PREFORMULATION AND BIOLOGICAL EVALUATIONS FOR THE INTRAVAGINAL DELIVERY OF GRIFFITHSIN FOR HIV PREVENTION

by

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HIV/AIDS persists as a global health concern, with the overwhelming majority of infections caused by sexual transmission. The lack of effective prevention modalities for women has prompted research of novel approaches to prevent disease spread. One such approach includes vaginal microbicides. The work presented within this dissertation project contributes significantly to the vaginal microbicide field, focusing on preformulation assessments, stability in the biological environment, and drug targeting of the biopharmaceutical microbicide candidate Griffithsin (GRFT).

We hypothesized that GRFT will undergo physical and chemical instabilities that will affect successful formulation and vaginal delivery of this microbicide candidate. We further hypothesized that GRFT will undergo binding interactions within cervicovaginal secretions that will negatively impacting GRFT-gp120 binding and that GRFT will not be able to inhibit HIV binding to the DC-SIGN receptor and transfer to CD4 cells because GRFT will not permeate cervical tissue.

Identification of degradation pathways for GRFT was conducted by performing preformulation experiments under selected conditions of temperature, light, shear, ionic strength, and oxidation. Analytical methods included HPLC, CD, UV-spectroscopy, ELISA, and SDS-PAGE. GRFT chemical modifications, including intact mass analyses and peptide sequencing, were evaluated in the presence of hydrogen peroxide and human cervicovaginal secretions. The

effects of human cervicovaginal secretions on GRFT-gp120 binding was also assessed with ELISA. Tissue permeability and localization of GRFT was evaluated using excised human ectocervical tissue.

Major findings from this dissertation indicate that: (1) GRFT is prone to oxidation, by both hydrogen peroxide exposure and human cervicovaginal secretion exposure; (2) Methionine at position 78 in the amino acid sequence of GRFT is oxidized; (3) GRFT-gp120 binding is inhibited in human cervicovaginal secretions containing normal microflora, but not in secretions with BV; and (4) GRFT does not permeate deep into or through human cervical tissue, but does adheres to the superficial epithelial tissue.

Overall, this dissertation has created more knowledge about a drug candidate in the microbicide field and will guide further development of GRFT. Further, the methodologies implemented throughout this dissertation can be used or adapted as part of a strategy to preclinically evaluate other vaginal microbicide candidates.

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PREFACE

Completing my graduate training would not have been possible without the support of many individuals – in both professional and personal settings. There are not enough written or spoken words to properly convey my extreme gratitude to all of the people who have affected me on my journey throughout graduate school. I wish to highlight the most significant relationships here.

My greatest gratitude goes to my advisor Dr. Lisa Cencia Rohan. Thank you for allowing me to obtain a summer internship in your laboratory in 2007 – this significantly sparked by interest in research and graduate school. Thank you for accepting me into your laboratory as a graduate student. Thank you for challenging me and believing in my abilities as a scientist. I truly cannot thank you enough.

I would like to acknowledge all of my committee members, both past and present: Dr. Billy Day, Dr. Sharon Hillier, Dr. Song Li, Dr. Bernard Moncla, and Dr. Raman Venkataramanan. Your guidance and support over the past years have been beneficial to improving my knowledge and skills in pharmaceutical sciences. Thank you all very much.

I would like to thank all of the members of the Rohan Lab, both past and present, for helping to shape the scientist that I have become. Thank you all for sharing your knowledge of science, techniques, and equipment with me. I would not have been able to conduct my research without your help. A special thanks goes to Marilyn Cost, Phillip Graebing, and Lin Wang.

DEDICATION

This dissertation is dedicated to all of the women around the world affected by HIV.

LIST OF ABBREVIATIONS

BSA   Bovine Serum Albumin

EFdA   4'-Ethynyl-2-fluoro-2'-deoxyadenosine

AIDS   Acquired Immunodeficiency Syndrome

ACN   Acetonitrile

AA   Amino Acid

ART   Antiretroviral Therapy

ARV   Antiretroviral(s)

ASPIRE   A Study to Prevent Infection with a Ring for Extended Use

BV   Bacterial Vaginosis

CADA   Cyclotriazadisulfonamide

CAP   Cellulose Acetate Phthalate

CD4   CD4 T lymphocyte

cDNA   Complementary DNA

CD   Circular Dichroism

CMC   Chemistry, Manufacturing, and Controls

CVL   Cervicovaginal Lavage

CV-N   Cyanovirin-N

DAPI   4',6-diamidino-2-phenylindole

DC   Dendritic Cell

DC-SIGN   Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-Integrin

DNA   Deoxyribonucleic Acid

EC$_{50}$	Half Maximal Effective Concentration
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
eMTCT	Elimination of Mother-to-Child-Transmission
EGCG	Epigallocatechin Gallate
FACTS	Follow-on African Consortium for Tenofovir Studies
FDA	Food and Drug Administration
FI	Fusion Inhibitor
FTC	Emtricitabine
GRFT	Griffithsin
H&E	Hematoxylin and Eosin
CMX157	Hexadecyloxypropyl Tenofovir
HCV	Hepatitis C Virus
HHA	Hippeastrum Hybrid Agglutinin
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
ICH	International Conference on Harmonization
IND	Investigational New Drug Application
INSTI	Integrase Strand Transfer Inhibitor
IVR	Intravaginal Ring
ICAM-1	Intercellular Adhesion Molecule 1
LC	Langerhans cell

LMBL	Lactobacillus Mannose-Binding Lectin
MPP	Mucus-Penetrating Nanoparticles
MTCT	Mother-to-Child-Transmission
MZC	MIV150, Zinc Acetate, and Carrageenan
N-9	Nonoxynol-9
NRTI	Nucleoside/Nucleotide Reverse Transcriptase Inhibitor
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
OTC	Over-The-Counter
OD	Optical Density
PK	Pharmacokinetics
PEHMB	Polyethylene Hexamethylene Biguanide
PEP	Post-Exposure Prophylaxis
PI	Protease Inhibitor
PPCM	Poly[1,4-phenylene-(1-carboxyl)methylene])
PRM-S	Pradimicin S
PrEP	Pre-Exposure Prophylaxis
PMTCT	Prevention of MTCT
RAI	Receptive Anal Intercourse
R&D	Research and Development
RNA	Ribonucleic Acid
RRT	Relative Retention Time
RT	Retention Time
RT	Room Temperature

SNA	Sambucus nigra agglutinin
STI	Sexually Transmitted Infection
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLS	Sodium Lauryl Sulfate
SRS	Sodium Rutin Sulfate
TasP	Antiretroviral Treatment as Prevention
TAF	Tenofovir Alafenamide Fumarate
TDF	Tenofovir Disoproxil Fumarate
TDF/FTC	Truvada
TMV	Tobacco Mosaic Virus
TFA	Trifluoroacetic Acid
TFV	Tenofovir
UNAIDS	Joint United Nations Programme on HIV/AIDS
US	United States
VCF	Vaginal Contraceptive Film
WHO	World Health Organization

1.0   INTRODUCTION

1.1   HIV/AIDS – GLOBAL HEALTH PROBLEM

1.1.1   HIV – Statistics

Recent global statistics have revealed that approximately 2.1 million people were newly infected with the Human Immunodeficiency Virus (HIV), and 1.5 million Acquired Immunodeficiency Syndrome (AIDS)-related deaths occurred in 2013 [1]. Although these figures reflect the ongoing pandemic, they have slowly, yet consistently, decreased over the past decade, which is reflective of HIV/AIDS management efforts, including the life-saving capacity of antiretroviral therapy. Currently, 35 million people are living with HIV worldwide.

In some countries, a reduction in sexually transmission of HIV has decreased over the past decade, but in other areas around the world the incidence of HIV has remained high despite the rollout of HIV treatment. Globally, sexual transmission remains a leading cause of HIV infection, and HIV prevalence among young women remains almost twice as high as among young men throughout sub-Saharan Africa, as seen in Figure 1 [2]. Physiological susceptibility and socioeconomical disadvantages make women more likely to become infected [3]. Abstinence, reduction in the number of sexual partners and concurrent sexual relationships, and correct, consistent condom use are highly effective against HIV acquisition but have proven to

be insufficient to combat this incurable disease [4-7]. Therefore, an opportunity for strengthening the toolbox of HIV prevention methods is evident. No HIV vaccination is available, but researchers continue to investigate the safety, immunogenicity, and efficacy of potential vaccines. In July 2012, Truvada® (one tablet orally once a day) was approved by the US Food and Drug Administration (FDA) for pre-exposure prophylaxis (PrEP) in combination with safer sex practices to reduce the risk of infection in adults at high risk of HIV acquisition. A Truvada tablet contains a fixed dose of emtricitabine (200mg) and tenofovir disoproxil fumarate (300mg), both of which are reverse transcriptase inhibitors. Additional PrEP products under preclinical and clinical development include those that are to be applied vaginally, rectally, or via injection. The sub-category of PrEP that is administered vaginally or rectally is termed microbicides. Development of vaginal microbicides will be discussed further and will play a major role in reducing the incidence of HIV acquisition in women.
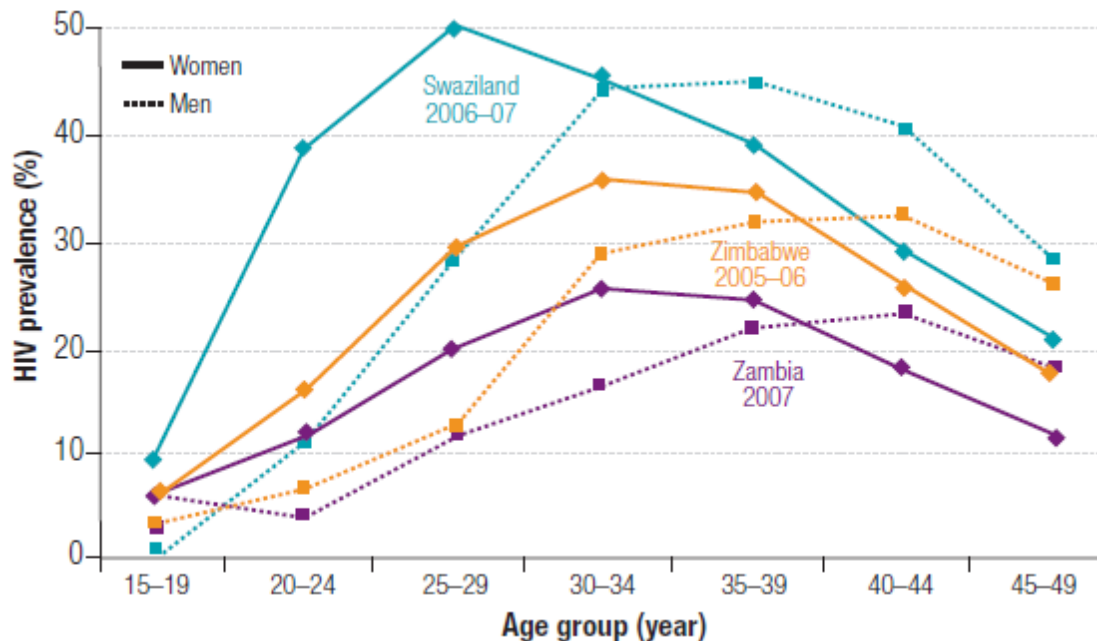
Figure 1. HIV Prevalence in Women and Men by age group; select sub-Saharan African countries.

1.1.2 HIV Pathogenesis

HIV is an (ribonucleic acid) RNA virus with an RNA nucleoprotein core surrounded by a lipid envelope derived from the host cell membrane and containing virally encoded envelope proteins. HIV is a retrovirus – a retrovirus uses an RNA genome to direct the synthesis of a deoxyribonucleic acid (DNA) intermediate, a situation consideration 'backward' or 'retro' from that used by most biological systems. Once HIV enters the body, the virus binds to CD4 receptors on the host cell surface by using the envelope protein gp120, which is altered by CD4 binding so that it also binds a specific chemokine co-receptor (CCR5 and CXCR4) on the cell. CD4 receptors are present on T-helper lymphocytes, monocytes, macrophages, dendritic cells, and brain microglia. This binding releases gp41, which then causes fusion of the viral envelope with the host cell membrane and release of the viral core into the host cell cytoplasm. The RNA genome is reverse transcribed into double-stranded complementary DNA (cDNA) by reverse transcriptase. Viral cDNA migrates to the host cell nucleus and is integrated into the host DNA (with viral integrase), becoming a provirus. Once HIV DNA is integrated with the host cell DNA, the virus begins to use the machinery of the cell to create long chains of HIV proteins (induction of transcription of provirus). The protein chains are the building blocks of more HIV. A viral enzyme, protease, cleaves the chains of HIV proteins. The resulting HIV proteins (Gag, Pol, and Env) combine with HIV RNA to form new virus. The newly made virus buds from the host cell [8-9]. An overall pictorial depiction of the HIV life cycle can be found in Figure 2.
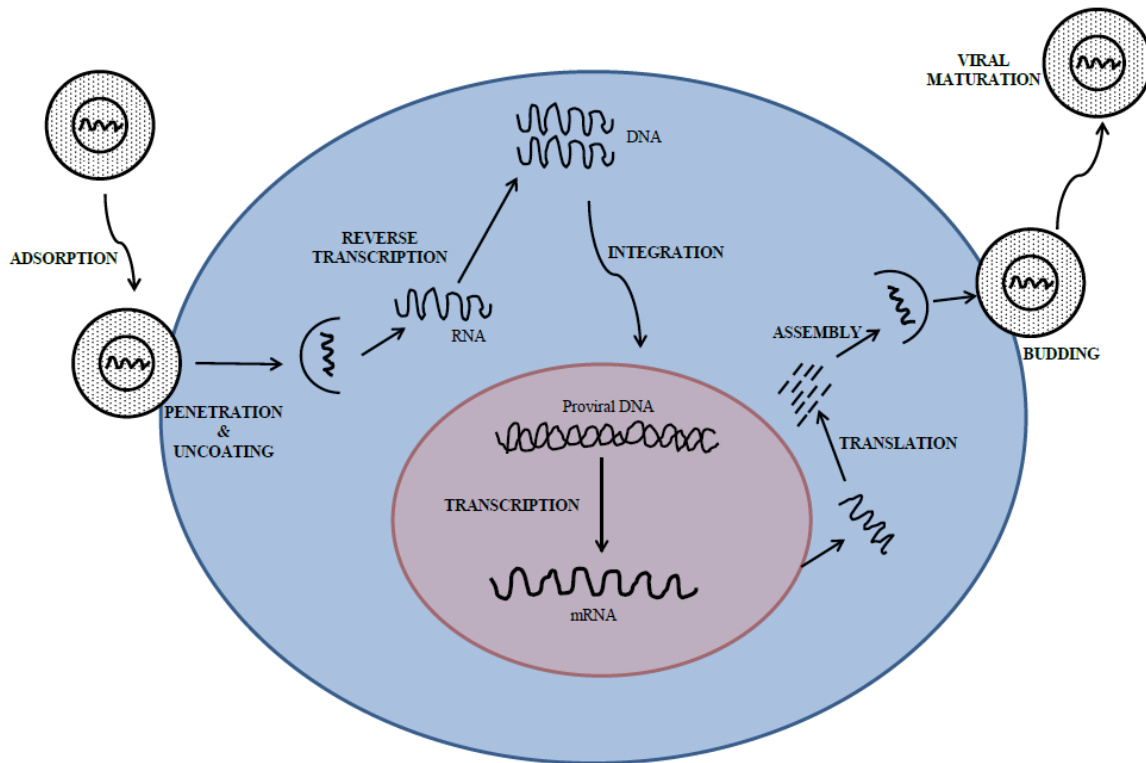
Figure 2. Life Cycle of HIV.

1.1.3   HIV – The Basics

Two types of HIV exist: HIV-1 and HIV-2. In most countries HIV-1 is the principle cause of AIDS. HIV-2, which is endemic to West Africa, is less virulent, causing a slower progression to AIDS. Overall, HIV attacks cells of the immune system, destroying or impairing their function, and results in progressive immune system deterioration or "immune deficiency". As a result of severe immunodeficiency, opportunistic infections can occur, since these infections thrive on a weakened immune system. This condition is called acquired immunodeficiency syndrome (AIDS). AIDS was first recognized as a disease in 1981, when an increasing number of homosexual men developed opportunistic infections and cancers. A few years later, HIV was

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identified as the causative factor. Over approximately three decades, HIV/AIDS has become one of the most devastating infectious diseases across the entire globe [8-9].

HIV can be transmitted from one person to another via exchanges of body fluids, such as blood, breast milk, semen, and vaginal secretions. Therefore, the virus can be transmitted through contaminated blood and the sharing of contaminated needles, syringes, or other sharp objects. HIV can be acquired through unprotected sex (both vaginal and anal) and through oral sex with an infected individual. An infected mother can spread HIV to her infant during pregnancy, childbirth, and breastfeeding. However, people cannot become infected during normal day-to-day contacts such as kissing, hugging, shaking hands, or sharing food or water [8-9].

There are multiple risk factors that increase an individual's likelihood of acquiring HIV. Some of these include receiving unsafe/unsterile injections, blood transfusion, or medical procedures; sharing contaminated drug solutions, needles, syringes, and/or other injecting items when injecting drugs; experiencing accidental needle sticks, including those among health workers; having unprotected vaginal or anal sex; and having another sexually transmitted infection (STI) such as syphilis, herpes, chlamydia, gonorrhea, and/or bacterial vaginosis [8-9].

Some individuals with early HIV infection will not experience any signs or symptoms (acute asymptomatic HIV infection). Therefore, these infections go undetected. The usual time to develop signs and symptoms of HIV is two to four weeks. A variety of signs and symptoms are associated with acute symptomatic HIV infection. The most common clinical manifestations are fever, fatigue, and myalgia/arthralgia. Others include lymphadenopathy, sore throat, rash, headache, some gastrointestinal complaints (nausea, diarrhea, anorexia, weight loss), and dry cough. None of the symptoms is specific for acute HIV infection, but certain features, especially

prolonged duration of symptoms and the presence of mucocutaneous ulcers, are suggestive of the diagnosis. Without treatment, HIV infected individuals could experience worsening of symptoms and develop a variety of opportunistic infections [8-9].

When acute/early HIV infection is suspected, an immunoassay (ideally a combination antigen/antibody immunoassay) and HIV virologic test/viral load (measuring RNA) should both be performed. An antibody-based immunoassay detects the presence of HIV antibodies in the blood. HIV antibodies take approximately three to six week to be produced and are not likely to be detected during this early time frame. An antigen-based test can detect the p24 antigen, which is viral core protein that can appear in the blood as viral RNA rises after HIV infection. People who are not infected with HIV have no viral load; therefore, having a viral load is generally indicative of HIV infection (a direct measure of HIV). Viral RNA can be detected within two weeks of initial infection. Timing of infection (acute/early versus established) can be determined by immunoassay reactivity and clinical presentation. A negative immunoassay and negative virologic test strongly suggest that HIV has not been acquired. A negative HIV immunoassay and a positive virologic test suggest early HIV infection [8-9].

HIV diagnosis guidelines are distinct from general HIV screening guidelines. General screening includes an immunoassay followed by a confirmatory immunoassay of a different type if the first one is positive. Virologic testing is only used to resolve disagreement between the two immunoassays. In July 2012, the US FDA approved the first over-the-counter (OTC) home-use rapid HIV test, called OraQuick® In-Home HIV Test (immunoassay). This test allows individuals to collect an oral saliva sample. The sample is then placed in a developer vial, and results (positive or negative) are obtained within 20 minutes. A positive result should prop the tested individual to seek additional/confirmatory testing in a formal medical setting [10-11]. One

single use kit is approximately $40 [12-13]. Another novel development in HIV testing is the use of a smartphone accessory. This $34 accessory/dongle plugs into the phone's audio jack and is compatible with both iPhone and Android smartphones. The test requires a single pin-prick of blood, and results are reported on the smartphone screen within 15 minutes. Smartphones are becoming popular mediums for low-cost medical diagnostic tests. By making this HIV test affordable, quick, and easy to use, it is hoped that HIV testing will increase in developing countries.

If left untreated, the majority of HIV-positive people will develop signs of HIV-related illness within five to ten years and will possibly develop AIDS. HIV is considered AIDS when a person's CD4 T lymphocyte (CD4) count is less than 200 cells/mm^3 and/or the person is diagnosed with an opportunistic infection [14-15]. Opportunistic infections include candidiasis of esophagus, trachea, bronchi, or lungs; invasive cervical cancer; extrapulmonary coccidioidomycosis; extrapulmonary cryptococcosis; cryptosporidiosis with diarrhea for > one month; cytomegalovirus of any organ other than liver, spleen, or lymph nodes; Herpes simplex with mucocutaneous ulcer for > one month   or bronchitis, pneumonitis, esophagitis; extrapulmonary Histoplasmosis; HIV-associated dementia; HIV-associated wasting; Isosporiasis with diarrhea for > one month; Kaposi's sarcoma; lymphoma; disseminated *Mycobacterium avium* or *M. kansasii*; disseminated *Mycobacterium tuberculosis*; pulmonary *Mycobacterium tuberculosis*; Nocardiosis; *Pneumocystis carinii* (*P. jiroveci*) pneumonia; recurrent bacterial pneumonia; progressive multifocal leukoencephalopathy; recurrent Salmonella septicemia (nontyphoid); extraintestinal Strongyloidiasis; and Toxoplasmosis of internal organ. Of all of the possible opportunistic infections, tuberculosis occurs most commonly [8-9]. Without medical treatment, those with AIDS usually survive for three years or less.

Currently, no cure for HIV exists. However, antiretrovirals (ARV) are available to help slow disease progression. The primary goals for initiating antiretroviral therapy (ART) include: 1) reduction of HIV-associated morbidity and prolonged duration/quality of life; 2) restoration and preservation of immunologic functioning; 3) suppression of HIV viral load; and 4) prevention HIV transmission [16]. It is recommended that ART be initiated when the infected individual's CD4 count is <500 cells/mm^3. Over 20 ARVs in six classes are approved for HIV therapy. These classes, which act directly on components of the HIV life cycle, consist of nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitors (PIs), fusion inhibitors (FIs), CCR5 antagonists, and integrase strand transfer inhibitors (INSTIs). The optimal ARV regimen for the treatment-naïve individual includes two NRTIs in combination with a third ARV from one of three drug classes: an NNRTI, a PI boosted with ritonavir, or an INSTI. Further therapeutic recommendations can be found throughout the AIDSinfo and World Health Organization (WHO) websites. With continued adherence to ART, the progression of HIV can be slowed to a near halt. ART is truly lifesaving, as reflected in the increasing number of people living with HIV globally, as seen below in Figure 3 [1] and as previously described UNAIDS. Additionally, individuals with HIV often need counseling and psychological support. Good nutrition, safe/clean water, and good hygiene can also increase quality of life.
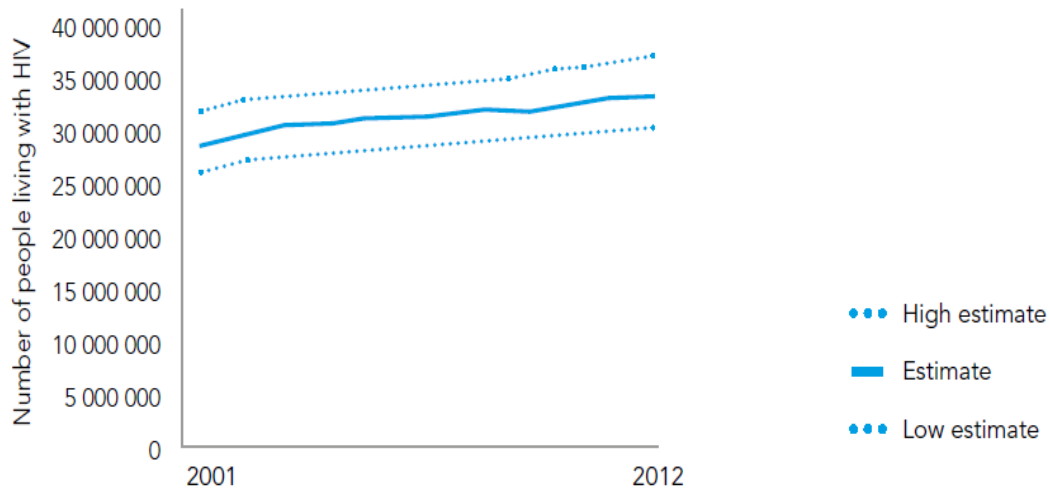
Figure 3. Total number of people living with HIV globally from 2001-2012.

HIV negative individuals can minimize acquisition of HIV by limiting exposures to risk factors. Important approaches to HIV prevention consist of 1) male and female condom use; 2) testing and counseling for STIs; 3) voluntary male circumcision; 4) harm reduction for injecting drug users; 5) elimination of mother-to-child transmission of HIV (eMTCT); and 6) ARV-related methods. Correct and consistent male or female condom use during vaginal or anal sex protects against the spread of STIs, including HIV. For individuals who think that they have or are being exposed to risk factors, STI testing, including HIV, is advisable so that they are made aware of their status and access prevention/treatment services as soon as possible. STI testing for partners is also highly recommended to expand the scope of prevention. As a one-time intervention, voluntary male circumcision provides life-long partial protection against STIs, including HIV. Intravenous drug abusers can reduce their risk of HIV acquisition by using sterile needles and syringes for each injection or by using opioid substitution therapy for those with opioid dependency [8-9].

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HIV transmission from a HIV-positive mother to her child during pregnancy, labor, delivery, or breastfeeding is called vertical transmission or mother-to-child transmission (MTCT). Without any intervention, MTCT rates are between 15-45%. There are multiple options for prevention of MTCT, which include providing ARVs to mothers and infants during pregnancy, labor and the post-natal period, or offering life-long treatment to HIV-positive pregnant women regardless of their CD4 count. In 2012, 62% of the approximately 1.5 million HIV-positive women in low- and middle-income countries received ARVs to prevent MTCT during pregnancy. 49% of HIV-positive women in low- and middle-income countries received ARVs to avoid MTCT during breastfeeding [1].

