mouse tissues were collected from mice undergoing natural cycling or estrus stage being synchronized with PMSG/Depo-Provera treatment. Six-week-old female Swiss Webster mice were used in the study which was approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) (protocol # IS00000654). The estrous cycle stage of each mouse was determined using vaginal cytology as described previously. Briefly, 20  $\mu$ L of normal saline was injected into mouse vaginal lumen with pipette. The vaginal lavage smear was taken out and spread onto a glass slide and observed under a bright light microscope. The cell morphology of mouse vaginal smear from different stages was list in Table 3.1. For estrous stage synchronization, mice were intraperitoneally (i.p.) injected with pregnant mare's serum gonadotropin (PMSG, Sigma, 5 IU/ mouse), and the synchronized estrous stage should be reached 24 hours after the administration. For diestrus stage synchronization, mice were subcutaneously (s.c.) injected twice with Depo-Provera (Pfizer, 3 mg/mouse) on Day 1 and Day 5, and the synchronized diestrus stage should be reached 7 days after the first time injection.

**Table 3.1 Cell types in mouse vaginal smear at different estrous stages**

<b>Estrous Cycle Stage</b>	<b>Cell types in the vaginal smear</b>
proestrus	small nucleated epithelial cells
estrus	larger anucleated cornified epithelial cells
metestrus	leukocytes, cornified, and nucleated epithelial cells
diestrus	predominantly consisted of leukocytes

3.2.2. Conventional RT-PCR of human and mouse tissues

Serially diluted liver cDNA was used as a standard to quantitate the mRNA level. Human liver tissue samples were obtained from 6 donors and pooled for RNA extraction. PCR was performed using the GoGreen Hot-start mastermix (Promega Inc.). The cDNA from 1, 5, 25 ng total RNA were used for liver standards and the cDNA transcribed from 50 ng of total RNA was used for all the cervicovaginal tissue samples. Human  $\beta$ 2-microglobulin served as the internal control. The PCR programs consist of a denaturation at 95 °C for 4 min, followed by 28 to 36 cycles of 95 °C 30 sec, 60 °C 30 sec, and 72 °C 30 s. The final extension was set at 72 °C for 3 min. The PCR products were then analyzed using agarose gel electrophoresis, and gel pictures were captured by using the ChemiDoc™ MP imaging system (Biorad Inc.). The information of primer sequences used in conventional PCR were summarized for human (Table 3.2) and mouse (Table 3.3).

**Table 3.2 Primer sequences for conventional PCR of human and macaque Phase I and Phase II metabolizing enzymes**

<b>Gene Name</b>	<b>GeneBank Accession no.</b>	<b>Primer sequence 5' to 3'</b>
CYP1A1	XM_007727	Forward: TAGACACTGATCATGGCTGCAG
		Reverse: GGGAAGGCTCCATCAGCATC
CYP1A2	NM_000761	Forward: CTTTGACAAGAACAGTGTCCG
		Reverse: AGTGTCCAGCTCCTTCTGGAT
CYP1B1	U56438	Forward: AACGTCATGAGTGCCGTGTGT
		Reverse: GGCCGGTACGTTCTCCAATC
CYP2B6	NM_000767	Forward: GCACTCCTCACAGGACTCTTG
		Reverse: CCCAGGTGTACCGTGAAGAC
CYP2D6	NM_000106	Forward: TCAACACAGCAGGTTCA
		Reverse: CCATCCATGTTTGCTTC
CYP2E1	AF182276	Forward: ACCTGCCCCATGAAGCAAACC
		Reverse: GAAACAACATCATGCGAGCC
CYP2C8	NM_000770	Forward: GTCCTGGTGCTGTGTCTCTC
		Reverse: TGGTGAAAGATTTGCAGATGTCC
CYP2C9	NM_000771	Forward: ACATTGACCTTCTCCCCACCAGCC
		Reverse: CAAATCCATTGACAACCTGGAGTGG
CYP2C19	NM_000769	Forward: ACTTGAGCTGGGACAGAGA
		Reverse: CATCTGTGTAGGGCATGTGG
CYP3A4	NM_017460	Forward: CCTTACACATACACACCCTTTGGAAGT
		Reverse: AGCTCAATGCATGATCAGAATCCCCGGTTA

UGT1A1	NM_000463	Forward: CATGCTGGGAAGATACTGTTGAT Reverse: GCCCGAGACTAACAAAAGACTCT
UGT1A3	NM_019093	Forward: ATGGCAATGTTGAACAATATG Reverse: GGTCTGAATTGGTTGTTAGTAATC
UGT1A4	NM_007120	Forward: ACGCTGGGCTACACTCAAGG Reverse: GACAGGTACTTAGCCAGCACC
UGT1A7	NM_019077	Forward: TGGCTCGTGCAGGGTGGACTG Reverse: TTCGCAATGGTGCCGTCCAGC
UGT1A8	NM_019076	Forward: CTGCTGACCTGTGGCTTTGCT Reverse: CCATTGAGCATCGGCGAAAT
UGT1A10	NM_019075	Forward: CCTCTTTCCTATGTCCCAATGA Reverse: GCAACAACCAAATTGATGTGTG
UGT2B4	NM_021139	Forward: TCTACTCTTAAATTTGAAGTTTATCCTGT Reverse: TCAGCCCAGCAGCTCACCACAGGG
UGT2B7	NM_001074	Forward: GACGTATGGCTTATTCGAAACTCCTGGAATTTTCAG Reverse: GCAATGTTATCAGGTTGATCGGCAAACAATGGAATC
UGT2B15	NM_001076	Forward: GTGTTGGGAATATTATGACTACAGTAAC Reverse: TCAGCCCAGCAGCTCACCACAGGG
UGT2B17	NM_001077	Forward: GTGTTGGGAATATTCTGACTATAATATA Reverse: CAGGTACATAGGAAGGAGGGAA
Human β-Actin	NM_0010179 92	Forward: CTGGCGGCACCACCATGTACCCT Reverse: GGAGGGGCCGACTCGTCATACT

**Table 3.3 Primer sequences used for conventional PCR of mouse Phase I and Phase II metabolizing enzymes**

<b>Gene Name</b>	<b>GeneBank accession no.</b>	<b>Primer sequence 5' to 3'</b>
Cyp1a1	NM_009992	Forward: GGCCAGACCTCTACAGCTTC
		Reverse: GCCAAAGCATATGGCACAG
Cyp1a2	NM_009993	Forward: GACGTCAGCATCCTCTTGCT
		Reverse: GGCACTTGTGATGTCTTGGA
Cyp1b1	NM_009994	Forward: AGCTGAGCTCGCTGTCTACC
		Reverse: GTCCGTCATGTCTCGAGGAG
Cyp2b10	NM_009998	Forward: GCCCAATGTTTAGTGGAGGA
		Reverse: GACTTCTCCTTCTCCATGCG
Cyp2c29	NM_007815	Forward: AGGAAAACCAAAGGCTCACC
		Reverse: TGCTTGCCAGATTTTCAAGT
Cyp2c40	NM_010004	Forward: AAAACAAATGGCTCACCCCTG
		Reverse: TTGCCAGGTGTTCAATGGTA
Cyp3a11	NM_007818	Forward: ATAGAGCTTTGCTGTCCCCC
		Reverse: CGGCTTTCCTTCATTCTGTC
Cyp19	NM_007810	Forward: CCTGACACCATGTCCGGTCACT
		Reverse: GGGCTTAGGGAAGTACTCGAG
Ugt1a1	NM_201645	Forward: CCAGCAGAAGGGGCACGAAGTTG
		Reverse: TGACCACGCGCAGCAGAAAAGAAT
Ugt1a2	NM_013701	Forward: TGCCCCTTCGAGGAATCTCAGG
		Reverse: CTCCCGCACAACATCCCTCATG
Ugt1a6	CCDS48314.1	Forward: CTGGCTGATGGTGGCTGACTG

		Reverse: ACTGAGGCCCAAAGCACTAGGAA
Ugt1a9	CCDS15138.1	Forward: CAGAGGGCATGAGGTTGTGGTA
		Reverse: TCCTGCAGTGTGAAAATGTTAGTT
Ugt2b5	NM_009467	Forward: ACCCCAGCAACTTTAGGACACAAT
		Reverse: TGATAGATCGCCTCGTAGACACCA
Mouse β-Actin	NM_175497	Forward: GCCACAGCACTCCATCGAC
		Reverse: GTCTCCGATCTGGAAAACGC

### 3.2.3. Real-time RT-PCR.

The total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The genomic DNA remaining in the total RNA preparations was removed using the Turbo DNase kit (Ambion). The reverse transcription was performed using the SuperScript III First Strand Synthesis Kit (Invitrogen). The real-time RT-PCR was conducted using the Ssofast Evergreen Mastermix (Bio-Rad), in the CFX Touch 96 thermocycler (Bio-Rad). Due to the high homology between human and macaque in the mRNA sequences of CYP1A1, CYP1B1 and GAPDH, the same primers were used to detect both human and macaque genes. For the PCR programs, the initial denaturation was set at 95°C for 30 s, and 40 cycles of amplification were run at 95°C for 5 s and 60°C for 5 s. A melt curve analysis was conducted upon the completion of the amplification cycles, to ensure the specificity of the PCR reaction. The PCR efficiency was confirmed to be within the range of 90%-110%, using serially diluted cDNA standards prepared from the liver or colon tissues. The mRNA levels of CYP1A1 and CYP1B1 in a given tissue sample were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in

the same sample, using the  $2^{-\Delta C_t}$  method. The information of primer sequences used in real time PCR were summarized in Table 3.4.

**Table 3.4 Information of the primers used for the real-time RT-PCR of CYP1A1, CYP1B1, and GAPDH in human and macaque tissue**

<b>Common Gene name</b>	<b>GeneBank accession number</b>	<b>Primer sequences 5' to 3'</b>
CYP1A1	NM_000499	Forward: TCGGCCACGGAGTTTCTTC Reverse: GGTCAGCATGTGCCCAATCA
CYP1B1	NM_000104	Forward: AAGTTCTTGAGGCACTGCGAA Reverse: GGCCGGTACGTTCTCCAAAT
GAPDH	NM_001256799	Forward: GGAGCGAGATCCCTCCAAAAT Reverse: GGCTGTTGTCATACTTCTCATGG

In the current study, standard curve served (serially diluted liver cDNA) as positive control and water without any cDNA was used as negative control. The standard curve prepared using serially diluted liver cDNA, it showed amplification in real time PCR, which means the positive control has given positive signal. If the liver expression level for an enzyme is very high, then the serially diluted cDNA will show lower levels. As long as the tissue sample signal falls within the range of liver samples, then the high expression level in the liver does not affect the tissue sample results. If tissue sample signal in PCR is out of the range of the liver sample standard curve, then the liver samples need to be diluted more, so that the tissue sample signal could be covered by the standard curve.

#### 3.2.4. Immunohistochemical staining

The immunohistochemical staining was performed by the Research Histology Service of the University of Pittsburgh. The human and macaque tissues were fixed in 10% neutral-buffered formalin for no less than 24 hours, and subsequently embedded in paraffin. Sections with 5  $\mu$ m thickness were made and de-paraffinized using xylene. The target antigens were retrieved after incubation with the pH 9 retrieval buffer (Dako) for 30 minutes. The slides were treated with 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes, and blocked using the Avidin block solution (Vector), Biotin block solutions (Vector), and serum, respectively. For CYP1A1 staining, the primary rabbit anti-human CYP1A1 polyclonal antibody (H-70 clone, sc-20772, Santa Cruz Biotechnology) was applied to the slides at 1:100 dilution, and incubated at 4 °C overnight. For CYP1B1 staining, the primary rabbit anti-human CYP1B1 polyclonal antibody (ab33585, Abcam) was applied at 1:750 dilution and incubated at 4 °C overnight. After the application of primary antibody, the slides were washed and incubated with the secondary antibody biotinylated goat anti-rabbit IgG (Vector) diluted in goat serum (1:200). The slides were incubated with the secondary antibody at room temperature for 30 minutes. The AEC chromogen (Skytec) was used to develop the red color on stained slides. In the negative control staining, the primary antibodies were replaced by the IgG purified from the serum of non-immunized rabbit. Human urinary bladder was used as positive control of CYP1A1. Since positive staining for CYP1B1 in human ectocervix was reported previously, ectocervical tissue was used as the positive control for CYP1B1 in this study.

### 3.2.5. Western blot

The human ectocervical tissue was homogenized using the Tissue Tearor homogenizer (Cole Parmer). The tissue homogenate was centrifuged at 10,000 g for 10 minutes, and the supernatant was transferred to a clean tube. The cytoplasm fraction and nucleus fraction were purified from the supernatant using the Nuclear Extraction Kit (Millipore). The total protein concentrations in the cytoplasm and nucleus preparations were measured using the Micro BCA Protein Assay Kit (Thermo). The purified fractions were then subjected to the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using the Mini-PROTEAN precast gels (Bio-Rad). After electrophoresis, the gels were electro-blotted onto a nitrocellulose membrane (Invitrogen) in an iBlot Dry Blotting System (Invitrogen). All the subsequent procedures were performed at room temperature. The blotted membranes were blocked in milk for 1 hour, then incubated with the primary antibodies of CYP1B1 (Abcam, ab33585) and GAPDH (Santa Cruz, sc-48167 for 1 hour. The dilutions were 1: 1000 for CYP1B1 antibody and 1: 500 for GAPDH antibody. The HRP-conjugated goat anti-rabbit IgG (1:2000, Cell Signaling #7074s) was incubated with the membrane for 1 hour. The Pierce ECL Plus Western blotting substrate (Thermo) was applied afterwards to detect HRP activity, and the chemiluminescence images were taken using the ChemiDoc MP imaging system (Bio-Rad).

### 3.2.6. Statistical Methods

The comparison in the mRNA levels between different types of tissue was performed using the one way analysis of variance (ANOVA) with Bonferroni post-hoc test.  $P < 0.05$  was considered as statistically significant.

### **3.3. Results**

#### **3.3.1. Conventional RT-PCR screening of CYP and UGT metabolizing enzymes in human cervicovaginal tissues**

Since there is little known about the CYP and UGT enzymes expression profile in human cervicovaginal tissues, conventional RT-PCR was used to quickly screen the CYPs and UGTs that are relevant to ARVs metabolism. In the study, 9 of CYP enzymes and 10 of UGT enzymes were examined, and the RT-PCR results in human ectocervical and vaginal tissue are summarized in Tables 11 and Table 3.6.

Most of Phase I enzymes relevant to anti-HIV therapy, including the CYP2B, 2C, 2D, and 3A families, plus several others generally important for xenobiotic metabolism, were examined. CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 were highly expressed in human liver. Relative to the expression found in liver, human ectocervix does not express most isoforms of Phase I enzymes, with the exception of CYP1A1 and 1B1 (Table 3.5 and Figure 3.1). On the other hand, CYP3A4, the most important Phase I enzyme involved in xenobiotic metabolism, was not detected in human ectocervix (Table 3.5). No detectable CYPs was observed in vaginal mRNA samples we examined.

In contrast to Phase I enzymes, many Phase II enzyme isoforms were found to be highly expressed in human ectocervix and vagina, including multiple isoforms in the UGT1A and 2B families as summarized in Table 3.6. Among the highly expressed enzymes, the examined enzymes have a similar expression level in both vagina and cervix. There was no obvious difference between ectocervix and vagina (Figure 3.2).

**Table 3.5 Summary of Phase I enzymes expression in human and mouse cervicovaginal tissues**

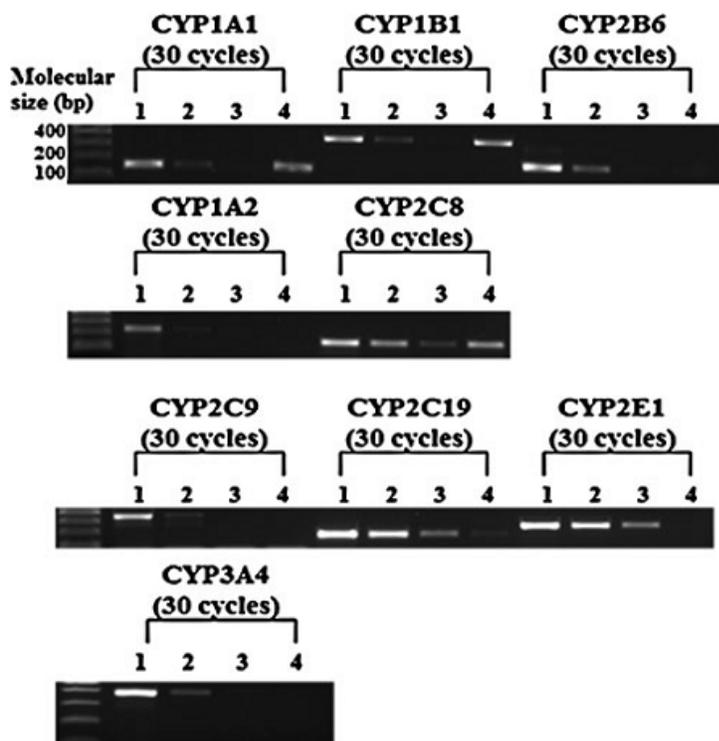
Human Phase I enzymes	Human Phase I enzyme level (% of human liver)	Mouse Phase I enzymes	Mouse Phase I enzyme level (% of mouse liver)	
	Human ectocervix		Mouse cervix	Mouse vagina
CYP 1A1	+++	Cyp1a1	++	++
CYP 1A2	-	Cyp1a2	-	-
CYP 1B1	+++	Cyp1b1	++	++
CYP 2C8	+	Cyp2b10	+	-
CYP 2C19	-	Cyp2c29	-	-
CYP 2B6	-	Cyp2c40	+	-
CYP 2C9	-	Cyp3a11	+	-
CYP 2E1	-			
CYP3A4	-			

mRNA level of each enzyme was qualified by conventional PCR and compared to their levels in human or mouse liver. “-” means  $\leq 2\%$  or undetectable, “+” means 2-10%, “++” means 10-50%, “+++” means 50-100%, “++++” means  $\gg 100\%$ . Human ectocervical tissues were obtained from 7 women undergoing hysterectomy for benign conditions. Human vaginal tissues were collected from 5 women with prolapse. Mouse tissues were collect form 6 of six-weeks-old female Webster mice.

**Table 3.6 Summary of Phase II enzymes expression in human and mouse cervicovaginal tissues**

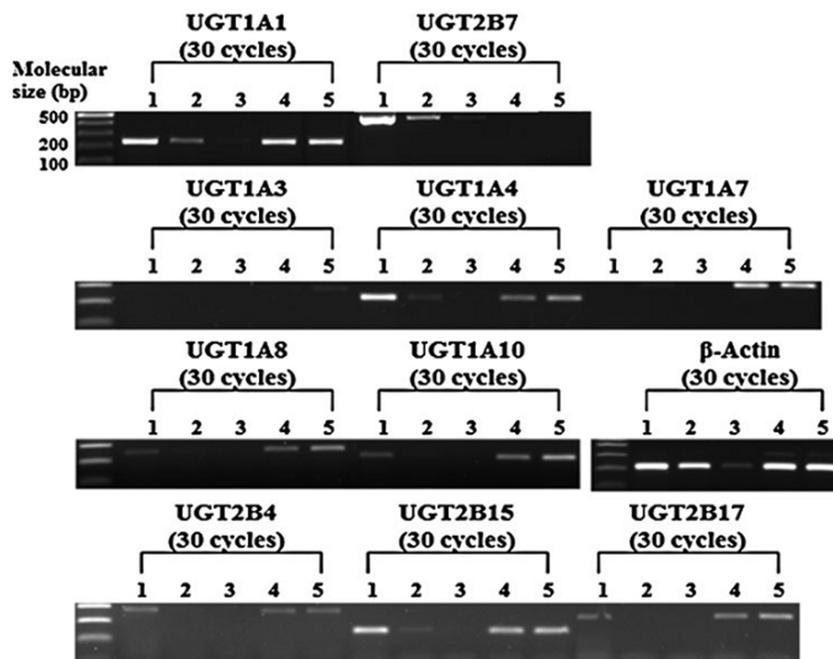
Human Phase II enzymes	Human Phase II enzyme level (% of human liver)		Mouse Phase II enzymes	Mouse Phase II enzyme level (% of mouse liver)	
	Human ectocervix	Human vagina		Mouse cervix	Mouse vagina
UGT1A1	+++	+++	Ugt 1a1	++	++
UGT1A3	-	-	Ugt 1a2	++	++
UGT1A4	++	++	Ugt 1a6	+++	+++
UGT1A7	++++	++++	Ugt 1a9	+	+
UGT1A8	+++	+++	Ugt 2b5	-	-
UGT1A10	+++	+++			
UGT2B4	+++	+++			
UGT2B7	-	-			
UGT2B15	+++	+++			
UGT2B17	+++	+++			

mRNA level of each enzyme was qualified by conventional PCR and compared to their levels in human or mouse liver. “-” means  $\leq 2\%$  or undetectable, “+” means 2-10%, “++” means 10-50%, “+++” means 50-100%, “++++” means  $\gg 100\%$ . Human ectocervical tissues were obtained from 7 women undergoing hysterectomy for benign conditions. Human vaginal tissues were collected from 5 women with prolapse. Mouse tissues were collect form 6 of six-weeks-old female Webster mice.