1.2 HIV PREVENTION WITH ANTIRETROVIRALS

Clearly, comprehensive and combination HIV prevention methods are of utmost importance to combat this global pandemic. One strategy involves the use of ARVs for HIV prevention. This involves three ARV approaches: antiretroviral treatment as prevention (TasP), post-exposure prophylaxis (PEP), and pre-exposure prophylaxis (PrEP). In 2011, results of a multi-centered trial (HPTN 052) indicated that if an HIV-positive individual is adherent to ART, then the risk of HIV transmission to his/her uninfected sexual partner is reduced by 96% [17]. As a result, in regards to serodiscordant couples, ART as TasP should be offered to the HIV-positive partner regardless of CD4 count [16]. ARV PEP reduces the likelihood of HIV acquisition after possible exposure following accidental occupational or non-occupational insult (whether consensual or assault). The current recommended duration of PEP is 28 days, with the first dose being administered as soon as possible and within 72 hours of suspected exposure [18-19]. The standard PEP ARV regimen should consist of two NRTIs. A three-drug regimen, comprising two

NRTIs plus a ritonavir-boosted PI, should be considered when ARV resistance is known or highly suspected. PrEP is the newest addition to the use of ARVs for HIV prevention. The effectiveness of oral PrEP has been investigated in several large clinical trials. Refer to Table 1.

Table 1. Clinical Trials Involving Oral PrEP

Study	PrEP Regimen	Study Population	Reduction in HIV Risk (overall)
iPrEx [20] Phase 3	Oral TDF/FTC tablet or placebo tablet; once daily	2499 HIV-negative men and transgender women who have sex with men	44%
FEM-PrEP [21] Phase 3	Oral TDF/FTC tablet or placebo tablet; once daily	1951 HIV-negative, sexually active, heterosexual women	None; early stop due to futility
Partners-PrEP [22] Phase 3	Oral TDF/FTC tablet, TDF tablet, or placebo tablet; once daily	4758 HIV-serodiscordant, heterosexual couples; HIV-negative partner takes PrEP regimen	67% (TDF) and 75% (TDF/FTC); stopped early due to significant results at interim
TDF2 [23] Phase 2b	Oral TDF/FTC or placebo tablet; once daily	1200 HIV-negative, sexually active, heterosexual men and women	63%; but not powered to demonstrate efficacy
VOICE [24] Phase 2b	Oral TDF/FTC tablet, oral TDF tablet, oral placebo tablet, vaginal TFV gel, or vaginal placebo gel; once daily	5029 HIV-negative, sexually active, heterosexual women	None; daily oral TDF, daily vaginal TFV, and daily vaginal placebo stopped early due to futility; daily oral TDF/FTC and oral placebo continued to planned end
PROUD [25]	Oral TDF/FTC tablet; once daily; open label	545 HIV-negative men who have sex with men	86%
IPERGAY [26] Phase 3	Oral TDF/FTC or placebo tablet; 2 tablets within 24 hours before and 2 tablets within 48 hours after sex	400 HIV-negative men who have sex with men	86%

In July 2012, Truvada (one tablet orally once a day) was approved by the US FDA as a PrEP regimen, based on the results of the phase 3 iPrEx and Partners-PrEP trials which displayed 44% and 75% reduced HIV risk with oral Truvada, respectively [20, 22]. A Truvada tablet contains a fixed dose of emtricitabine (200mg) and tenofovir disoproxil fumarate (300mg), and both are reverse transcriptase inhibitors. PrEP is recommended for those at high risk of HIV

acquisition and should always be suggested as part of a comprehensive HIV prevention package. Additionally, of important note, are the highly favorable results of the PROUD (**PR**e-exposure **O**ption for reducing HIV in the **U**K: an open-label randomization to immediate or **D**eferred daily Truvada for HIV negative gay men) and IPERGAY (**I**ntervention **P**réventive de l'**E**xposition aux **R**isques avec et pour les hommes **Gay**s or Action to Prevent Risk Exposure By and For Gay Men) trials that showed a 86% reduction in the risk of HIV prevention [25-26] . Both studies were completed after the US FDA approval of Truvada for PrEP.

1.2.1   HIV PrEP – In Development

Despite the addition of oral Truvada to available HIV prevention modalities, the development of more than one PrEP product is warranted. Unlike the oral PrEP trials with men who have sex with men and HIV serodiscordant couples, daily oral PrEP did not reduce HIV risk among young, unmarried African women in either the VOICE or FEM-PrEP studies due to low product adherence [21, 24]. This is one reason, among many, for PrEP research to continue in order to investigate the safety and efficacy of other PrEP agents and products. Continued research will broaden agent options and dosage form options, which will ultimately increase acceptability, adherence, and effectiveness of PrEP regimens across different populations at risk. Refer to Tables 2 and 3 to view the agents and products under preclinical and clinical development for HIV PrEP. The oral Truvada tablet is currently in Phase 4, post marketing surveillance for HIV prevention. Two notable vaginal microbicide products in clinical testing are the tenofovir 1% vaginal gel and dapirivine vaginal ring. Both reverse transcriptase-based products are currently in Phase 3 clinical trials.

Table 2. Investigational PrEP Agents/Products – Preclinical and Clinical Development

Drug Candidate(s)*	Mechanism(s) of Action	Dosage Form(s) and Route(s) of Delivery (if known)	Farthest Phase of testing completed/ongoing/in development
Small Molecule			
Tenofovir disoproxil fumarate (TDF)	NRTI	Tablet – Oral Ring – Vaginal	Oral Tablet – Phase 3 Vaginal Ring – Phase 1
Tenofovir (TFV)	NRTI	Gel – Vaginal, Rectal Ring – Vaginal Film – Vaginal Tablet – Vaginal	Vagina Gel – Phase 3 Reduced Glycerin Gel, Vaginally – Phase 1 Reduced Glycerin Gel, Rectally – Phase 2 Vaginal Tablet, Film, and Ring – Phase 1
UC-781 (development discontinued)	NNRTI	Gel – Vaginal, Rectal Film – Vaginal	Vaginal Gel – Phase 1 Rectal Gel - Preclinical Vaginal Film - Preclinical
Dapivirine	NNRTI	Gel – Vaginal, Rectal Ring – Vaginal Film - Vaginal	Vaginal Gel – Phase 2 Rectal Gel – Phase 1 (in development) Vaginal Ring – Phase 3b (in development Vaginal Film – Phase 1
Maraviroc	CCR5 inhibitor	Tablet – Oral Gel – Vaginal, Rectal Ring – Vaginal	Oral Tablet – Phase 2 Vaginal Gel – Phase 1 (in development) Rectal Gel – Phase 1 (in development) Vaginal Ring – Phase 1
IQP-0528	NNRTI and inhibits HIV entry (unknown mechanism)	Gel – Vaginal Film - Vaginal	Vaginal Gel – Phase 1 (in development) Vaginal Film – Preclinical
MK-2048	INSTI	Ring – Vaginal	Vaginal Ring – Phase 1 (in development)
Vicriviroc (MK-4156)	CCR5 inhibitor	Ring - Vaginal	Vaginal Ring – Phase 1 (in development)
Raltegravir	INSTI	Gel - Vaginal	Vaginal Gel - Preclinical
DS003 (BMS793)	gp120 inhibitor	Ring – Vaginal Film – Vaginal Tablet - Vaginal	Vaginal Ring, Film, and Tablet - Preclinical
MIV150	NNRTI	Gel – Vaginal Ring – Vaginal	Vaginal Gel – Phase 1 Vaginal Ring - Preclinical
Rilpivirine	NNRTI	Long Acting Injection – Intramuscular	Intramuscular Injection – Phase 2 (in development)
Cabotegravir (GSK 744)	INSTI	Long Acting Injection – Intramuscular, Subcutaneous	Intramuscular Injection – Phase 2a Subcutaneous Injection – Phase 1
Acyclovir	HSV-2 suppressive therapy	Tablet – Oral	Oral Tablet – Phase 3
CMPD167	CCR5 inhibitor	Solution – Oral Gel – Vaginal, Rectal Ring – Vaginal	Oral Solution, Vaginal/Rectal Gel, and Vaginal Ring - Preclinical

Drug Candidate(s)*	Mechanism(s) of Action	Dosage Form(s) and Route(s) of Delivery (if known)	Farthest Phase of testing completed/ongoing/in development
Oligonucleotide/Peptide/Protein			
ISIS 5320	gp120 inhibitor	Injection - Subcutaneous	Subcutaneous Injection - Preclinical
Griffithsin	gp120 inhibitor	Gel – Vaginal, Rectal Film – Vaginal Nanofiber - Vaginal	Vaginal Gel, Film, and Nanofiber – Preclinical Rectal Gel – Phase 1 (in development
5P12-RANTES	CCR5 inhibitor	Gel – Vaginal, Rectal	Vaginal Gel – Phase 1 (in development) Rectal Gel – Preclinical
RC-101	gp41 inhibitor	Ring – Vaginal Film – Vaginal	Vaginal Ring and Film – Preclinical
Monoclonal Antibody			
TMB-355	CD4 receptor inhibitor	Long Acting Injection – Subcutaneous	Subcutaneous Injection – Phase 1
2nd generation TMB-355 (glycosylation)	CD4 receptor inhibitor and gp120 inhibitor		Preclinical
VRC01	gp120 inhibitor	Gel – Vaginal	Vaginal Gel - Preclinical
4E10	gp41 inhibitor	Injection – Intravenous	Intravenous Injection – Preclinical
2F5	gp41 inhibitor	Injection – Intravenous	Intravenous Injection – Preclinical
2G12	gp120 inhibitor	Injection – Intravenous	Intravenous Injection – Preclinical
b12	gp120 inhibitor	Gel – Vaginal Injection – Intravenous	Vaginal Gel and Intravenous Injection – Preclinical
Combination Agents/Products			
Tenofovir disoproxil fumarate/Emtricitabine (TDF/FTC)	NRTIs	Tablet – Oral	Oral Tablet – Phase 4
TFV/FTC	NRTIs	Tablets - Vaginal	Vaginal Tablets – Phase 1
Dapivirine/Maraviroc	NNRTI/CCR5 inhibitor	Gel – Vaginal Ring – Vaginal Film - Vaginal	Vaginal Gel and Film – Preclinical Vaginal Ring – Phase 1
MK-2048/Vicriviroc (MK-4176)	INSTI/CCR5 inhibitor	Ring - Vaginal	Vaginal Ring – Phase 1 (in development)
Maraviroc/Emtricitabine	CCR5 inhibitor/NRTI	Tablets – Oral	Oral Tablets – Phase 2
Dapivirine/Darunavir	NNRTI/PI	Gel – Vaginal Ring - Vaginal	Vaginal Gel and Ring – Preclinical
Tenofovir/Maraviroc	NRTI/CCR5 inhibitor	Gel – Rectal Film – Vaginal Tablets – Oral	Rectal Gel – Preclinical Vaginal Film – Preclinical Oral Tablets – Phase 2
Dapivirine/DS003	NRTI/gp120 inhibitor	Film - Vaginal	Vaginal Film - Preclinical
MIV150, Zinc Acetate, and Carrageenan (MZC)	NNRTI + 2 broad spectrum antivirals (carrageenan is a polymer)	Gel – Vaginal Ring – Vaginal	MZC Vaginal Gel – Phase 1 MZC Vaginal Ring – Preclinical MZC and Levonorgestrel Vaginal Ring – Phase 1 (in development)
Griffithsin/Tenofovir	gp120 inhibitor/NRTI	Gel - Rectal	Rectal Gel - Preclinical
4E10, 2F5, 2G12, and b12 cocktail	gp41 inhibitors/gp120 inhibitors	Gel - Vaginal	Vaginal Gel - Preclinical
VRC01/4E10	gp120 inhibitor/gp41 inhibitor (with one HSV neutralizing monoclonal antibody; called mapp66)	Ring – Vaginal Film – Vaginal	Vaginal Ring and Film – Preclinical
Herbal/Extract-based			
Praneem	unknown	Tablet - Vaginal	Vaginal Tablet – Phase 2
Basant	unknown	Cream - Vaginal	Vaginal Cream – Preclinical
Device			
Thin Film Polymer; no drug	Biodegradable device	Injection - Subcutaneous	Subcutaneous Injection – Preclinical

Table 3. Investigational PrEP Agents – Early Preclinical Development

*Attachment, Fusion, and Entry Inhibitors**	DS001 (L167) DS004 (L872) DS005 (L882) DS007 (L-644 peptide) Tenofovir Alafenamide Fumarate (TAF) Hexadecyloxypropyl Tenofovir (CMX157) C52L Cyclotriazadisulfonamide (CADA) compounds Feglymycin Cyanovirin-N (CV-N) (and bioengineered Lactobacillus expressing CV-N) D-peptides Epigallocatechin Gallate (EGCG) K5 derivatives Lactobacillus Mannose-Binding Lectin (LMBL) Nanobodies® Optimized Dendrimers Polyethylene hexamethylene biguanide (PEHMB) and other biguanide-based compounds Poly[1,4-phenylene-(1-carboxyl)methylene]) (PPCM) RANTES peptides (and bioengineered Lactobacillus expressing RANTES derivatives) sCD4-17b anti-ICAM-1 (intercellular adhesion molecule 1) antibody delivered by Lactobacillus Sodium Rutin Sulfate (SRS) Pradimicin S (PRM-S) Syndecan Antagonists and Agonists T-1249
*Replication Inhibitors (NRTI, NNRTI, INSTI, PI)**	Dolabelladienetriol 4'-Ethynyl-2-fluoro-2'-deoxyadenosine (EfdA) Saquinavir, Lopinavir, and Ritonavir
*Combination Agents/Multiple or Novel Mechanisms/Novel Technology**	Hippeastrum hybrid agglutinin (HHA)/KRV2110/Enfuvirtide (T20) Pyrimidinediones NCp7 Thioesters Nisin Novasomes® xREPLAB Dissolving Tampon

*Tables 2 and 3 are adapted from information on the development of PrEP agents on the following websites: AVAC, MTN, HPTN, CONRAD, IPM, POP Council.

Preclinical development can include, but is not limited to, drug discovery activities for said indication (during early preclinical), preformulation assessments and dosage formulation development, *in vitro* research for safety/biocompatibility and efficacy, and *in vivo* safety and efficacy testing in animal models. Usually, in early phase clinical trial (Phase 1 and 2) testing for vaginal microbicides (for example), the female participants are HIV-negative, sexually-active, non-pregnant, pre-menopausal, and 18-45 years old. Some early phase clinical trials of PrEP

have incorporated a variety of study populations, study designs, and study product regimens. To gain insight into 'special' female populations, Phase 1 and 2 testing of selected PrEP study products is occurring in women who are adolescent, post-menopausal, pregnant, or lactating. Another 'special' study population under investigation is high risk HIV-negative, but HSV-2-positive participants. Penile tolerance of mucosally applied microbicide products is also being assessed. Additionally, for microbicides, various dosing regimens (daily, coitally-dependent, monthly, etc.) and varying routes of delivery (cross over study examining vaginal and rectal route of administration for one product) are under evaluation. These are just a few examples of the variety of study populations, designs, and regimens of PrEP in early phase clinical trials. The AVAC website (www.avac.org) is an excellent, comprehensive resource for further details on the HIV PrEP preclinical and clinical trials pipeline. For the purposes of this thesis dissertation, focus will be given to vaginal microbicide development for the prevention of female sexual acquisition of HIV.

1.3   SEXUAL (MUCOSAL) TRANSMISSION OF HIV – MALE-TO-FEMALE TRANSMISSION

Heterosexual transmission accounts for the twenty-five percent of HIV-1 infection, yet HIV is not easily acquired via this route with an estimated transmission incidence between discordant couples to be 0.0005-0.0050 for a sexual act (male-to-female transmission) [14, 27]. Several factors drive the susceptibility and likelihood of female sexual (vaginal) acquisition including the duration of viral shedding, genital health, a high frequency of sexual intercourse, and compromise to the mucosal lining. Genital abrasions, lesions, ulcerations, and inflammation

caused by various STIs and/or other vaginal infections or conditions may increase the risk of HIV-1 transmission. Additionally, hormonal status, nutrient levels, or the use of certain vaginal preparations may increase a female's vulnerability to infection [28]. Determining the true male-to-female transmission risk is not possible due to the multitude of variables involved. Therefore, as stated by Shattock et al, any HIV prevention strategy must assume that each sexual contact has the ability to transmit the virus [28].

Upon deposition of HIV-containing seminal fluid within the vagina, infected cells or free viruses may become trapped within the cervicovaginal fluid, specifically within the cervical mucus [29-30]. This entrapment may allow longer contact time of infected cells or free virus with the mucosa. Conversely, the mucus entrapment may halt transmission by the cells and viruses and further increase the likelihood of attack by innate anti-viral substances [31].

Male-to-female sexual transmission of HIV may occur via several mechanisms. Uncertainty remains as to whether these pathways collectively or individually explain transmission [28, 31-32]. HIV can be transmitted through both the multilayered squamous epithelium of the vagina and ectocervix and the single layer columnar epithelium of the endocervix [27, 33]. Refer to Figure 4 [34].

Despite stratified mucosal epithelial cells being CD4 negative, Langerhans cells (LCs) and T lymphocytes which reside in this area are CD4 positive, marking themselves as targets for HIV [28, 32, 35]. The virus can penetrate this mucosa via thin gaps between the squamous epithelial cells [31]. This route may result in the virus coming into direct contact with LCs and T cells. Breaches in the epithelium may allow the virus to contact the deeper, basal epithelial cells that are vulnerable to viral binding, endocytosis, and transcytosis. These breaches may also allow the virus access to subepithelial targets within the stroma [28]. The stromal tissues are densely

populated with dendritic cells (DCs), macrophages, and T cells that express CD4 and chemokine co-receptors, CCR5 or CXCR4 [36-37]. DCs also express C-type lectin receptors, including DC-SIGN [38]. Each is vulnerable to HIV infection. Once HIV-1 gains contact with these target cells, establishment of infection is possible [28]. Additionally, inflammation may facilitate HIV infection by thinning and disrupting the cellular lining, recruiting a pool of target cells for local HIV expansion, and interfering with innate antimicrobial activity [39]. After HIV expands locally, dissemination of infection occurs. Movement of virus to lymph nodes and secondary lymphoid organs generates a systemic infection. Given the role of heterosexual transmission in the spread of HIV, the susceptibility of the cervicovaginal mucosa, and the fact that women have a higher vulnerability to infection than men, the vagina provides an optimal route/target for HIV prevention therapy.
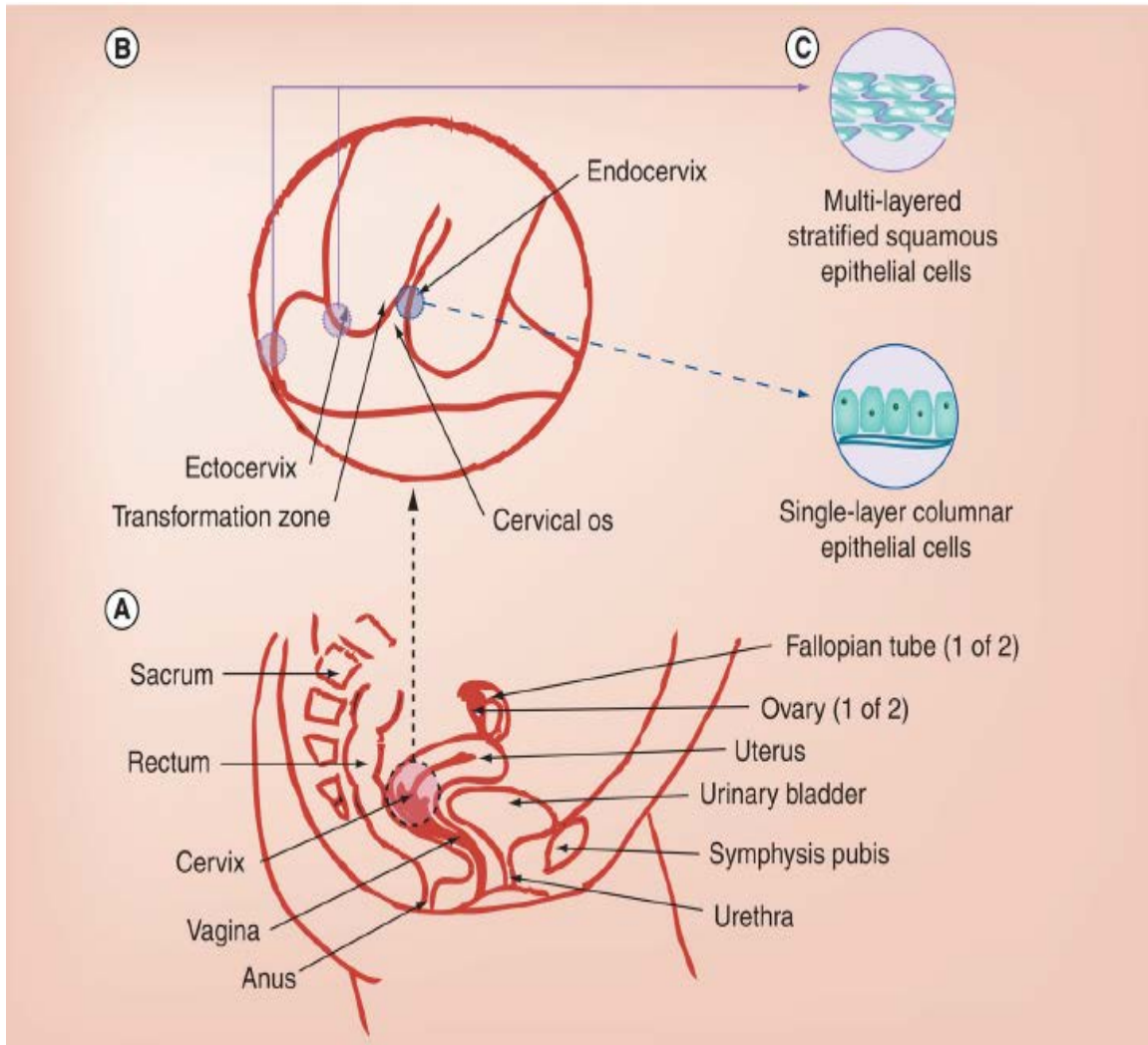
Figure 4. Female Genital Tract Anatomy.
(A) Represents a side view of the gross anatomy of the female genital tract with respect to other organs. (B) Represents tissue types and areas of the upper vagina and cervix. (C) Display of cells composing the vaginal and ectocervical tissue and display of cells composing the endocervical tissue.

1.4   HUMAN VAGINAL ANATOMY AND PHYSIOLOGY

The vagina is situated between the rectum, urethra, and bladder and is the gateway from the

vulva, the outer genitalia, to the cervix, the opening of the uterus, which is a central organ of the

reproduction in a female [40]. Refer to Figure 4 [34]. The vagina provides several important

functions within the female body. These include: (1) facilitating the entrance of seminal fluid; (2)

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providing an outlet for menstrual fluids during menstruation; (3) providing structure for the lower portion of the birth canal; and (4) providing an opening for internal examination of the female reproductive system [41].

The vagina is a fibromuscular tube which exists in a relaxed state, with the anterior and posterior walls being slack and remaining in contact with each other, yet it is easily distensible. The lateral walls of the vagina are fairly rigid, and the vagina is characterized as a potential space but not an actual open space. In a woman standing upright, the vagina is similar in shape to a convex curve, with the upper portion of the vagina being almost at a right angle to the lower portion [40-42].

The dimensions of the vagina vary greatly from woman to woman. In a reproductive aged woman, the anterior vaginal wall averages a length of 6 to 8 cm. The posterior wall can reach a length of 14 cm, with the length of the cervix included [43]. The vaginal diameter ranges from 2.4 to 6.5 cm, and the largest diameter is achieved at a vaginal depth of 2 to 5 cm from the introitus (vaginal opening) [43]. The surface area of the vagina ranges from 65.73 to 107.07 cm^2, with a mean of 87.46 cm^2 [44]. These surface area data do not include the area of the vaginal rugal folds, therefore, representing an underestimation of true surface area.

The shape of the vagina also varies from woman to woman and can be categorized as parallel sides, conical, heart, slug, and pumpkin seed [43, 45]. When compared among races, the pumpkin seed shape was found to be specific to African-American women [45]. These varying vaginal dimensions, sizes, and shapes may be reflected in the acceptability of certain vaginal products, such as gels and tablets, and may build justification for the need of multiple vaginal dosage forms for HIV prevention products. Further, given the variability of the vagina among women, design of products which attempt to cover the entire vaginal surface by the particular

formulation may be challenging. Clearly, a formulation that covers the largest surface area will also cover the smallest, but the opposite is not true [44]. It has yet to be determined if full vaginal/cervical coverage by a vaginal microbicide is necessary.

The vagina and ectocervix, the portion of the cervix that is exposed to the vaginal environment, are lined by a nonkeratinized, stratified squamous epithelium (Figure 4 [34]). The vaginal mucosal layer is composed of multiple rugal folds, increasing the surface area. The squamous epithelium is constantly renewed and desquamated during the premenopausal years and contains three cellular zones: (1) germinal or basal cell layer; (2) midzone or stratum spinosum, the dominant portion of the epithelium; and (3) superficial zone, consisting of the most mature cell population [40]. The superficial cells help to protect the underlying epithelial cells and subepithelial vasculature from trauma and infection. The squamous cells feature gap junction nexuses that represent an open channel system between adjacent cells through which certain molecules and electrolytes can transverse [40].

The endocervix is lined by a single layer of columnar epithelium and invaginates the underlying stroma (Figure 4 [34]). The transformation zone is located on the exposed portion of the cervix and is found in most women of reproductive age [40]. This area is the region where the columnar endocervical epithelium meets the squamous epithelium of the ectocervix. The transformation zone is thought to be a highly immunologically active region important in HIV infection.