**Figure 3.1 RT-PCR of major Phase I metabolizing enzymes in human cervicovaginal tissues**

Lanes 1, 2, and 3: serially diluted pooled human liver (from 6 donors) cDNA transcribed from 100, 10, and 1 ng of total RNA. Lane 4: Human ectocervix cDNA (50 ng) pooled from seven patients.  $\beta$ -2-microglobulin ( $\beta$ 2mg) was used as internal control to indicate cDNA loading in PCR.

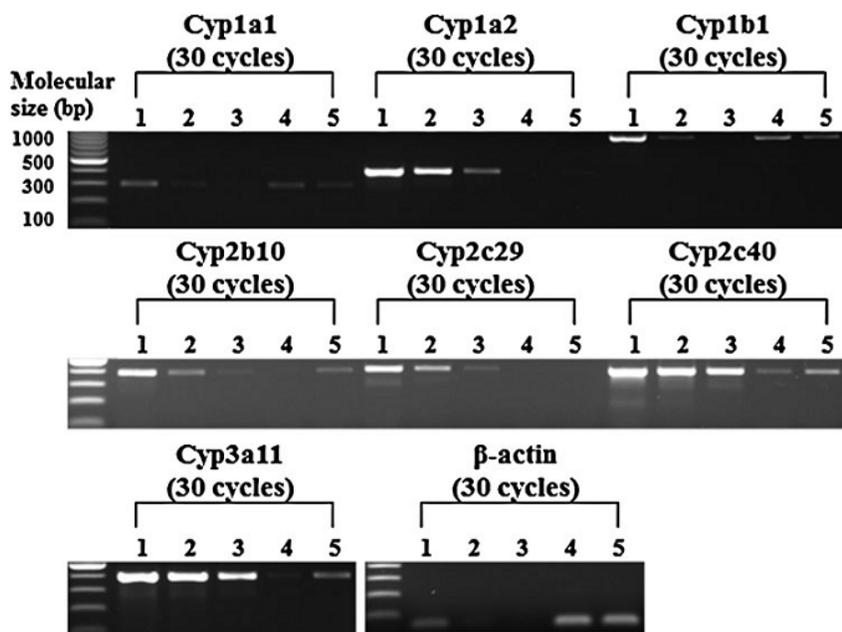


**Figure 3.2 RT-PCR of major Phase II metabolizing enzymes in human cervicovaginal tissues**

Lanes 1, 2, and 3: serially diluted pooled human liver cDNA transcribed from 100, 10, and 1 ng of total RNA. Lane 4: Human ectocervix cDNA (50 ng) pooled from seven patients. Lane 5: Human vagina cDNA (50 ng) pooled from five patients.  $\beta$ 2-microglobulin ( $\beta$ 2mg) was used as internal control to indicate cDNA loading in PCR.

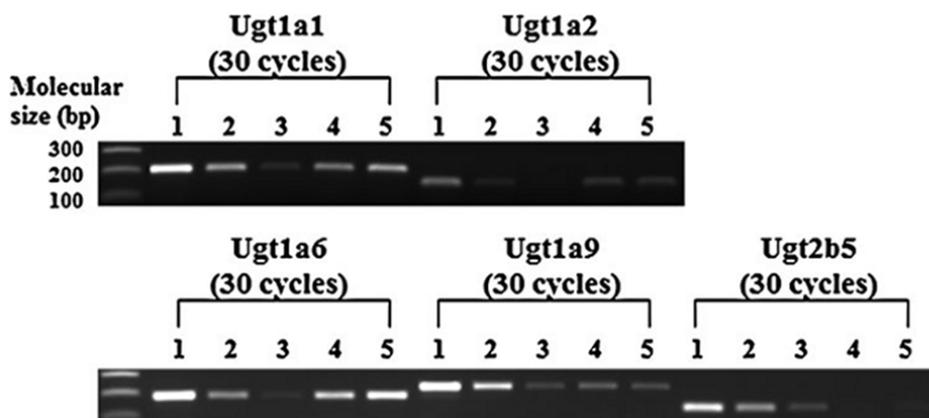
### 3.3.2 Conventional RT-PCR screening of CYP and UGT metabolizing enzymes in mouse cervicovaginal tissues

The expression of mouse enzymes that are orthologous to human CYP1A1, CYP1B1, and UGT1A1 was examined in cervix and vagina (Table 3.5 and Table 3.6). Mouse Cyp1a1, Cyp1b1, and Ugt1a1 are moderately expressed as compared to the mouse liver levels. In addition to these three enzymes, several other commonly studied Cyp and Ugt isoforms were also examined in mouse cervicovaginal tissue. Ugt1a2 and 1a6 were found to be highly expressed. No profound difference between mouse cervix and vagina was observed in terms of expression level (Figure 3.3 and Figure 3.4).



**Figure 3.3 RT-PCR of major Phase I metabolizing enzymes in mouse cervicovaginal tissues**

Lanes 1, 2, and 3: serially diluted liver cDNA pooled from five mice transcribed from 100, 10, and 1 ng of total RNA. Lane 4: cervix (50 ng) pooled from three mice. Lane 5: vagina (50 ng) pooled from five mice.  $\beta$ 2-microglobulin ( $\beta$ 2mg) was used as internal control to indicate cDNA loading in PCR.



**Figure 3.4 RT-PCR of major Phase II metabolizing enzymes in mouse cervicovaginal tissues**

Lanes 1, 2, and 3: serially diluted liver cDNA pooled from five mice transcribed from 100, 10, and 1 ng of total RNA. Lane 4: cervix (50 ng) pooled from three mice. Lane 5: vagina (50 ng) pooled from five mice.

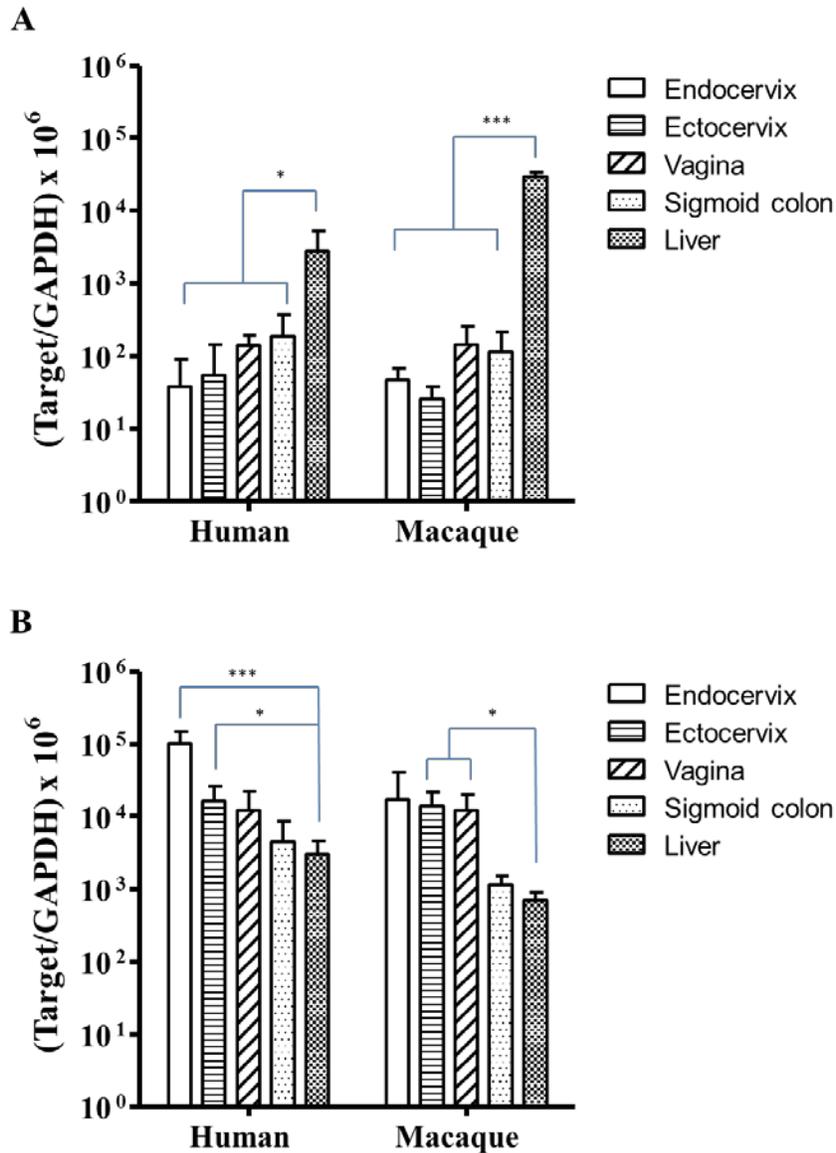
### 3.3.3. The mRNA levels of CYP1A1, 1B1 in the genital tract and colorectal tissues of premenopausal women and macaques

From conventional RT-PCR results, CYP1A1 and CYP1B1 were the most highly expressed and relevant enzymes in human ectocervical tissue. Therefore, the real-time PCR was conducted to confirm and examine mRNA levels of these two enzymes in both human and macaque genital tract and colorectal tissues. In human tissues, the CYP1A1 mRNA levels in endocervix, ectocervix, vagina and sigmoid colon were significantly lower than their levels in the liver in which the CYP enzyme expression and functionality have been well studied, while there was no significant difference among endocervix, ectocervix, vagina and colon (Figure 3.5A). The CYP 1B1 mRNA level in endocervix was significantly higher than that in any other type of tissue, while there was no difference among ectocervix, vagina, sigmoid colon and liver (Figure 3.5B). Although CYP1A1 and 1B1 were considered to metabolize similar pools of substrates, their

relative mRNA levels were different from each other, and were tissue-dependent. The mRNA levels of CYP1A1 and 1B1 were similar in human liver, while the CYP1B1 was expressed at a significantly higher level compared to 1A1 in the female genital tract and colonic tissues (Figure 3.5, A and B).

In macaque tissues, similar mRNA expression patterns were observed for both CYP1A1 and 1B1 (Figure 3.5, A and B). However, differences were also observed between macaque and human. The CYP1A1 level in macaque liver was significantly higher than the liver CYP1B1 level (Figure 3.5, A and B). In addition, the CYP1B1 level was not significantly different among all the types of macaque tissue examined (Figure 3.5B).

It should be pointed out that the quantification approach used in real time PCR study did not directly compare the absolute quantity of mRNA copies. Instead, relative quantification (CYP level is normalized to GAPDH level in the same organ) was used when the mRNA levels were compared between different organs or across species. By normalizing the CYP level to the GAPDH level in the same cDNA sample, we could know the CYP expression relative to a highly expressed housekeeping gene in the same tissue and species which, in our opinion, may be more biologically meaningful than directly comparing the absolute mRNA copy numbers. Our method of comparison was also used in previous publications by our group and other researchers who studied gene expression across different tissues [236].

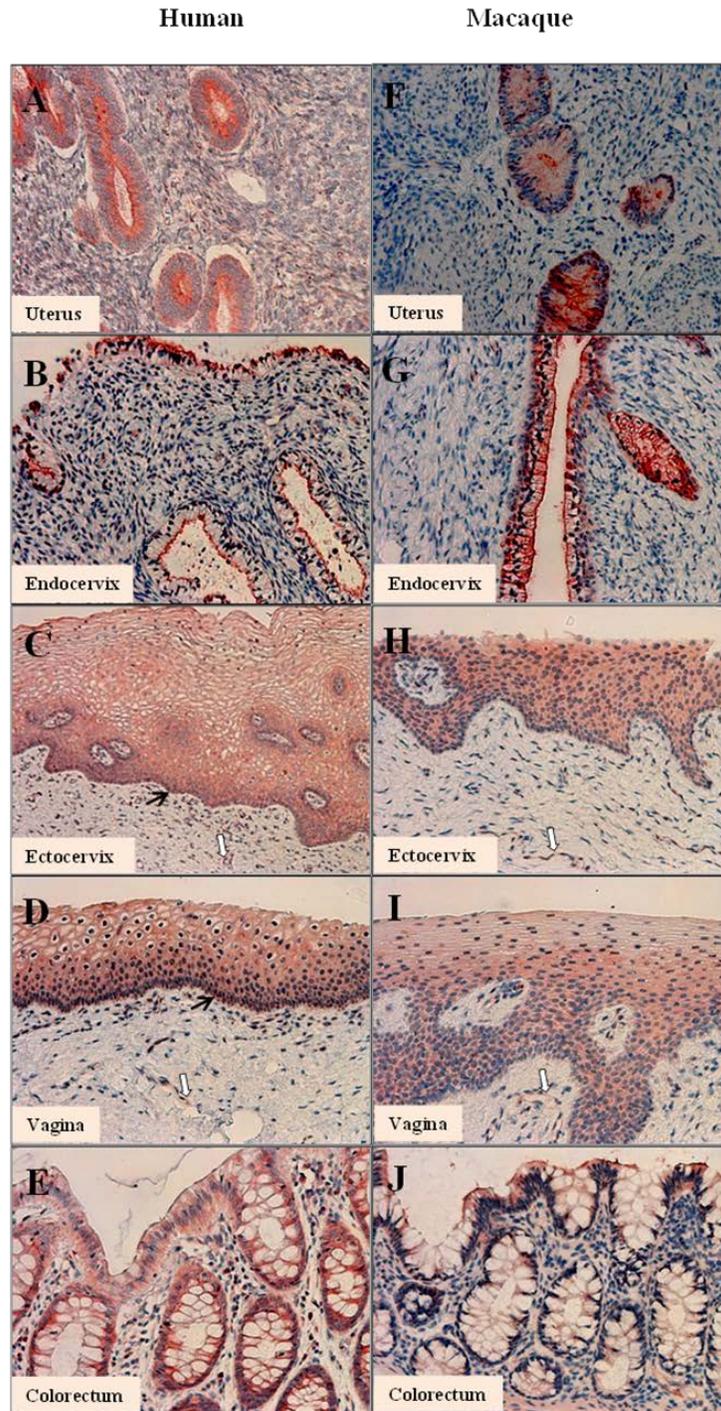


**Figure 3.5 The mRNA levels of CYP1A1 and CYP1B1 in the genital tract and colon tissue from humans and pigtailed macaques**

The tissues for endocervix, ectocervix, vagina, sigmoid colon and liver were from human donors (endocervix: n= 4; ectocervix: n= 6; vagina: n= 5; sigmoid colon: n= 5; liver: n= 6). 50-200 mg of tissue was collected from each donor and used for RNA extraction and later RT-PCR analysis. The threshold cycle numbers (Ct) of enzymes and GAPDH of each sample were measured in triplicates, and the average Ct was used to reflect the Ct of a tested gene. All tested gene levels were generated using  $2^{-\Delta Ct}$  method and normalized to GAPDH and multiplied by  $10^6$ . The data shown represent mean  $\pm$  standard deviation of all samples.

#### 3.3.4. The protein localization of CYP1A1 and 1B1 in the genital tract and colon tissues from humans and macaques

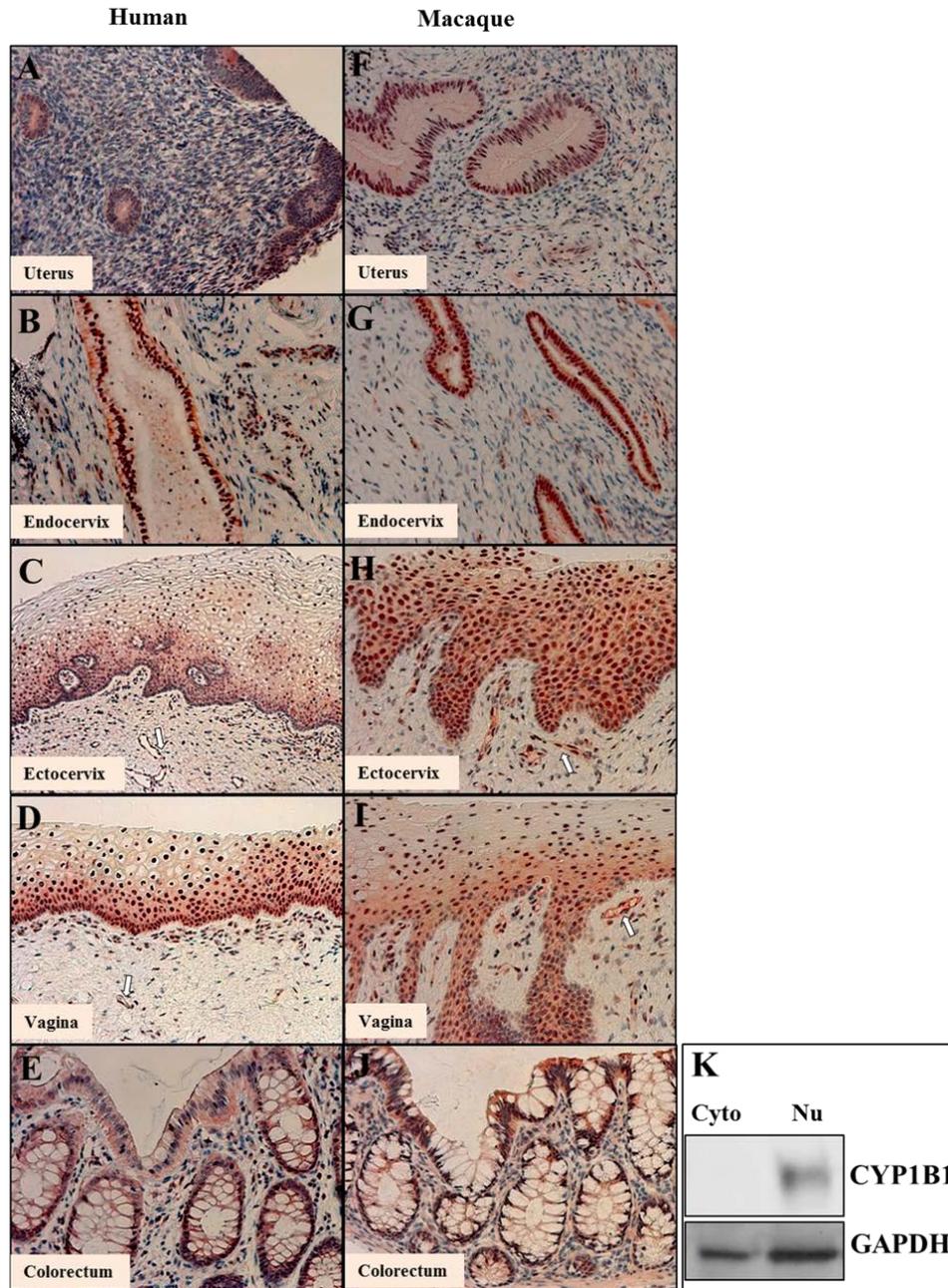
In human uterus and endocervix, the CYP1A1 protein was localized on the plasma membrane of the glandular (columnar) epithelial cells, as revealed by the immunohistochemical staining (Figure 3.6, A and B). In human ectocervix and vagina, weak cytoplasmic staining of CYP1A1 can be found in the basal layers of the squamous epithelium (Figure 3.6, C and D, black arrow). However, the positive staining pattern obtained for CYP1A1 was not confirmed when western blot was performed on the tissue fraction extracted from the ectocervix, which might due to the low protein concentration. In human sigmoid colon, the staining was overall weak on the luminal epithelial cells. The signal appeared stronger on the plasma membrane of the glandular epithelial cells (goblet cells) (Figure 3.6E). The staining patterns of CYP1A1 in macaque genital tract tissues were very similar to those of human tissues (Figure 3.6, F-I). Both human and macaque ectocervix and vagina appeared to have some positive staining on the vascular endothelial cells (Figure 3.6, C, D, H and I, white arrow). In addition, the staining of the glandular epithelial cells in macaque colorectum was very weak (Figure 3.6J). It should be noted that, due to limited patient resources, sufficient quantity of human uterus samples were not available to perform transcript quantification. The uterus was solely investigated using immunohistochemistry staining to study the CYP1A1 and CYP1B1 localization profile in the tissue.



**Figure 3.6 Localization of CYP1A1 protein in the genital tract and colon tissue from humans and pigtailed macaques**

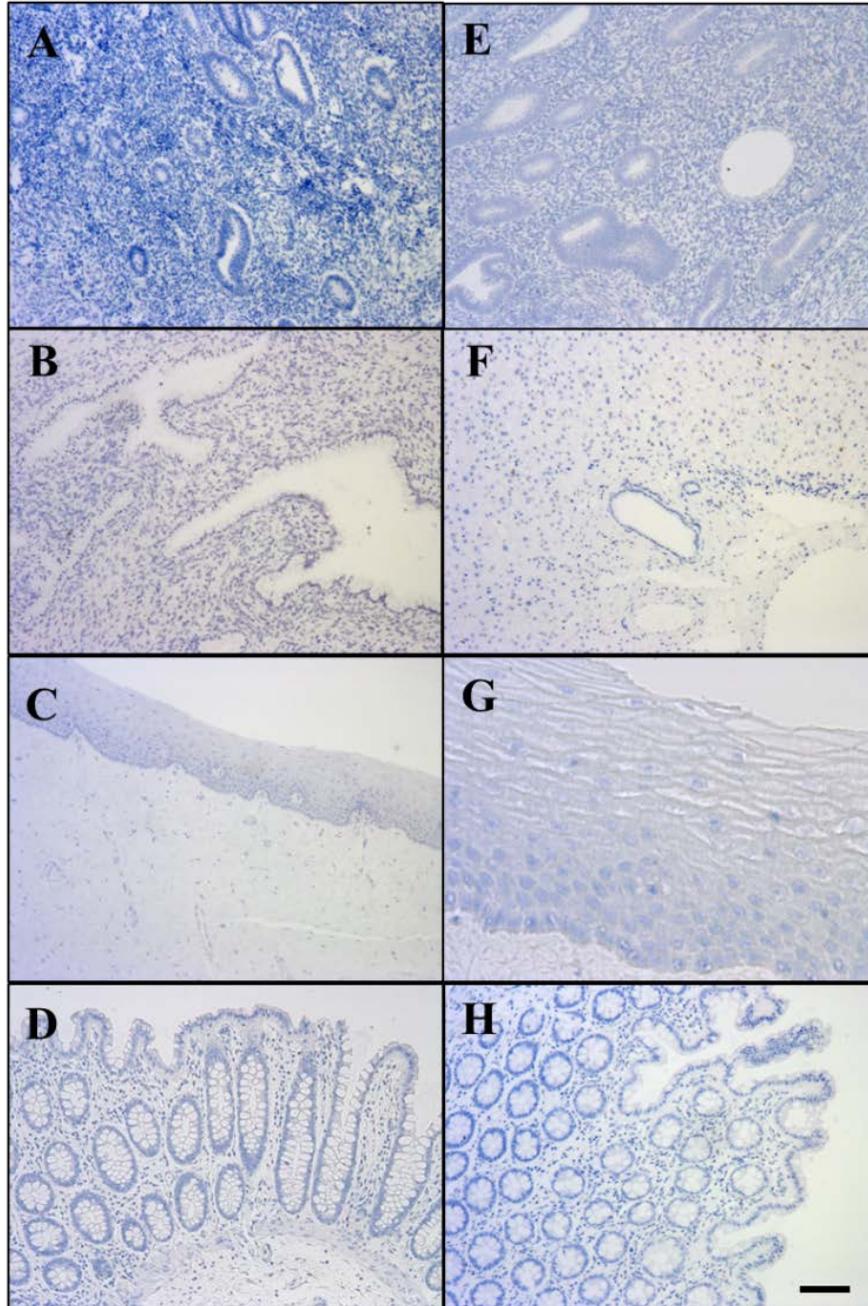
**A-E:** human uterus, endocervix, ectocervix, vagina and colorectal tissue. **F-J:** macaque uterus, endocervix, ectocervix, vagina and colorectal tissue. Black arrow: basal layers of the squamous epithelium, white arrows: vascular endothelial cells. Magnification: 10 × for **C**, 20 × for **A, B, D, E, F, G, H, I, J**.

In human genital tract tissues, weak CYP1B1 staining was observed in the glandular (columnar) epithelial cells in the uterus (Figure 3.7A). Intense nucleus staining was observed in the glandular epithelial cells of endocervix (Figure 3.7B), as well as throughout the entire squamous epithelial cells of the ectocervix and vagina, not just the basal layers (Figure 3.7, C and D). In human sigmoid colon, the CYP1B1 staining was primarily found in the epithelial cells (Figure 3.7E). Interestingly, the CYP1B1 staining was concentrated in the nucleus and distinguished from the surrounding cytoplasm. This nucleus distribution pattern could be clearly observed in human endocervix, ectocervix and vagina (Figure 3.7, B-D). The nucleus localization of CYP1B1 in the lower genital tract tissues was confirmed in Western blot, using human ectocervix as the representative genital tract tissue (Figure 3.7 K). The nucleus fraction extracted from the ectocervical tissue showed a much more intense band, compared to the cytoplasmic fraction prepared from the same type of tissue. In macaque genital tract tissues, the staining patterns of CYP1B1 were very similar to those of human tissues (Figure 3.7, F-I). However, the macaque uterus appeared to have stronger staining than human uterus (Figure 3.7F). In addition, similar staining of CYP1B1 was observed in the macaque colorectum compared to human tissue (Figure 3.7J).



**Figure 3.7 Localization of CYP1B1 protein in the genital tract and colon tissue from humans and pigtailed macaques**

**A-E:** human uterus, endocervix, ectocervix, vagina and colorectal tissue. **F-J:** macaque uterus, endocervix, ectocervix, vagina and colorectal tissue. White arrows: vascular endothelial cells. Magnification:  $10\times$  for **C** and **H**,  $20\times$  for **A**, **B**, **D**, **E**, **F**, **G**, **I**, **J**. **K:** Western blot analysis of CYP1B1 in cytoplasmic and nuclear fractions from human premenopausal ectocervix. Upper panel: CYP1B1; lower panel: GAPDH as internal reference; Cyto: cytoplasmic fraction; Nu: nuclear fraction.

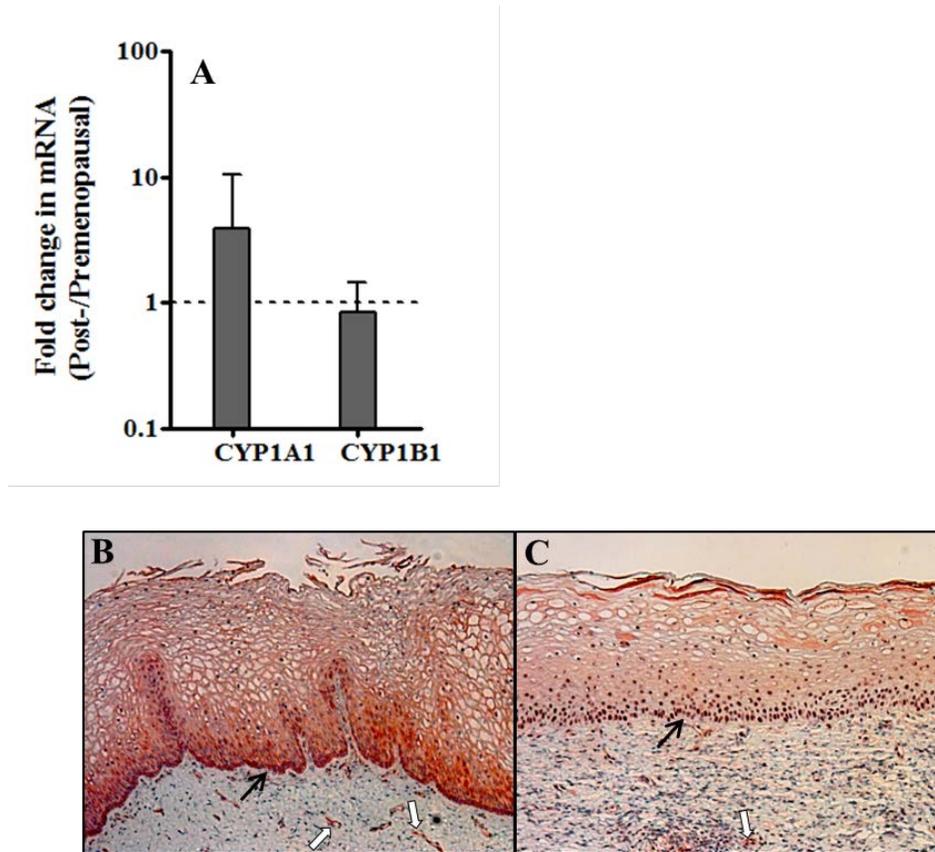


**Figure 3.8 Representative negative controls for CYP1A1 (A-D) and CYP1B1 (E-H)**

A-D: human uterus, human endocervix, human ectocervix, human colon. E-H: human uterus, human liver, human ectocervix, human colon. Scale bar is 50  $\mu\text{m}$  for A, B, C, D, F, and H; 100  $\mu\text{m}$  for E; 200  $\mu\text{m}$  for G.

### 3.3.5. The effect of menopause on the expression of CYP1A1 and CYP1B1 in human ectocervix

The effect of menopause was examined in the postmenopausal human ectocervix. Compared to the premenopausal tissues, the CYP1A1 mRNA level appeared to be higher in the postmenopausal tissues, but no statistical significance was observed. The mRNA levels of CYP1B1 was not significantly different between pre- and postmenopausal ectocervical ectocervix (Figure 3.9). However, the CYP1A1 and 1B1 protein in the postmenopausal tissues appeared to be more condensed in the basal layers of the epithelial cells, compared to premenopausal tissues.



**Figure 3.9** The effect of menopause on the expression of CYP1A1 and CYP1B1 in human ectocervix

**A:** fold changes in mRNA levels of CYP1A1 and CYP1B1 in human ectocervix. **B and C:** protein localization of CYP1A1 (**B**) and CYP1B1 (**C**) in postmenopausal human ectocervix. Black arrow: basal layers of the squamous epithelium, white arrows: vascular endothelial cells. Magnification:  $10 \times$  for **B** and **C**.

### **3.4. Discussion and conclusions**

The data presented in the study evaluated the expression profile of CYP and UGT enzymes in the human ectocervix and vagina. Since the mRNA expression of enzymes does not necessarily correlate with their activity in microbicide absorption and disposition, the information on protein localization and mRNA/protein regulation will facilitate the prioritization and experimental design of the functional studies of these enzymes.

The highly expressed enzymes in the lower genital tract may have an impact on the cervicovaginal exposure of various microbicide candidates (Table 3.7). For antiretroviral drug metabolism studies involving Phase II UGT enzymes, most studies were focused on UGT1A1 and 2B7, while the metabolism mediated by other highly expressed UGTs in cervicovaginal tissue such as UGT1A7, 1A10, 2B15, and 2B17 were poorly characterized. It is possible that as the antiretroviral drugs will be subjected to more thorough screening, more microbicide candidates will be included in this table. It should be noted that the mRNA level of CYP and UGT enzymes were examined in cervical and vaginal tissues and compared to their levels in liver tissue. However, for some isoforms, such as CYP1A1 and 2B6, the liver is not the organ with the highest expression. Also, the enzyme expression level in other tissues might be underestimated due to the over-high level in liver.

Of note, some enzymes isoforms may impact microbicide PK-PD indirectly. For example, CYP1A1 is not directly involved in the inactivation of antiretrovirals, however, the CYP1A1 genotype was reported to impact the treatment outcome of highly active antiretroviral therapy (HAART) among female smokers [237, 238]. The genotype with higher activity is associated

with impaired HAART effectiveness. Since this enzyme could convert smoke-derived compounds to DNA-damaging agents that promote HIV-1 gene expression and replication, the proposed explanation is that higher CYP1A1 activity contributes to a higher concentration of DNA-damaging agents in HIV-infected tissues. Whether the cervicovaginal CYP1A1 could impact the microbicide efficacy among female smokers would be an interesting topic for future research.

**Table 3.7 Antiretroviral drugs that could be affected by the highly expressed metabolizing enzymes in human cervicovaginal tissues**

Super-family	Name of transporter/enzyme	Expression level in human ectocervix/vagina	Antiretroviral drugs as substrates	Drug class
Phase I CYP enzymes	CYP1A1	+++	Affects the effectiveness of HAART in female smokers	RTI, PI
Phase II UGT enzymes	UGT1A1	+++/>+++	Efavirenz, Raltegravir, Elvitegravir, Dolutegravir	RTI, II

From the IHC staining results (Figure 3.6 and Figure 3.7), CYP1A1 and CYP1B1 express mostly in cervical and vaginal epithelium of both human and macaque, therefore for the real time PCR study, the tissue epithelial layer was isolated using a Thomas-Stadie-Riggs tissue slicer (described in the method section). If the API of microbicide product is a substrate of these highly expressed CYPs and UGTs enzymes, they might be quickly metabolized and deactivated before reaching the immune cells and lymphnode resident in deeper part of stromal tissue. If this is the

case, formulation modification can be used to facilitate the delivery of API to target cells. Prodrug strategy can also be utilized to overcome the elimination of the active drug molecules.

In addition, the expression and localization profile of the CYP1A1 and CYP1B1 in human tissues has implications for the future research of these two enzymes in many aspects. CYP1B1 appeared to be the most important member of CYP1 family in female lower genital tract (CYP1A1, CYP1A2, and CYP1B1 are considered three major members of the CYP1 family [239]). In this study, the CYP1B1 expression level was found to be significantly higher than the CYP1A1 level in the female genital tract and colorectal tissue in both human and macaque (Figure 3.5). CYP1A2 expression was not detectable in pooled human ectocervix as reported here and in our previous publication [236]. Taken together, these observations suggested that CYP1B1 was the most important member for the CYP1 enzyme activities in female genital tract.

Furthermore, the tissue-dependent subcellular localization patterns of CYP1A1 and 1B1 indicate that these enzymes may target different subcellular compartments in different types of tissues. The plasma membrane localization of CYP1A1 in the endocervix was not observed before, however the plasma membrane localization of other CYP isoforms, including CYP1A2, CYP2B has been reported [234, 235, 240]. Marie-Anne et al. studied CYP1A2 localization in rat hepatocytes and found that newly synthesized CYP1A2 followed the intracellular vesicular flow to the plasma membrane, and that the plasma membrane CYPs were mainly located on the external surface [234]. This localization indicates that the major metabolic site of CYP1A1 in the endocervix might be the plasma membrane, and those substrates that are preferentially

distributed into the lipid bilayer membrane may be subject to most extensive metabolism by CYP1A1.

Although the majority of the CYPs are expressed in the cytoplasm, Muskhelishvili et al has reported that the CYP1B1 protein is located in the nucleus, in both neurons and ectocervix [241]. This finding correlates with those of this study, in that CYP1B1 was found to be concentrated in the nucleus of endocervical, ectocervical, and vaginal epithelium. This indicates that, among all the substrates, those that can enter the nucleus would be preferentially metabolized by CYP1B1 in the female lower genital tract. However, Carnell et al claimed that no evidence was obtained for CYP1B1 expression in the nucleus with protein expression present exclusively in the cytoplasm of the prostate carcinoma cells [149]. This discrepancy in CYP1B1 protein subcellular localization may be due to the differences in CYP1B1 physiologic function between tissues. Another possibility is the specificity of the antibody. The CYP1B1 antibodies used in published reports came from various sources and target different epitopes. It is possible that different levels of antibody specificity may contribute to the differences observed in localization patterns.

Although the CYP1A1 and CYP1B1 mRNA levels were not significantly different in postmenopausal ectocervix, the protein localization appeared to be altered in postmenopausal tissues. Future investigations on the factors that can modulate CYP1A1/1B1 expression and activity are warranted, for better delineation of the CYP1A1/1B1 functionality in reproductive and colorectal pathology, as well as for anticancer pharmacology. It should be noted that, in addition to age and menopausal status, which were the only available patient data for the specimens procured for these studies, other factors such as stage of the sexual cycle, ethnic group,

and clinical history may impact the findings presented. The impact of these additional factors should be evaluated in the future studies.

The comparison in expression patterns of cervicovaginal enzymes between human and animals has implications for the utilization of these animal models in the microbicide testing and functionality study of cervicovaginal enzymes, as well as guide the rational selection of preclinical models in future study of CYP1A1 and CYP1B1. Since the pigtailed macaque tissue showed consistent expression patterns of the CYP1A1 and CYP1B1 to human tissues, the macaque is a good model of choice in future investigations. It should be noted that the difference observed in mRNA does not necessarily reflect a difference in enzyme expression/activity. Although compared to other animal models, such as the mouse, the macaque is considered a more clinically relevant model for the female genital tract in pharmaceutical research, it is possible that enzymes involved in the metabolism of drugs in humans might perform differently in the macaque. Animals express different amounts of enzyme isoforms compared to human. For example, there are 103 of sub family CYP genes in mice whereas only 57 in humans. It is possible that enzymes involved in the metabolism of an antiretroviral in humans would belong to a completely different subfamily in animal models. Further work is needed to investigate whether there are interspecies difference at both a protein and activity level. Taken together, the discoveries obtained from this study will likely facilitate the examination of the regulation of CYP1A1 and 1B1, which will in turn lead to the development of novel approaches that utilize CYP1A1 and 1B1 in the treatment or prevention of various diseases in the female genital and colorectal tissues.

## **Conclusions**

To summarize, this study is the first to provide a systematic evaluation of the expression profile of CYP and UGT enzymes in female genital tissues of human, mice and macaque. The mRNA of several enzyme isoforms, such as CYP1A1, CYP1B1 and UGT1A1, are highly expressed in the tested tissues. Although some of them are not directly involved in the metabolism of currently available ARVs, they may impact microbicide PK-PD indirectly. In addition, we found that CYP1A1 and CYP1B1 were expressed in the female genital and colorectal tissues of human and macaque. However, the mRNA level and protein localization of these CYP enzymes depended on the type of tissue examined. The resemblance between human and pigtailed macaque in the expression patterns of CYP1A1 and CYP1B1 suggests the utility of the macaque model for the future studies.

## **Acknowledgements**

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acknowledge the University of Pittsburgh Health Sciences Tissue Bank for helping with the tissue acquisition. We also thank the patients for their willingness to provide tissue for research.

## **4. EXPRESSION, REGULATION, AND FUNCTION OF PHOSPHORYLATING ENZYMES IN TISSUES AND CELLS RELEVANT TO HIV-1 SEXUAL TRANSMISSION**

### **4.1. Introduction**

Nucleoside analogues are very important in both HIV treatment and prevention. After being taken up into cells, nucleoside analogues are subsequently phosphorylated into the active metabolites, i.e. nucleoside triphosphate (diphosphates for nucleotide), which compete in the cell with the endogenous substrate dATP for incorporation into viral DNA and stop HIV synthesis. To be effective, NTRIs, such as tenofovir (TFV), require intracellular phosphorylation to its active form (Figure 1.3). However, the cellular kinase(s) responsible for phosphorylation of TFV has not yet been clearly established. Several groups have studied the enzymatic activity of several phosphorylating enzymes (PEs) for tenofovir activation *in vitro* and *ex vivo*, and found that isoforms of adenylate kinases (AKs), nucleoside diphosphate kinases (NDPKs), and creatine kinases (CKs) may be involved in TFV activation [140-146]. However there is limited data available regarding the expression of PEs in the female genital and colorectal tissue relative to immune cells.

In addition, the concentration of intracellular TFV diphosphate (TFV-DP) in blood was highly correlated with reduction in HIV in a clinical trial [18]. Models have shown that differences in dATP in the intestinal vs. the genital tract combined with tenofovir levels observed in cervicovaginal versus colon tissues could substantially impact the effectiveness of TFV based pre-exposure prophylaxis depending on the route of transmission [242]. As TFV requires phosphorylation by nucleotide kinases, it has been suggested that studying the expression

variation and kinetics of the enzymes that are responsible for TFV activation are important for promoting the TFV-based microbicide development [29, 243, 244].

In this chapter, we aim to study the mRNA expression of 7 TFV-related PEs in tissues and cells relevant to HIV sexual transmission, and examine the effect of pathophysiological factors on the expression and activity of these PEs. We first systematically compared the mRNA expression of PEs in the cervicovaginal and colorectal tissues of human and animal models (macaque, rabbit, mouse), as well as in the cell lines derived from human cervicovaginal tissues (Ect1/E6E7, VK2/E6E7) and T cell (PM1). Since a major target of HIV is CD4<sup>+</sup> T cells, we also studied the expression of these PEs in CD4<sup>+</sup> cells isolated from female blood. In addition, we examined the effect of two hormonal contraceptives and two proinflammatory cytokines on PE expression and TFV-converting activity, in cervicovaginal epithelial cell lines and a T cell line. Finally, we tested whether the change in TFV activation under hormone and cytokine treatments is correlated with the alteration in TFV efficacy, using the T cell line PM1. Such data is important as it will help establish the expression profile of important PEs in the tissues and cells relevant to HIV sexual transmission, and will facilitate the identification of the pathophysiological factors that affect PE expression and activity in antiretroviral-based HIV prevention. Overall, the results generated in this chapter have wide implications on the improvement of PrEP products for HIV prevention.

## **4.2. Materials and methods**

#### 4.2.1. Acquisition of human and animal tissues

The human female genital tissues were obtained from women undergoing hysterectomy for benign conditions. Human liver tissues were obtained from donors (age: 15-75) without hepatic malignancies. Human sigmoid colon tissue were obtained from female (age: 20-79) undergoing colectomy. The colon tissues used were histologically examined without malignancies. All human ectocervical tissues used in this study were obtained through the University of Pittsburgh Health Sciences Tissue Bank as per approved IRB protocol PRO09110431 and were de-identified and collected through an Honest Broker. The epithelial layer of ectocervical tissue was isolated using a Thomas-Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ) and epithelial thickness was measured prior to the studies as described elsewhere [179].

The three pigtailed macaques used in this study were 12.6, 18.7 and 17.6 years old, and were of reproductively active. The macaques were maintained in Washington National Primate Research Center at the University of Washington, which was supported by the Office of Research Infrastructure Programs of the National Institutes of Health under Grant Number P51OD01042, and in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. The macaque tissues (uterus, endocervix, ectocervix, vagina, colorectum, and liver) were acquired through the Tissue Distribution Program, which was approved by the Institutional Animal Care and Use Committee.