The connective tissue of the cervical stroma may be divided into two zones. One zone has a superficial, subepithelial location and is rich in interstitial fluid. The second is a deep, dense collagen layer. The superficial endocervical stroma also contains many capillaries, particularly abundant beneath the endocervical epithelial lining. These blood vessels do not have direct

contact with the overlying squamous or columnar epithelial membranes. Therefore, metabolic exchanges including oxygen supply of the cervical epithelium must occur via diffusion [40].

The thickness of the vaginal epithelium varies as a result of changes in estrogen levels during the menstrual cycle. This estrogenization has important consequences for drug permeation through the tissue and may influence targeting and the pharmacokinetics (PK) of vaginally delivered drugs [42, 46].

In addition to the epithelium and stroma (sub-mucosa), two more layers, the muscularis and tunica adventitia constitute the walls of the vagina. The muscularis layer is made up of smooth muscle, collagen, and elastic fibers that allow for substantial stretching without tearing during child birth [42]. The tunica adventitia is a thin layer of dense connective tissue that helps to anchor the vagina to the lateral and anterior walls of the pelvis. Blood vessels, lymphatic vessels, and nerves are located in the tunica adventitia.

1.4.1 Cervicovaginal Secretions

The fluid that collects within the vagina originates from endometrial or salpingeal mucous membranes and from serum transudate. The transudate originates from blood vessels surrounding this area, transverses the vaginal wall, and mixes with vulval secretions from sebaceous and sweat glands and from the Skene's glands. Endometrial and oviductal fluids may also contribute to the vaginal fluid. Incorporated into this fluid are cervical mucus (a major component), sloughed vaginal epithelial cells, and microorganisms comprising the innate microflora. This mixture of cervicovaginal secretions can also contain various components such as amino acids, proteins, carbohydrates, enzymes, enzyme inhibitors, ions, and lipids [40].

Cervical mucus consists of water and a matrix of mucins, which are high molecular weight glycoproteins. Sources of mucus secretions include the goblet cells within the columnar epithelium of the endocervix and the Bartholin's glands. The squamous epithelium of the vagina and ectocervix is not secretory and does not produce mucus. The physical characteristics, composition, and volume of mucus secretions of the endocervical epithelium are dependent on the menstrual cycle, which deems this secretion production as estrogen dependent. At the time of ovulation, the amount of cervical secretions increase, resulting in an increase in the overall volume of fluid; however, the approximate volume of fluid in the vagina for an adult premenopausal woman is 0.5mL [47]. An increase in pH and a decrease in viscosity are also evident. Cervical mucus has a complex net-like structure resembling interlacing microfibers. Orientation and pore size are influenced by circulating hormones. Under estrogen stimulation (during ovulation), mucus pore size is larger than when under progesterone stimulation. Additionally, during ovulation mucus fibers run parallel to each other and are long and thick, making the mucus less viscoelastic. Such organization is suitable sperm entry. Conversely, the nonovulatory mucus structure becomes dense and compact, which is unfavorable for sperm entry. Mucus-penetrating nanoparticles (MPP) have been utilized to assess the pore size of human cervicovaginal mucus. This assessment found the average pore size to be 340 ± 70 nm [48]. These dynamic changes in fluid volume and physical makeup can modify the drug release profiles from intravaginal formulations and alter drug targeting *in vivo*. Changes in mucus network pore size also have the capacity to affect drug targeting. For example, acidic environments cause mucin aggregation and decrease pore size, which is turn causes increasing viscosity and a physical barrier to drug permeation through the secretions.

Mucus can maintain an unstirred layer adjacent to epithelial surfaces despite the shearing action that occurs during vaginal intercourse. The depth of the unstirred layer is determined by the balance between the rate of secretion and the rate of shedding/degradation. Drugs delivered vaginally which have targets within the mucosa must move through and penetrate the unstirred layer before it is shed or degraded [49].

Normal vaginal fluid of an adult premenopausal woman has a pH between 4 and 4.5. This pH is maintained by the commensal *Lactobacillus* sp. which produces lactic acid from glucose in the vaginal fluid as well as glycogen in the sloughed epithelial cells of the mucosa. In addition to the acidic environment, the production of hydrogen peroxide by some *Lactobacillus* sp. offers protection from overgrowth of various pathogens, including those causing bacterial vaginosis (BV) [50]. Further, a high pH can increase risk or be indicative of an infection. The pH of vaginal fluid in adult women rises during menstruation and after frequent acts of vaginal intercourse, since both vaginal transudate and ejaculate have higher pH. These alterations in pH can manipulate the release profile of pH-sensitive drugs from vaginal delivery formulations [42].

The vaginal microflora found within cervicovaginal secretions is dynamic and may consist of both gram positive and gram negative bacteria species from both cocci and bacilli classes [51-52]. Anaerobic microorganisms are usually present in small quantities. The menstrual cycle, bacterial species present, and vaginal infections caused by various pathogenic microorganisms have the ability to alter the constituent present and the enzymatic composition of the vagina and vaginal fluid. The enzymatic activity within the vaginal lumen/fluid and within the epithelium can affect drug transport and drug stability [42].

Further, the act of coitus affects the volume and composition of vaginal fluids due to tension-induced changes and the introduction of semen. These alterations have the ability to affect the drug release profile within the vaginal compartment [42].

Despite the multitude of challenges to drug delivery presented by the anatomy and physiology of the vagina, the vaginal route of drug delivery is used for the administration of both locally-and systemically-acting agents.

1.5   VAGINAL DRUG DELIVERY

The vaginal route of drug delivery has been recognized since ancient Egyptian times, with the use of various substances as vaginal contraceptives. Agents commonly delivered vaginally include antimicrobials, spermicides, and agents used for contraception, hormone replacement, and cervical ripening/labor induction. Commonly utilized dosage formulations for these indications include solid dosage forms such as suppositories and tablets and semi-solid forms such as creams and gels. Intravaginal rings (IVRs), vaginal films, and foams are also utilized. Vaginal delivery platform selection may depend upon multiple variables, spanning from drug properties to clinical requirements to user acceptability [42].

Several advantages of the vaginal drug delivery route have been defined [41-42, 46, 53]. The vagina can provide an accessible route of delivery, due to ease of self-insertion. Vaginal delivery represents a non-invasive route of delivery, by avoiding pain, tissue damage, and possible infections associated with the parenteral route. Drugs delivered vaginally are able to avoid hepatic first-pass metabolism. The vagina has a well-developed blood supply for drugs needing systemic delivery. Further, the vagina provides great permeability for drugs with certain physiochemical characteristics. Drug absorption depends on the physiochemical properties of the

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agent in question, specifically molecular weight, dissolution characteristics, and ionization properties. Vaginal drug absorption may occur by diffusion and/or active transport. This delivery route also allows for avoidance of the incidence and severity of various side effects, such as gastrointestinal and hepatic effects, associated with oral and parental drug delivery.

The lower genital tract is the initial site of HIV infection during male-to-female sexual transmission. The advantages of vaginal delivery also hold true for HIV PrEP agents. Furthermore, by locally delivering anti-HIV products to this area, systemic exposure can be limited as compared to the oral or parental routes of drug delivery. Limiting systemic exposure by local vaginal delivery would limit the amount and severity of side effects associated with systemic exposure to the anti-HIV products. Besides providing an adequate route of delivery for the HIV preventative product, a vaginal dosage formulation may provide additional benefits to the female user. With regard to vaginal gels, once inserted the gel could provide a physical barrier to HIV epithelial penetration. Additionally, a gel may provide lubrication which may decrease the likelihood of genital abrasions induced by the act of vaginal intercourse and may increase sexual pleasure experienced by both female and male partner. Upon disintegration, the same may be said for both vaginal tablets and quick-dissolving polymeric films; however, this characteristic do not hold true for oral preparations.

Although threshold tissue or cervicovaginal fluid drug concentrations for the prevention of HIV acquisition has yet to be determined, a vaginal microbicide formulation may elicit a PK benefit by creating a higher drug concentration within the female genital tract, in comparison to an oral formulation. In fact, after vaginal tenofovir 1% gel use, tenofovir diphosphate concentrations are approximately 1000-fold higher in vaginal tissue samples than after oral Truvada use [54]. Additionally, the concentration of tenofovir in undiluted cervicovaginal fluid

after vaginal tenofovir 1% gel use was examined. The women with tenofovir concentrations greater than 1000 ng/mL had a significantly lower HIV incidence rate, in comparison to placebo gel users. This concentration greatly exceeds the undiluted cervicovaginal fluid concentration seen after oral Truvada use [54]. Similar trends were found in cervicovaginal tissue samples in a separate cross-over clinical study comparing the vaginal tissue PK of tenofovir vaginal gel and oral tablets [55]. Therefore, vaginal delivery of PrEP may prove advantageous given that the female lower genital tract is the initial site of male-to-female HIV acquisition.

1.6   VAGINAL PREP FOR HIV PREVENTION IN FEMALES

1.6.1   Microbicide Dosage Formulation Considerations

Topical PrEP or microbicides are products that can be applied vaginally or rectally to protect the user from HIV infection and possibly STIs. Note: Rectal microbicides will not be discussed. The effectiveness of any given microbicide product is dependent on both the anti-HIV activity (efficacy) of the product and the user's willingness and ability to use the product as instructed (acceptability and adherence). Further, Morrow et al. showed that the success of microbicide products depends on both drug-related variables and HIV "dose"-related variables [56]. The importance of product formulation in the development of a successful microbicide is evident. The appropriate drug delivery strategy for each microbicide drug candidate will depend upon aspects such as the physiochemical characteristics of the candidate and its mechanism of action against HIV transmission [57]. For example, if the drug binds to the receptor or co-receptor of the target host cells to prevent HIV-1 infection, then the method of delivery must be able to get

the drug to the site of action within the tissue. Considering these concepts early within the microbicide product development process would prove beneficial.

Several dosage formulation strategies are under development and investigation in the microbicide field [58-60]. Gels, or semisolid dosage forms, were the initial vaginal drug delivery formulation for microbicide products [37-42, 53-69, 73]. Some dosing strategies for microbicide gels are application as a single daily dose (coitally independent dosing) or a single dose before and/or after vaginal intercourse (coitally dependent dosing). Advantages of vaginal gels include familiarity of the gel dosage form for this route of delivery, resulting lubrication effect (enhanced sexual pleasure), and their relative ease of manufacture. Despite vaginal gel utilization within trials, reports of leakage or messiness are extremely common [61-65]. Further, gel and/or gel applicator acceptability within PrEP trials may prove to be factor(s) in the disconnect between the subjective and objective measures of gel use [24, 66].

Unlike other dosage formulation, IVRs offer a unique delivery method. IVRs offer a sustained or controlled release (coitally-independent release) of microbicide within the female genital tract for at least one month. IVR types under investigation include matrix, reservoir, and coated pod systems [60]. IVRs are to be placed within the upper third of the vagina, and the drug release rate depends on excipients, IVR design, and composition.

The stresses of one's social environment, environmental conditions, and personal choice factor into the acceptability and adherence of a microbicide product. Based not only on the physiochemical properties of a given microbicide but also on the varied characteristics of potential consumers, multiple options for microbicide dosage forms are necessary. Vaginal quick-dissolving polymeric thin films and tablets may aid in fulfilling these diverse needs by providing another platform for vaginal drug delivery. These types of delivery formulations

quickly disintegrate and dissolve upon contact with fluids. The acceptability of the Vaginal Contraceptive Film (VCF) containing nonoxynol-9 has been investigated. The VCF was found to be the most favorable vaginal product when compared to other vaginal products including tablet, suppository, and gels [67-69]. The women favoring the VCF reported that it was easy to use, comfortable, discreet, and less messy (than gels) [67, 69]. Other women liked the tight or fit feeling that the film provided within the vagina [68]. Conversely, other studies have shown the vaginal film to be the least favored among various vaginal products [70-71]. Concerns of vaginal film use that surfaced during these studies included fears that the film would not dissolve and would accumulate in the body [70]. Additionally, some women disliked the plastic appearance of the film and did not trust its efficacy [71]. Vaginal microbicide films containing tenofovir and dapivirine (separately, as single entity films) have under gone early phase clinical testing. Additionally, research efforts are underway to develop coitally independent vaginal films for HIV prevention.

There is not a single vaginal microbicide dosage formulation that will fit the product preferences of every potential female user [56]. However, acceptability assessments that focus on product-related factors may lack important evaluations, for example, that pertain to HIV risk perception which may factor into the acceptability/adherence of a vaginal microbicide product. Ideally, product-related, user-related, and contextual factors should be integrated into acceptability/adherence assessments of microbicide products. Further, these types of assessments might best be utilized within the preclinical and early clinical settings before moving microbicide products forward into advanced clinical safety and efficacy trials [56].

The development of a vaginally applied microbicide has been the major focus of microbicide research over the past two decades, yet women around the world, in both developed

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and developing countries, practice receptive anal intercourse (RAI) [72-73]. In fact, a US national survey found that 36% of female responders aged 25-44 have engaged in anal sex with an opposite-sex partner [72]. Although the absolute frequency of RAI may be low, the increased risk per sexual act is such that unprotected RAI may play an important role in spreading HIV infection in women. Personal lubricants are used in multiple fashions, including both vaginal and rectal application to decrease friction and to enhance sexual experience for both participants. Therefore, there is no guarantee that a woman, who engages in both vaginal and anal sexual intercourse, will solely use a vaginal microbicide product for the indicated route, even with proper and clear written instructions/labeling and verbal counseling. Development of a microbicide that is effective and safe in both mucosal compartments should be considered [74].

1.6.2   Vaginal Microbicides – Past, Present, and Future

First Generation Microbicides

The products first developed as vaginal microbicides contained agents that do not specifically affect HIV and may be effective against other pathogens. These "first generation" or non-specific microbicides include surfactants/detergents, acidifying agents, and anionic polyanions that would be delivered vaginally and have bioactivity within the vaginal lumen. Surfactants disrupt membranes of cells, viruses, and bacteria. Three surfactants tested clinically were products containing nonoxynol-9 (N-9) (gel, sponge, film), SAVVY or C31G gel, and sodium lauryl sulfate (SLS) gel. Results of phase 2/3 trials concluded that N-9 formulations were not effective at reducing the rate of male-to-female vaginal HIV transmission [75-77]. In fact, the gel product was found to increase HIV infection risk in women with frequent use (use of more than 3.5

vaginal applications per day) and in those who had high incidences of vaginal lesions [77]. Another surfactant that was assessed clinically is SAVVY or C31G (gel) [78-79]. These phase 3 studies were terminated early due to futility [78]. The third surfactant studied was SLS. SLS is the chemical component in one gel formulation of the Invisible Condom® gel product. Both phase 1 safety and acceptability and phase 2 extended safety trials have found Invisible Condom® formulations to be safe, well-tolerated, and acceptable [80-82]. To date, no clinical efficacy trials have been published pertaining to this product.

Acidifying agents help to restore or maintain the protective acidic pH of the vagina. Acidifying agents clinically assessed for safety and effectiveness against male-to-female HIV acquisition include BufferGel and Acidform. Both BufferGel and Acidform are acid-buffering bioadhesive vaginal gels. Acidform and BufferGel are formulated to pH values of approximately 3.55 and 3.9, respectively. BufferGel was found to be well tolerated, but not effective for the prevention of HIV vaginal transmission in a phase 2/2b trial [83]. Acidform vaginal gel was found to have favorable formulation properties [84]. However, no clinical effectiveness trials pertaining to the prevention of male-to-female vaginal transmission of HIV-1 have been reported for Acidform.

The last class of non-specific microbicide candidates includes the anionic polyanions Carraguard gel, cellulose sulfate gel, cellulose acetate phthalate (CAP) gel, VivaGel, and PRO2000 gel. These agents carry a negative charge which results in a charge interaction with viral envelope proteins interfering with attachment of virus to CD4 positive cells. A phase 3 study did not find Carraguard to be effective against the vaginal transmission of HIV; however, the gel was found to be safe [66]. Cellulose sulfate gel effectiveness was tested in two phase 3 trials [85-86]. Both studies were stopped early due to increased HIV infection at an interim

analysis [86]. CAP gel safety and acceptability was assessed in a phase 1 trial [65]. This trial was terminated early, only enrolling five women, due to complaints of wetness and leakage after vaginal application. Upon conducting further osmolarity and rheologic assessments, it was found that the gel was hyperosmolar in comparison to vaginal fluid and became less viscous upon vaginal application which explains the unacceptable events experienced by the participants [65]. Several phase 1 trials have been performed to assess the vaginal safety of VivaGel [87-89]. Two studies found that genitourinary adverse events were more common among women using VivaGel [88-89]. However, another study found VivaGel to be safe and well tolerated, with no systemic absorption [87].

PRO2000 gel was assessed in two large-scale effectiveness trials [83, 90]. The phase 2b trial found that 0.5% PRO2000 gel was safe and reduced the risk of HIV infection by 30%. However, this study result was not statistically significant [83]. To provide further insight into the effectiveness of PRO2000 gel for the prevention of male-to-female HIV-1 acquisition, a phase 3 trial was conducted to evaluate the efficacy of 0.5 and 2% PRO2000 gel [90]. The 2% PRO2000 gel was terminated early due to futility. At study end, the 0.5% PRO2000 gel was found to not reduce the risk of male-to-female HIV transmission [90]. Table 4 below highlights the major clinical trials for first generation vaginal microbicides.

Table 4. First Generation Vaginal Microbicides

Name	Dosage Form(s)	Dosing Regimen	Clinical Trial Results
Surfactants/Detergents: disrupt membranes of cells, viruses, and bacteria.			
Nonoxynol-9 (N-9) [75-77]	Vaginal Sponge, Vaginal Film, Vaginal Gel	Product inserted into vagina prior to each penile-vaginal sexual act.	N-9 formulations do not reduce the rate of male-to-female vaginal HIV transmission (Phase II/III). N-9 sponge: over three-fold increased risk of vulvitis and genital ulcers. N-9 gel: increased HIV risk in women with frequent use (> 3.5 applications/day) or vaginal epithelial disruptions/lesions.
SAVVY® or C31G [78-79]	Vaginal Gel	Product inserted into vagina prior to each penile-vaginal sexual act.	Phase III studies discontinued early due to futility; insufficient number of HIV infections to obtain desired study power. SAVVY gel use was associated with increased reporting of reproductive tract adverse events (Ghana study).
Sodium Lauryl Sulfate (SLS) [80-82]	Vaginal Gel	Product inserted into vaginal once, twice, or thrice daily.	Safety, tolerability, and acceptability of gel confirmed (Phase I/II). No clinical effectiveness trials have been conducted.
Acidifying Agents: help to maintain or restore the protective acidic pH of the human vagina.			
BufferGel [83]	Vaginal Gel	Product inserted into vagina within one hour prior to each penile-vaginal sexual act.	Gel is well-tolerated but not effective for the prevention of male-to-female vaginal HIV transmission (Phase II/IIb).
Acidform [84]	Vaginal Gel	Product insert into vagina daily before bedtime (Phase I).	Gel was found to have favorable properties (Pre-clinical and Phase I). No clinical effectiveness trials have been conducted.

Table 4. First Generation Vaginal Microbicides (continued)

Name	Dosage Form(s)	Dosing Regimen	Clinical Trial Results
Anionic Polyanions: agents that carry a negative charge. This negative charge interacts with viral envelope proteins and prohibits the attachment of virus to CD4+ cells.			
Carraguard® [66]	Vaginal Gel	Product inserted into vagina one hour prior to each penile-vaginal sexual act.	Gel was found to be safe but not effective (Phase III).
Cellulose Sulfate [85-86]	Vaginal Gel	Product inserted into vagina one hour prior to each penile-vaginal sexual act.	Two Phase III studies were stopped early due to increased HIV infection at interim analyses.
Cellulose Acetate Phthalate [65]	Vaginal Gel	Product inserted into vagina once daily.	Phase I trial discontinued – gel was found to be hyperosmolar to vaginal fluid and become hypoviscous upon vaginal application; major complaints of vaginal wetness/leakage.
PRO2000 [83, 90]	Vaginal Gel	Product inserted into vagina within one hour prior to each penile-vaginal sexual act.	PRO2000 (0.05%) gel was safe and reduced the risk of HIV infection by 30%. This effectiveness result was not statistically significant (Phase IIb). PRO2000 (0.05 and 2%) gel was safe but does not offer protection against HIV acquisition. PRO2000 2% gel use was discontinued early because of low probability of benefit (Phase III).
VivaGel® (SPL7013) [87-89]	Vaginal Gel	Product inserted into vagina once or twice daily.	VivaGel was generally well tolerated, but low-grade related genitourinary adverse events were more common among women using VivaGel (Phase 1 studies; twice daily dosing). Another study found VivaGel to be safe and well tolerated, with no systemic absorption (Phase 1 study; once daily dosing).

Second Generation Microbicides

Due to the lack of efficacy in clinical trials for non-specific microbicide candidates, focus in the field has now shifted to vaginal microbicide candidates that directly and specifically act against HIV (Table 5). Two second generation vaginal microbicide candidates most advanced in the development pipeline are tenofovir vaginal gel and dapivirine intravaginal ring. Both APIs are reverse transcriptase inhibitors (RTIs). Upon HIV entering a cell, the viral enzyme, reverse transcriptase, converts the viral RNA into DNA, which can then be integrated into the host cell's DNA. A reverse transcriptase inhibitor prevents this step in the HIV life cycle from occurring.

Tenofovir is the most clinically studied microbicide candidate that is a reverse transcriptase inhibitor. Specifically, tenofovir is a nucleotide reverse transcriptase inhibitor (NRTI). A phase 2b trial assessing the safety and effectiveness of tenofovir 1% gel in the prevention of male-to-female HIV transmission was performed in South Africa [91]. This trial is known as CAPRISA 004. Women were instructed to insert gel within 12 hours before and again within 12 hours after vaginal intercourse (pericoital dosing; coitally dependent). The results of this trial showed that tenofovir gel use was associated with an overall 39% decrease in HIV-1 acquisition. Additionally, among women with high gel adherence, the tenofovir gel reduced HIV infection by 54% when compared to placebo gel [91]. Other findings from this study include: (1) No significant renal, hepatic, hematologic, or genital adverse event concerns were associated with gel use; and (2) 97.4% of participants found gel use to be acceptable, with 97.9% stating that they would use the gel in the future if it did prevent HIV [91]. For those women in the tenofovir gel group that did seroconvert, no tenofovir resistance manifested. The results of CAPRISA 004 have paved the way for future vaginal microbicide trials by providing proof of concept for antiretrovirals as microbicides for HIV prevention. Pericoital administration of

tenofovir 1% vaginal gel was studied in the Follow-on African Consortium for Tenofovir Studies (FACTS) 001 Trial in South Africa. Unfortunately, tenofovir gel was not found to be effective in this trial due to low adherence to gel.

The Vaginal and Oral Interventions to Control the Epidemic (VOICE) trial is a Phase 2b five-group study that examined the safety and efficacy of daily oral tenofovir disoproxil fumarate, oral Truvada, oral placebo, tenofovir 1% vaginal gel, and placebo vaginal gel in HIV-negative women in Malawi, South Africa, Uganda, and Zimbabwe. This daily use regimen of a vaginal microbicide (topical PrEP) and oral PrEP differs from the coitally dependent frequency utilized in CAPRISA 004 and FACTS 001. The daily oral TDF, daily vaginal TFV, and daily vaginal placebo arms were stopped early due to futility. Only the daily oral TDF/FTC and oral placebo continued to planned end. After study conclusion, none of the products were found to reduce HIV infection due to low adherence to product [24].

Tenofovir is not the only ARV that is being clinically evaluated. Many other second generation antiretroviral agents are also under development as potential microbicide candidates. A dapivirine-containing intravaginal ring is under investigation as a potential vaginal microbicide product in a large scale phase 3 study. This study is termed ASPIRE – A Study to Prevent Infection with a Ring for Extended use (MTN-020). This is the first trial in Africa assessing an IVR that contains an antiretroviral agent. This study is closed to accrual, and results are expected in 2015. Additionally, a phase 3B open-label follow-on extension study of the ASIPRE study is under development (MTN-025; HIV Open-label Prevention Extension, HOPE). Further, early phase clinical trials involving the delivery of specific antiretroviral agents (tenofovir and dapivirine) in vaginal polymeric films are currently ongoing.

Multiple additional antiretrovirals are in preclinical testing as topical PrEP for HIV. These include agents that target cell membrane receptors, including agents that target the CD4 receptor, CXCR4 and CCR5 co-receptors, and C-type lectin receptor. Additionally, agents that bind to HIV envelope glycoproteins are under preclinical investigation. These include gp120 and gp41 binding agents. Various NRTIs and NNRTIs besides tenofovir and dapivirine are under investigation as potential microbicides. Viral integrase and protease inhibitors are also under preclinical evaluation as HIV prevention agents. Agents span small molecules to peptides to proteins including monoclonal antibodies [57-58, 92]. Refer to Tables 2 and 3.

Antiretroviral treatment for HIV-1 infected individuals (antiretroviral-naïve patient) includes the use of combination therapy, namely one NNRTI + two NRTIs or ritonavir-boosted/unboosted protease inhibitor (PI) + two NRTIs [93]. Combining more than one antiretroviral agent with different mechanisms of action within a single vaginal microbicide formulation may be a viable option for HIV prevention. Therefore, combining APIs in a single formulation may increase activity across viral subtypes, reduce the development of HIV resistance, and prevent other STIs [94]. Microbicide drug candidate combinations are currently under development and evaluation [95]. Also refer to Table 2.

Future vaginal microbicide preclinical and clinical studies will involve more exploration into the pharmacokinetics and pharmacodynamics of antiretroviral agents and vaginal formulations during vaginal delivery. These studies will assist in elucidating a dosing strategy pertaining to a specific API and/or vaginal formulation. For example, more knowledge will be gained in order to specify the amount of drug within a given formulation and frequency of use for a female human user. Additionally, future studies will also involved the investigation of sustained and controlled release vaginal microbicide formulations and various drug targeting

strategies. For example, a specific targeting strategy for a vaginally delivered antiretroviral may involve transport to specific tissues or cells composing or surrounding the vaginal compartment or neighboring area, such as lymph nodes.

Table 5. Second Generation Microbicides

Microbicide Candidate[#]	Mechanism(s) of Action	Dosage Form(s) and Route(s) of Delivery	Farthest Phase of testing completed/ongoing/in development
Tenofovir Disoproxil Fumarate (TDF)	NtRTI	Tablet – Oral Ring – Vaginal	Oral Tablet – Phase 3 Vaginal Ring – Phase 1
Tenofovir (TFV)	NtRTI	Gel – Vaginal, Rectal Ring – Vaginal Film – Vaginal Tablet – Vaginal	Vagina Gel – Phase 3 Reduced Glycerin Gel, Vaginally – Phase 1 Reduced Glycerin Gel, Rectally – Phase 2 Vaginal Tablet, Film, and Ring – Phase 1
Tenofovir Disoproxil Fumarate (TDF) /Emtricitabine (FTC)	TDF – NtRTI FTC – NTRI	Tablet – Oral	Oral Tablet – Phase 4
Dapirivine	NNRTI	Gel – Vaginal, Rectal Ring – Vaginal Film – Vaginal	Vaginal Gel – Phase 2 Rectal Gel – Phase 1 (in development) Vaginal Ring – Phase 3b (in development) Vaginal Film – Phase 1
Dapirivine/Maraviroc	Dapivirine – NNRTI Maraviroc – CCR5 inhibitor	Gel – Vaginal Ring – Vaginal Film - Vaginal	Vaginal Gel and Film – Preclinical Vaginal Ring – Phase 1
MK-2048/Vicriviroc (VCV, MK-4176)	MK-2048 – integrase inhibitor VCV – CCR5 inhibitor	Ring – Vaginal	Vaginal Ring – Phase 1 (in development)
[#]These second generation candidate microbicide products have been/are/will be evaluated through the MTN.			

1.7   IDEAL VAGINAL MICROBICIDE

During the development of a vaginal microbicide, it is important to take into consideration characteristics which the drug (API) should possess in order to be a leading candidate. If the microbicide candidate acts against viral surface glycoproteins, then the API should be active within the vaginal lumen, and cervicovaginal tissue permeability is not required. However, if the API elicits anti-HIV activity that is host cell dependent, then tissue permeability of the API is required. The API should possess chemical and physical stability such that it can be successfully formulated into a vaginal dosage form. The microbicide drug candidate must be safe toward the cervicovaginal environment. Conversely, the cervicovaginal environment and semen should not affect the stability or anti-HIV activity of the compound. Table 6 represents a compilation of ideal microbicide agent characteristics. These target parameters should be taken into consideration when designing and evaluating new agents.

Table 6. Ideal Drug Candidate (API) Characteristics for a Vaginal Microbicide

1. If API acts against viral gp120 or gp41, then the API should be active within the vaginal lumen. Tissue permeability is not necessary for activity.
2. If the API acts against CCR5 receptor, reverse transcriptase, integrase, protease and/or another host cell dependent mechanism, then the API should be able to penetrate the cervicovaginal tissue to reach these targets. Cervicovaginal tissue permeability is necessary for activity.
3. The API should have potential to be formulated into a vaginal dosage form. API should not experience chemical or physical instabilities within formulation.
4. The API should be safe toward the cervicovaginal environment. This consists of safety to the cells that compose the cervicovaginal tissues and compatibility with the components of the cervicovaginal secretions, namely the commensal bacteria.
5. The environment(s)/event(s) involved in male-to-female sexual transmission of HIV should not negatively impact the API. This includes confirmation that API and cervicovaginal secretion and semen are compatible and do not cause API chemical instability, physical instability, or decreased anti-HIV efficacy.
6. If API is able to permeate through the cervicovaginal tissue and is able to reach the systemic circulation, there should be limited systemic exposure. Limiting systemic exposure is important to protect the user from off-target API side effects and drug resistance.
7. No HIV resistance profile
8. Lack drug-drug interactions, specifically for other drugs that are delivered vaginally or related to contraception.

9. Since women around the world partake in RAI, and since unprotected RAI has an increase HIV acquisition risk (as compared to unprotected vaginal intercourse), the microbicide API should either: 1) When applied vaginally, offer protection from potential HIV infection via RAI, or 2) Have the ability to be a dual compartment product – a product that can be applied and be safe and effective both vaginally and rectally.
    • The API should be safe toward the rectal environment. This consists of safety to the cells that compose the rectal tissues and compatibility with the components of the rectal fluid.
    • The rectal environment should not negatively impact the API. This includes confirmation that API and rectal fluid are compatible and do not cause API chemical instability, physical instability, or decreased anti-HIV efficacy.

10. If API is a biopharmaceutical agent, it should lack immunogenicity.

In addition to the attributes listed in Table 6, there are other important considerations for an ideal vaginal microbicide formulated product. For example, all excipients used within the formulation should be safe toward the cervicovaginal environment. Safety consists of no harm to the cells that compose the cervicovaginal tissues and no harm to the components of the cervicovaginal secretions that would negatively impact commensal bacteria or pH. There is no one ideal dosage formulation for a vaginal microbicide, and there never will be because each consumer's definition of 'ideal' differs. Women at high risk of sexual acquisition of HIV need dosage form choices. Not a single female in the world has the same personal or social

environment, which affect her product preferences. Additionally, the use of a vaginal microbicide product should not negatively impact the user's quality of life.

1.8 GRIFFITHSIN – A MUCOSAL PREP CANDIDATE

One potential compound under investigation as a vaginal microbicide is Griffithsin, also known as GRFT. Interestingly, GRFT has characteristics of both first and second generation microbicides. GRFT is a lectin and, unlike first generation microbicides, binds specifically to a component of the HIV envelope, gp120, to prevent HIV infection [96]. Therefore, this compound would be active within the vaginal lumen, which is the location of the anti-HIV activity of first generation microbicides. Additionally, GRFT also has the ability to inhibit HIV binding to the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-Integrin (DC-SIGN) receptor and transfer to CD4 cells [97]. Further, GRFT is a broad spectrum antiviral, for its bioactivity is not limited to HIV. In addition to binding to the terminal mannose residues of N-linked glycans on the surface of HIV-1 [96, 98-99] and HIV-2 [99], GRFT is also active against hepatitis C virus (HCV) [100-101], and severe acute respiratory syndrome coronavirus (SARS-CoV) [102].

GRFT exists as a proteinaceous homodimer, with each monomer containing 121 amino acids and a molecular mass of approximately 12,732 Da [96]. Refer to Figure 5 for the amino acids sequence of the alga-derived GRFT monomer [96, 103]. GRFT consists of 10 basic; 10 acidic; 37 nonpolar, hydrophobic; and 64 polar, uncharged amino acid residues. See Figure 6 for further structural details [104].

```
1                          18
SLTHRKFGGSGGSPFSGL

19                                                    56
SSIAVRSGSYLDXIIIDGVHHGGSGGNLSPTFTFGSGE

57                                                        100
YISNMTIRSGDYIDNISFETNMGRRFGPYGGSGGSANTLSNVKV

101                        121
IQINGSAGDYLDSLDIYYEQY
```

Figure 5. Griffithsin Amino Acid Sequence.

The protein monomer of the alga-derived GRFT contains 121 amino acids – 10 basic; 10 acidic; 37 nonpolar, hydrophobic; and 64 polar, uncharged amino acid residues. The GRFT monomer has a molecular mass of 12,732 Da.
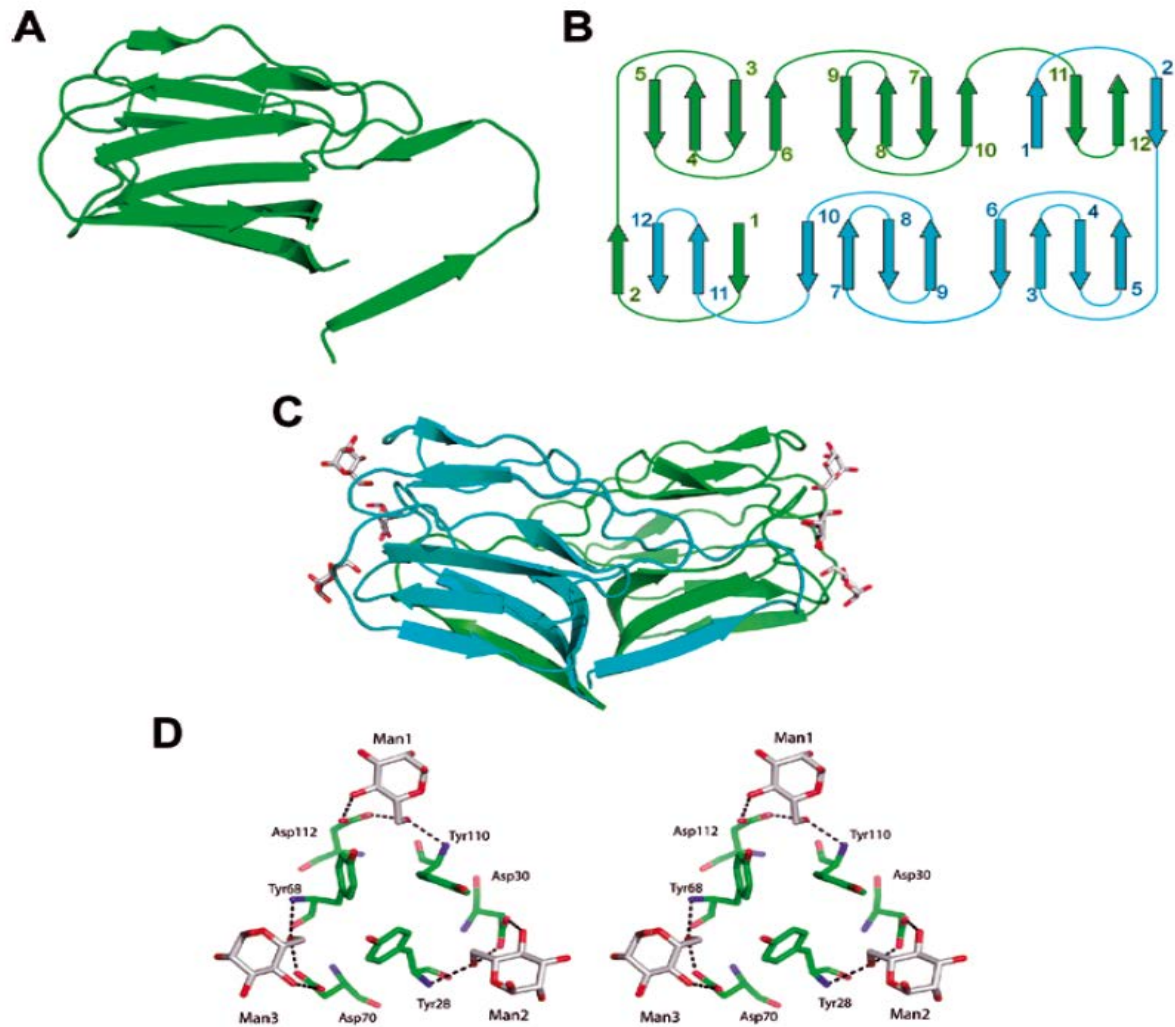
Figure 6. Structure of GRFT.
(A) Monomer of GRFT. (B) Topology of the domain swapped dimer in which the first two β-strands of one chain are associated with ten strands of the other chain (and vice versa). (C) A complex of GRFT with mannose. The six carbohydrate binding sites are very similar and are arranged in two groups of three on each monomer. (D) Mannose-binding sites. Hydrogen bonds between protein and carbohydrates are marked by dashed lines.

This lectin, carbohydrate-binding protein, was originally isolated from marine red alga *Griffithsia* sp. [96], and is now mass produced via expression in *Nicotiana benthamiana* leaf tissues using a recombinant tobacco mosaic virus (TMV) vector system [98]. Both the native and recombinant forms of this lectin potently inhibit CXCR4- and CCR5-tropic HIV infection and transmission *in vitro*, requiring concentrations only in the mid- to-high picomolar range [105], and this potency can be explained by GRFT's ability to have tight multivalent and multisite

interactions with high-mannose N-linked glycoproteins [106-107]. Additionally, GRFT has been found to be safe to human cervical cells and tissue explants and in the rabbit vaginal irritation model [98, 108]. Given GRFT's favorable preclinical antiviral bioactivity and safety profile thus far, further development of GRFT as a microbicide candidate is warranted. However, special consideration for the vaginal delivery of this proteinaceous microbicide candidate is necessary. Within the scope of this dissertation, the focus will be on the GRFT preformulation as it relates to pharmaceutical development and vaginal microbicide delivery.

1.9   HYPOTHESES AND SPECIFIC AIMS

Preformulation evaluations are essential for pharmaceutical research and development (R&D). Within these assessments, the prospective API is exposed to a variety of environments in order to determine its susceptibility to instabilities/degradation or potential obstacles for drug targeting. Outcomes from these experiments are paramount in preparing appropriate drug formulation by providing knowledge of processing, storage, and usage conditions to avoid or against which to protect the active pharmaceutical ingredient. Testing conditions such as pH, acidic and alkaline hydrolysis, photolysis, oxidative effects, and temperature will highlight the potential chemical instabilities of the API. Proteinaceous compounds, like GRFT, are special cases in that they can show physical instability issues due to conformational changes, like denaturation or aggregation, due to agitation or repeated freezing and thawing. Further, drugs intended to be delivered via the vaginal route also pose additional obstacles to successful delivery and targeting due to the pH of this environment and the presence of bacteria, such as Lactobacillus sp., enzymes such as lysozyme and aminopeptidase [109-110], other polypeptides, salts, organic acids, peroxides, and oligosaccharides [111-112].

GRFT has two mechanisms of action against HIV infection. Its main activity is due to its binding to viral gp120 and thereby prevention of HIV attachment to host cells. This process occurs within the vaginal lumen. This protein also inhibits HIV binding to the DC-SIGN receptor and transfer to CD4 cells, which would require GRFT to permeate cervicovaginal epithelial tissue. In terms of vaginal dosage formulation and vaginal drug delivery, this protein may undergo physical and chemical instabilities prior to and during administration that may impact drug activity. Additionally, during vaginal administration and due to its lectin nature, GRFT can undergo non-specific binding to components of the cervicovaginal mucins, namely mucins. These binding interactions will impact its bioactivity. Further, given GRFT's proteinaceous nature, it may not permeate the cervicovaginal epithelium in order to elicit its secondary mechanism of action against HIV infection.

Hypotheses

We hypothesize that GRFT will undergo physical and chemical instabilities that will affect successful formulation and vaginal delivery of this microbicide candidate. We further hypothesize that GRFT's non-specific binding within cervicovaginal secretions will decrease gp120 binding and that its inability to permeate into cervical tissue will prevent co-localization with dendritic cells. These hypotheses will address ideal vaginal microbicide drug candidate characteristics and will be studied within the following three specific aims.

Specific aim 1. Identify degradation pathways for GRFT by performing preformulation studies under selected conditions (Chapters 2 and 3).

Specific aim 2. Evaluate chemical modifications and GRFT-mucin binding in the presence of cervicovaginal secretions from human females (Chapters 3 and 4).

Specific aim 3. Assess GRFT cervical tissue permeability and localization with excised human cervical tissue and two methods of GRFT visualization (Chapter 5).

2.0   PREFORMULATION EVALUATIONS OF GRIFFITHSIN

2.1   INTRODUCTION

Griffithsin (GRFT) binds to viral gp120 [96] and can inhibit HIV binding to the DC-SIGN receptor and transfer to CD4 cells [97]. Griffithsin is a proteinaceous homodimer. Each monomer has a molecular mass of approximately 12,732 Da and contains 121 amino acids [96]. Therefore, the homodimer has a molecular mass of approximately 25,464 Da and contains 242 amino acids. See Figures 5 and 6 in Chapter 1 for structural details pertaining to GRFT.

When undertaking research and development for the intravaginal product of a biopharmaceutical microbicide candidate, one must consider performing preformulation evaluations that will guide the formulation development and optimize vaginal product delivery. These evaluations should address chemical and physical instabilities that may affect the peptide or protein of interest while in formulation and *in vivo*. Chemical and/or physical instabilities that are indentified during preformulation studies may negatively impact product stability (shelf life) and *in vivo* safety and anti-HIV activity.

Some examples of instability are as follows: 1) protein modifications due to covalent chemical changes; 2) protein modifications due to conformational changes; 3) proteolytic inactivation of the protein within the cervicovaginal environment (vaginal lumen); 4) off-target/non-specific protein binding; and/or 5) low penetration of the protein drug into the mucosal tissue (if permeability is necessary for anti-HIV activity). Items (1), (2), and (3) will be

addressed in Chapters 2 and 3. Item (4) will be addressed in Chapter 4. Item (5) will be addressed in Chapter 5.

Modifications to the primary, secondary, tertiary, and quaternary structures of GRFT can lead to protein instability. The primary structure of a protein is its amino acid sequence. GRFT's primary sequence is reflected in Figure 5 in Chapter 1. GRFT contains 10 basic; 10 acidic; 37 nonpolar, hydrophobic; and 64 polar, uncharged amino acids. A protein's secondary structure consists of local structures that are stabilized by hydrogen bonds. These can be described as α-helices, β-sheets, and turns. GRFT consists of β-sheets [104]. A protein's tertiary structure is described as the overall shape of a single protein molecule, i.e. the spatial relationship of the secondary structure(s) to one another. GRFT's tertiary structure is described as a β-prism-I motif [113], which has been observed with other proteins and lectins [114]. GRFT's tertiary structure consists of three repeats of an anti-parallel four-stranded β-sheet that form a triangular prism. Additionally, a protein may have a quaternary structure which is a structure formed by several protein molecules (polypeptide chains) usually called protein subunits in this context, which function as a single protein complex. GRFT does have a quaternary structure – GRFT forms a domain swapping homodimer, with the first two β-sheets of one polypeptide chain associating with ten β-sheets of the other polypeptide chain and vice versa [103-104].

The purpose of the studies presented in this chapter was to determine the stability of GRFT under several conditions and to describe the degradation pathways. In the studies, several preformulation evaluations were performed for GRFT to provide information needed to develop vaginal formulations of GRFT for use as a vaginal microbicide product. Chemical and physical stability were evaluated by high performance liquid chromatography (HPLC), circular dichroism (CD), UV-spectroscopy, enzyme-linked immunosorbent assay (ELISA), and sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the compatibility of GRFT with commensal vaginal bacteria was determined. Overall, these results will be important to guide further development of GRFT as a vaginal microbicide product.

2.2   MATERIALS AND METHODS

Recombinant Griffithsin drug substance was manufactured by Kentucky Bioprocessing LLC (KBP; Owensboro, KY) and provided to our laboratory by Dr. Kenneth Palmer of the University of Louisville School of Medicine. The protein was supplied as a solution in phosphate buffered saline. Phosphate buffered saline 10X molecular biology grade was purchased from Mediatech, Inc (Manassa, VA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were obtained from Fisher Scientific (Pittsburgh, PA). Hydrogen peroxide (30%), amiopeptidase, lysozyme, and proteinase K were obtained from Sigma (St. Louis, MO). A MilliQ (Millipore; Milford, MA) water filtration system operating at 18.2 MΩcm was used for water.

2.2.1   High Performance Liquid Chromatography

The High Performance Liquid Chromatography (HPLC) system (Waters Corporation; Milford, CA) was equipped with an autosampler model 717plus, a quaternary pump controller model 600, and a fluorescence detector model 2487, using an excitation wavelength of 273 nm and an emission wavelength of 303 nm. Separation of GRFT from products/degradants was achieved using a Phenomenex Jupiter C18 5µ 300Å (4.6x250 mm) column (Phenomenex; Torrance, CA) protected by a Gemini C18 (4x3 mm) guard cartridge (Phenomenex; Torrance, CA), both maintained at ambient temperature. The mobile phase gradient consisted of mobile phase A

(0.1% TFA in water (v/v)), and mobile phase B (0.05% TFA in acetonitrile (v/v)) pumped at a flow rate of 1.0 mL/min. The gradient was proportioned as follows (minute, %B): 0, 12; 15, 20; 16, 50; and 20, 12. Linearity of the detector response curve was over the range of 10 µg/mL to 500 µg/mL. Empower software was used to control the HPLC system.

2.2.2 UV-Spectroscopy

UV spectroscopy with a Cary 50 Bio UV-Visible Spectrophotometer was employed for the evaluation of ionic strength effects on GRFT stability (potential for insoluble aggregation). UV scans of GRFT in buffer solutions were performed from 220 to 800 nm. Cary WinUV software was used to control the spectrophotometer.

2.2.3 Circular Dichroism

CD was employed to detect conformational changes in the secondary structure of GRFT (far-UV). CD measurements were performed on a Jasco J-815 CD spectrophotometer equipped with a 0.1 cm path length quartz cell. Spectra were recorded between 200-300nm at 25°C. Solution background scans were subtracted from experimental scans over the same wavelength range.

2.2.4 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for select GRFT samples. An SDS-PAGE SOP was developed by Dr. Kenneth Palmer's laboratory and utilized for these experiments. In brief, BioRad precast Mini-PROTEAN® TGX Any kD

gels, BioRad Precision Plus Protein™ Kaleidoscope™ Standards, and 1X Tris/Glycine/SDS Running buffer were used. GRFT samples were diluted in specified solutions to a concentration of 1-8µg protein (0.033mg/mL – 0.267 mg/mL for 30 µL well size). Thirty µL of sample was then combined with 30uL Laemmli sample loading buffer and boiled for 5 minutes. Samples were cooled on ice. 15uL of sample was loaded into a single well. At the end of the run, protein band detection was performed by Coomassie staining (Bio-Safe Coomassie Stain). Gel images were collected with a Gel DocTM EZ System (Bio-Rad).

NOTE: The BioRad website does not specify the composition of the BioRad precast Mini-PROTEAN® TGX Any kD gel. The website states that the Any kD gel is a "unique formulation…which offers optimal resolution of proteins in the 10-100 kD molecular weight range and is ideal for 2-D PAGE." The website does state that the NuPAGE® Novex 12% Bis-Tris gel is equivalent to the BioRad precast Mini-PROTEAN® TGX any kD gel. The NuPAGE Novex System is based upon a Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel, with a separating gel that operates at pH 7.0. NuPAGE® Novex Bis-Tris gels are available in three acrylamide concentrations: 10%, 4-12%, and 12%.

2.2.5   gp120 Binding ELISA

An Enzyme-linked Immunosorbent Assay (ELISA) was employed to evaluate the gp120 binding activity of select GRFT samples. Nunc Maxisorp 96-well plates were used for this experiment. The MaxiSorp surface is a hydrophilic/hydrophobic mix that binds to a wide variety of biomolecules. Briefly, gp120 is bound to the wells of a 96-well plate overnight at 4°C. HIV-1 gp120 CM was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Cat# 2968). The solution of gp120 was removed after overnight incubation, and a blocking

solution was applied for two hours at room temperature. The wells were then washed and incubated for one hour with various dilutions of GRFT samples. Gp120 binding was visualized by goat anti-GRFT primary antibody (one hour incubation) and HRP-labeled rabbit anti-goat secondary antibody (one hour incubation). TMB substrate was applied to the wells after washing of the secondary antibody. Wells were allowed to develop (blue color) for approximately three minutes before the application of sulfuric acid to stop the reaction (yellow color). Gp120 binding was measured by optical density at 450nm.

2.2.6 Physical and Chemical Stability Assessments

For all of the stability assessments described below, samples were prepared and analyzed in triplicate. HPLC with fluorescence detection was the main mode of analysis, with CD, SDS-PAGE, and UV-spectroscopy utilized where specified.

Thermal Stability

Griffithsin (450µg/mL) in milliQ water was exposed to 5°C, 25°C/60%RH, 30°C/65%RH, 40°C/75%RH, and 65°C in controlled temperature/humidity Carson 6010 environmental chambers for 28 days. GRFT concentration was monitored via HPLC with fluorescence detection.

Photolysis

Griffithsin (450µg/mL) in milliQ water was exposed to 30°C/65%RH for 28 days. Experimental containers were exposed to the D65 lights/lamps with the controlled temperature/humidity chamber. The D65 lamp/light is a commonly used standard illuminant and is also called a daylight illuminant. The D65 light/lamp is intended to represent average daylight. Negative

control containers were protected from light with aluminum foil. GRFT concentration was monitored HPLC-fluorescence detection.

Freeze/Thaw Cycling

Griffithsin (450µg/mL) in milliQ water was exposed to five freeze/thaw cycles at both -80°C/25°C and -20°C/25°C. GRFT concentration was monitored HPLC-fluorescence detection.

Agitation

Griffithsin concentration, via HPLC-fluorescence detection, was monitored over 72 hours of agitation (Vortex Genie®-2, speed 3) at 25°C.

Ionic Strength

Griffithsin solutions were prepared at concentrations ranging from 250µg/mL to 31.25µg/mL in water and phosphate buffers (pH 4, 7, and 10) at low (50mmol/kg) and high (500mmol/kg) ionic strengths. UV spectroscopy with a Cary 50 Bio UV-Visible Spectrophotometer was employed for this stability evaluation.

Oxidation

Oxidation of GRFT was evaluated by exposing GRFT to 0.02% hydrogen peroxide (H_2O_2). This concentration is based on the amount of H_2O_2 secreted by H_2O_2–producing *Lactobacillus* sp. and the quantity of these bacteria that are generally present within the human vagina [115-116]. The final concentration of GRFT in solution was 450 µg/mL and was stored at ambient temperature. Samples were assayed by HPLC-fluorescence detection every 30 minutes for a total of approximately 24 hours. Note: Two oxidative products (Peak B and Peak C) were observed and prompted further investigation into GRFT oxidation (Chapter 3). GRFT parent peak will be denoted Peak A, for ease of further explanation, where approximate, throughout the text.