Mice tissues were collected from mice undergoing natural cycling or estrus stage being synchronized with PMSG/Depo-Provera treatment. Six-week-old female Swiss Webster mice were used in the study which was approved by the University of Pittsburgh Institutional Animal

Care and Use Committee (IACUC) (protocol # IS00000654). The estrous cycle stage of each mouse was determined using vaginal cytology as described previously. Briefly, 20  $\mu$ L of normal saline was injected into mouse vaginal lumen with pipette. The vaginal lavage smear was taken out and spread onto a glass slide and observed under a bright light microscope. The cell morphology of mouse vaginal smear from different stages was list in Table 3.1. For estrous stage synchronization, mice were intraperitoneally (i.p.) injected with pregnant mare's serum gonadotropin (PMSG, Sigma, 5 IU/ mouse), and the synchronized estrous stage should be reached 24 hours after the administration. For diestrus stage synchronization, mice were subcutaneously (s.c.) injected twice with Depo-Provera (Pfizer, 3 mg/mouse) on Day 1 and Day 5, and the synchronized diestrus stage should be reached 7 days after the first time injection.

In addition, the rabbit tissues were obtained from euthanized New Zealand White rabbits (6-8 weeks old) which were a kind gift from Dr. Eric Romanowski of the Eye & Ear Institute of University of Pittsburgh. The rabbits were used for the testing of topical drug products for the treatment of eye infection. The infection and drug action were contained only in the cornea. All tissue was collected under the regulations of the Institutional Animal Care and Use Committee (IACUC) of University of Pittsburgh.

#### 4.2.2. Cell culture and conditions

Human CD4+ T cells were isolated from normal human peripheral blood mononuclear cells (PBMCs). PBMCs were from three HIV-1-negative blood female donors were purchased from Central Blood Bank (Pittsburgh, PA). PBMCs were purified by differential centrifugation, and stored in the gas phase of liquid nitrogen until needed. After the isolation,  $1 \times 10^8$  PBMC were used

to isolate CD4<sup>+</sup> T cells by negative selection using the Dynabeads Untouched Human CD4<sup>+</sup> T Cell Kit (Invitrogen 11346D). From the purified CD4<sup>+</sup> T cells,  $1 \times 10^7$  cells were saved for flow cytometry, while the remainder was frozen as cell pellets for RNA extraction.

The human ectocervical (Ect1/E6E7) and vaginal epithelial (VK2/E6E7) cell lines were purchased from American Type Culture Collection (ATCC). The cells were cultured in keratinocyte-serum free medium (GIBCO-BRL 17005-042) with 0.1 ng/mL human recombinant EGF, 0.05 mg/mL bovine pituitary extract, 44.1 mg/L calcium chloride (final concentration 0.4 mM) with 50 IU/mL penicillin and 50 µg/mL streptomycin at 37 °C under 5% of carbon dioxide (CO<sub>2</sub>). PM1 cells, which are a human T cell line, were obtained from the NIH AIDS Reagent Program (Germantown, MD). PM1 cells were cultured in 90% RPMI 1640 medium without phenol red (Corning 17-105-CV) supplemented with glutamax, 10% fetal bovine serum (ThermoFisher), 100 IU/ml penicillin and streptomycin sulfate.

To evaluate the effect of hormones and cytokines on PEs mRNA expression, VK2/E6E7 and PM1 cells were treated with MPA or P4; or IL-1β or IL-8 (PeproTech AF-200-01B, AF-200-08M), or vehicle solution used to create the stocks in EtOH or cell culture medium for 48 hours. Then, cells were collected and subjected to RT-PCR analysis of the mRNA levels of PEs. To evaluate the effect of hormones and cytokines on TFV-DP formation, VK2/E6E7 and PM1 cells were treated with MPA or P4; or IL-1β or IL-8; or vehicle solution alone with TFV solution for 48 hours. Then, cells were lysed and total protein concentration was quantified using BCA protein assay kit (ThermoFisher). The intracellular TFV and TFV-DP were measured by LC-MS/MS as previously described [244]. For TFV efficacy study, PM1 cells were culture in

96-well plate with final density of 20,000 cells/well, and were treated with MPA, P4, IL1 $\beta$  or IL8 along with TFV solution. Cells were then challenged with 3,000 TCID<sub>50</sub> of HIV-1<sub>BaL</sub> and cultured for 4 days. Culture supernatant was then collected and tested for HIV-1 replication using the p24gag enzyme-linked immunosorbent assay (Perkin Elmer Life and Analytical Sciences, Inc., Waltham, MA, USA).

#### 4.2.3. Real-time RT-PCR.

The total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The genomic DNA remaining in the total RNA preparations was removed using the Turbo DNase kit (Ambion). The reverse transcription was performed using the SuperScript III First Strand Synthesis Kit (Invitrogen). The real-time RT-PCR was conducted using the Ssofast Evergreen Mastermix (Bio-Rad), in the CFX Touch 96 thermocycler (Bio-Rad). Due to the high homology between human and macaque in the mRNA sequences of PEs and GAPDH, the same primers were used to detect both human and macaque genes. For the PCR programs, the initial denaturation was set at 95°C for 30 s, and 40 cycles of amplification were run at 95°C for 5 s and 60°C for 5 s. A melt curve analysis was conducted upon the completion of the amplification cycles, to ensure the specificity of the PCR reaction. The PCR efficiency was confirmed to be within the range of 90%-110%, using serially diluted cDNA standards prepared from the liver or colon tissues. The mRNA levels of PEs in a given tissue sample were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the same sample, using the 2<sup>- $\Delta$ Ct</sup> method. The information of primer sequences used in real time PCR were summarized in Table 4.1-4.3

In the current study, a standard curve served (serially diluted liver cDNA) as positive control and water without any cDNA was used as negative control. The standard curve was prepared using serially diluted liver cDNA, it showed amplification in real time PCR, which means the positive control has given a positive signal. If the liver expression level for an enzyme is very high, then the serially diluted cDNA will show lower levels. As long as the tissue sample signal falls within the range of liver samples, then the high expression level in the liver does not affect the tissue sample results. If tissue sample signal in PCR is out of the range of the liver sample standard curve, then the liver samples need to be diluted more, so that the tissue sample signal could be covered by the standard curve.

**Table 4.1 Primer sequences for real time PCR of phosphorylating enzymes in human and macaque tissues**

Gene Name	GeneBank Accession no.	Primer sequence 5' to 3'
adenylate kinase (AK2)	NM_001625	F-GCAGAACCCGAGTATCCTAAAG G R-TTCCCAGCATCCATAGTTGCC
adenylate kinase AK3L1 (AK4)	NM_001005353	F: CAAGGCCAGCACCGAAGTT R: GATCACATGGTCTGGAACCAAAA
nucleoside diphosphate kinase 1 (NME1 or NDPKA)	NM_198175	F: GCGTTTTGAGCAGAAAGGAT R: CGGTCCTTCAGGTCAACGTA
nucleoside diphosphate kinase 2 (NME2 or NDPKB)	NM_002512	F: AAAGACCGACCATTCTTCCC R: ATCACTCGGCCTGTCTTCAC
creatine kinase, brain (CKB)	NM_001823	F: GCTGCGACTTCAGAAGCGA R: GGCATGAGGTCGTCGATGG
creatine kinase, mitochondrial 1 (CKMT1)	NM_001015001. 1	F: CACCTGTCATCCACACCAAG R: GGCAGGTCCAGAGCAAGTAG
creatine kinase, mitochondrial 2 (CKMT2)	NM_001099736	F: CCAAGCGCAGACTACCCAG R: GGTGTCACCTTGTTGCGAAG
Human GAPDH	NM_001256799	F: GGAGCGAGATCCCTCCAAAAT R: GGCTGTTGTCATACTTCTCATGG

**Table 4.2 Primer sequences for real time PCR of phosphorylating enzymes in mouse tissues**

Gene Name	GeneBank Accession no.	Primer sequence 5' to 3'
adenylate kinase (AK2)	NM_001033966	F: GGCTTCCGAACCGGAGATTC R: CAGACACAAAAGTTTTTCAGCCAG
adenylate kinase AK3L1 (AK4)	NP_001171075	F: ACCGTTTGCGAAAGGATCG R: TTGCCACGTCACCAACTTCC
nucleoside diphosphate kinase 1 (NME1 or NDPKA)	NM_008704	F: AGGAGCACTACACTGACCTGA R: GGTTGGTCTCTCCAAGCATCA
nucleoside diphosphate kinase 2 (NME2 or NDPKB)	NP_001070997	F: ATCGACCTGAAAGACCGTCCT R: TCAGTTCTTCGGGCTTAAACC
creatine kinase, brain (CKB)	NM_021273	F: AGTTCCTGATCTGAGCAGC R: GAATGGCGTCGTCCAAAGTAA
creatine kinase, mitochondrial 1 (CKMT1)	NM_009897	F: TGTCTTCAAGAGTCAGAACTGGC R: AGCATCCACCACAACACGTT
creatine kinase, mitochondrial 2 (CKMT2)	NM_198415	F: AAGCTGTCTGAGATGACGGAG R: GGGACACTGGCTTATCAAACA
Mouse Gapdh	NM_008084	F: GCCCAGAACATCATCCCTGC R: CCGTTCAGCTCTGGGATGACC

**Table 4.3 Primer sequences for real time PCR of phosphorylating enzymes in rabbit tissues**

Gene Name	GeneBank Accession no.	Primer sequence 5' to 3'
adenylate kinase (AK2)	XM_002720703	F: ACTGTGAGGCAGGCAGAAAT R: CGTGGTAGGAACGTCCACTT
nucleoside diphosphate kinase 1 (NME1 or NDPKA)	NM_001204749	F: AGTGATGCTCGGGGAGACTA R: GAATGATGTTCTGCCAACC
nucleoside diphosphate kinase 2 (NME2 or NDPKB)	NM_001204750	F: GCAGCACTACATCGACCTGA R: ATCACTCGGCCTGTCTTCAC
creatine kinase, brain (CKB)	NM_001082261	F: GTTCATGTGGAACCCTCACC R: TTGAGCACCTCGGAGAACTT
creatine kinase, mitochondrial 1 (CKMT1)	NM_001171313	F: CATGTGGAATGAACGTCTGG R: TTTGATGTGCACTCCTGCTC
creatine kinase, mitochondrial 2 (CKMT2)	NM_001163070	F: ATCAACGAGGAGGACCACAC R: GACAGAAGCGCTCAAAGACC
Rabbit Gapdh	NM_001082253	F: ATCACTGCCACCCAGAAGAC R: GTGAGTTTCCCGTTCAGCTC

#### 4.2.4. Flow Cytometry

The following mouse anti-human monoclonal antibody (mAb) was used for staining: CD4/FITC. The antibody was purchased from BD Biosciences (San Jose, CA) and titrated under assay conditions to determine an optimum saturating dilution 1.0 (1.0µg or 20µL). Cells were first washed with Dulbecco's Phosphate-Buffered Salines (DPBS, Thermo Fisher Scientific, Waltham, MA) and centrifuged at 800x g for 5 minutes. The supernatants were decanted completely and

the cells were stained with LIVE/DEAD® Fixable Aqua Dead (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Thermo Fisher Scientific, Waltham, MA) stain fluorescence for 15 minutes. The cells were vortexed gently in the residual fluid and then transferred to labeled 5mL round bottom tubes (Falcon® 5mL Round Bottom Polystyrene Test Tube, Corning Inc., Corning, NY) containing 20µL of mAb CD4/FITC. The tubes were vortexed and incubated for 20 minutes at room temperature protected from light. 1mL of BD FACS™ lysing solution (BD Biosciences, San Jose, CA) was then added to the tubes. The tubes were vortexed and incubated for 10 minutes at room temperature protected from light. The tubes were then centrifuged at 800xg for 5 minutes and after decanting the supernatants, the cells were washed with 1mL of DPBS and centrifuged again. Following the final decanting step, the cells were resuspended in 250µL DPBS and ready to be run.

Flow cytometric analysis was performed on a BD™ LSRFortessa cytometer (BD Biosciences, San Jose, CA) using BD™ FACSDIVA software (BD Biosciences, San Jose, CA). The acquisition was restricted to the lymphocyte gate based on characteristic properties of the cells in the forward and side scatter. The FCS files were analyzed using FlowJo V10 (FLOWJO, LLC, Ashland, OR). For each graph, two factors were compared using all processed events. Cells were stained with LIVE/DEAD Fixable Aqua stain fluorescence (Life Technologies, Eugene, OR) to define viable cells. CD4/FITC mAb verses Live/Dead viability stain was used to gate on viable CD4 positive cells. Side scatter area verses CD4/FITC mAb was used to gate on all CD4 positive cells.

#### 4.2.5. Statistical analysis

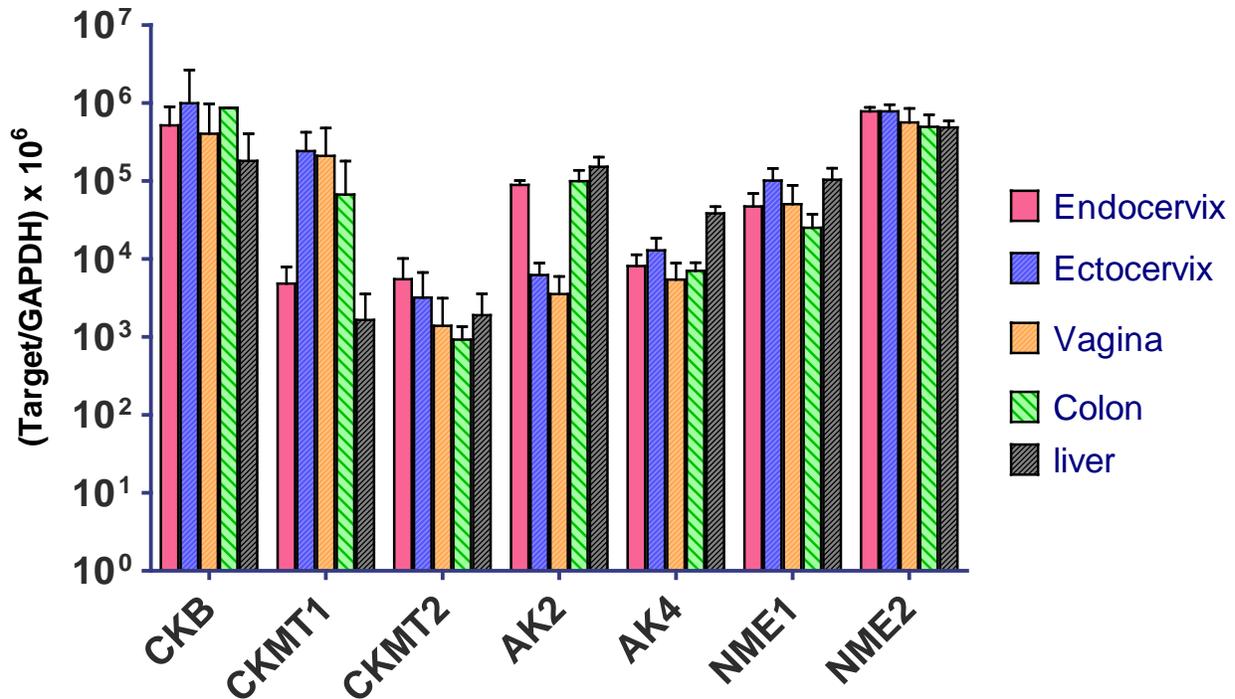
Statistical analyses were performed in Excel (Microsoft Corporation) and Prism (GraphPad Software, Inc.). One way analysis of variance (ANOVA) with Bonferroni post-hoc test was used to compare the enzyme mRNA expression in different tissue types. Student t-test was used for the comparison of drug/metabolite concentrations in different treatments.  $P < 0.05$  was considered as statistically significant.

### **4.3. Results**

#### 4.3.1. Real-time RT-PCR analysis of a select panel of PEs in human tissues

Since the PEs expression profile have not been well established for human cervicovaginal tissues, real time RT-PCR was used to examine mRNA levels of the PEs that were most relevant to TFV activation. Seven PEs were examined, and these results are summarized in Figure 4.1. Human lower genital tract and colon tissue had similar mRNA expression levels for CKB, CKMT2, NME1, and NME2 to levels of these in liver tissue. However, ectocervical, vaginal, and colon tissues showed a dramatically higher level of CKMT1 as compared to that of endocervical and liver tissue ( $p < 0.05$ ). In contrast, endocervical, colon and liver tissues had higher expression of AK2 as compared to levels found for the ectocervical and vaginal tissues (Figure 4.1). Statistical analyses were conducted to compare the expression of each enzyme across different types of human tissues. The mRNA level of CKMT1 was significantly lower in endocervical and liver tissue than that of ectocervical and vaginal tissues ( $p < 0.05$ ). Colon tissue also show higher expression level of CKMT1 as compared to endocervical and liver tissue, however there was no significant difference among these three tissue types, AK4 was significantly lower in ectocervical and vaginal tissues than the level found in other tissues ( $p < 0.005$ ). Meanwhile, liver

had slightly higher but statistically significant mRNA expression of AK4 than other tissues examined ( $p < 0.001$ ). For other tested PEs, there was no difference between genital tract tissues and colon/liver tissues.



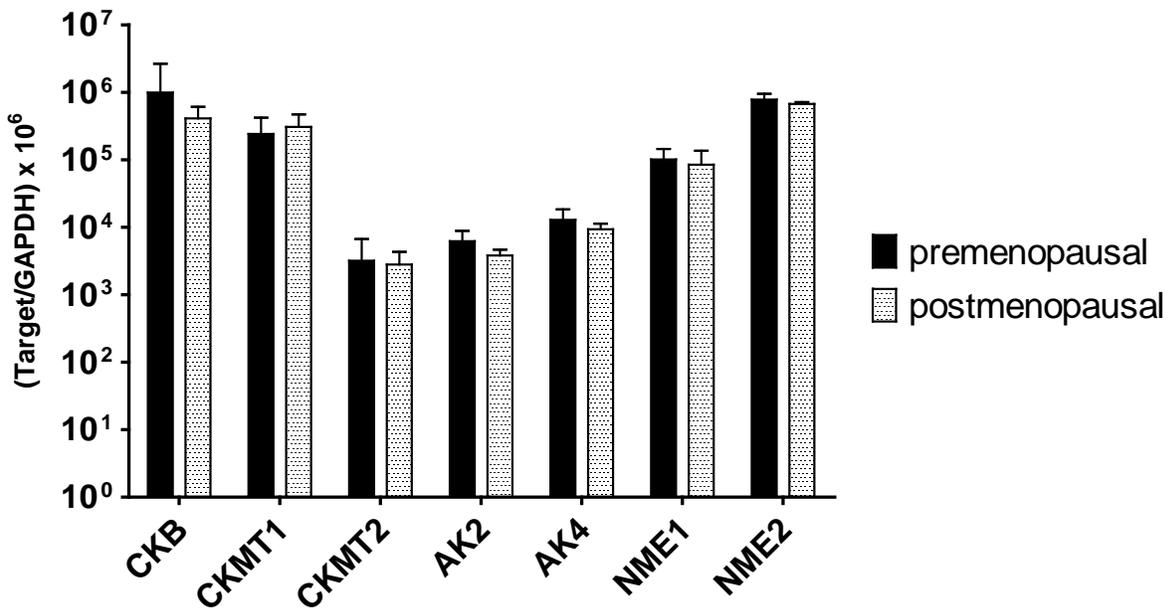
**Figure 4.1 Real-time RT-PCR analysis of a select panel of PEs in human tissues**

The tissues for endocervix, ectocervix, vagina, sigmoid colon and liver were from human donors (endocervix:  $n = 4$ ; ectocervix:  $n = 6$ ; vagina:  $n = 5$ ; sigmoid colon:  $n = 5$ ; liver:  $n = 6$ ). 50-200 mg of tissue was collected from each donor and used for RNA extraction and later RT-PCR analysis. The threshold cycle numbers (Ct) of enzymes and GAPDH of each sample were measured in triplicates, and the average Ct was used to reflect the Ct of a tested gene. All tested PE gene levels were generated using  $2^{-\Delta Ct}$  method and normalized to GAPDH. The data shown represent mean  $\pm$  standard deviation of all samples. Bonferroni's Multiple Comparison Test was performed to compare the enzyme expression between different tissues and only the enzymes which showed significant difference are listed (Table 4.4)

**Table 4.4 Bonferroni's Multiple Comparison Test of PE's expression in human tissue**

	<b>Bonferroni's Multiple Comparison Test</b>	<b>Mean Diff.</b>	<b>t</b>	<b>Significant ? P &lt; 0.05?</b>	<b>Summar y</b>	<b>95% CI of diff</b>
CKB	colon vs liver	0.6885	3.31	Yes	*	0.01818 to 1.359
	colon vs vagina	0.6976	3.273	Yes	*	0.01075 to 1.384
AK2	colon vs ectocervix	0.09338	5.302	Yes	***	0.03845 to 0.1483
	colon vs vagina	0.09606	5.454	Yes	***	0.04113 to 0.1510
	liver vs endocervix	0.06344	3.379	Yes	*	0.004879 to 0.1220
	liver vs ectocervix	0.1465	8.724	Yes	****	0.09413 to 0.1989
	liver vs vagina	0.1492	8.883	Yes	****	0.09681 to 0.2016
	endocervix vs ectocervix	0.08307	4.424	Yes	**	0.02451 to 0.1416
	endocervix vs vagina	0.08575	4.567	Yes	**	0.02719 to 0.1443
AK4	colon vs liver	-0.03146	9.812	Yes	****	-0.04146 to -0.02146
	liver vs endocervix	0.03036	8.884	Yes	****	0.01970 to 0.04102
	liver vs ectocervix	0.02554	8.355	Yes	****	0.01601 to 0.03507
	liver vs vagina	0.03304	10.81	Yes	****	0.02351 to 0.04258
NME1	colon vs liver	-0.07905	3.687	Yes	*	-0.1459 to -0.01217
	colon vs ectocervix	-0.07624	3.556	Yes	*	-0.1431 to -0.009370

The effect of menopause on PEs mRNA expression was also examined by comparing enzyme levels in pre- and postmenopausal human ectocervix. There was no significant difference observed across all 7 PEs examined (Figure 4.2).



**Figure 4.2 Real-time RT-PCR analysis of a select panel of PEs in human ectocervical tissue from premenopausal and postmenopausal women**

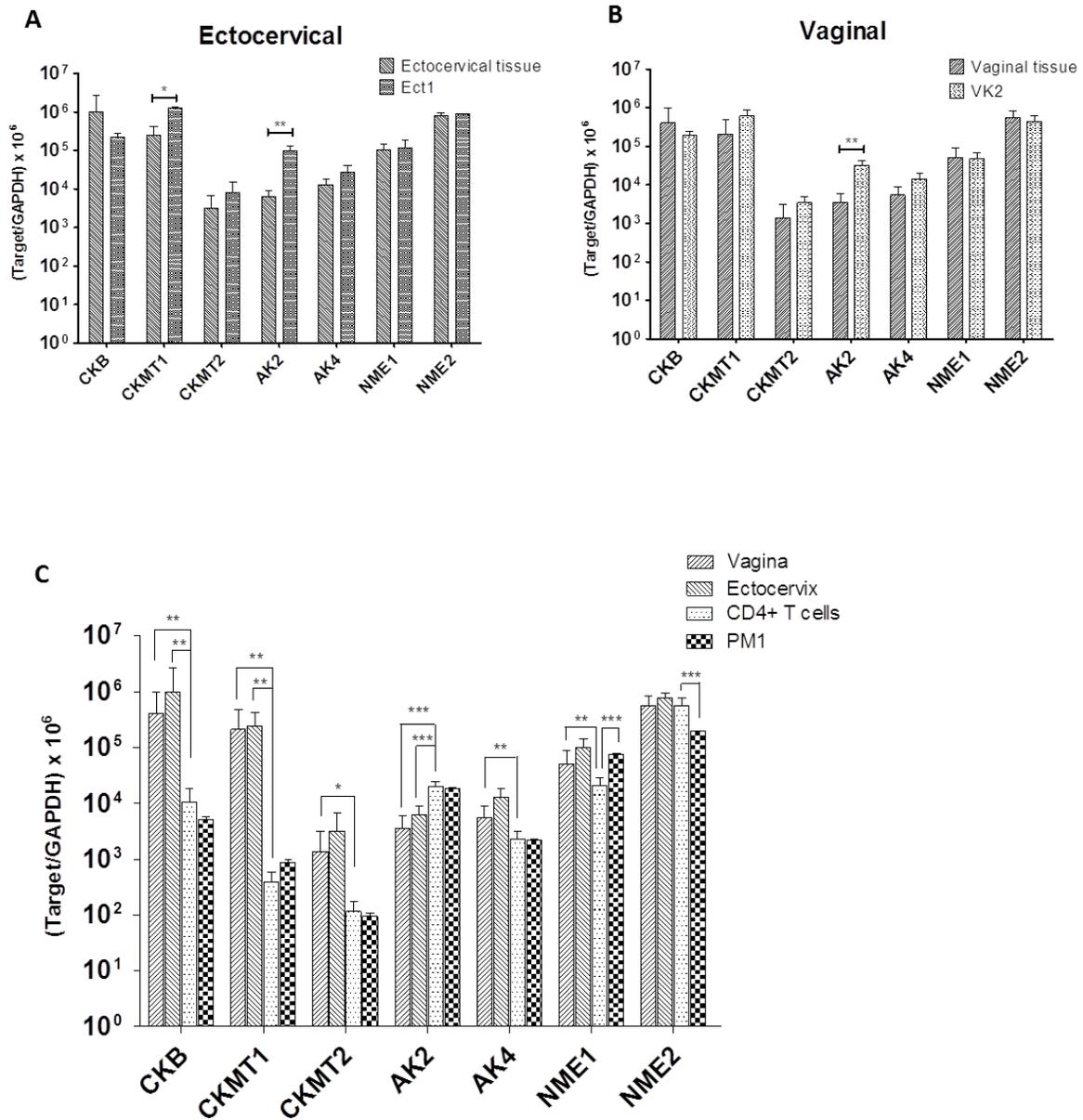
#### 4.3.2. Real-time RT-PCR analysis of a select panel of PEs in human cell/ cell lines

The same panel of PEs was tested in cell lines derived from human ectocervical epithelium (Ect1/E6E7), and vaginal epithelium (VK2/E6E7). Considering the CD4+ T cells are one of the major target cells of HIV, we also screened the PE expression in CD4+ T cells isolated from human blood (female donor), as well as a human T cell line, PM1.

As shown in Figure 4.3, although CKMT1 and AK2 had significant higher level in Ect1 cell line (Figure 4.3A), and AK2 had higher level in VK2 cell line than those found in the human tissues (Figure 4.3B), the levels of most of the tested enzymes found in the cervicovaginal epithelial cell lines were not significantly different from those in corresponding tissues.

However, when comparing PEs levels between cervicovaginal tissues and the CD4+ T cells, the majority of enzymes we tested, including CKB, CKMT1, CKMT2, AK4, and NME1 showed significantly decreased mRNA level in T cells as compared to their levels in cervicovaginal tissues (Figure 4.3C). However, AK2 had highest level in CD4+ T cells, and NME2 had similar level among cervicovaginal tissues and T cells.

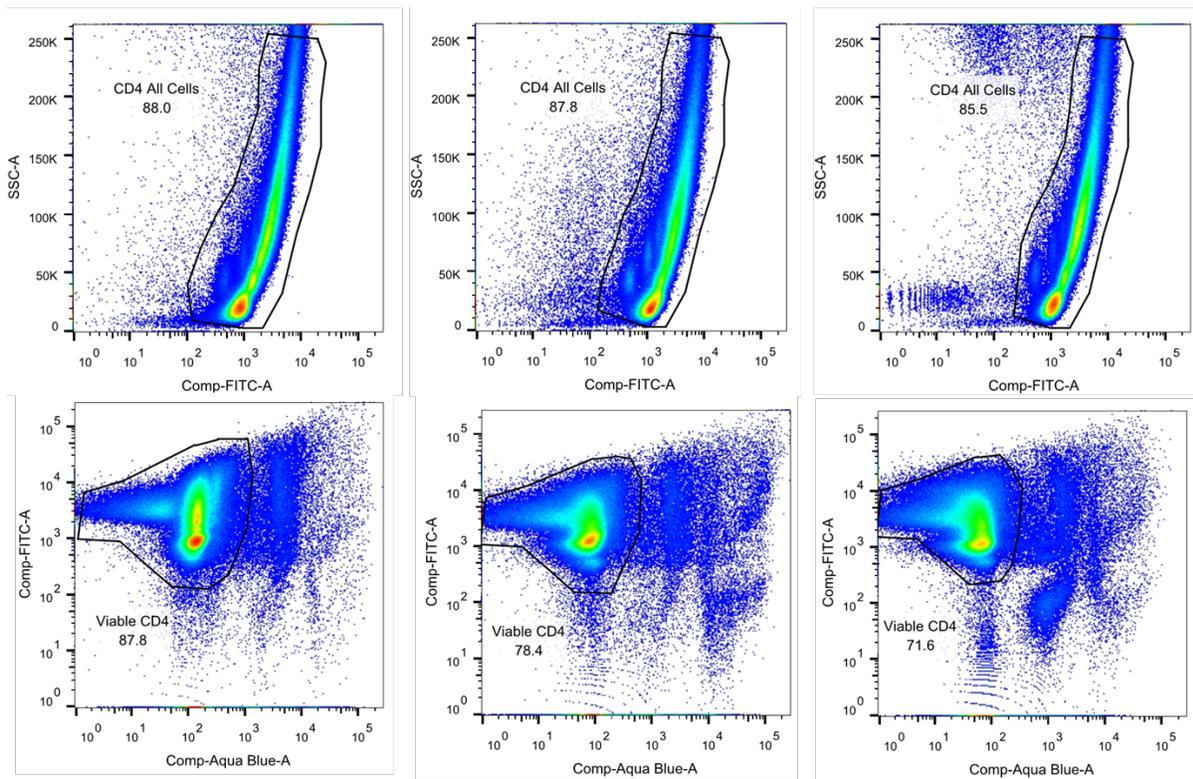
In the PM1 cell line, the majority of tested PEs had similar mRNA expression levels as compared to CD4+ T cells (Figure 4.3C). Though PM1 cell line had significantly higher level of NME1 and lower level of NME2 compared to CD4+ T cells, the difference were slight and less than 10 fold (Figure 4.3C). Therefore, in the following studies, PM1 was used to examine the effects of HC and ILs on TFV activity.



**Figure 4.3 Real-time RT-PCR analysis of a select panel of PEs in in human cervicovaginal tissue, CD4+ T Cell and cell lines**

The threshold cycle numbers (Ct) of enzymes and GAPDH of each sample were measured in triplicates, and the average Ct was used to reflect the Ct of a tested gene. All tested PE gene levels were generated using  $2^{-\Delta Ct}$  method and normalized to GAPDH. The data shown represent mean  $\pm$  standard deviation of all samples. A, mRNA expression of PEs between human ectocervical tissue and ectocervical epithelial cell line Ect1; B, mRNA expression of PEs between human vaginal tissue and vaginal epithelial cell line VK2; C, mRNA expression of PEs in human cervicovaginal tissues, CD4+ T cells and T cell line PM1.

Before doing the real time PCR, human CD4+ T cells were isolated from normal female PBMC (n=3), and the purity and viability were examined using flow cytometry. As shown in Figure 4.4, the purity of three groups were 88.0, 87.8, and 85.5 (%), and the viability were 87.8, 78.4 and 71.6 (%) respectively.



**Figure 4.4 CD4+ T cell purity and viability in the isolated cell suspensions**

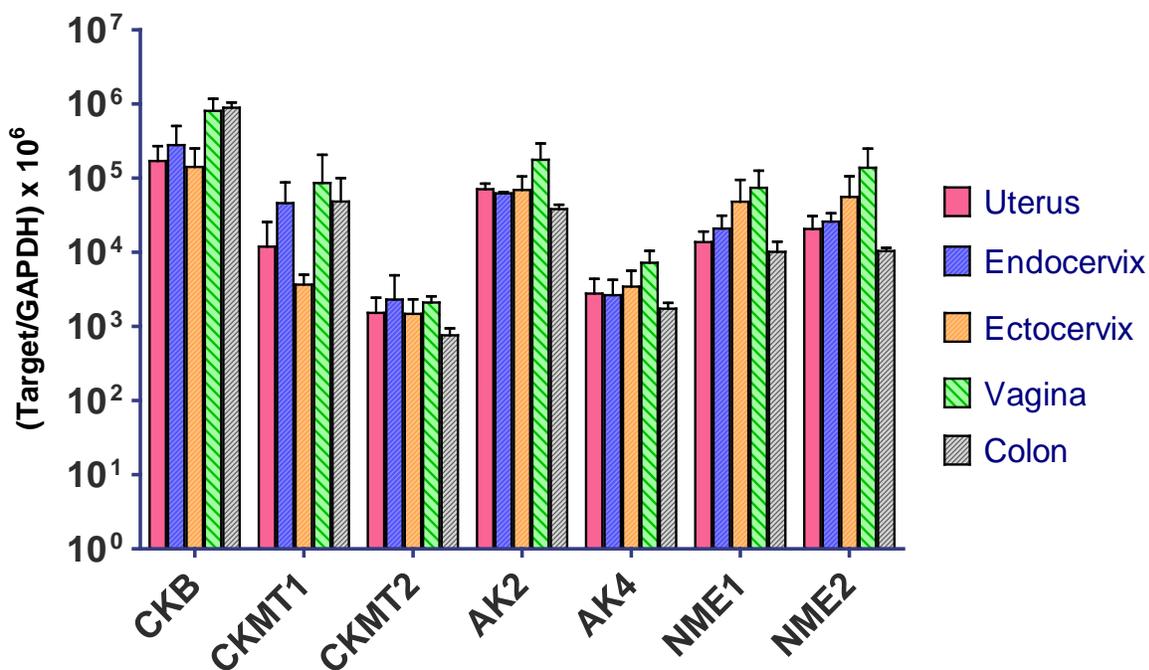
The upper panel is the percentage of CD4+ T cell in the isolated cell suspensions from 3 female blood donors, and the lower panel is the CD4+ T viability (%) for each sample. For each graph, two factors were compared using all processed events. Side scatter area versus Live/Dead viability stain was used to gate on all viable cells. CD4/FITC mAb versus Live/Dead viability stain was used to gate on viable CD4 positive cells. Side scatter area versus CD4/FITC mAb was used to gate on all CD4 positive cells.

#### 4.3.3. mRNA levels of selected PEs in cervicovaginal and colon tissues from pigtailed macaque, rabbit, and mouse

Besides human tissues, we also compared the PEs mRNA levels (using GAPDH as internal control) across different species. The purpose of this comparison is to inform model selection for future studies of PEs function based on the mRNA expression data. It might be not practical to find a model that has identical expression pattern of multiple PEs, but such comparisons will help the researchers make informed decision and will help correlating pharmacokinetic/pharmacodynamics (PK/PD) data between human subjects and animal models.

##### 4.3.3.1. mRNA levels of a select panel of PEs in pigtailed macaque tissues

The macaque model is a common and established animal model in the field of microbicides [234]. In this study, the same panel of PEs was examined in pigtailed macaque tissues. In macaques, the seven enzymes were also similarly expressed in endocervix, ectocervix, vagina, and colorectum compared to macaque liver levels (Figure 4.5). Similar to human tissues, CKMT2 and AK4 had relatively low mRNA expression level as compared to other tested PEs, and had similar mRNA levels for these enzymes across macaque genital tract tissues (Figure 4.5). Bonferroni's Multiple Comparison Test was performed to compare the enzyme expression between different tissues, however no statistical difference ( $P < 0.05$ ) was observed.



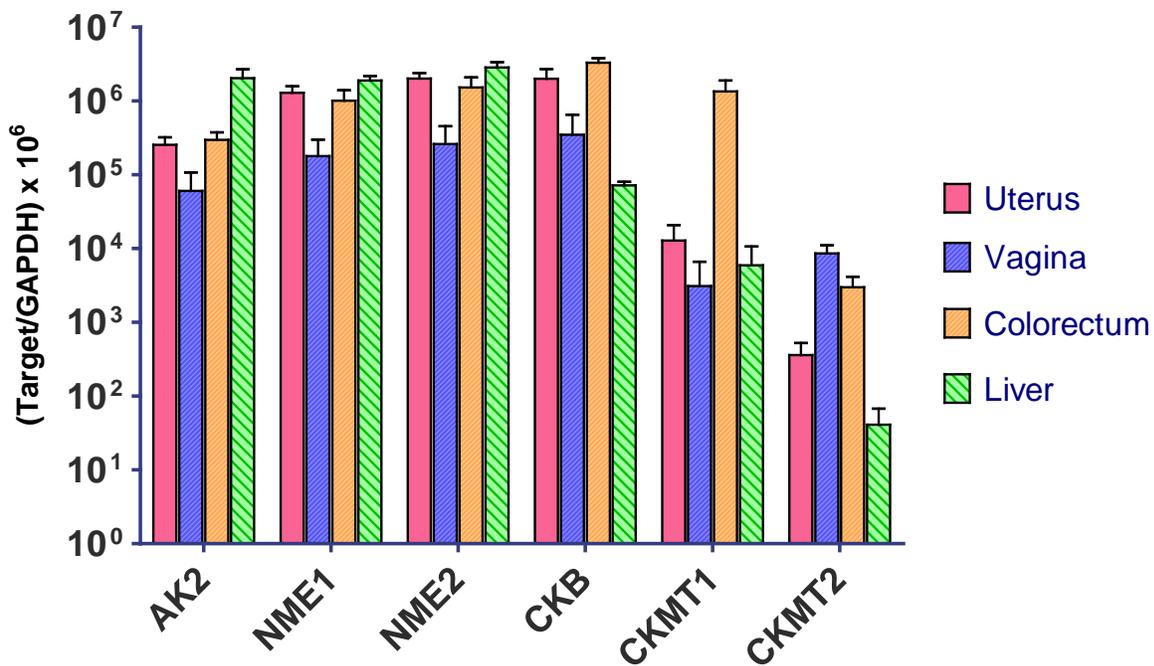
**Figure 4.5 Real-time RT-PCR analysis of a select panel of PEs in macaque tissues**

The tissues for endocervix, ectocervix, vagina, sigmoid colon and liver were from 3 macaques. 50-200 mg of tissue was collected from each region for RNA extraction. The threshold cycle numbers (Ct) of enzymes and GAPDH of each sample were measured in triplicates, and the average Ct was used to reflect the Ct of a tested gene. All tested PE gene levels were generated using  $2^{-\Delta Ct}$  method and normalized to GAPDH. The data shown represent mean  $\pm$  standard deviation of all samples. Bonferroni's Multiple Comparison Test was performed to compare the enzyme expression between different tissues, no statistical difference was observed.

#### 4.3.3.2. mRNA levels of a select panel of PEs in rabbit tissues

The rabbit model is commonly used for the safety testing of vaginally and rectally applied microbicides. The New Zealand White rabbit was recommended by the US FDA for the safety testing of vaginally or rectally administered microbicides[245]. Since the safety/toxicity profile of drug products partly depends on drug pharmacokinetics in the tissues, it is necessary to examine PEs expression pattern in rabbit tissues in comparison to human. Since there is no corresponding enzyme of human AK4 in rabbit, only six PEs (AK2, NME1, NME2, CBK,

CKMT1, CKMT2) were examined in rabbit tissues. Refer to Figure 4.6. Three out of six PEs, including AK2, NME1, and NME2, showed significantly lower expression level in rabbit vagina than in cervix, colorectum, and liver ( $p < 0.05$ ). CKB had a significantly lower levels in vagina than in cervix and colorectum ( $p < 0.05$ ), while CKMT1 had significantly higher level in colorectum than cervix and vagina ( $p < 0.05$ ). For CKMT2, its level was much higher in vagina than other types of tissue examined ( $p < 0.05$ ).



**Figure 4.6 Real-time RT-PCR analysis of a select panel of PEs in rabbit tissues**

The tissues for uterus, cervix, vagina, colorectum and liver were from 3-7 rabbits (uterus: n= 3, cervix n= 3; liver: n= 3; colorectum: n= 6; vagina: n= 7). 50-200 mg of tissue was collected from each region for RNA extraction. The threshold cycle numbers (Ct) of enzymes and GAPDH of each sample were measured in triplicates, and the average Ct was used to reflect the Ct of a tested gene. All tested PE gene levels were generated using  $2^{-\Delta Ct}$  method and normalized to GAPDH. The data shown represent mean  $\pm$  standard deviation of all samples. Rabbit endocervix and ectocervix were difficult to distinguish, therefore the rabbit genital tract part connecting vagina and uterine horns was obtained to represent the whole cervix. The uterine horn adjacent to cervix was obtained to represent the tissue similar to endocervix. Bonferroni's Multiple

Comparison Test was performed to compare the enzyme expression between different tissues and only the enzymes which showed significant difference are listed (Table 4.5).