2.2.7 Biologically-Relevant Assessments

Vaginal Fluid Simulant

Vaginal fluid simulant (VFS) was manufactured as previously described [117]. GRFT (450μg/mL) concentration in VFS at 37°C was monitored over four days with HPLC-fluorescence detection.

Cervicovaginal Enzymes

Griffithsin (450μg/mL) was exposed to enzymes commonly present in human cervicovaginal secretions for up to six hours at 37°C (aminopeptidase 100U/mL, lysozyme 100,000U/mL, and proteinase K 100U/mL). GRFT concentration was monitored via HPLC-fluorescence detection.

Commensal Vaginal Bacteria Viability

Griffithsin stock material was tested for compatibility with commensal vaginal bacteria utilizing the Standard Microbicide Safety Test, as previously described [118]. Strains tested were *L. crispatus* ATCC 33197, *L. jensenii* ATCC 25258, and *L. jensenii* LBP 28Ab. Bacterial suspensions were prepared in N-(2-acetamido)-2-aminoethanesulfonic acid buffer. GRFT was added in the suspensions and incubated at 37°C for 30 minutes. Samples were taken at time zero and 30 minutes. Lactobacillus viability was determined by standard plate count and calculated as the log difference between the time zero plate count and the 30 minute plate count for the experiment. If the calculated viability value is decreased by 10-fold or greater, then the viability of the specified bacterial strain has been compromised, and test compound has failed this assessment.

2.2.8   Statistical Analyses

GRFT concentration over time is reported as GRFT recovery (% of time zero) ± standard deviation for all of the analyses involving GRFT stability assayed with HPLC. This data was analyzed using one-way analysis of variance (ANOVA). p values of < 0.05 were considered statistically significant.

2.3   RESULTS

2.3.1   Physical and Chemical Stability

Thermal Stability

GRFT recovery over 28 days exposure was measured by HPLC-F for the following thermal conditions: 5°C, 25°C/60%RH, 30°C/65%RH, 40°C/75%RH and 65°C. For all thermal conditions except 65°C, GRFT loss was only 10% of time zero during the exposure time. At day 28, there is no difference in GRFT recovery between any of these four conditions (p = 0.07). There is no difference in the thermal stability of GRFT at these four conditions over 28 days exposure. Refer to Figure 7. Further, at 65°C, GRFT recovery quickly declined within one day and was undetectable at 14 days exposure (detectable up until seven days exposure). No GRFT degradation/product peaks were noted on HPLC chromatograms. 65°C is a highly thermally stressed situation that is unlikely to be encountered by this API during formulation manufacturing or dosage form administration/use. GRFT, given its proteinaceous nature, is likely

to have undergone denaturation during the duration of exposure at this elevated temperature. Loss of GRFT was expected and confirmed in this thermal experiment.
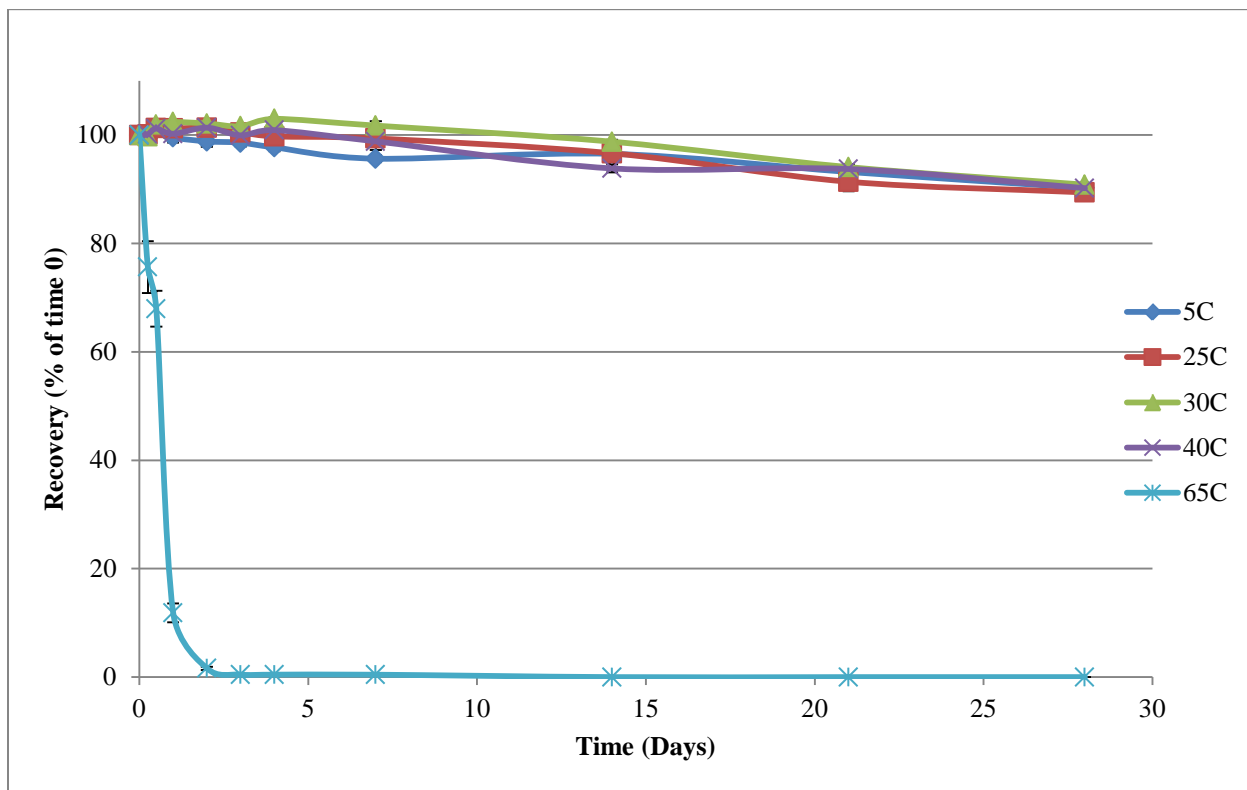
Figure 7. GRFT stability over 28 days at 5°C, 25°C/60%RH, 30°C/65%RH, 40°C/75%RH and 65°C. GRFT (450μg/mL) in milliQ water was exposed to 5°C, 25°C/60%RH, 30°C/65%RH, 40°C/75%RH, and 65°C in controlled temperature/humidity Carson 6010 environmental chambers for 28 days. GRFT concentration was monitored via HPLC with fluorescence detection.

The log concentration versus time profiles for GRFT exposed to 5°C and 65°C were plotted (Figure 8). Since there is not statistical difference between GRFT at 5°C, 25°C/60%RH, 30°C/65%RH, or 40°C/75%RH, only one of these conditions (5°C) will be shown in Figure 8. The slope of both curves was calculated to confirm results of Figure 7 and to estimate the GRFT degradation rate (rate constant, k) under these two conditions. Assuming first order kinetics, rate constant (k) can be calculated with the slope of the curve from the graph of log concentration versus time.

Slope = -k/2.303

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Additionally, the half-life ($t_{1/2}$) of GRFT can also be determined.

$$t_{1/2} = 0.693/k$$

As seen in Figure 8, at 5°C, the slope of the line was small. The rate constant was 0.00345 days^{-1} (0.000144 hr^{-1}), and the half-life was approximately 201 days, indicating the potential for very slow degradation over a long period of time. At 65°C, biphasic degradation was apparent. From time 0 to two days, the rate constant was 2.18 days^{-1} (0.0908 hr^{-1}), and half-life was approximately 7.63 hours (0.318 days); GRFT quickly degraded over this short time frame. From three days to seven days, the rate constant was 0.006 days^{-1} (0.000240 hr^{-1}), and the half-life was approximately 120 days.
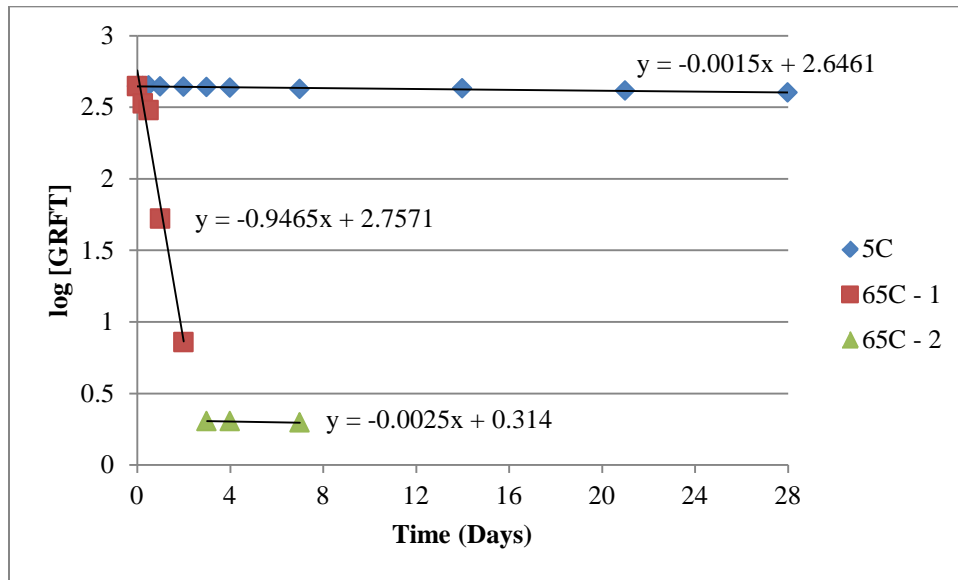
Figure 8. GRFT stability over 28 days at 5°C and 65°C, log[GRFT] versus time profile of results from Figure 7.

To further evaluate GRFT stability at 65°C, the following secondary analyses were employed: CD, SDS PAGE, and gp120 ELISA. Comparison of CD spectra (as shown in Figures 9 and 10) indicate that GRFT conformation at 25°C exposure has a maximum lower absorbance at 220nm and consists of β-sheets. However, no CD spectra is observed for GRFT solution

exposed to 65°C, which suggests conformational change in which secondary structures of β-sheets no longer exists (denaturation).
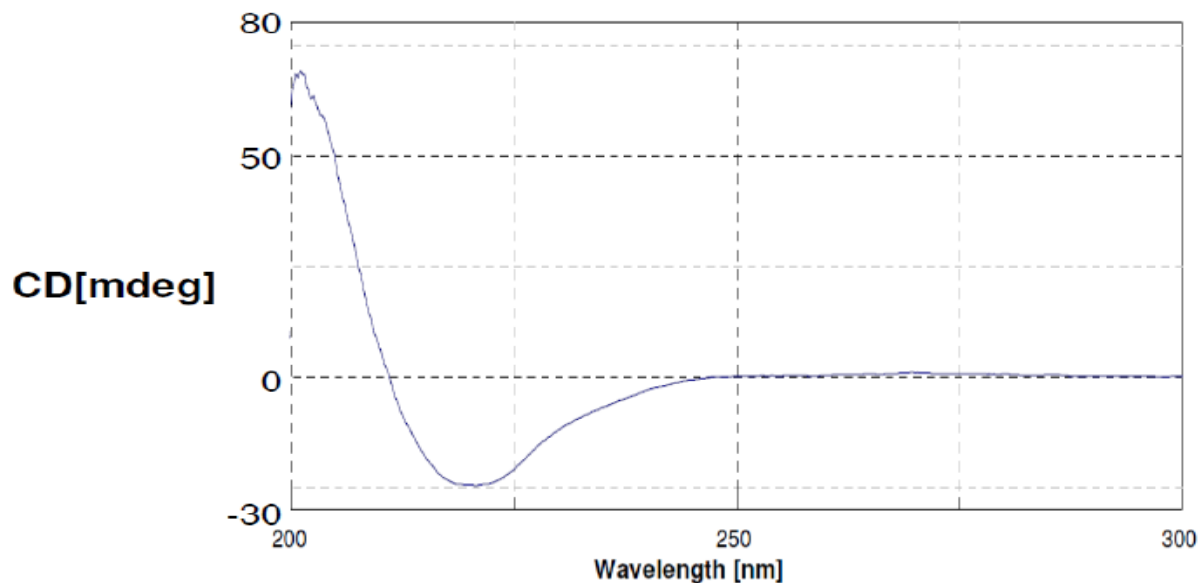
Figure 9. CD Spectrum of GRFT solution exposed to 25°C for 28 days.
Circular dichroism was performed on a sample of GRFT solution exposed to 25°C at the 28 day time point of the thermal stability experiment.
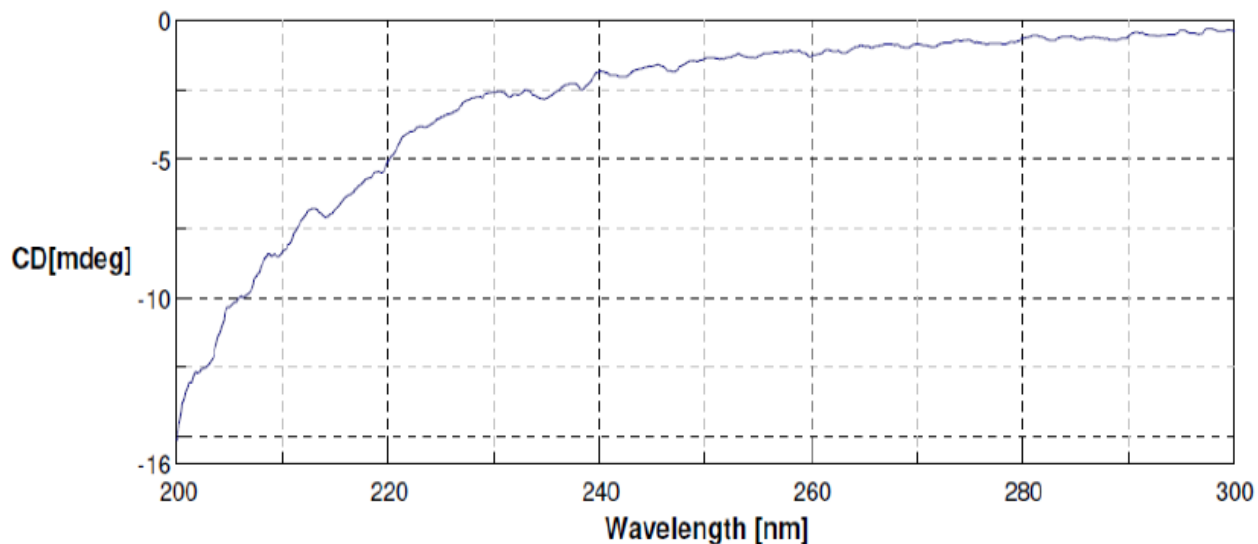
Figure 10. CD Spectrum of GRFT solution exposed to 65°C for 28 days.
Circular dichroism was performed on a sample of GRFT solution exposed to 65°C at the 28 day time point of the thermal stability experiment.

With SDS PAGE analysis of GRFT controls and thermally stressed samples, further stability data can be gathered. Refer to Figure 11. GRFT control (Lanes 3 and 11) displays a sharp band between the 10 and 15 KD markers. This is banding pattern is characteristic of GRFT using this gel type (BioRad precast Mini-PROTEAN® TGX Any kD gel) and these molecular weight markers (BioRad Precision Plus Protein™ Kaleidoscope™ Standards). A GRFT sample that has been thermally stressed at 65°C for one day (Lane 9) displays a band that is starting to smear between 10 and 15 kD and darkening at the top of the lane/well. A GRFT sample that has been thermally stressed at 65°C for one year (Lanes 5 and 7) does not display any GRFT band, but a faint smearing from below 10 kD to around 15kD marker. Additionally, darkening at the top of these two lanes/wells is evident. Smearing and no sharp GRFT band can indicate degradation of the protein. Darkening at the top of the lane/well indicates protein aggregation.
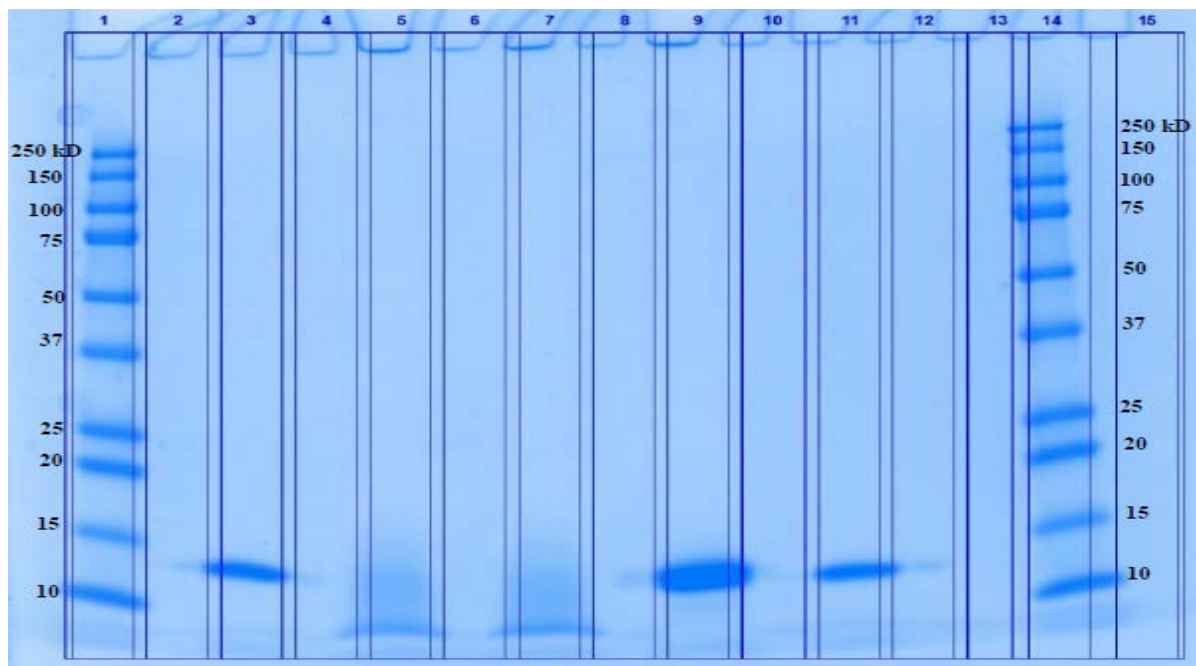
Figure 11. Thermal profile of GRFT on SDS-PAGE.
Separation of protein standards and GRFT exposed to 65C, detected by Coomassie blue staining on a BioRad precast Mini-PROTEAN® TGX Any kD gel. Lanes 2, 4, 6, 8, 10, 12, 13, and 15 are blank. Lanes 1 and 14 contain protein molecular weight markers. Lanes 3 and 11 contain GRFT 0.125mg/mL in milliQ water control sample; sample was prepared fresh from stock. Lane 9 contains GRFT sample thermally stressed for 24 hours as 65°C. Lanes 5 and 7 contain GRFT sample thermally stressed for one year at 65°C. All GRFT controls and samples are of the same GRFT drug lot.

To investigate the gp120 binding ability of GRFT after being thermally stressed, an ELISA was utilized. Within this study, the gp120 binding potential of the thermally stressed protein sample was compared to the same GRFT drug Lot (unstressed, stored at 2-8°C, refrigerated) and two other GRFT drug Lots (same manufacturing process, different manufacture date, unstressed, stored at 2-8°C, refrigerated). Figure 12 displays the results of this ELISA experiment. These results show that upon exposure to the thermal stress of 65°C for one month, GRFT loses its ability to bind to gp120 *in vitro*. GRFT has lost the function of its binding pockets to bind to gp120.
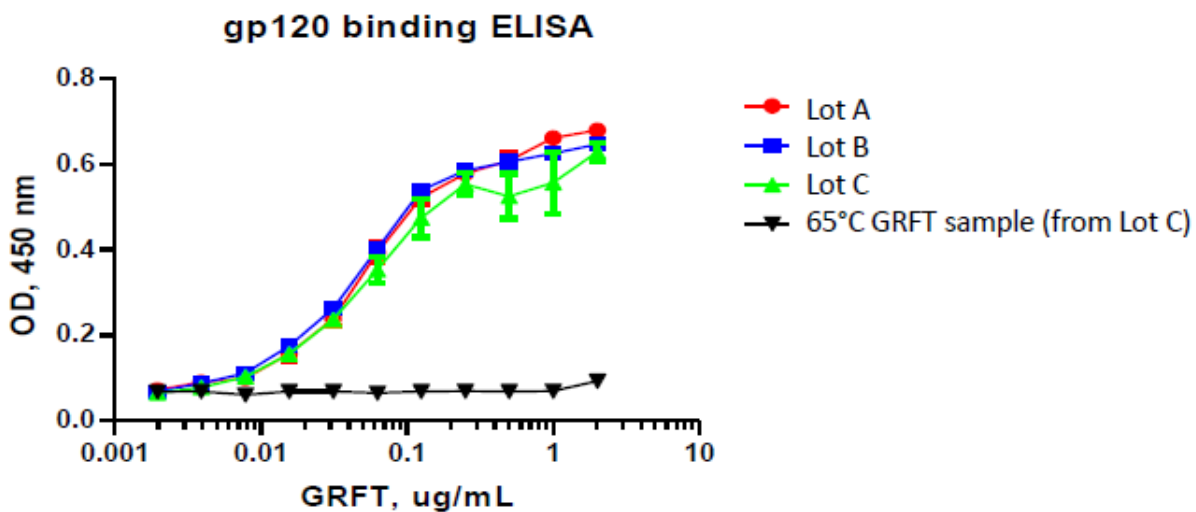
Figure 12. GRFT-gp120 Binding Curves.
The binding curves for GRFT from a gp120 binding ELISA are compared for three different drug lots (Lot A, B, and C) and **GRFT exposed to 65°C from the thermal stability experiment.**

Photolytic Stability

The photostability of GRFT solution was explored at 30°C. Exposure to light did not impact the stability of GRFT at 30°C, as shown in Figure 13. % Recovery day 28 values for the GRFT exposed to light and not exposed to light is 87% ± 1% and 90% ± 0.4%, respectively.
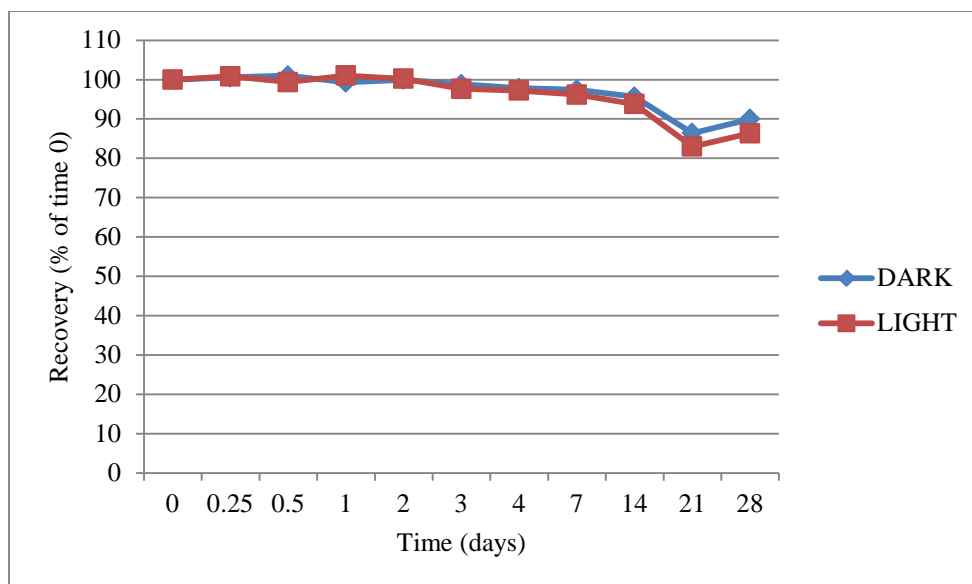
Figure 13. Photolytic stability of GRFT in milliQ water at 30°C.
GRFT (450μg/mL) in milliQ water was exposed to 30°C/65%RH for 28 days. Experimental containers were exposed to the lamps with the controlled temperature/humidity chamber. Negative control containers were protected from light with aluminium foil. GRFT concentration was monitored HPLC-fluorescence detection.

Freeze/Thaw Cycling

Griffithsin was not prone to physical instability caused by freeze/thaw cycling or agitation. After five freeze/thaw cycles at -80°C/25°C and -20°C/25°C (separately), GRFT recovery was 102.0%±0.23% and 99.0%±0.90%, respectively. Refer to Figure 14.
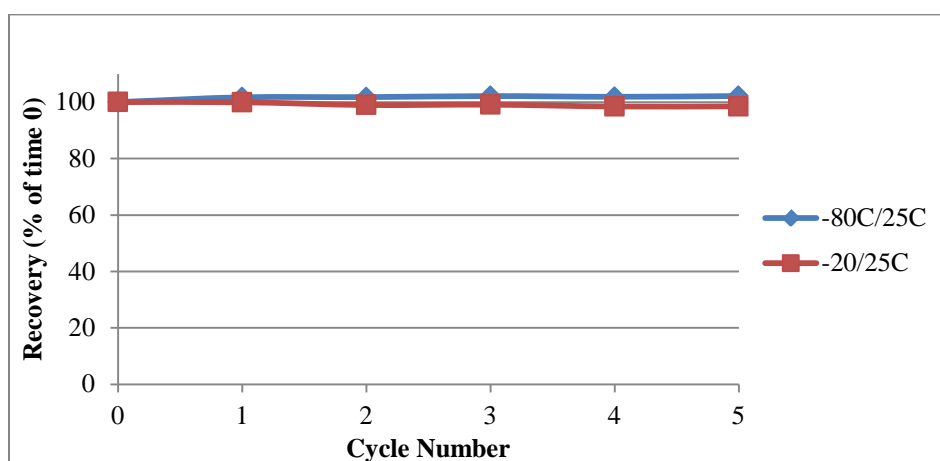
Figure 14. GRFT Recovery after Five Freeze/Thaw Cycles -80°C/25°C and -20°C/25°C.
GRFT (450μg/mL) in milliQ water was exposed to five freeze/thaw cycles at both -80°C/25°C and -20°C/25°C. GRFT concentration was monitored HPLC-fluorescence detection.

Agitation

After 72 hours (three days) of agitation at room temperature, GRFT recovery remained at 97%±0.88%. Therefore, GRFT was not prone to physical instability that may be caused by agitation/shaking. See Figure 15.
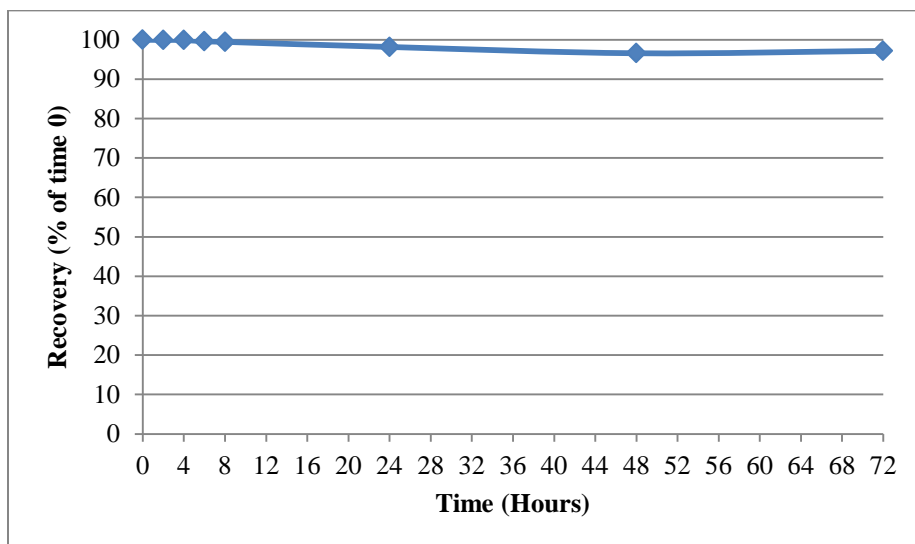
Figure 15. Effect of agitation on GRFT over 72 hours (three days) at 25°C.
GRFT concentration, via HPLC-fluorescence detection, was monitored over 72 hours of agitation (Vortex Genie®-2, speed 3) at 25°C.

Ionic Strength

The effect of ionic strength on the stability of GRFT was evaluated by UV-spectroscopy and the results for GRFT 250µg/mL can be found in Figure 16. Figure 16A represents results for low ionic strength, and Figure 16B represents high ionic strength results. No pronounced increases in absorbances are evident at either ionic strength. These results indicate insoluble protein aggregates are not present under these conditions.
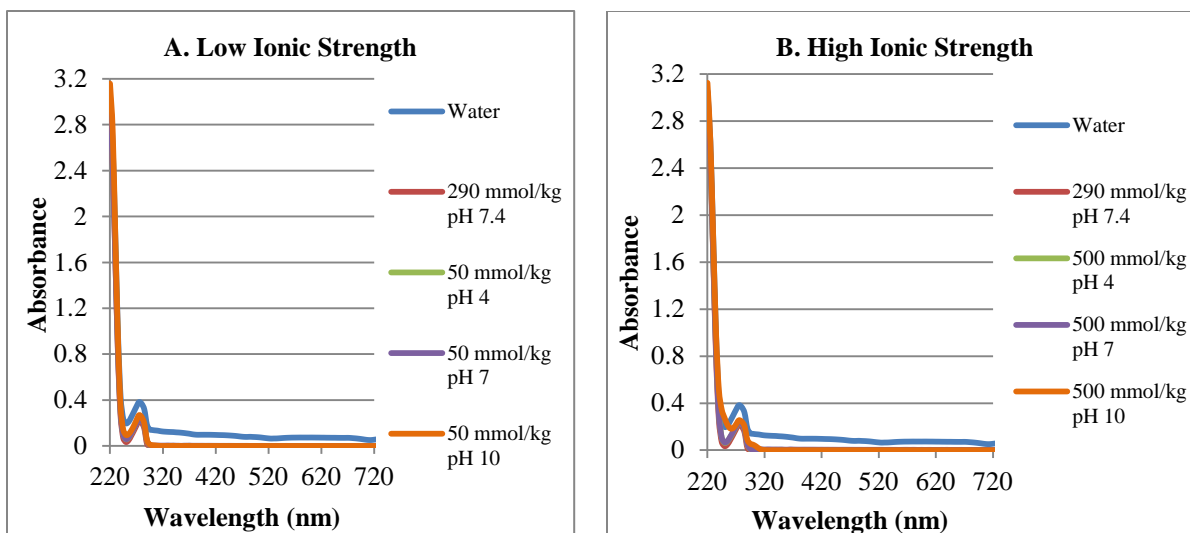
Figure 16. UV Scans to determine the effect of pH and ionic strength.
GRFT solution in water, pH 4, pH 7, and pH 10 buffers were analyzed by UV-spectroscopy to determine formation of insoluble aggregates. (A) Represents GRFT solutions prepared in low ionic strength buffers (50mmol/kg. (B) Represents GRFT solutions prepared in high ionic strength buffers (500mmol/kg).

2.3.2   Biologically-Relevant Assessments

Vaginal Fluid Simulant

The stability of GRFT in vaginal fluid simulant was monitored. This biological fluid simulant was used due to its historic use in *in vitro* experiments of vaginally applied products. GRFT drug substance remained stable over four days (p = 0.08), with a recovery of 98% ± 1.4%. Refer to Figure 17.
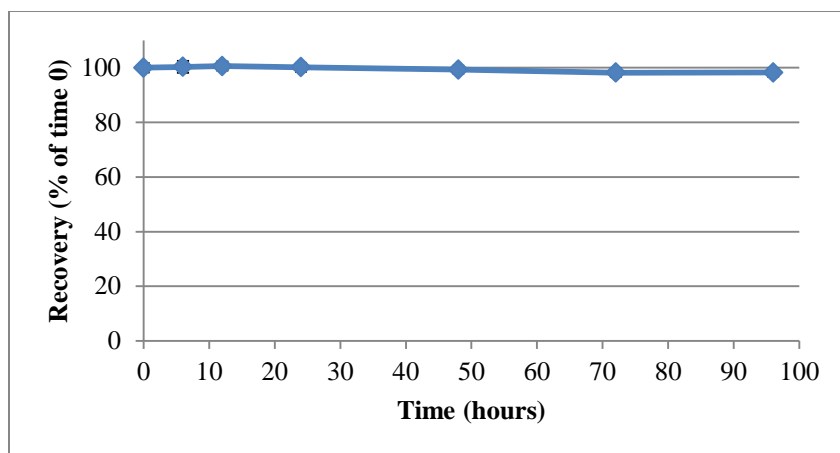
Figure 17. GRFT stability in VFS at 37°C over 96 hours (four days) exposure.
GRFT (450µg/mL) concentration in VFS at 37°C was monitored over four days with HPLC-fluorescence detection.

Cervicovaginal Enzymes

GRFT is a hardy protein and was not highly prone to the enzymatic degradation of aminopeptidase, lysozyme, or proteinase K. Recovery of GRFT from these individual enzyme solutions ranged from 90-103% as seen in Figure 18. When comparing log [GRFT] over time, as seen in Figure 19, no difference in profiles is evident. The rate constant value for the control, lysozyme, and aminopeptidase condition is $0.008hr^{-1}$ and for the proteinase K condition is $0.013hr^{-1}$.
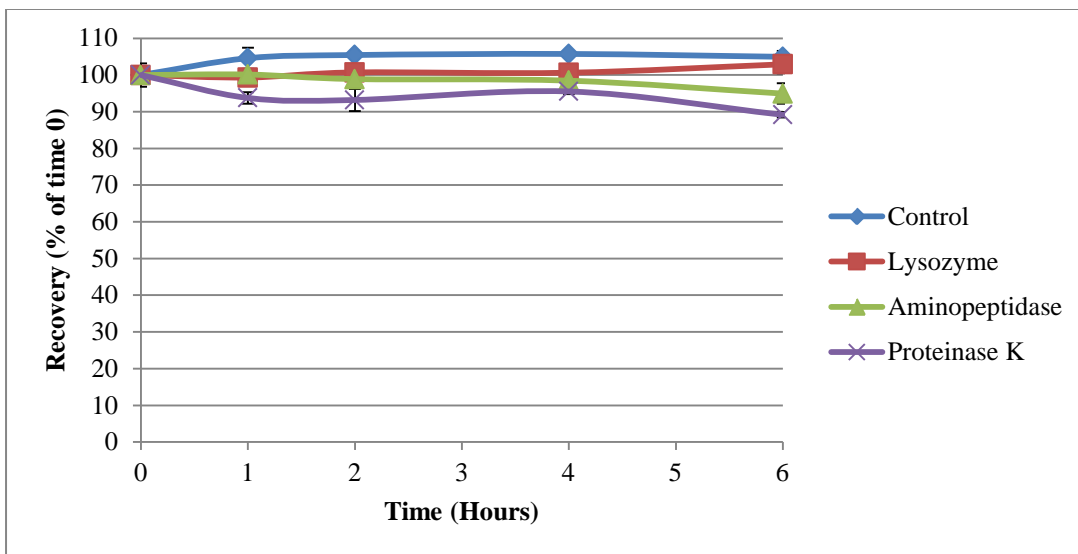
Figure 18. GRFT stability in the presence of lysozyme, aminopeptidase, and proteinase K over 6 hours at 37°C.
GRFT (450μg/mL) was exposed to enzymes commonly present in human cervicovaginal secretions for up to six hours at 37°C (aminopeptidase 100U/mL, lysozyme 100,000U/mL, and proteinase K 100U/mL). GRFT concentration was monitored via HPLC-fluorescence detection.
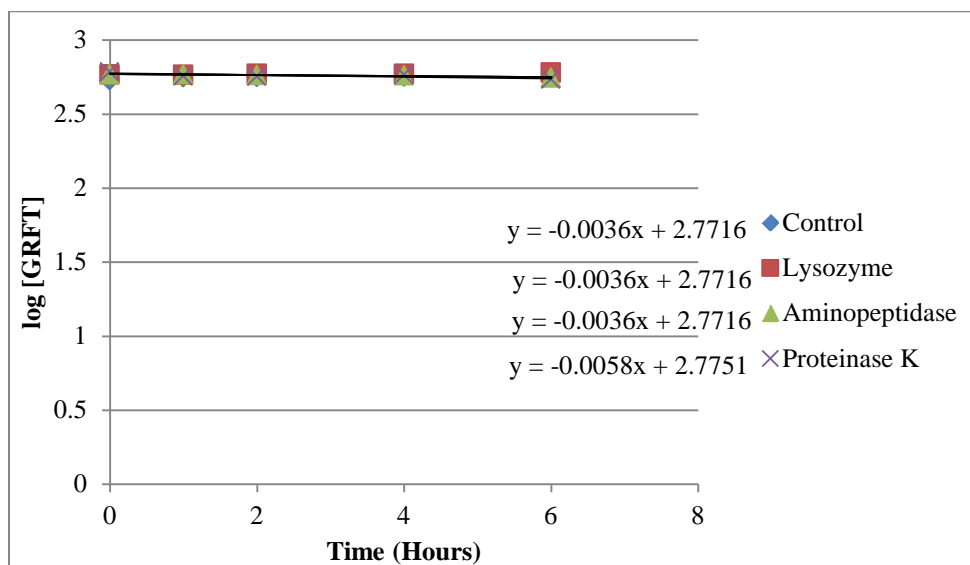
Figure 19. GRFT stability in the presence of lysozyme, aminopeptidase, and proteinase K over 6 hours at 37°C, log[GRFT] versus time profile from the data in Figure 18.

Lactobacillus Compatibility

GRFT was exposed in *in vitro* studies to three strains of *Lactobacillus* that are present within the normal, healthy human vaginal environment. GRFT was found to be compatible with lactobacilli, as indicated by a < 10-fold change in bacterial growth across all three commensal bacterial strains (Table 7).

Table 7. GRFT Compatibility with Lactobacilli

Lactobacillus Strain	Viability Reduction (log difference)
L. crispatus ATCC 33197	0.0211
L. jensenii ATCC 25258	0.0588
L. jensenii LBP 28Ab	0.0298

Oxidation

Per HPLC analysis, GRFT was found to completely degrade over approximately 24 hours when exposed to 0.02% H_2O_2. Refer to Figures 20 and 21. At baseline, Stock GRFT API substance, consisted of approximately 92% GRFT (denoted as GRFT Parent Peak A, RRT 1.000) and 8% impurity (denoted as Peak B, RRT 0.900). After 24 hours, this material consisted of approximately 8% Peak B and 92% Peak C (RRT 0.690).
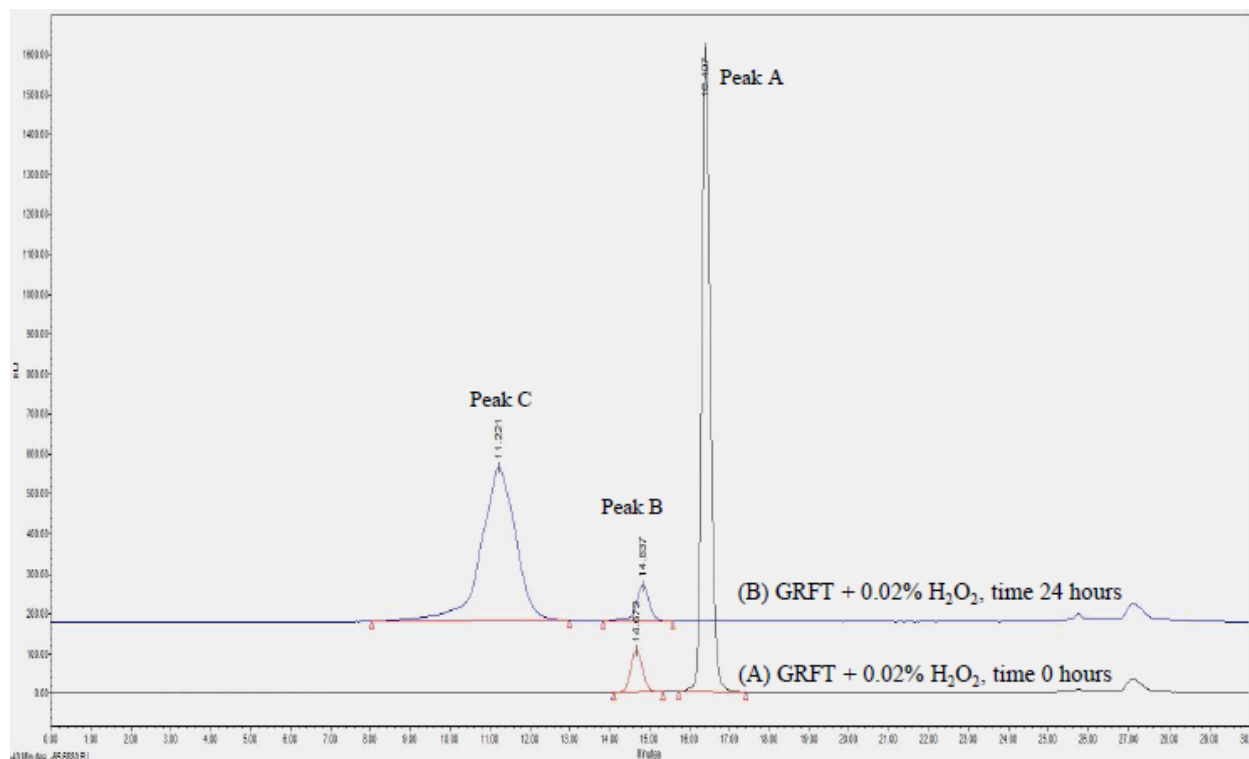
Figure 20. HPLC chromatograms of GRFT.
(A) HPLC chromatogram of GRFT time 0. (B) HPLC chromatogram of GRFT exposure to 0.02% hydrogen peroxide after 24 hours. Oxidation of GRFT was evaluated by exposing GRFT to 0.02% hydrogen peroxide (H2O2). The final concentration of GRFT in solution was 450 μg/mL and was stored at ambient temperature. Samples were assayed by HPLC-fluorescence detection every 30 minutes for a total of approximately 24 hours.
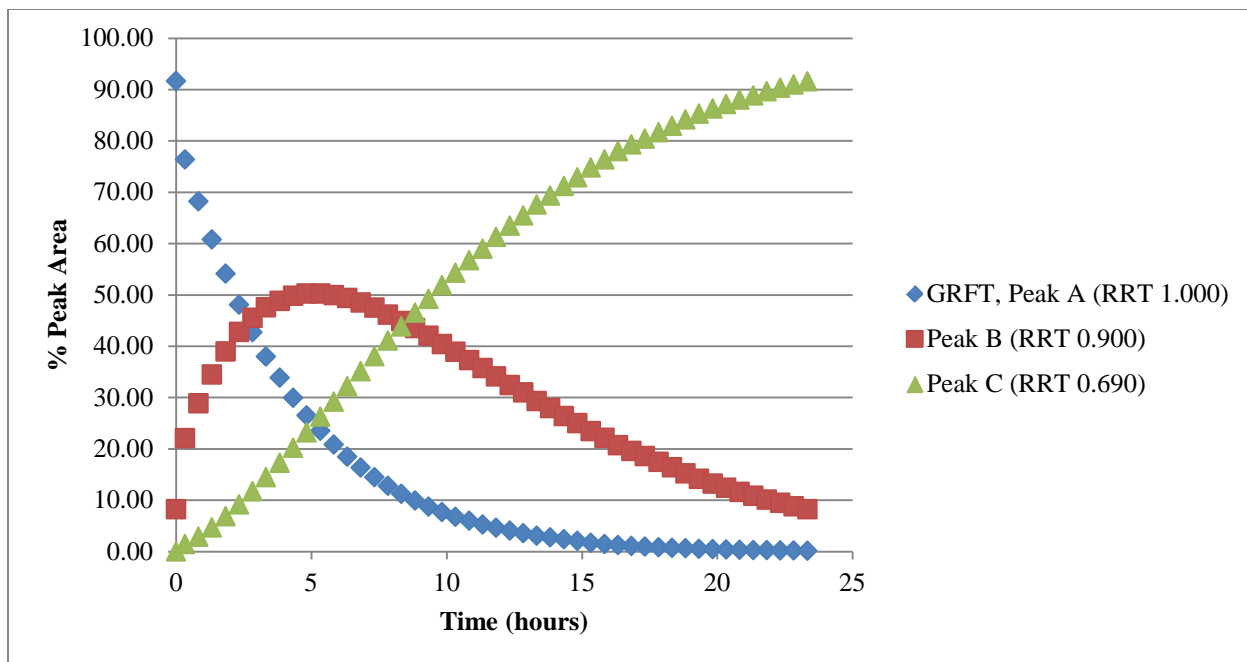
Figure 21. GRFT and oxidative degradant % peak areas over 24 hours exposure to 0.02% hydrogen peroxide.
Samples were assayed by HPLC-fluorescence detection every 30 minutes for a total of approximately 24 hours.

The log GRFT concentration versus time profile was plotted for GRFT to estimate the rate of oxidative degradation due to 0.02% hydrogen peroxide exposure (Figure 22). The calculated rate constant, k, was 0.269 hr^{-1}, and the half-life was 2.581 hr.
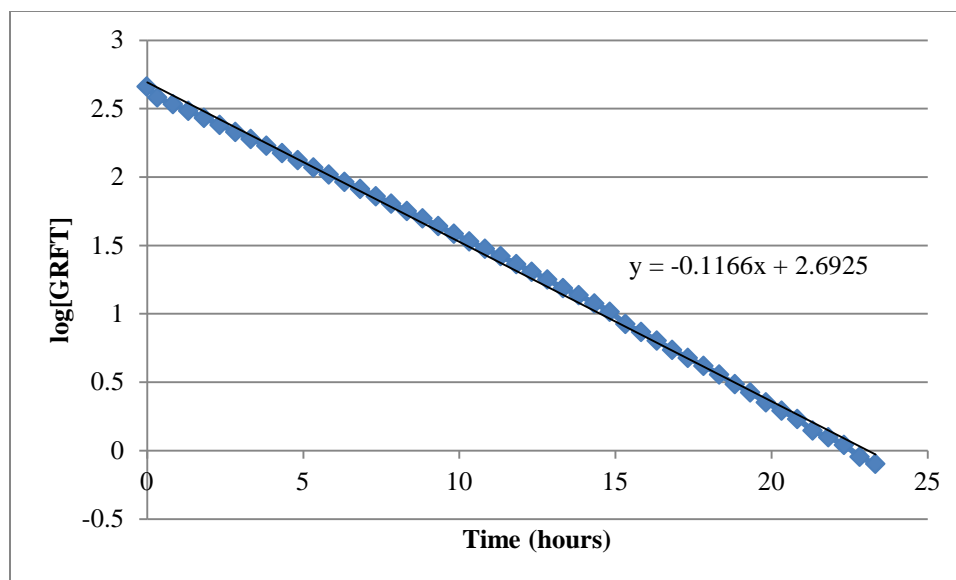
Figure 22. log[GRFT] versus time, 24 hours exposure to 0.02% hydrogen peroxide from data in Figures 20 and 21.

2.4   DISCUSSION AND CONCLUSIONS

Degradation studies are performed as a part of the overall preformulation strategy to achieve two goals: 1) The first goal is to ensure that the analytical methods being used in the preformulation study can detect/separate the various specific degradation products. 2) The second goal is to obtain additional information on the potential degradation pathways of the protein. The specific purpose of these studies within this chapter was to determine the stability of GRFT under several conditions. It is important for the active pharmaceutical ingredient (API) to maintain its chemical and physical stability in the dosage form prior to use and when exposed to biological conditions upon delivery into the body and to the target site of action. Therefore, standard preformulation assessments of thermal stability, photostability, aggregation and denaturation potential, ionic strength effects, and susceptibility to oxidation were performed. Additional vaginal delivery-specific preformulation assessments were also investigated. These included

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GRFT's stability/compatibility with vaginal fluid simulant, cervicovaginal enzymes, and commensal vaginal bacteria.

Thermal stability studies on GRFT drug substance are performed to determine the chemical and physical stability of GRFT API when exposed to various temperature conditions including those relevant to storage or use. The purpose of thermal stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a varying temperature/humidity. The resulting data helps to establish a retest period for the drug substance or a shelf life for the drug product and recommended storage conditions for drug substance/drug product. The storage conditions used in the thermal stability studies are based on ICH (International Conference on Harmonization) guidelines for general drug cases and drugs that are to be refrigerated: long term ($25°C+2°C/60\%RH+5\%RH$), intermediate ($30°C+2°C/65\%RH+5\%RH$), accelerated ($40°C+2°C/75\%RH+5\%RH$), stressed ($65°C$), and long term refrigerated ($5°C+3°C$) [119].

The stability of proteins to thermal stress is an important variable to those who will formulate the protein into a usable dosage formulation. Changes in temperature may accompany processing required to generate the desired dosage form and serves as a convenient thermodynamic variable to probe the stability and shelf life for the protein pharmaceutical through accelerated protein stability testing. Typical temperature for maximum protein stability is between -10°C and 35°C for most proteins [120-121]. Temperature induced destabilization is driven by the gain in conformation entropy of the protein chain and usually begins with partial unfolding of the protein. Although the unfolding is typically reversible, many proteins will undergo chemical reactions or other irreversible denaturation processes such as aggregation,

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leading to loss of protein function. This is especially true at elevated temperatures where reaction kinetics and collision rates are increased.

Per ICH guidelines, the photostability of new drug substances and drug products should be evaluated to demonstrate that light exposure does not result in unacceptable changes in the API [122]. Photostability evaluations are most relevant for drugs/products formulated as solutions where there is potential for storage under lighted conditions, potential for photodegradation. These preformulation studies can be used for forced degradation testing and/or confirmatory testing. Forced degradation studies are used to evaluate the overall photosensitivity of the drug for methods development purposes and/or degradation pathway elucidation. Confirmatory studies should be undertaken to provide necessary information for handling, manufacturing, packaging, and labeling. Photostability analysis for GRFT API was performed for both purposes of degradation elucidation and to gather information necessary for handling, manufacturing, packaging, and labeling for future product.

The preformulation evaluation of oxidative stress was also studied for GRFT. Oxidation can be a complex process with many possible reaction reactants and catalysts. For preformulation and formulation development of proteins, there are many areas for concern. These include the ability to cause oxidation of specific amino acid residues through metal ion catalysis, by hydrogen peroxide, photochemically, through organic solvent, or formulation excipients. Therefore, many areas of storage, process, handling, and formulation manufacture are a potential oxidation source. Oxidation has the potential to alter the biological activity of a protein drug. The molecular mechanism of altered bioactivity can arise from: (1) oxidation of a critical residue at or near the enzyme active site or the receptor binding site of the protein, or (2) drastic change in the protein structure due to amino acid oxidation [120].

Further, studies to determine GRFT's susceptibility to protein denaturation and aggregation were performed. Protein denaturation refers to a disruption of the higher-order structure, such as secondary and tertiary structure of a protein. Denaturation, which may be reversible or irreversible, can be caused by thermal stress, extremes of pH, shear/shaking, and exposure to denaturing chemicals. Denaturation typically involves unfolding of the protein. In some cases, the unfolded protein can be transformed back to its native state by using denaturants such as guanidine hydrochloride or urea, followed by dialysis. If the protein cannot easily recover its native state by refolding, denaturation is considered to be irreversible. In many cases, this can lead to aggregation and precipitation. Therefore, it is important to perform studies that have the potential to cause denaturation (agitation studies) to determine the ruggedness of the protein solution [121].