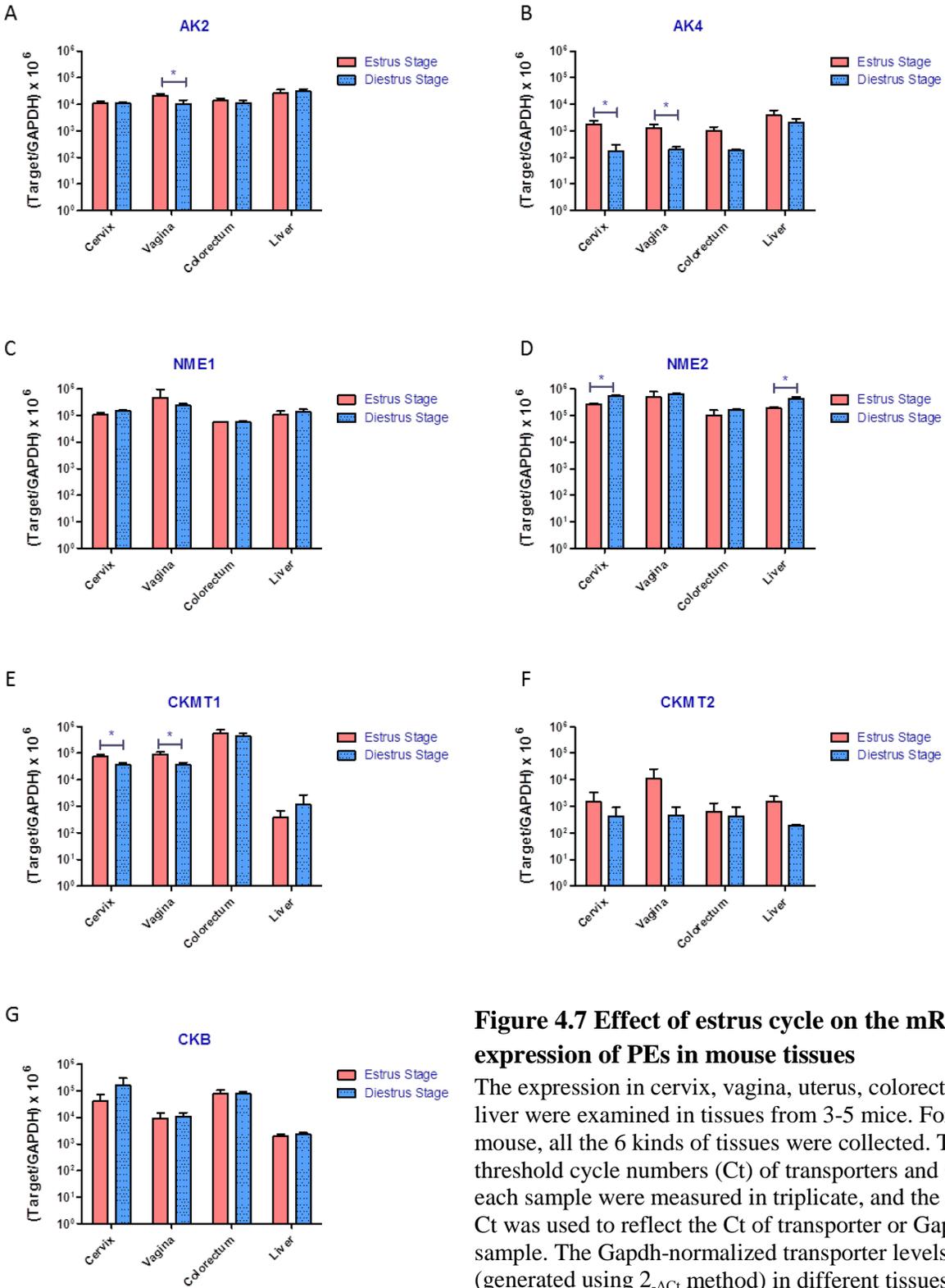
**Table 4.5 Bonferroni's Multiple Comparison Test of PE's expression in rabbit tissue**

	<b>Bonferroni's Multiple Comparison Test</b>	<b>Mean Diff.</b>	<b>t</b>	<b>Significant? P &lt; 0.05?</b>	<b>Summary</b>	<b>95% CI of diff</b>
AK2	Uterus vs Liver	-1.787	7.416	Yes	***	-2.576 to -0.9972
	Vagina vs Liver	-1.98	8.787	Yes	***	-2.719 to -1.242
	Colorectum vs Liver	-1.743	7.734	Yes	***	-2.481 to -1.004
NME1	Uterus vs Vagina	1.115	5.032	Yes	**	0.3889 to 1.841
	Vagina vs Colorectum	-0.8322	4.057	Yes	*	-1.504 to -0.1601
	Vagina vs Liver	-1.711	7.721	Yes	***	-2.437 to -0.9846
	Colorectum vs Liver	-0.8784	3.965	Yes	*	-1.604 to -0.1524
NME2	Uterus vs Vagina	1.75	5.221	Yes	**	0.6516 to 2.848
	Vagina vs Colorectum	-1.267	4.082	Yes	*	-2.283 to -0.2500
	Vagina vs Liver	-2.585	7.715	Yes	***	-3.683 to -1.487
	Colorectum vs Liver	-1.319	3.935	Yes	*	-2.417 to -0.2205
CKB	Uterus vs Vagina	1.656	4.778	Yes	**	0.5201 to 2.791
	Uterus vs Colorectum	-1.303	3.76	Yes	*	-2.438 to -0.1673
	Uterus vs Liver	1.932	5.215	Yes	**	0.7179 to 3.145
	Vagina vs Colorectum	-2.958	9.222	Yes	***	-4.009 to -1.907
	Colorectum vs Liver	3.234	9.335	Yes	***	2.099 to 4.370
CKMT1	Uterus vs Colorectum	-1.338	5.827	Yes	**	-2.090 to -0.5855
	Vagina vs Colorectum	-1.348	6.339	Yes	***	-2.044 to -0.6511
	Colorectum vs Liver	1.345	5.857	Yes	***	0.5924 to 2.097
CKMT1	Uterus vs Vagina	-0.008257	7.266	Yes	***	-0.01198 to -0.004533
	Vagina vs Colorectum	0.005633	5.354	Yes	**	0.002185 to 0.009080
	Vagina vs Liver	0.008576	7.547	Yes	***	0.004852 to 0.01230

#### 4.3.3.3. mRNA levels of a select panel of PEs in mouse tissues

The mRNA levels of AK2, AK4, NME1, NME2, CKMT1, CKMT2, and CKB were also examined in Swiss Webster mice treated with Depo-Provera for estrous stage synchronization.

This synchronized mouse model is used by researchers in the field of vaginal microbicides to evaluate the safety of vaginal products. The effects were tissue- and enzyme-dependent, however, in mouse female genital tract, some general tendencies appeared to exist. The mRNA levels of several PEs tended to be higher at estrus stage and lower at the diestrus stage. For example, in mouse vagina, the mRNA levels of AK2, AK4, and CKMT1 were higher at estrus stage compared to their levels at diestrus (Figure 4.7A, B, E). CKMT2 also showed 10 folds, yet not significantly, higher level in vaginal tissue at estrus stage than diestrus (Figure 4.7F). AK4 showed generally higher level at estrus stage in all tested type of tissues including cervical, vaginal, colorectal and liver (Figure 4.7B). NME1 and NME2 seemed to have similar level across all tissue types at both stages (Figure 4.7C and D). Mouse lower genital tract and colorectal tissue had higher CMKT1 level as compared to that found in mouse liver (Figure 4.7E), while CKMT2 showed relatively low but similar level across tissues (Figure 4.7F).



**Figure 4.7 Effect of estrus cycle on the mRNA expression of PEs in mouse tissues**

The expression in cervix, vagina, uterus, colorectum and liver were examined in tissues from 3-5 mice. For each mouse, all the 6 kinds of tissues were collected. The threshold cycle numbers (Ct) of transporters and Gapdh of each sample were measured in triplicate, and the average Ct was used to reflect the Ct of transporter or Gapdh in this sample. The Gapdh-normalized transporter levels (generated using  $2^{-\Delta Ct}$  method) in different tissues were plotted in the figure shown above. The data shown represent mean  $\pm$  standard deviation of all samples in a given group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

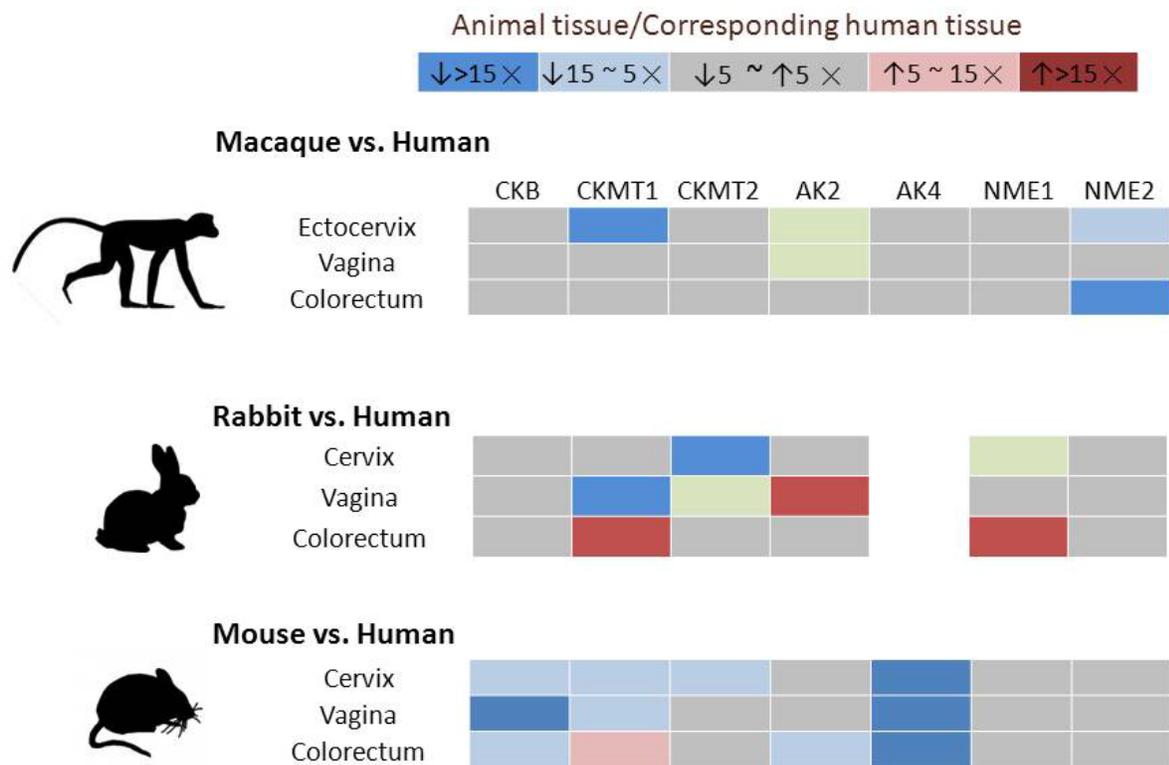
#### 4.3.3.4. mRNA expression of PEs across different species

We also compared the PEs mRNA levels (using GAPDH as the internal control) across different animal species. The purpose of this comparison was to inform model selection for future studies of PEs function based on the mRNA expression data. It may not be practical to find a model that has identical expression pattern of multiple PEs, but such comparisons will help researchers to make more informed decisions and will help correlating pharmacokinetic/pharmacodynamics data between animal models and humans.

In Figure 4.8, the enzyme expression in animal tissues from genital tract and colorectum was compared to the expression level in human tissue. Color codes are utilized to represent the relative enzyme level between animal and human. If the enzyme level is similar to human, gray color is used in the table. If the mRNA level is higher in animal, pink or red color were used, and if the level is lower in animal than human, blue color is used. As shown in Figure 4.8, in terms of cervicovaginal and colorectal PE expression pattern, the pigtailed macaque appeared to be the model with highest degree of similarity to human (most macaque tissue enzymes within  $\pm 5$  folds changes). However there still are several differences. For example, in macaque ectocervix, CKMT1 and NME2 showed lower mRNA level than those in human ectocervix. AK2 had 5-15 fold higher level in both ectocervical and vaginal tissues than their levels in human, while NME2 had more than 15-fold lower level in macaque colorectum.

Conversely, the PE mRNA expression in rabbit tissues appears much more different than human tissue (Figure 4.8). In rabbit cervix, CKMT2 levels were more than 15-fold lower than human levels while NME1 was 5~15 fold higher than human. Rabbit vagina had higher levels of CKMT2 and AK2, and a lower level of CKMT1, however, rabbit colorectum had more than 15-fold higher level of CKMT1 and NME1 as compared to their level in human (Figure 4.8).

The mRNA levels of AK2, AK4, NME1, NME2, CKMT1, CKMT2, and CKB were also examined in Swiss Webster mice treated with Depo-Provera for diestrous stage synchronization. As shown in Figure 4.8, several enzymes' level in mouse tissue, such as CKB and AK4, were lower than those of human's. However, the CKMT1 mRNA level had more than 15-fold lower in mouse colorectal tissues as compared to human data.



**Figure 4.8 Comparison in mRNA levels of a select panel of PEs in animal models**

The Gapdh-normalized PEs mRNA levels in animal tissues were normalized to the GAPDH-normalized enzyme mRNA levels in corresponding human tissues, and the ratios were expressed using different colors indicating different degrees of similarity. Grey color, 5 fold lower or higher than the human tissue level; light blue and light red, 5-15 fold lower or higher; dark blue and dark red, more than 15 fold lower or higher. Human data was shown in Figure 4.1. Pigtailed macaque data were shown in Figure 4.5. New Zealand White rabbit data was shown in Figure 4.6. Mouse data (diestrus stage) was shown in Figure 4.7.

#### 4.3.4. RNA expression of PEs after MPA, P4 or interleukins treatment

To test the effect of MPA and P4 on PEs, the vaginal epithelial cell line VK2 and human T cell line PM1 were cultured with MPA (1 $\mu$ M) or P4 (1 $\mu$ M), and the mRNA of the PEs were analyzed using real time PCR and compared to control group. However, certain enzymes respond to MPA or P4 treatment differently than others, and there is not one single effect across all PEs. The

enzyme mRNA data after MPA or P4 treatment are summarized in Table 4.6 and only the enzymes which showed significantly changes are listed. The red color means the change (increase or decrease after treatment) was greater than 50 percent, if there was less than a 50% change, the color is black. Several enzymes exhibited altered levels after the treatment, however, like mentioned before, there is not a consistent response across different enzymes or cells. For example, MPA treatment in VK2 cells causes both enzyme increases and decreases. Similar results were observed after IL-1 $\beta$  (10 ng/mL) and IL-8 (10 ng/mL) treatment (Table 4.7). Because, unfortunately, at this point, it is not confirmed exactly which enzyme or which group of enzymes are specifically responsible for the TFV conversion. Based on mRNA data alone, we are not able to predict how these changes will impact enzyme function or TFV-DP formation. Also, the expression does not necessarily correlate with enzyme activity. Therefore, the following study was done directly looking at TFV phosphorylation conversion, which is TFV-DP.

**Table 4.6 Effect of MPA and P4 on enzyme mRNA expression**

		MPA	P4
VK <sub>2</sub> (Vaginal epithelial cell)	↑	AK2	-
	↓	CKB	CKMT2
PM <sub>1</sub> (T cell)	↑	CKMT2, CKB	CKB
	↓	AK4, NME1	CKMT1, NME1

\* VK2 and T cells were cultured with either (1 $\mu$ M) or P4 (1 $\mu$ M) or ethanol for 48 hours and PEs mRNA levels were measured by real time PCR and compared to the control group. The red color means the change (increase or decrease after treatment) was greater than 50 percent, if there was less than a 50% change, the color is black.

**Table 4.7 Effect of interleukins on enzyme RNA expression**

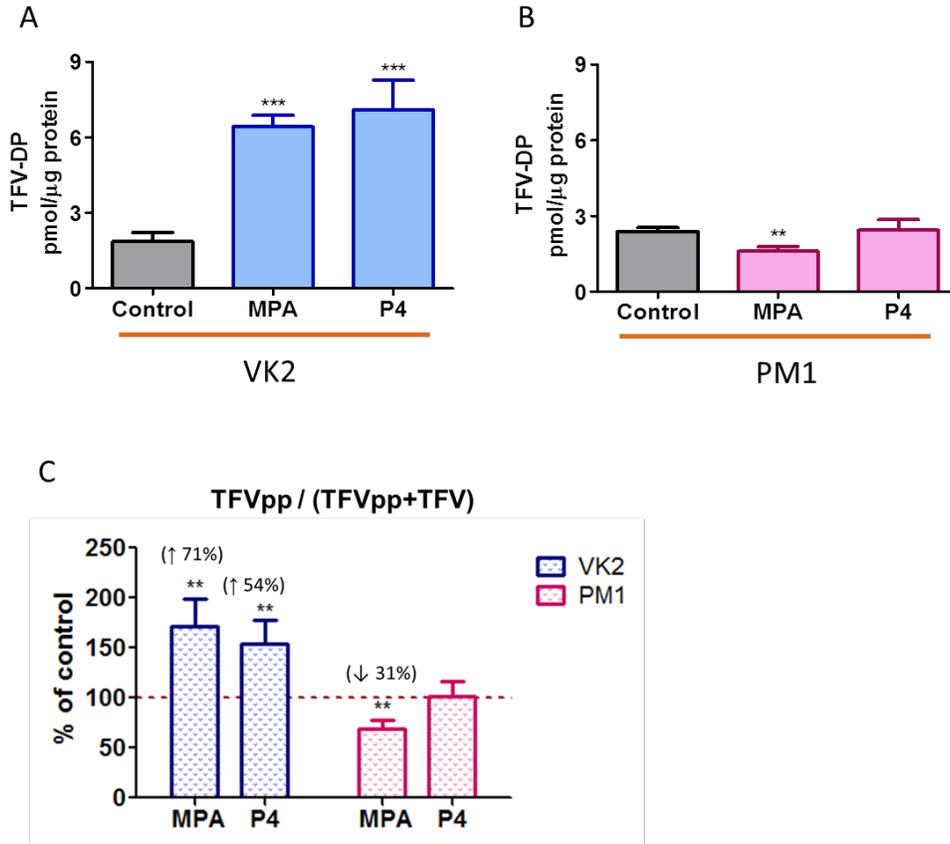
		IL-1 $\beta$	IL-8
<b>VK2</b> (Vaginal epithelial cell)	↑	AK4, NME1	AK4, NME1, NME2
	↓	-	-
<b>PM1</b> (T cell)	↑	-	-
	↓	CKMT1, NME1, NME2	CKMT1, NME1, NME2

\* VK2 and T cells were cultured with IL-1 $\beta$  (10 ng/mL) and IL-8 (10 ng/mL) or culture medium for 48 hours and PEs mRNA levels were measured by real time PCR and compared to the control group. The results were listed as compared with control group. The red color means the change (increase or decrease after treatment) was greater than 50 percent, if there was less than a 50% change, the color is black.

#### 4.3.5. MPA and P4's effect on TFV conversion

Based on the PEs mRNA expression data, the epithelium cell line VK2 and T cell line PM1 were selected to test the MPA and P4's effect on TFV conversion. As shown in Figure 4.9A, after MPA (1 $\mu$ M) and P4 (1 $\mu$ M) treatment, the TFV-DP concentration in VK2 cells were 6.42 $\pm$ 0.46 and 7.09 $\pm$ 1.19 pmol/ $\mu$ g protein, respectively, which were significantly increased as compared to the TFV-DP concentration in the control group (1.87 $\pm$ 0.36 pmol/ $\mu$ g protein, p<0.001). On the contrary, MPA (1 $\mu$ M) slightly but significantly decreased TFV-DP in PM1 cells (p<0.01) while no effect was observed with P4 treatment in PM1 cells (Figure 4.9B). The percentage of TFV-DP in the total TFV with TFV-DP in the cell homogenate as compared to control group was shown in Figure 4C. In VK2 cells, compared to the control group, MPA (1 $\mu$ M) and P4 (1 $\mu$ M) treatment significantly increased the ratio of TFV-DP over total TFV-DP and TFV by 71% and 54% respectively while MPA (1 $\mu$ M) decreased this ratio by 31% in PM1 cells (Figure 4.9C).

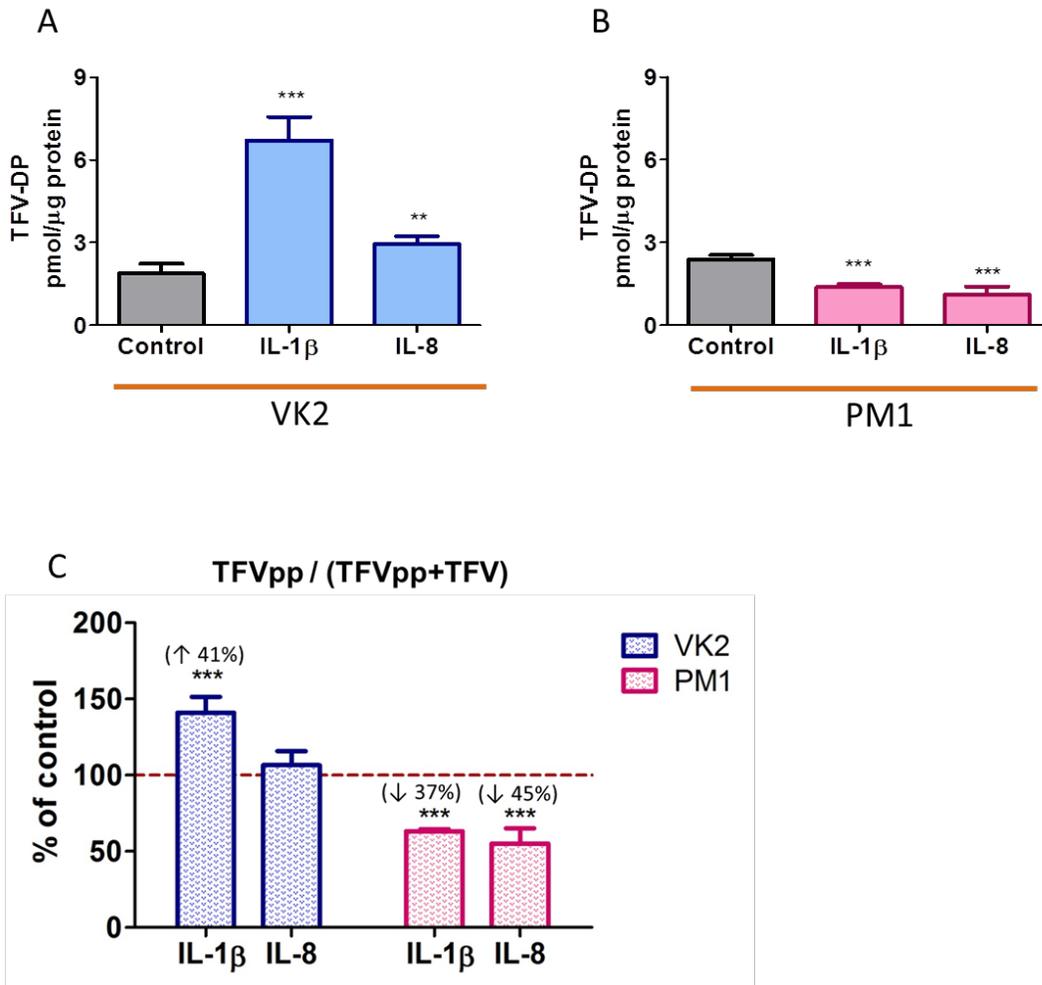
This ratio reflected the capability of intracellular PEs in converting TFV into TFV-DP. The difference in the ratios between VK2 and PM1 indicated that the effect of hormones on PE activity is cell type-specific.