Protein aggregates can result in an increase in immunogenicity, and a decrease in bioactivity which may be both a formulation and delivery challenge. Factors such as temperature may induce aggregation formation, and freezing is a drug/product handling consideration. At various stages in processing/manufacturing/handling of protein, freezing bulk protein solutions may be necessary. Although the storage condition of final drug product is generally 2-25°C, there is always the possibility of inadvertent exposure to freezing temperatures during shipping and storage. Freezing might occur during shipments of protein solutions, since packages may end up in uncontrolled temperature cargo bays or in refrigerated storage units having colder areas that are below freezing. Therefore, it is important to perform freeze-thaw cycling studies to determine the ruggedness of the protein solution. Freeze-thaw studies are considered relevant because product may encounter hostile environment conditions en route to its destination or accidentally in the hands of those administering such a product.

Additional studies of potential GRFT aggregation were performed, specifically investigating solution ionic strength. The ionic strength of a solution is a measure of the concentration of ions in that solution. For UV-spectroscopy, changes in absorbance baseline of the UV spectrum in non-absorbing regions (>300nm), indicates the presence of aggregates. This type of method is only qualitative, but indicates aggregation formation. As previously stated, aggregates can result in an increase in immunogenicity, and a decrease in bioactivity, presenting formulation development and protein delivery challenges. Protein monomers can aggregate by physical association with one another without any changes in primary structure or by formation of a new covalent bond (irreversible aggregation). Both mechanisms can generate both soluble and insoluble aggregates, depending on the protein, environmental conditions, and the stage of the aggregation process. In the case of GRFT, since this protein is a homodimer, there may be the potential to create dimers of dimers. Some external factors such as temperature, pH, and protein concentration may induce the aggregation formation. If aggregation is concentration, pH, and ionic strength dependent, all of those factors may impact maximum GRFT concentration that can be used in the formulation as well as the formulation pH and ionic strength [121].

In addition to the standard preformulation evaluations, vaginal delivery-specific preformulation assessments were investigated. These included GRFT's stability/compatibility with vaginal fluid simulant, cervicovaginal enzymes, and commensal vaginal bacteria. Vaginal fluid simulant was used due to its historic use in *in vitro* experiments of vaginally applied products. This media can be used for drug API or product formulation permeability/release studies, disintegration studies, dissolution studies, and various other *in vitro* experiments. Therefore, it is paramount to evaluate GRFT's stability in this biological simulant to determine this simulant utility in future in vitro experiments involving preformulation and formulation

development of GRFT. GRFT, given its proteinaceous nature, has the potential to be enzymatic degraded. In order to establish the cervicovaginal enzymatic degradation potential of GRFT, this protein was exposed to enzymes commonly present in the normal human vaginal environment [109-110, 123]. Further, a vaginally applied product, like a vaginal GRFT microbicide, should not exert any harmful effects on the innate commensal microflora of the cervicovaginal environment. To this end, the compatibility of GRFT with *Lactobacillus* sp. was established.

During all of the standard preformulation and biologically-relevant *in vitro* assessments that were performed, except two, GRFT proved to be safe toward commensal cervicovaginal bacteria and chemically and physically stable. Concerning the vaginally- relevant assessments, GRFT was found to have compatibility with vaginal fluid simulant, per HPLC analysis. This result provides evidence that supports this simulant's utility in future *in vitro* experiments for the development of GRFT as a vaginally applied entity. Enzymes commonly found within the human vagina did not degrade GRFT; this result is supportive of the findings of Moncla et al [124]. Of the nine proteases tested by this group, GRFT was resistant to digestion by eight of them. Further, GRFT was found to be safe toward three stains of commensal Lactobacillus bacteria present in the normal human vagina, using the Standard Microbicide Safety Test [118]. Taken together, these observations provide evidence of the vaginal safety of GRFT and the continued development of a vaginally-applied GRFT microbicide product.

The thermal stress of 65°C for 28 days and the oxidative stress of 0.02% H_2O_2 over 24 hours were unstable conditions for GRFT API. When GRFT was stressed with 0.02% H_2O_2, it was found to be highly susceptible to oxidative degradation. 65°C (149$^°$F) is a highly thermally stressed situation that is unlikely to be encountered by this API during formulation handling, processing, or dosage form administration/use. During the manufacturing of GRFT containing

products, such elevated temperatures should be avoided. GRFT, given its proteinaceous nature, is likely to have undergone denaturation and aggregation, as evidenced by HPLC, CD, and SDS PAGE, during the duration of exposure at this elevated temperature. Further, the *in vitro* gp120 binding analysis showed that the physical degradation of this protein negatively impacts its ability to bind to its target molecule. This is a negative outcome that can reflect decreased bioactivity. However, as previously stated the likelihood of GRFT exposure to this elevated temperature is extremely small and was evaluated to push the thermal storage limits for this protein.

Oxidative degradation surfaced as GRFT's major pathway of chemical instability, as evidenced by HPLC analysis. This chemical instability is of major concern to the development of a GRFT-containing pharmaceutical product for vaginal delivery. This route of drug delivery contains sources of oxidative potential, including hydrogen peroxide-producing *Lactobacillus* sp. that take part in the maintenance of a health cervicovaginal environment [50]. Management of GRFT amino acid oxidation can be approached as follows: (1) Use antioxidants to prevent GRFT oxidation; or (2) Identify the amino acid(s) that are oxidized, and modify these oxidizable amino acid(s) to non-oxidizable amino acids. In an effort to protect GRFT from oxidative forces, several antioxidant excipients were tested for their potential to prevent oxidation. Theses antioxidant excipients included: methionine (0.09 and 0.25%), cysteine (0.09 and 0.25%), glutathione (0.09 and 0.25%), vitamin E TPGS (0.009%), disodium EDTA (0.05%), ascorbic acid (0.1%). None of the tested antioxidants afforded any significant protection from oxidation to this protein. Note: Antioxidant data are not shown. This was not part of my dissertation project. These experiments were conducted by Phillip Graebing of the Rohan Lab.

In terms of commonly oxidized amino acids, there are five. These include cysteine (C), methionine (M), histidine (H), tryptophan (W), and tyrosine (Y) [120]. Figure 23 shows the amino acid sequence of GRFT and highlights the amino acids that may be prone to oxidation.

Figure 23. Primary structure of GRFT, with potential sites of oxidation highlighted in red.

Oxidation is very important to take into consideration for the vaginal delivery of this protein. *In vivo*, oxidation can be a result of exposure to the hydrogen peroxide-producing Lactobacillus sp. The production of hydrogen peroxide by some *Lactobacillus* sp. offers protection from overgrowth of various pathogens, including those causing bacterial vaginosis (BV) [50]. Other sources of oxidative potential within the human cervicovaginal environment include enzymes such as oxidases [125-127] and peroxidases [128-129]. Considering GRFT's susceptibility to exogenous hydrogen peroxide exposure, it is likely to undergo oxidation due to endogenous exposure. Potential GRFT oxidation is of great interest to the further evaluation of this vaginal microbicide candidate. The next chapter of this dissertation, Chapter 3, is devoted to further exploring GRFT oxidation – specifically GRFT's stability in human cervicovaginal secretions and the identification of the specific amino acid(s) that are oxidized.

ACKNOWLEDGEMENTS

3.0   GRIFFITHSIN OXIDATION

3.1   INTRODUCTION

As shown in Chapter 2, GRFT is highly prone to the chemical instability of oxidation caused by exogenous hydrogen peroxide exposure. Oxidation is very important to take into consideration for the vaginal delivery of this protein. Human cervicovaginal secretions are a complex mixture of serum transudate and its constituents, cervical mucus, sloughed vaginal epithelial cells, microflora, amino acids, proteins, carbohydrates, enzymes, enzyme inhibitors, ions, and lipids [40]. Further, pertaining to vaginal drug delivery, the enzymatic activity within the vaginal lumen/fluid and within the epithelium can affect drug transport and drug stability [42]. *In vivo*, oxidation can be a result of exposure to the hydrogen peroxide-producing *Lactobacillus* sp. that the predominant bacteria of a health cervicovaginal environment [50]. Other sources of endogenous oxidative potential within the human cervicovaginal environment include enzymes such as oxidases [125-127] and peroxidases [128-129]. Oxidases catalyse an oxidation reaction using molecular oxygen. Peroxidases catalyse an oxidation reaction using hydrogen peroxide.

Considering GRFT's susceptibility to exogenous hydrogen peroxide exposure, this protein is likely to undergo oxidation due to endogenous oxidative stresses as well. Oxidation has the potential to alter the biological activity of this protein, when delivered vaginally. The molecular mechanism of altered bioactivity can arise from: (1) oxidation of a critical residue at

or near the enzyme active site or the receptor binding site of the protein, or (2) drastic change in the protein structure due to amino acid oxidation [120].

The objective of these studies was to further explore GRFT oxidation. This will be accomplished by: (1) determining the stability of GRFT in human cervicovaginal secretions, and (2) identifying the amino acid(s) of GRFT that are prone to oxidization. Analyses that were performed to accomplish these goals include HPLC, mass spectrometry (mass analysis and peptide sequencing), SDS PAGE, and gp120 binding ELISA. Overall, the results generated will be important to establish the chemical stability (potential for oxidation) of GRFT when vaginally administered in order to further guide development of a vaginal product containing GRFT.

3.2   MATERIALS AND METHODS

Recombinant Griffithsin drug substance was manufactured by Kentucky Bioprocessing LLC (KBP; Owensboro, KY) and provided to our laboratory by Dr. Kenneth Palmer of the University of Louisville School of Medicine. Recombinant GRFT was exposed to 0.02% hydrogen peroxide and human cervicovaginal lavage (CVL), respectively. Exposures were performed at ambient temperature and for approximately four to five hours. GRFT was assayed via HPLC-F. GRFT and oxidative products (Peak A/GRFT, Peak B, and Peak C) from both conditions were collected and further characterized using mass spectrometry (intact mass analysis). Recombinant GRFT and one oxidative product (Peak C) were further examined with peptide sequencing.

3.2.1 Hydrogen Peroxide Exposure

Oxidation of GRFT was evaluated by exposing GRFT to 0.02% hydrogen peroxide (H_2O_2). This concentration is based on the amount of H_2O_2 secreted by H_2O_2–producing *Lactobacillus* sp. and the quantity of these bacteria that are generally present within the human vagina. The final concentration of GRFT in solution was 500 µg/mL and was sampled at ambient temperature. Samples were assayed by HPLC-fluorescence detection. Note: GRFT parent peak will be denoted Peak A. Two oxidative products are denoted Peak B and Peak C. Exposure time was approximately five hours, due to time constraints of a work day and the unknown stability of collected oxidative products. The peak fractions were manually collected after passing through the HPLC detector, directly before entering the waste container. These sample collections were used for mass spectrometry, SDS PAGE, and gp120 ELISA.

3.2.2 Human Cervicovaginal Lavage Exposure

Human cervicovaginal lavages (CVLs) were obtained at the University of Pittsburgh under IRB Protocol PRO11020218. CVLs were from healthy, HIV negative, premenopausal women. Samples were stored at -80°C until they were thawed for immediate testing. GRFT was exposed to human CVL at room temperature and monitored via HPLC-fluorescence detection over approximately 2.5 hours due to CVL volume constraints. The final concentration of GRFT in solution was 500 µg/mL and was sampled at ambient temperature. Note: GRFT parent peak are denoted Peak A. Two oxidative products are denoted Peak B and Peak C. The peak fractions were manually collected after passing through the HPLC detector, directly before entering the

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waster container. These sample collections were used for mass spectrometry, SDS PAGE, and gp120 ELISA.

3.2.3 High Performance Liquid Chromatography

The HPLC system (Waters Corporation; Milford, CA) was equipped with an autosampler model 717plus, a quaternary pump controller model 600, and a fluorescence detector model 2487, using an excitation wavelength of 273 nm and an emission wavelength of 303 nm. Separation of GRFT from degradants was achieved using a Phenomenex Jupiter C18 5µ 300Å (4.6x250 mm) column (Phenomenex; Torrance, CA) protected by a Gemini C18 (4x3 mm) guard cartridge (Phenomenex; Torrance, CA), both maintained at ambient temperature. The mobile phase gradient consisted of mobile phase A (0.1% TFA in water (v/v)), and mobile phase B (0.05% TFA in acetonitrile (v/v)) pumped at a flow rate of 1.0 mL/min. The gradient was proportioned as follows (minute, %B): 0, 12; 15, 20; 16, 50; and 20, 12. Linearity of the detector response curve was over the range of 10 µg/mL to 500 µg/mL.

3.2.4 Mass Spectrometry – Intact Mass

In preparation for intact mass analysis, 5% formic acid was added to each sample. Corresponding blanks were also assayed. For intact mass analysis, a Bruker micrOTOF with a capillary ESI source and Dionex U3000 HPLC system were utilized. The HPLC column was a PRLP-S column (Thermo Fisher, 1000Å, 5µm, 100x0.3 mm) maintained at 40°C temperature. The mobile phase gradient consisted of mobile phase A (2.5% acetonitrile/0.1% formic acid in water), and mobile phase B (80% acetonitrile/20% water/0.1% formic acid) pumped at a flow

rate of 3.5 µL/min. The gradient was proportioned as follows (minute, %B): 0, 5; 10, 30; 50, 75; 65, 100; and 75, 5. The intact mass analysis was performed in positive ion mode, with the acquisition range of *m/z* 50-3000. The *m/z* values were converted into molecular mass values with the MaxEnt Deconvolution program. The mass accuracy of this instrument is 0.01% of the analyte mass. Therefore, for GRFT parent monomer of 12.7 kDa, the mass accuracy is 1-2 Da. For GRFT parent homodimer of 25.4 kDa, the mass accuracy is 2-4 Da.

3.2.5   Mass Spectrometry – Peptide Sequencing

GRFT parent and Peak C (from hydrogen peroxide exposure) underwent peptide sequencing. Prior to this analysis, samples were concentrated, denatured with 1% Rapigest, and diluted with 100mM ammonium bicarbonate to 0.1% Rapigest. Then protease (trypsin or chymotrypsin) at 2:1 protein:protease (w:w) was added to each sample. After incubation overnight at 37°C, 5% formic acid was added to each sample to hydrolyze the Rapigest. The samples were then concentrated, the Rapigest was removed, and the sample was desalted with zip tips (Millipore). For peptide sequencing analysis of GRFT, a Thermo LTQ XL and Dionex U3000 HPLC system were utilized. For peptide sequencing of Peak C, a Thermo LTQ Orbitrap XL and Waters nano-Acquity UPMC system were used. The same column system was used for both: PicoChip ReproSil-Pur C18-AQ 3µm 120Å HPLC column maintained at ambient temperature. The mobile phase gradient consisted of mobile phase A (0.1% formic acid in water), and mobile phase B (0.1% formic acid in acetonitrile) pumped at a flow rate of 300 nL/min. The gradient was proportioned as follows (minute, %B): 0, 2; 50, 40; 51, 95; 52, 95; 52.5, 2; and 60, 2.

3.2.6   SDS-PAGE of GRFT and Oxidative Products

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for GRFT and oxidative product samples (HPLC Peaks A, B, and C from both exposure types) to compare molecular masses of protein samples. An SDS-PAGE SOP was developed by Dr. Kenneth Palmer's laboratory and utilized for these experiments. In brief, BioRad precast Mini-PROTEAN® TGX Any kD gels, BioRad Precision Plus Protein™ Kaleidoscope™ Standards, and 1X Tris/Glycine/SDS Running buffer were used. GRFT samples were diluted in specified solutions to a concentration of 1-8µg protein (0.033mg/mL – 0.267 mg/mL for 30 µL well size). 30ul of sample was then combined with 30µL Laemmli sample loading buffer and boiled for 5 minutes. Samples were cooled on ice. 15µL of sample was loaded into a single well. At the end of the run, protein band detection was performed by Coomassie staining (Bio-Safe Coomassie Stain). Gel images were collected with a Gel DocTM EZ System (Bio-Rad).

3.2.7   gp120 Binding of GRFT and Oxidative Products

Samples of Peaks A, B (relative retention time, RRT, 0.900), and C (RRT 0.690) were created via hydrogen peroxide exposure and human CVL exposure (separately) and subsequent HPLC separation and collection of eluent. These collected peaks were evaluated for their ability to bind to gp120 via a gp120 binding ELISA. Briefly, gp120 is bound to the wells of a 96-well plate overnight at 4°C. HIV-1 gp120 CM was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Cat# 2968). Nunc MaxiSorp 96-well plates were used for this experiment. The MaxiSorp surface is a hydrophilic/hydrophobic mix that binds to a wide variety of biomolecules. The solution of gp120 was removed after overnight incubation, and a blocking solution was applied for two hours at room temperature. The wells were then washed and incubated for one hour with various dilutions of GRFT samples. gp120 binding was visualized

by goat anti-GRFT primary antibody (one hour incubation) and HRP-labeled rabbit anti-goat secondary antibody (one hour incubation). TMB substrate was applied to the wells after washing of the secondary antibody. Wells were allowed to develop (blue color) for approximately three minutes before the application of sulfuric acid to stop the reaction (yellow color). gp120 binding was measured by optical density (OD) at 450nm. OD values are then converted into % maximum binding values using the following equation:

$$(OD_{sample\ value}/OD_{GRFT250ng/mL})*100 = \%\ maximum\ binding\ value$$

Half maximal effective concentration (EC_{50}) values were determined using GraphPad Prism 6 software and compared with Student's t-test. P values of ≤ 0.05 will be considered statistically significant.

3.3   RESULTS

3.3.1   Hydrogen Peroxide and CVL Exposure

To investigate the stability profile of GRFT within a biologically relevant environment, GRFT was exposed, *in vitro*, to human CVL and compared to GRFT's stability profile upon hydrogen peroxide exposure. As seen in Figure 24, generation of degradants due to CVL exposure was similar to those generated from hydrogen peroxide exposure, based on HPLC relative retention times (RRT) – GRFT Peak A (RRT 1.000), Peak B (RRT 0.900), and Peak C (RRT 0.690).

Due to this similarity, GRFT degradant peaks resulting from hydrogen peroxide exposure and CVL exposure were further characterized through intact mass analyses, gp120 binding, and

SDS PAGE. Further, the amino acid sequence of recombinant GRFT and Peak C were determined.
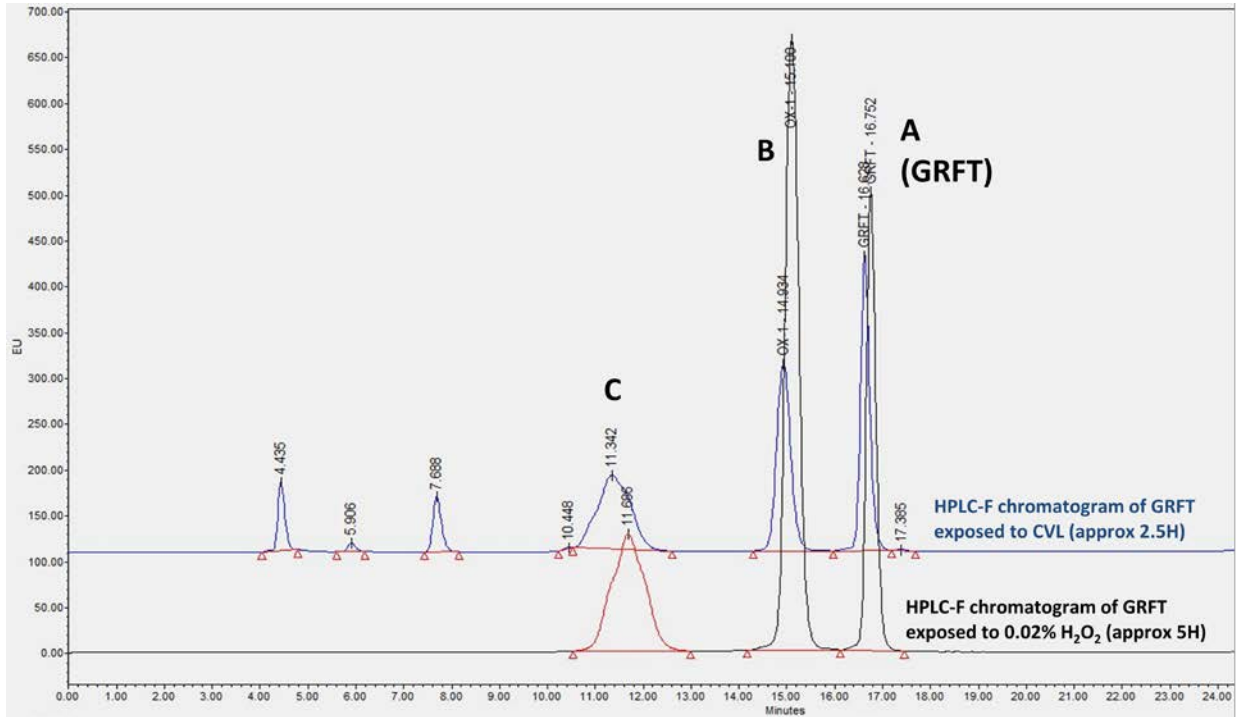
Figure 24. HPLC chromatograms of GRFT exposed to hydrogen peroxide and to human CVL.
Chromatograms of GRFT exposed to hydrogen peroxide and to human CVL (separately) are overlaid in
order to visually compare the chromatographic similarity. The bottom (black line) chromatogram represents
GRFT in 0.02% H2O2 at five hours. The upper (blue line) chromatogram represents GRFT in human CVL
at 2.5 hours of exposure. Peak A is GRFT. Peak B and C are oxidative products of GRFT.

3.3.2 Mass Spectrometry – Intact Mass

Due to the complex nature of human cervicovaginal secretions, there was initial concern that proteins in the CVL may interfere with detection of GRFT proteins in this sample matrix. However, no interference was present. The follow table (Table 8) shows the approximate concentrations of the protein samples evaluated for intact mass. These concentrations are based

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on HPLC peak areas. Despite lack of interference with CVL proteins, during preparing for intact

mass analyses, it was determined the GRFT-CVL samples needed to be concentrated. Therefore,

a potential miscalculation in GRFT protein concentrations in the CVL may have occurred. This

may be due to GRFT proteins binding to components, namely mucins [130-131] or cellular

debris [108], of CVL and not being available for HPLC detection/quantification. The non-

specific/off target bind potential of GRFT is further discussed within Chapter 4 of this

dissertation.

Table 8. GRFT Samples for Intact Mass Analysis

Sample	Approx. Conc. (µg/mL)
GRFT H_2O_2 Stress Peak A	14.5
GRFT H_2O_2 Stress Peak B	20.4
GRFT H_2O_2 Stress Peak C	6.3
GRFT CVL Stress Peak A	7.7
GRFT CVL Stress Peak B	11.2
GRFT CVL Stress Peak C	13.4

A summary of intact mass analyses can be found in Table 9 below.

Table 9. Summary of GRFT Intact Mass Analyses

	Homodimer	Heterodimer – One Monomer Oxidized	Monomer	Oxidized Monomer
GRFT H_2O_2 Peak A	X		X	
GRFT H_2O_2 Peak B		X	X	X
GRFT H_2O_2 Peak C				X
GRFT CVL Peak A			X	
GRFT CVL Peak B			X	X
GRFT CVL Peak C				X

Refer to Figures 25-30 for intact mass spectra for protein masses of Peaks A, B, and C

from H_2O_2 and CVL exposures.
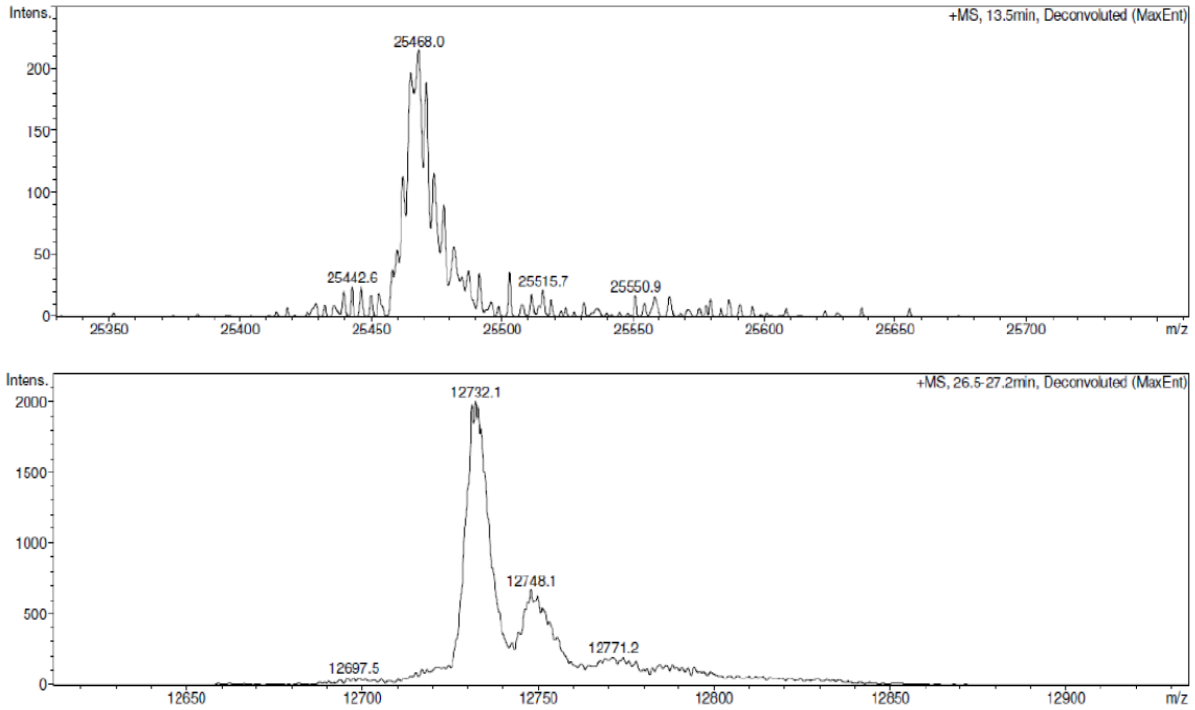
Figure 25. Intact Mass Analysis of GRFT exposed to hydrogen peroxide, Peak A.
HPLC-F Peak A eluent from GRFT-hydrogen peroxide exposure was collected, and the protein mass was determined with mass spectrometry.
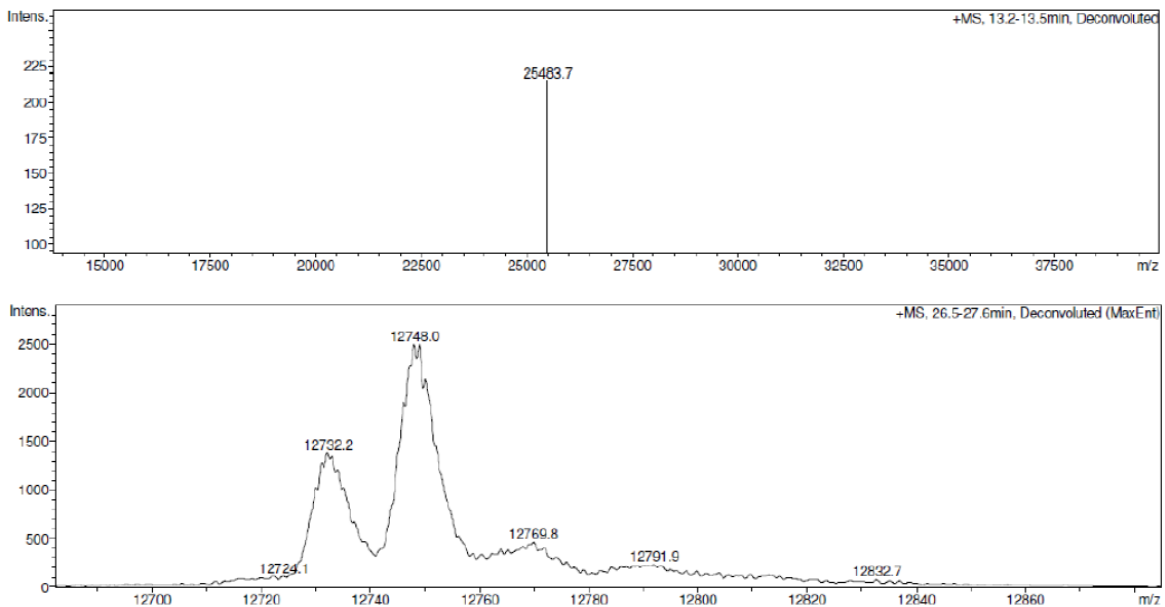
Figure 26. Intact Mass Analysis of GRFT exposed to hydrogen peroxide, Peak B.
HPLC-F Peak B eluent from GRFT-hydrogen peroxide exposure was collected, and the protein mass was determined with mass spectrometry.