**Figure 4.9 Effect of MPA and P4 on TFV conversion in vaginal epithelial cell line and T cell line**

Cells were cultured with MPA (1μM) or P4 (1μM), or vehicle solution used to dissolve contraceptives along with TFV (1 mM) in cell culture medium for 48 hours. The intracellular TFV and TFV-DP were both measured by LC/MS/MS and normalized to protein concentration. A. effect of MPA and P4 on TFV conversion in vaginal epithelial cell line VK2; B. effect of MPA and P4 on TFV conversion in human T cell line PM1. C. the ratio of TFV diphosphate to the total concentration of TFV and TFV-DP. Student T-test was used to examine the statistical difference between different groups. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. IL-1β and IL-8's effect on TFV conversion

Since bacterial vaginosis (BV) is the most common cause of vaginitis in reproductive-aged women [246] and the increased levels of IL-1 $\beta$  and IL-8 have been found in vaginal fluid obtained from women with BV [162, 163], we decided to examine the potential impact of these cytokines' effect on TFV conversion. As shown in Figure 4.10A and 4.10B, TFV-DP concentrations were significantly increased in VK2 cells ( $p < 0.001$  and  $p < 0.01$  respectively), while significantly decreased in PM1 cells ( $p < 0.001$ ) after IL-1 $\beta$  (10 ng/mL) and IL-8 (10 ng/mL) treatment. Interestingly, IL-8 treatment did not increase the ratio of TFV-DP over total TFV-DP and TFV in VK2 cells (Figure 4.10C). Similar to contraceptive hormones, the proinflammatory cytokines had exerted differential effects on PE activity in different cell lines, as reflected by the differential changes in the conversion ratios between VK2 and PM1 cell lines (Figure 4.10C).

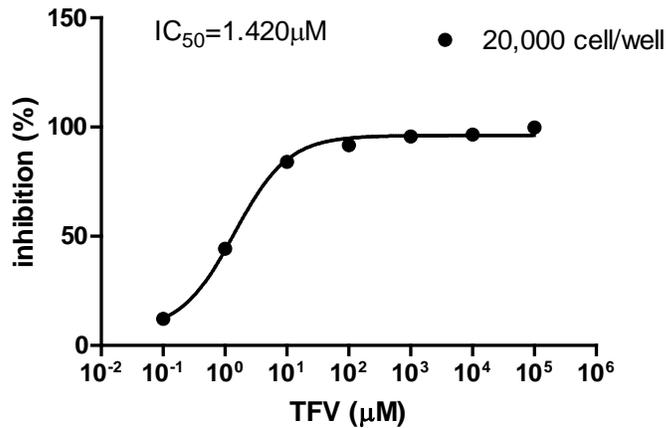


**Figure 4.10 Effect of IL-1 $\beta$  and IL-8 on TFV conversion in vaginal epithelial cell line and T cell line**

Cells were cultured with IL1 $\beta$  (10 ng/mL) or IL8 (10 ng/mL), or cell culture medium alone with TFV (1 mM) for 48 hours. The intracellular TFV and TFV-DP were both measured by LC/MS/MS and normalized to protein concentration. A, effect of IL1 $\beta$  or IL8 on TFV conversion in vaginal epithelial cell line VK2; B, effect of IL1 $\beta$  or IL8 on TFV conversion in human T cell line PM1. C, the ratio of TFV diphosphate to the total concentration of TFV and TFV diphosphate. Student T-test was used to examine the statistical difference between different groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### 4.3.6. MPA and P4's effect on TFV activity

To decide the TFV concentration that should be used in the activity study, the IC<sub>50</sub> of TFV against HIV<sub>BaL</sub> was measured in PM1 cells (Figure 4.11). We decided use both 1 and 1000μM of TFV in the following study.



**Figure 4.11 TFV dose responses in PM1 cell line**

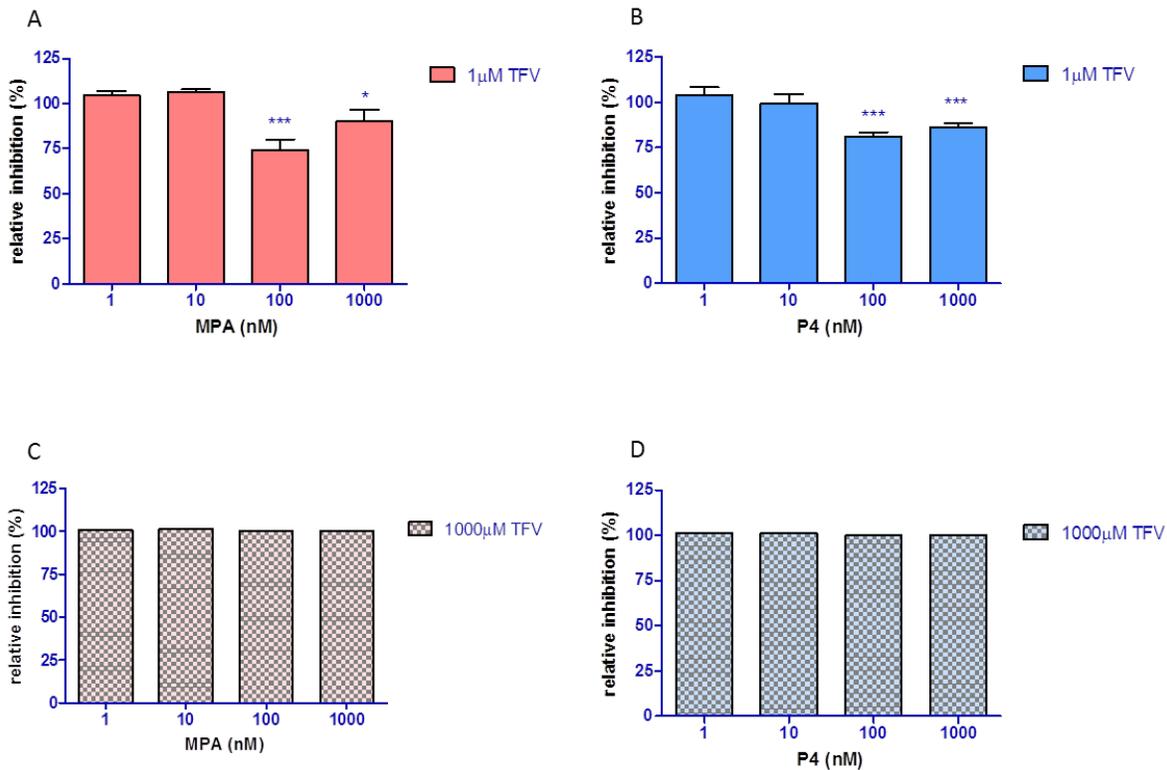
PM1 cells were culture in 96-well plate with final density of 20,000 cell/well, and were treated with serial diluted TFV solution (10<sup>-2</sup> to 10<sup>6</sup> μM). Cells then were challenged with 3,000 TCID<sub>50</sub> of HIV-1<sub>BaL</sub> and cultured for 4 days. The supernatant was then collected and tested for HIV-1 replication using the p24gag enzyme-linked immunosorbent assay. IC<sub>50</sub> was calculated using Graphpad software.

To determine whether the MPA and P4 treatment could affect TFV anti-HIV activity, PM1 cells were treated with MPA or P4 with TFV, and then challenged with HIV-1<sub>Bal</sub>. HIV p24 protein was quantified using a p24 protein ELISA and compared with TFV treatment only group. Clinically, immediately after injection of 150 mg/ml MPA, plasma levels were  $1.7 \pm 0.3$  nmol/l and increase to  $6.8 \pm 0.8$  nmol/l two weeks after, and then fell to the initial levels by after 12 weeks (<https://www.medicines.org.uk/emc/medicine/11121>). In the current study, we tested the MPA at 1 to 1000nM. As shown in Figure 4.12A, MPA at 1 and 10 nM levels did not change TFV activity in PM1 cells. However when MPA concentration was increased to 100 and 1000 nM, TFV (1 $\mu$ M) activity was significantly decreased by ~25% ( $p < 0.001$ ) and 10% ( $p < 0.05$ ) respectively, which was consistent with the decreased TFV-DP concentration after MPA treatment (Figure 4.9B). Interestingly, when TFV concentration was increased to 1000  $\mu$ M, none of the MPA treatment affected TFV activity (Figure 4.12C). Similar results were found for P4 treated PM1 cells, where at 100 and 1000 nM level, P4 treatment significantly decreased TFV (1 $\mu$ M) activity by ~20% ( $p < 0.001$ ) and 16% ( $p < 0.001$ ) respectively (Figure 4.12B), but the impact of P4 was no longer observed when TFV concentration was increased to 1000  $\mu$ M (Figure 4.12D).

#### 4.3.7. IL-1 $\beta$ and IL-8 effect on TFV activity

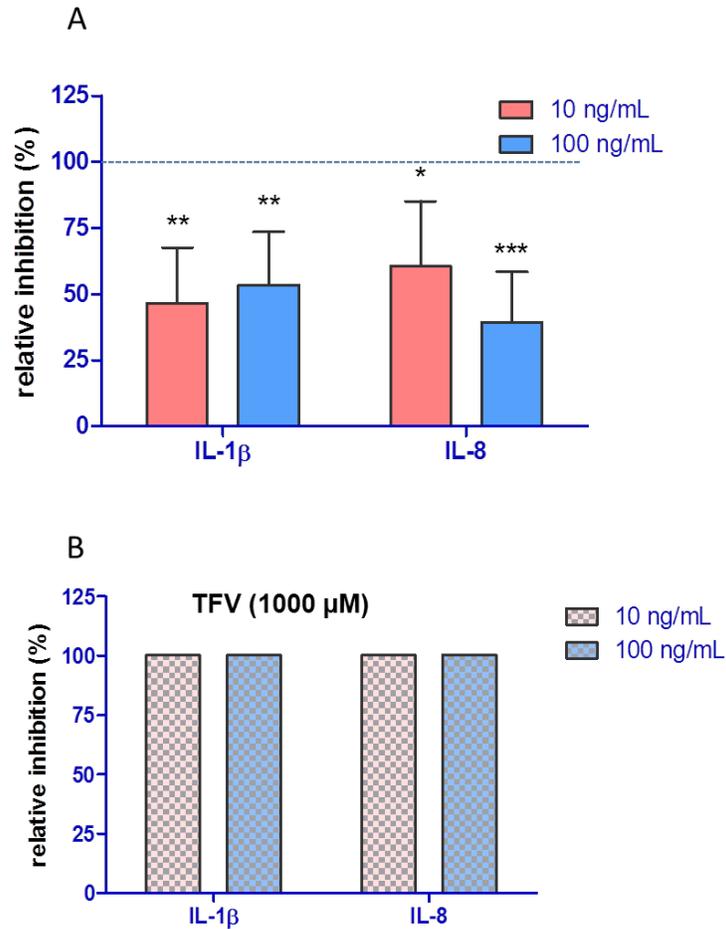
Similarly to the hormones experiment, we also determined if interleukins could impact TFV activity in PM1 cells. As shown in Figure 4.13A, IL-1 $\beta$  at 10 and 100 ng/mL levels significantly decreased TFV (1  $\mu$ M) activity by  $53.4 \pm 21.1\%$  ( $p < 0.01$ ) and  $46.5 \pm 20.1\%$  ( $p < 0.01$ ) respectively. In addition, IL-8 at 10 and 100 ng/mL levels significantly decreased TFV (1  $\mu$ M)

activity by  $39.4 \pm 24.6\%$  ( $p < 0.05$ ) and  $60.7 \pm 19.2\%$  ( $p < 0.001$ ) respectively. These decreased TFV anti-HIV activities were consistent with decreased TFV-DP concentration after interleukins treatment which was shown in Figure 4.10B. Once again, when TFV concentration was increased to  $1000 \mu\text{M}$ , none of the interleukins treatment affected TFV activity (Figure 4.13B).



**Figure 4.12 MPA and P4's effect on TFV activity in PM1 cell line**

PM1 cells were culture in 96-well plate with final density of 20,000 cell/well, and were treated with serial diluted MPA or P4 alone with TFV in cell culture medium. Cells then were challenged with 3,000 TCID<sub>50</sub> of HIV-1Bal and cultured for 4 days. The supernatant was then collected and tested for HIV-1 replication using the p24gag ELISA. A, effect of MPA on TFV (1 $\mu\text{M}$ ) activity; B, effect of P4 on TFV (1 $\mu\text{M}$ ) activity; C. effect of MPA on TFV (1000 $\mu\text{M}$ ) activity; D. effect of P4 on TFV (1000 $\mu\text{M}$ ) activity. Student T-test was used to examine the statistical difference between different groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



**Figure 4.13 Interleukins' effect on TFV activity in PM1 cells**

PM1 cells were culture in 96-well plate with final density of 20,000 cell/well, and were treated with serial diluted IL-1 $\beta$  or IL-8 alone with TFV in cell culture medium. Cells then were challenged with 3,000 TCID<sub>50</sub> of HIV-1Bal and cultured for 4 days. The supernatant was then collected and tested for HIV-1 replication using the p24gag ELISA. A, effect of IL-1 $\beta$  and IL-8 on TFV (1 $\mu$ M) activity; B, effect of IL-1 $\beta$  and IL-8 on TFV (1000  $\mu$ M) activity. Student T-test was used to examine the statistical difference between different groups. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### **4.4. Discussion and conclusions**

In this study, we evaluated of the expression profile of TFV-related PEs in human cervicovaginal and colon tissues, important animal models, as well as epithelial / T cell lines used in the preclinical testing of many drug in the HIV prevention pipeline. We found that the majority of TFV-related PEs showed significantly decreased mRNA level in T cells as compared to their levels in cervicovaginal tissues. Furthermore, our results indicated that MPA and P4 treatments changed PEs activity toward different directions in cervicovaginal epithelial cell line (VK2) and a T cell line (PM1). Additionally, we found that TFV (1 $\mu$ M) activity against HIV-1BaL in T cell line was significantly decreased when treated with MPA (0.1 $\mu$ M) or P4 (0.1 $\mu$ M).

As an acyclic nucleotide analog of adenosine monophosphate, TFV requires cellular phosphorylation to become pharmacologically active, the phosphorylation happened in both local mucosal tissue cell and peripheral blood immune cell could contribute to total effective TFV-DP concentrations [146]. If mucosal tissue phosphorylation is a critical contribution to mucosal tissue cell TFV-DP concentrations, the kinases responsible for TFV conversion are expressed in cells and tissues associated with HIV infection [140, 141]. Bumpus et al characterized nucleotide kinase expression in PBMC, vaginal tissue, and colorectal tissue and found that AK2 performed the first TFV phosphorylation step in PBMC, vaginal, and colorectal tissues [146]. They also found that pyruvate kinase isozymes were able to convert TFV-MP to TFV-DP in PBMC and vaginal tissue, while CKM catalyzed this conversion in colorectal tissue. However, to date, the expression profiles of nucleotide kinases in peripheral blood mononuclear cells (PBMC), cervicovaginal tissue, and colorectal tissue have not been systematically

characterized. Also, there is limited data regarding these kinases activity towards TFV conversion in cells related to HIV sexual transmission.

The current studies provide information on the mRNA expression of PEs in human female genital tract and colon tissues, immune cells, as well as several preclinical animal models used in microbicide research. This information is important for understanding the dynamics of PEs expression in these tissue sites, and is needed for experimental design and data interpretation of further studies concerning TFV activation and PK/PD in these tissues and cells. We have also examined the effect of progestins and proinflammatory cytokines on TFV-DP formation in vaginal epithelial and immune cells. Our data provides proof of concept that TFV activation may differ in immune cells and local tissue under different conditions.

In our study, For the mRNA expression in human tissues, seven tested PEs were generally present in all tissue types examined, which is consistent with their fundamental role in the intracellular physiological events cell proliferation, differentiation and development, signal transduction, such as regulating the overall cellular energy balance, maintaining an equilibrium between the concentrations of different nucleoside triphosphates, and enhancing skeletal, cardiac, and smooth muscle contractility [247, 248]. As nucleoside monophosphate and diphosphate kinases catalyze the reaction that convert monophosphates to their corresponding diphosphate/triphosphate form, these enzymes play a crucial role in the cellular metabolic process, including RNA and DNA synthesis[249]. There was no difference in PE mRNA expression between pre- and post-menopausal women. If AK2 and AK4 are the key enzymes responsible for the first activation step of TFV, which is the rate-limiting step, it is reasonable

that AK2 and AK4 trend to show relatively lower mRNA expression as compared to other PEs that are reported responsible for the final activation of TFV. In addition, mRNA of AK2 was significantly lower in human vaginal and ectocervical tissue than colorectal tissue, which may result in different TFV metabolism in vaginal and rectal tissues. It has been reported that a different TFV-DP level was observed between vaginal and rectal tissues after a single oral dose of Truvada [29]. Although the TFV-DP concentration difference in vaginal and rectal tissue might be due to the dissimilarity in tissue permeability, it also could be due to the different PEs activities in these sites. It should be noted that the PEs functionality and activity in the CD4<sup>+</sup> T cells isolated from PBMC are not necessarily the same with those of the immune cells residing in the cervicovaginal tissue, and further work is needed to confirm the functional activity of the PEs in CD4<sup>+</sup> T cells isolated from human cervicovaginal tissue for a better understanding on the PE function/regulation in female lower genital tract *in vivo*.

It should be noticed that in the real time PCR study, we used same primers of tested enzyme for both human and macaque due to a high degree of homology between human and macaque species for tested enzyme genes. We have run BLAST on NCBI to compare the similarity of tested enzyme gene mRNA sequence between human and macaque. We found that all mRNA sequence showed > 95% identity between human and macaque. In addition, the melting curve analysis after real-time PCR and agarose gel electrophoresis of the amplicons showed that the amplicons from both species have the same melting curve pattern and same size. Given that the majority of genes are highly conserved between human and macaque, we think the use of the same set of human enzyme primers in macaque samples is appropriate for our purpose. However, we should also admit that the substrate spectrum and DNA binding specificity of macaque PEs

might be quite different from those of humans. If we observe an enzyme is expressed at the same level in human and macaque tissues, this does not guarantee that this enzyme has the same activity in regulating same genes in human and macaque.

Human tissue is used to evaluate many topical microbicide drugs for potency [47]. We show good expression of the seven PEs tested. However, one limitation was the epithelium was not completely separated from the lamina propria. Although the most part of stromal tissue was isolated using a Thomas-Stadie-Riggs tissue slicer, part of lamina propria was still attached to the epithelium. However, our results showed that the mRNA expression of PEs in ectocervical (Ect1) and vaginal (VK2) epithelial cell line were very similar to their levels found in the corresponding human tissues, suggesting that the majority of the PE expression noted in the tissue was likely from the epithelium. These data also show consistency of PE expression between fresh tissue and the epithelial cell lines, which supports the use of the cell lines for further testing. It should be pointed out that Bonferroni post-hoc test was used in comparing enzyme expression across different type of tissues. However, since the significance cut off of this test is  $\alpha/n$ , the Type II error rate is really high for each test run, it can overcorrect for Type I errors.

Although the mRNA level of several important PEs in PM1 cells and purified T cells was much lower than that in human tissue and tissue-derived epithelial cells, the PE activity in TFV-DP formation appeared to be similar between the tested cell types, as the TFV-DP concentration and TFV-DP over total TFV ratio were similar between VK2 and PM1 cells. However, we should

note that equal concentration of TFV was supplemented to the culture media of VK2 and PM1. In clinical use of topical microbicides, the drug concentration decreases as the tissue depth increases, and the concentration of drug available to submucosal immune cells is actually much lower than that to epithelial cells. Therefore the TFV-DP concentration in those tissue-associated immune cells in women should be much lower than the TFV-DP concentration measured in cervical/vaginal tissue biopsies, which mainly consist of tissue epithelial cells.

Besides human tissues and cell lines, the mRNA expression of PEs were also examined in the tissues of pigtailed macaques and rabbits. These animal models have been used for the efficacy and/or safety testing of PrEP products especially topical microbicides [31-33, 164, 165]. Synchronized mice are representative of the mouse model utilized in microbicide research. The results have shown positive expression of the PEs in animal tissues with species difference which will help researchers in making informed decision, as well as correlating PK/PD data between human subjects and animal models. With the three animal models tested in the study, macaque genital tract anatomy is more similar to human, and the PEs expression pattern is most close to human. The New Zealand White rabbit has been widely used to evaluate the safety of vaginal and rectal microbicide products [164]. As drug concentration in the tissue greatly impact the safety and toxicity of drug product, it is necessary to examine metabolizing enzyme expression level in rabbit tissues in comparison to human. The anatomy of rabbit lower genital tract is different from that of human and macaque. Human endocervix is single-layer epithelium, while ectocervix and vagina consist of stratified squamous cells. However, rabbit vaginal and cervical epitheliums are both lined with single-layer columnar cells.

Polarized cervical mucosal tissue model has been largely utilized to evaluate API and microbicide product safety, efficacy, and drug localization [182, 199, 250]. In this model, human tissue is oriented with the apical side face up and the product can directly interact with the epithelium which mimics the microbicide product using *in vitro*. However, in some microbicide clinical studies, the efficacy of microbicide products has been examined in the *ex vivo* challenge model, where the cervical or vaginal tissue biopsies are taken from the product-exposed participants (NHPs or humans), and cultured *ex vivo* for several days, followed by the HIV challenge, and measurement of viral proteins such as p24 as an indicator of HIV replication [251]. Since the biopsies are small pieces of tissue, the drug concentration measured in these samples mainly reflect the drug concentration in the epithelial layers and upper part of the lamina propria. It does not necessarily reflect the drug concentration in the HIV target cells, and the PK/PD results obtained using the tissue biopsies may tend to overestimate the drug's capability of penetrating the tissue and preventing HIV infection *in vivo*. Therefore, it is important to understand if the PEs function similarly in both immune cells and epithelial cells. Biswas et al previously reported that several nucleotidases, such as NT5E and NT5C2, showed different expression patterns in human T cells and cervix epithelial cells [148]. This supported the hypothesis that certain PEs may also have different expression levels in different cell types. In the current study, we found that several PEs, including AK4, CKB, CKMT1, CKMT2, and NME1, showed significant lower mRNA expression in CD4+ T cells than cervicovaginal tissue, which may lead to different TFV activation kinetics in tissue and residing T cells.

Considering the majority of potential female microbicide users are of reproductive age and very likely in the use of some type of contraceptives, we investigated whether the HCs impact PEs

activity and NRTIs conversion. In the current study, we found that TFV-DP level was increased in vaginal epithelial cells but decreased in PM1 cells after MPA/P4 treatment, suggesting the TFV-relevant phosphorylating enzyme activity might be changed toward different directions in these two cell lines with the presence of MPA and P4. In addition, we found that both MPA and P4 at 100nM and 1000nM could inhibit TFV activity in PM1 cells, although these levels are 10-1000 times higher than their plasma level achieved after dosing, it is possible that their cumulative concentration in the local tissue is higher than the blood concentration. Similar results were found for IL-1 $\beta$  and IL-8 treatment.

Furthermore, we have also examined if the MPA and P4, as well as interleukins treatment could impact PEs mRNA expression on VK2 and PM1 cell lines. We found that several enzymes exhibited altered levels after the treatment (data is not shown), however, there was not a consistent response across different enzymes or cells. Although MPA (1 $\mu$ M), P4 (1 $\mu$ M), as well as IL-1 $\beta$ (10ng/mL) and IL-8(10ng/mL) treatment caused both enzyme mRNA increases and decreases, however, based on mRNA data alone, we are not able to predict which PEs are responsible for altered TFV-DP level after MPA or P4 treatment.

Although the effect of tested hormones and cytokines on PE mRNA level remains unclear, these agents did exert an impact on the activity of PEs in cultured VK2 and PM1 cells. This is reflected by the increase or decrease in the TFV-DP concentration and TFV-DP over total TFV ratio after treatment. In addition, the same hormone or cytokine may have differential effects in different cell types, because the TFV-DP concentration and TFV-DP ratio changed in opposite

directions in VK2 and PM1 cells after treatment. It should be noted that the plasma level of MPA or P4 after administration of contraceptives is usually much lower than the concentrations used in the cell experiments (1  $\mu$ M), while the concentrations of IL1 $\beta$  and IL8 in women with reproductive tract BV infection are similar to the concentrations used in our cell assay (10 ng/mL). Although the effect of MPA and P4 at physiological concentrations was not tested in this study, the effect of these hormones on TFV effectiveness, a metric directly linked to clinical endpoint, was examined at both physiological and supra-physiological concentrations.

The viral assays suggested that the decreased TFV-DP formation in PM1 cells was linked to altered TFV inhibition of HIV-1 infection. At concentrations below 0.1  $\mu$ M MPA / P4 treatment or 10 ng/mL cytokine treatment, TFV was less efficacious in protecting the PM1 cells from HIV-1 infection. The effect of progestins and cytokines depended on the concentration of the agent (hormones or cytokines) and TFV. The relative inhibition of HIV-1 by TFV was reduced by 10-20%, when MPA / P4 was above 0.1  $\mu$ M, or when IL1 $\beta$  / IL8 was above 10 ng/mL. MPA and P4 had no observable effect on TFV efficacy when their concentrations were reduced to 10 and 1 nM. The increase in TFV concentration appeared to abrogate the negative impacts of progestins and cytokines on TFV efficacy. When TFV concentration was increased from 1  $\mu$ M to 1000  $\mu$ M, the TFV effectiveness was not affected by hormones or cytokines regardless of their concentration. Notably, the observed decrease in TFV effectiveness after hormone and cytokine treatment might be due to a number of reasons, not just altered TFV-DP formation in PM1 cells. For example, reports have shown that hormonal contraceptives and proinflammatory cytokines could alter the HIV receptor abundance and intracellular signaling in immune cells [99, 252, 253]. Clinically, immediately after single injection of 150 mg/ml MPA, plasma levels were  $1.7 \pm$

0.3 nmol/l and increase to  $6.8 \pm 0.8$  nmol/l two weeks after, and then fell to the initial levels by after 12 weeks (<https://www.medicines.org.uk/emc/medicine/11121>). This level is much lower than 100 nM of the MPA level tested in our study, however, the cumulated MPA concentration in the genital tract tissue has not been reported and might be higher than its plasma concentration. In addition, reports show that the high dose of MPA (1.5g/day) has been used in women with breast cancer, and the plasma concentrations of MPA can be as high as 12  $\mu\text{g/ml}$  [254, 255]. In those cases, the PEs activity towards NRTIs activation could possibly be impacted.

Finally, our *in vitro* observations have implications on the clinical PrEP studies which evaluated TFV and other NRTIs. First, the majority of these studies focused on female participants and in some studies, women were required to use hormonal contraceptive [256]. Some women may have been asymptomatic for BV which has been associated with proinflammatory cytokine upregulation in their genital tract [100]. Although the effect of MPA and P4 on TFV-DP formation was not tested at lower physiological concentrations (under 1000 nM), the 1000 nM MPA and P4 as well as the pathophysiological levels of IL1 $\beta$  and IL8 (10 ng/mL) affected TFV-DP formation in VK2 and PM1 cells. In general, more TFV-DP was made in VK2 cells as compared to PM1 cells. This suggests that the TFV-DP in clinical trial biopsies, which is comprised primarily of epithelial cells, may not correlate with the concentration in the submucosal immune cells, which are targets for HIV infection. Thus, our results provide insight into the discordant results with TFV-based products effectiveness across studies [11, 12, 15-24]. Second, based on our results, the MPA and P4 at physiological levels (1 and 10 nM) are not likely to affect TFV efficacy, but IL1 $\beta$  and IL8 at levels often seen in BV (10 ng/mL) may reduce TFV-DP formation and TFV effectiveness in female genital tract. In those situations

where TFV effectiveness is reduced by disease-related factors such as upregulated proinflammatory cytokines, the negative impact of these agents might be diminished by increasing ARV concentration.

### **Conclusion**

In conclusion, we found that the majority of TFV-related PEs showed significantly decreased mRNA level in T cells as compared to their levels in cervicovaginal tissues. Furthermore, our results indicated that MPA and P4 treatments changed PEs activity toward different directions in cervicovaginal epithelial cell line (VK2) and a T cell line (PM1). Additionally, we found that TFV (1 $\mu$ M) activity against HIV-1bal in T cell line was significantly decreased when treated with MPA (0.1 $\mu$ M) or P4 (0.1 $\mu$ M). The information generated from this study will facilitate the understanding of PEs in TFV pharmacokinetics. Based on our *in vitro* results, TFV effectiveness is not likely reduced in participants taking progestin-based contraceptives, but may be reduced in participants with asymptomatic infections.

### **Acknowledgments**

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## 5. SUMMARY AND PERSPECTIVES

### 5.1 Summary of major findings and contribution to the field of microbicides

HIV remains a global health challenge with an estimated two million people who are newly infected each year. Topical microbicide products, such as vaginal gels containing antiretroviral drugs, are currently being developed to reduce the incidence of acquiring HIV-1 infections. To date several clinical studies of preexposure prophylaxis (PrEP) for HIV-1 prevention have evaluated tenofovir, a nucleoside reverse transcriptase inhibitor (NRTI), as a vaginal gel. However, the results from these studies have been inconsistent and there is an urgent need to understand what factors may influence the effectiveness of microbicides.

From a drug delivery perspective, there is limited understanding regarding the safety of vaginal product excipients in the context of HIV-1 prevention, such as the excipients' effect on cervicovaginal tissue integrity and permeability. Obtaining comprehensive information in this area of research will not only help us select excipients in formulation development, but will also facilitate the development of novel delivery strategies that aim to enhance drug penetration through modulating tissue integrity. From a pharmacological perspective, there is limited data available regarding the expression and activity of enzymes that are responsible for metabolizing or activating antiretroviral drugs in the female genital tract. Additionally, it is necessary to examine the effect of the menstrual cycle, exogenous hormones, and inflammation, on enzyme expression and activity. These factors could also contribute to the effectiveness of vaginal microbicide products.

This dissertation describes the data from several research projects with the overall aim of understanding what factors, including both product-related and patient-related factors, may impact vaginal microbicide effectiveness. These projects included the evaluation of vaginal formulations and formulation ingredients, as well as the role of metabolizing enzymes in the PK/PD of vaginally administered anti-HIV drugs. In this dissertation, we hypothesize that cervicovaginal tissue integrity and metabolizing enzymes are critical determinants of tissue exposure and efficacy of antiretroviral drugs and metabolites in HIV-1 prevention. Therefore, the modulators of tissue integrity and metabolizing enzymes could affect antiretroviral drug exposure and efficacy in cervicovaginal tissues. Based on this hypothesis, three studies were designed.

First, we tested the effects of a number of generally-regarded-as-safe excipients on human cervical tissue integrity, viability and morphology. We found that the excipients which increased paracellular permeability of cervicovaginal tissue did not significantly reduce TFV activity in HIV-1 prevention in an ex vivo explant model. In addition, we have demonstrated that TEER, morphology, permeability, and MTT-based tissue viability do not necessarily change in parallel with each other and the use of a single measurement cannot accurately reflect the effect of excipients on cervicovaginal tissue integrity.

While doing the excipients study, we started to think what other factors may also impact drug activity in the tissue and we considered the metabolizing enzyme. Therefore, in the second project, we provided a systematic evaluation of the expression profile of CYP and UGT enzymes in female genital tissues of human, mice and macaque. We demonstrated that the mRNA of

several enzyme isoforms, such as CYP1A1, CYP1B1 and UGT1A1, are highly expressed in the human female genital tract. In addition, we found the resemblance similarities between human and pigtailed macaque in the expression patterns of CYP1A1 and CYP1B1, suggesting the utility of the macaque model for the future genital tissue CYPs studies. However, these highly expressed CYP and UGT enzymes are not directly involved in the metabolism of currently available ARVs. As we already know that NTRIs, such as TFV, require intracellular phosphorylation to its active form, we decided to study the phosphorylation enzymes that are responsible TFV activation.

In the fourth chapter, we started to examine the mRNA expression of phosphorylating enzymes in tissues and cells relevant to TFV for the prevention of HIV sexual transmission. We systematically compared the mRNA expression of PEs in the cervicovaginal and colorectal tissues of human and animal models (macaque, rabbit, mouse), human CD4+ cells, as well as in the cell lines derived from human cervicovaginal tissues (Ect1/E6E7, VK2/E6E7), and a T cell line (PM1). The tested PEs showed various mRNA expression levels in the examined tissue types. Also, when comparing PE levels between cervicovaginal tissues and the CD4+ T cells, the majority of enzymes we tested, including CKB, CKMT1, CKMT2, AK4, and NME1 showed significantly decreased mRNA level in T cells as compared to their levels in cervicovaginal tissues. In addition, our data suggested that the most tested enzymes showed similar mRNA levels in VK2 and PM1 cell lines as compared to vaginal epithelial tissue and CD4+ T cells.

Considering the wide utilization of contraceptive hormones and high incidence of bacterial vaginosis among potential microbicide users, we further examined the effect of MPA and P4, as

well as two proinflammatory cytokines on PE expression and TFV-converting activity, in cervicovaginal epithelial cell line (VK2) and a T cell line (PM1). We found that the effect of medroxyprogesterone acetate or progesterone treatment on phosphorylating enzyme activity varied between cervicovaginal epithelial cells and T cells.

Inspired by these findings, we moved forward and tested whether the changes in TFV activation under hormone and cytokine treatments is correlated with the alteration in TFV efficacy, using the T cell line PM1. We found that MPA and P4 at concentrations above 0.1  $\mu\text{M}$ , as well as IL1 $\beta$  and IL8 at concentrations above 10ng/mL significantly decreased HIV-1BaL inhibition in T cell line when 1 $\mu\text{M}$  TFV was added. However, the effects of hormones and cytokines were revoked when TFV concentration was raised to 1 mM.

Taken together, these studies provide valuable information on the effect of excipients on multiple aspects of cervicovaginal tissue integrity and will facilitate the efforts toward optimized vaginal PrEP products for HIV-1 prevention. In addition, the TFV effectiveness against HIV-1 could potentially be affected by hormone and cytokines, and increasing TFV concentration maybe an effective way of counteracting the negative effects of high-level contraceptive hormones and proinflammatory cytokines.

## **5.2 Summary of limitations**

Although this dissertation has made several contributions to the microbicides research there are some limitations.

In chapter 2, we studied the effect of excipients on cervical tissue integrity using different testing platforms. Those excipients are commonly used in vaginal products. However, for most of the excipients tested in the study, only one concentration was evaluated, which is the medium level of their common levels in pharmaceutical formulations. This may cause the over-estimate or under-estimate of the excipients' effect on the tissue integrity. Therefore an extra study is needed to test if those findings in chapter 2 are concentration dependent. Also, it is still unknown the mechanism of the increased  $P_{app\text{-mannitol}}$  after exposure to the excipients, which need to be further investigated. In addition, although we evaluated excipients from various categories such as antioxidants, preservatives, and solvents/cosolvents, there are other excipients that have not been studied but also largely used in vaginal products, such as gelling agents and humectants including various polymers and lipids. Another limitation in the experiments that using human tissues (Chapter 2) is that due to the low sample size, the statistical power maybe too low and may negatively affect the statistically significant finding in the study.

In chapter 3, we studied the expression of several CYP and UGT enzymes in human female genital tract using real time qRT-PCR and western blot, however, the mRNA and protein expression do not always correlate well with their activity. Therefore functional and kinetic studies are needed to explore the role of CYP1A1, 1B1 and UGTs in xenobiotics metabolism in cervicovaginal tissue for both orally and topically administrated drugs.

The conventional and real-time RT-PCR studies reported in this dissertation (Chapter 3 and Chapter 4) have some limitations. Some primers used in the conventional PCR did not span exon

boundary, which might increase the risk of amplification from genomic DNA and lead to false positive results. To address this limitation, DNase was used in the preparation of RNA of tissue samples for real-time RT-PCR experiment to remove the residual genomic DNA contamination. In addition, the size of the PCR products was not confirmed by DNA electrophoresis in some of the real-time RT-PCR studies. To address this limitation, the melt curve analysis was checked following the amplification. The single melting peak suggested the formation of single PCR product. However it is possible that a non-specific amplification occurred instead of the target-specific amplification, and the melt curve analysis still showed single amplification product. If this was the case, then positive detection of some enzyme may be false positive results. This limitation can be addressed by examining the protein expression and/or enzyme activity using the well characterized enzyme substrate. Another limitation of this study is the difficulty in obtaining human and macaque tissues. A limited number of human cervicovaginal tissues were used in PCR experiments, and some experiments lack the most appropriate control tissues. For example, CYP1A1 and CYP1B1 are highly expressed in kidney, but human kidney tissue could not be obtained in the study. The positive control used for all enzymes in our study was human liver, some enzymes, such as CYP3A4 and CYP2D6, have very high level expression in liver, and therefore a much lower signal compared to the liver in conventional PCR does not necessarily mean negative expression. Thus, using liver as positive control for these enzymes might cause the possibility that the enzymes were expressed at high level in cervicovaginal and colorectal tissues but primers were inefficient to detect the expression. This limitation can be addressed by compare our findings with published data. Also the examination of protein expression can also be utilized.

In addition, due to the limited recourse and access to patient's information, although the cervical and vaginal tissues used in the study were histologically benign, the long-term medication treatment of the patients were not clear, which might impact the enzyme expression.

In chapter 4, we studied the expression of several TFV-related phosphorylating enzymes in the tissues and cells relevant to sexual transmission, however, it is unknown if any other enzymes involve in TFV activation. Therefore, we may not cover all the enzymes that can converse TFV to its active form. Also, although VK2 and PM1 cell lines were used as representatives for vaginal epithelial and T cells respectively, they are immortalized cells, and the enzymes may not express and function in the same way as they are in primary vaginal epithelial cell and CD4+ T cells. Therefore, the enzyme's expression and function in primary vaginal epithelial cell and CD4+ T cells may need to be further investigated and compared to the immortalized cell lines. In addition, the effect of MPA and P4 on PE expression and function was only tested within 48 hours in vaginal epithelial cell line and T cell line, however in the real situation, hormonal contraceptives are usually used in a long time period. Therefore, the long term effects of those contraceptive hormones are need to be studied. Similar limitations can be applied to interleukins studies as well.

In this dissertation, we used same primers of tested enzyme for both human and macaque due to a high degree of homology between human and macaque species for tested enzyme genes. We have run BLAST on NCBI to compare the similarity of tested enzyme gene mRNA sequence

between human and macaque. We found that all mRNA sequence showed > 95% identity between human and macaque. In addition, the melting curve analysis after real-time PCR and agarose gel electrophoresis of the amplicons showed that the amplicons from both species have the same melting curve pattern and same size. Given that the majority of genes are highly conserved between human and macaque, we think the use of the same set of human enzyme primers in macaque samples is appropriate for our purpose. However, we should also admit that the substrate spectrum and DNA binding specificity of macaque PEs might be quite different from those of humans. If we observe an enzyme is expressed at the same level in human and macaque tissues, this does not guarantee that this enzyme has the same activity in regulating same genes in human and macaque.

Bonferroni post-hoc test was used in comparing enzyme expression across different type of tissues. This multiple-comparison post-hoc correction is used when performing many independent or dependent statistical tests at the same time and can reduce the type I error. One potential problem when running many student t-tests simultaneously is that the probability of a significant result increases with each test. The significance cut off of Bonferroni post-hoc test is  $\alpha/n$ , therefore reduce the probability of false positive result. However, it should be noted that this post-hoc test does suffer from a loss of power as type II error probability is high for each test [257].

### **5.3 Proposed future studies**

More studies are needed to achieve further understanding of excipient and formulation's effect on microbicide effectiveness, and enzyme expression, regulation and function in the tissues and cells relevant to HIV-1 sexual transmission.

1. For the effect of excipients on cervicovaginal tissue integrity, excipients could be evaluated at different levels covering their common concentration range in vaginal products. Also, it would be good to evaluate combinations of excipients in these models to see if this is a combined effect that induces a change in tissue morphology. Additionally, other excipients that are commonly used in vaginal product should be also tested, such as some polymers and lipid. The combinations of excipients can be also evaluated. These experiments can be designed and analyzed through DoE (JMP) software. Furthermore, the mechanism of the altered  $P_{app}$  in ectocervical tissue caused by some excipients can be further explored by using IHC staining of tight junction proteins and other technologies.
2. For CYP and UGT enzyme study, functional and kinetic studies with vaginal microsome need to be explored. Well established CYP1A1 and 1B1 substrates can be incubated in vaginal microsome with and without enzyme inhibitors, and kinetic parameters can be calculated. These results can be compared with microsome activity with human liver microsomes, which will help understand the role of CYPs and UGTs in xenobiotics metabolism in cervicovaginal tissue for both orally and topically administrated drugs. In

addition, similar kinetic experiment can be conducted with animals' (such as macaque) vaginal microsome and compared to human data.

3. Expression and activity of phosphorylating enzymes should be evaluated in purified vaginal epithelial cells and immune cells isolated from human cervicovaginal tissues. To further explore the effect of hormones, contraceptives and inflammation on enzyme expression and activity, different concentrations of hormones from various categories can be tested in both immortalized and primary cells. Also, the effect of long term (e.g. 7 days) treatment of hormones and interleukins on PE's expression and activity should also be evaluated due to the reason discussed above. In addition, the PE activity could also be tested in T cells isolated from the cervicovaginal tissues of human subjects who take HCs or experience BV infections, these data can be compared with the PE activity data generated from HCs or interleukin treated cell lines. To further explore the mechanism with regard to how HCs and inflammation impact PE's expression and function, cell lines can be used for testing nuclear receptors gene expression and protein expression, which are related with PEs' expression. Finally, in vivo studies can be conducted using animal models to examine the PEs' function. As discussed in Chapter 4, the macaque model seems the best model to use, however, considering the availability and cost, the mouse model might be a good model to start with. Taken together, these studies will give us a better idea on how these factors can impact AVRs effectiveness and what we can do to ensure their effectiveness under various conditions.

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