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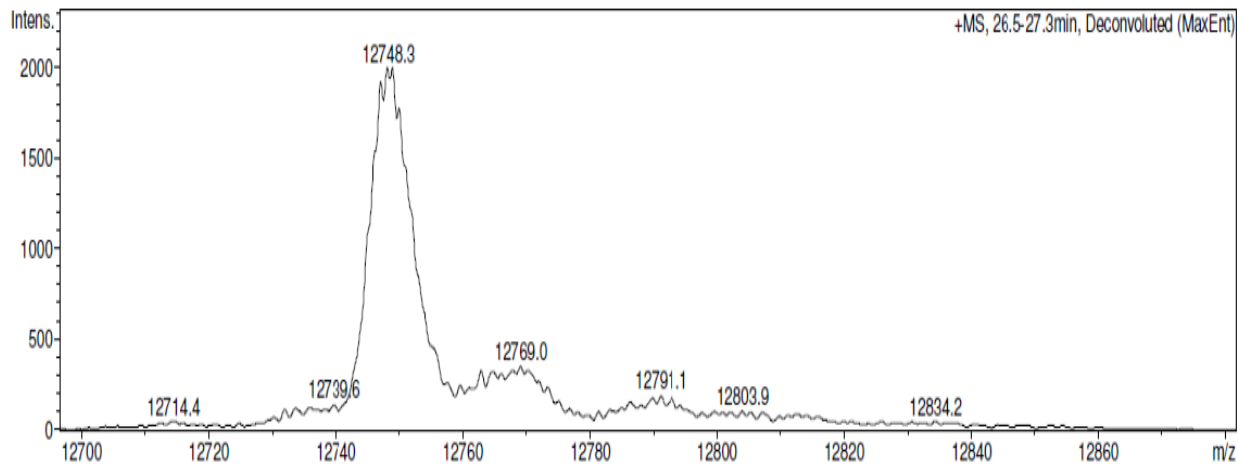
Figure 27. Intact Mass Analysis of GRFT exposed to hydrogen peroxide, Peak C.
HPLC-F Peak C eluent from GRFT-hydrogen peroxide exposure was collected, and the protein mass was determined with mass spectrometry.
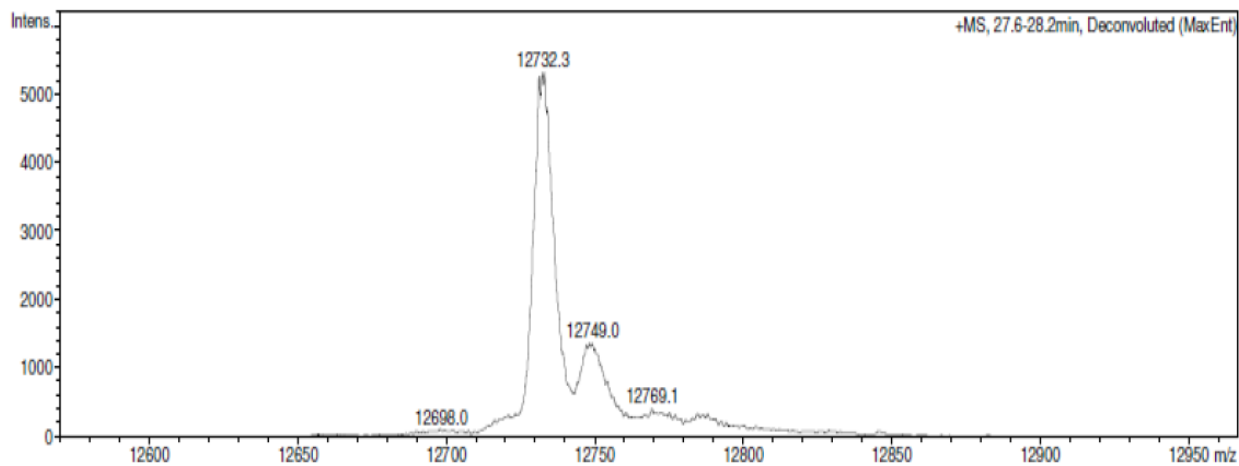
Figure 28. Intact Mass Analysis of GRFT exposed to human CVL, Peak A.
HPLC-F Peak A eluent from GRFT-human CVL exposure was collected, and the protein mass was determined with mass spectrometry.
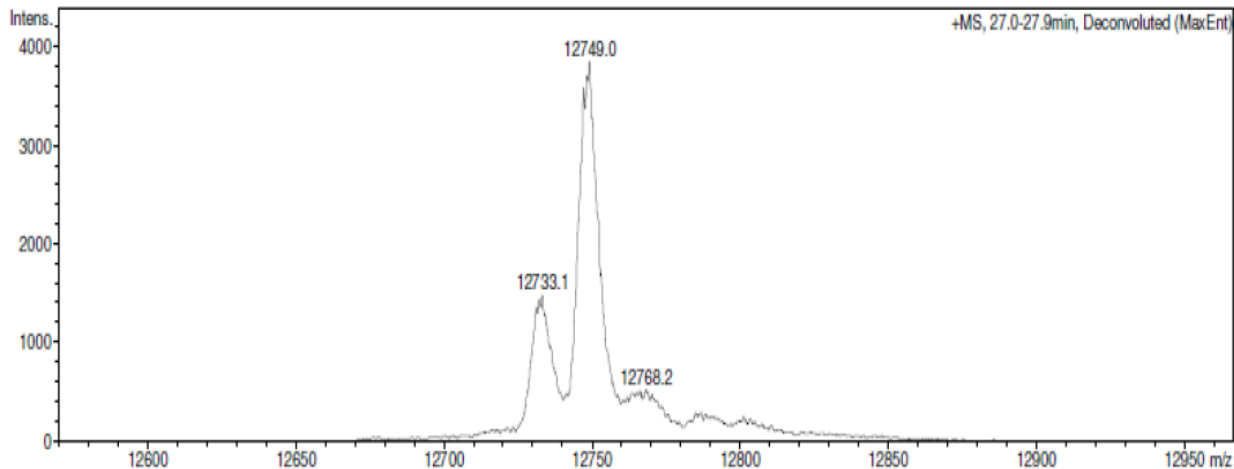
Figure 29. Intact Mass Analysis of GRFT exposed to human CVL, Peak B.
HPLC-F Peak B eluent from GRFT-human CVL exposure was collected, and the protein mass was determined with mass spectrometry.
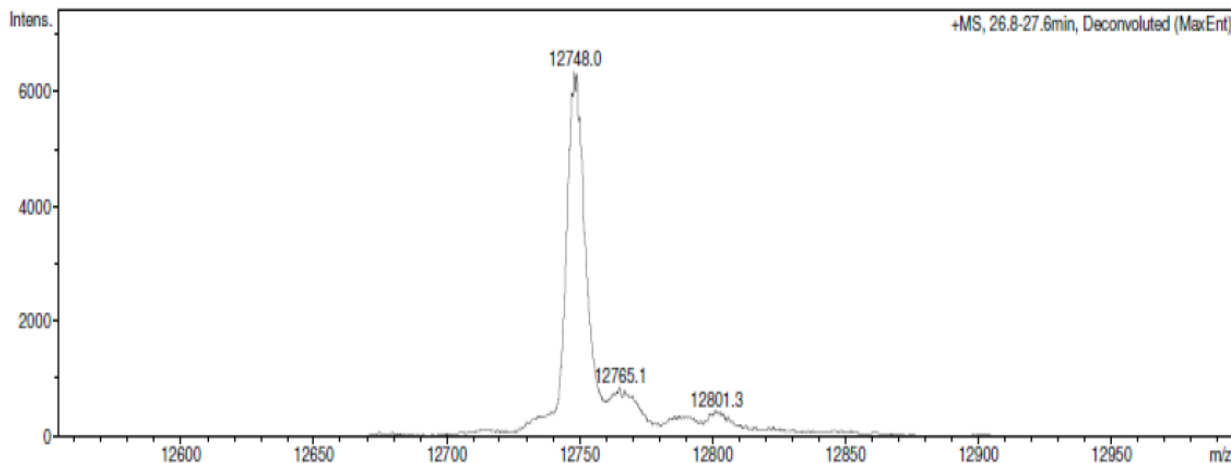
Figure 30. Intact Mass Analysis of GRFT exposed to human CVL, Peak C.
HPLC-F Peak C eluent from GRFT-human CVL exposure was collected and the protein mass was determined with mass spectrometry.

In the GRFT sample exposed to H_2O_2, Peak A (GRFT parent) contained the following molecular masses: 25468.0 and 12732.1Da. 28468.0 Da corresponds to the GRFT parent homodimer. 12732.1 Da corresponds to the GRFT parent monomer. Refer to Table 9 and Figure 25. In the GRFT sample exposed to H_2O_2, Peak B contains the following molecular masses:

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25483.7, 12732.2, and 12748.0 Da. 25483.7 Da corresponds to a GRFT heterodimer – one monomer has no oxidized amino acids, and one monomer contains one oxidized amino acid. 12732.2 Da corresponds to the GRFT parent monomer, and 12748.0 Da corresponds to the oxidized GRFT monomer (one amino acid is oxidized). The intensity of 12748.0 is approximately double that of 12732.2. Refer to Table 9 and Figure 26. In the GRFT sample exposed to H_2O_2, Peak C contained only 12748.3 Da. This mass corresponds to the oxidized GRFT monomer. Refer to Table 9 and Figure 27. In the GRFT sample exposed to CVL, Peak A (GRFT parent) contains the following molecular mass: 12732.3 Da, the GRFT parent monomer. Refer to Table 9 and Figure 28. In the GRFT sample exposed to CVL, the following molecular masses were present in Peak B: 12733.1 and 12749.0 Da, the GRFT parent monomer and oxidized GRFT monomer, respectively. Intensity of 12749.0 Da in comparison to 12733.1 Da was approximately three times greater. Refer to Table 9 and Figure 29. In the GRFT sample exposed to CVL, Peak C contains one molecular mass, 12748.0 which represents the oxidized GRFT monomer. Refer to Table 9 and Figure 30.

3.3.3   Mass Spectrometry – Peptide Sequencing

Several other important key pieces of information regarding GRFT have also been generated through peptide sequencing. The amino acid sequence of recombinant GRFT was determined to be the same as the published sequence of GRFT isolated from red alga *Griffithsia* sp.[96].

SLTHRKFGGSGGSPFSGLSSIAVRSGSYLDXIIIDGVHHGGSGGNLSPTFTFGSGEY
ISNMTIRSGDYIDNISFETNMGRRFGPYGGSGGSANTLSNVKVIQINGSAGDYLDSLDIYY
EQY

GRFT Peak C from H_2O_2 exposure also underwent peptide sequencing. This oxidative product contains an oxidized methionine at position 78, as indicated by bold and underlined font.

SLTHRKFGGSGGSPFSGLSSIAVRSGSYLDXIIIDGVHHGGSGGNLSPTFTFGSGEY ISNMTIRSGDYIDNISFETN**M**GRRFGPYGGSGGSANTLSNVKVIQINGSAGDYLDSLDIYY EQY

3.3.4 SDS-PAGE of GRFT and Oxidative Products

Figure 31. Oxidation profile (hydrogen peroxide exposure) of GRFT on SDS-PAGE.
Separation of proteins, detected by Coomassie blue staining on a BioRad precast Mini-PROTEAN® TGX Any kD gel. The first lane contains Peak A (GRFT). The second lane contains Peak B, and the third lane contains Peak C. The last lane contains protein molecular weight markers.

SDS PAGE was also employed to investigate the molecular masses of H_2O_2-oxidative products.

Peaks A, B, and C from hydrogen peroxide exposure displays faint, but sharp bands between the

10 and 15 KD markers. This protein banding pattern is characteristic of GRFT or proteins with a very similar molecular mass using this gel type (BioRad precast Mini-PROTEAN® TGX Any kD gel) and these molecular weight markers (BioRad Precision Plus Protein™ Kaleidoscope™ Standards). In regards to protein concentration: Peak B (20.4 µg/mL) > Peak A (14.5 µg/mL) > Peak C (6.3 µg/mL). In regards to actual amount (µg) of protein loaded onto the gel, the same pattern exists: Peak B (0.153 µg) > Peak A (0.109 µg) > Peak C (0.0473 µg). These details are shown in both Table 8 and Figure 31. Further, the quantitative mass spectrometry mass analysis data support this qualitative SDS PAGE data.

3.3.5   gp120 Binding of GRFT and Oxidative Products

To determine if oxidation of GRFT affects its gp120 binding ability, a gp120 binding ELISA was employed. Results from the gp120 binding ELISA experiments can be found in Figure 32. All H_2O_2 oxidative samples show comparable % binding curves to each other and in comparison to GRFT standard. However, CVL oxidative samples display binding curves that have shifted down and to the right, indicating decreased GRFT-gp120 binding in all three samples. The half maximal effective concentration (EC_{50}) of GRFT during these two exposures was also determined. The EC_{50} represents the GRFT concentration which produces a response (% GRFT binding to gp120) that is halfway between the baseline of the curve (bottom of the curve) and the maximal response of the curve (top of the curve). The EC_{50} for GRFT-CVL oxidative samples was 2.110 nM (53.73 ng/mL), which is approximately 3X that of the GRFT-H_2O_2 oxidative samples (0.728 nM; 18.54 ng/mL) and statistically different ($p < 0.0001$). The sample protein calculations that were used for these samples during intact mass analyses were used for the ELISA experiments. Therefore, as previously mentioned in addition to oxidation, in the presence

of CVL, GRFT may be undergoing non-specific binding with glycoproteins of cervicovaginal mucus and cells, which may cause a decrease in GRFT-gp120 binding, as reflected in Figure 32.
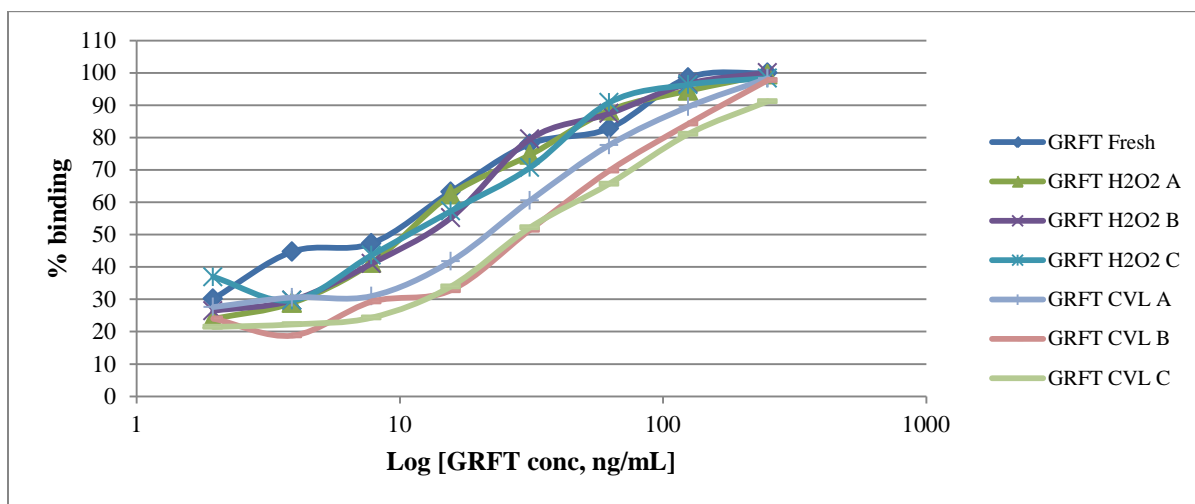
Figure 32. % maximum binding of GRFT oxidative products from hydrogen peroxide and human CVL exposures.
The binding curves for GRFT from a gp120 binding ELISA compare GRFT in normal saline (GRFT Fresh) to each peak eluent (Peaks A, B, and C) from the two oxidative exposure conditions (hydrogen peroxide and human CVL).

3.4   DISCUSSION AND CONCLUSIONS

When GRFT was stressed with 0.02% H_2O_2, it was found to be highly susceptible to oxidative degradation. Upon GRFT exposure to human CVL, GRFT displayed a similar degradation pattern. To investigate these conditions, intact mass analysis and peptide sequencing were performed on GRFT and GRFT oxidative products after exposure to both H_2O_2 and human CVL. The same chemical modification that occurred to GRFT due to 0.02% H_2O_2 also occurred to GRFT when it is exposed to human CVL, as shown by LC-MS intact mass analyses of Peak A (GRFT), Peak B, and Peak C. Pertaining to GRFT-related monomers, all three fractions in both exposures contained the same pattern of molecular mass(es). Peak A contained the unoxidized monomer. Peak B mainly contained oxidized monomer and a small portion of unoxidized

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monomer. Peak C only contained the oxidized monomer. The oxidized monomer is 16 Da more than the unoxidized monomer, indicating that only one amino acid is oxidized. SDS PAGE of Peaks A, B, and C from hydrogen peroxide exposure display molecular weight bands similar to the molecular weight of GRFT – a single oxidation (16Da) would not affect the GRFT/GRFT oxidative product/degradant band (approximately 12.7 kDa) in this type of qualitative analysis. These results support our hypothesis that GRFT is chemically unstable (is oxidized) upon exposure to human cervicovaginal secretions. To investigate which amino acid was oxidized in this protein, peptide sequencing was performed on Peak C obtained from the 0.02% H_2O_2 exposure.

1   18
SLTHRKFGGSGGSPFSGL

19   56
SSIAVRSGS**Y**LDXIIIDGV**HH**GGSGGNLSPTFTFGSGE

57   100
YISNM**T**IRSGD**Y**IDNISFETN**M**GRRFGP**Y**GGSGGSANTLSNVKV

101   121
IQINGSAGD**Y**LDSLDI**YY**EQ**Y**
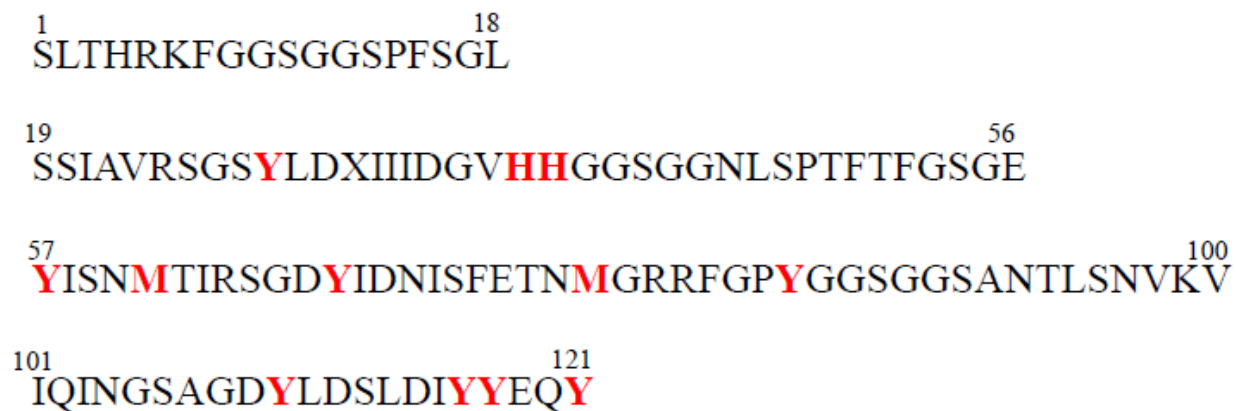
Figure 33. Primary structure of GRFT with potential sites of oxidation highlighted in red.

Given the knowledge of oxidizable amino acids, GRFT primary structure (Figure 33), and GRFT tertiary structure, it is most likely that either the methionine (Met) at position 61 or 78 is oxidized due to their orientation within the GRFT tertiary structure. Further, Met 78 is more surface exposed than Met 61, and therefore, probably more likely prone to oxidation in solution. Peptide sequencing of Peak C from hydrogen peroxide exposure indicated an oxidized Met 78. This result further supports our hypotheses regarding the GRFT oxidation profile – this study confirmed predicted results.

During preparing for intact mass analyses, CVL samples containing GRFT needed to be concentrated due to the extremely low intensities for masses in the non-concentrated samples. Further, the GRFT oxidative products from CVL exposure displayed decreased gp120 binding in comparison to their H_2O_2 counterparts. GRFT is a lectin and binds to high-mannose N-linked glycoproteins [96, 106-107]. Therefore, the potential exists for GRFT to bind, non-specifically to membrane bound and unbound mucins within the cervicovaginal environment, which can negatively impact anti-HIV activity [130-131]. This degradation pathway and decreased gp120 binding activity of CVL exposed oxidative products needs to be taken into consideration. Unfortunately, no anti-oxidants protect GRFT from oxidative degradation [132]. Therefore, an alternative approach to eliminate this degradation and concern for decreased bioactivity *in vivo*, would be to mutant Met 78 to a non-oxidizable amino acid during protein manufacture.

During preformulation assessments of GRFT, a microbicide candidate, oxidative degradation was found to be this protein's major pathway of instability. Upon exposure to human CVL, the oxidation of GRFT was also confirmed. With exogenous (hydrogen peroxide) and endogenous (CVL) oxidative stresses, the amino acid, methionine, at position 78 is oxidized to methionine sulfoxide. Although a number of potential antioxidants commonly used in pharmaceutical products were evaluated, none were shown to prevent oxidation of GRFT. For this reason, in order to eliminate GRFT oxidative potential within formulation and upon intravaginal delivery, the most promising strategy may be modification of Met 78 to a non-oxidizable amino acid. This strategy is currently being explored with the laboratory of Dr. Kenneth Palmer at the University of Louisville and Dr. Barry O'Keefe at NCI.

ACKNOWLEDGEMENTS

4.0   EVALUATION OF THE EFFECT OF HUMAN CERVICOVAGINAL SECRETIONS ON GRIFFITHSIN BINDING TO GP120

4.1   INTRODUCTION

4.1.1   Human Cervical Mucus

Major contributions to cervical mucus are water (95-98%) and mucins (2-5%). Mucins are high molecular weight, heavily glycosylated proteins. They can possess molecular masses of between 10-40MDa, with 80% of the mass due to branched polysaccharides or oligosaccharides (carbohydrate/glycan) chains. Mucins are one type of glycoprotein. A glycoprotein is a glycoconjugate in which one or more glycans (polysaccharides or oligosaccharides) are covalently attached to a protein backbone, usually via an O or N linkage. Both O and N linked glycans are found on mucins. N-linked glycans are linked to a protein backbone via an amide bond to asparagine (Asn), in an Asn-X-Ser (serine) or in an Asn-X-Thr (threonine) motif, where X is any amino acid residue except proline. O-linked glycans are linked to the hydroxyl group of serine or threonine of the protein backbone. Glycoproteins containing N-linked glycans are of interest to this project (discussed throughout this dissertation chapter) [133].

All N-linked glycans are based on the common core pentasaccharide (five sugars), $Man_3GlcNAc_2$. $Man_3GlcNAc_2$ = 3 Mannose + 2 GlcNAc (2 glucose that have N-acetyl group on

them). There are three main classes of N-linked glycans: high-mannose (oligomannose), hybrid, and complex. Refer to Figure 34 [133]. High-mannose glycans contain unsubstituted terminal mannose sugars. These glycans typically contain between five and nine mannose residues attached to the chitobiose (GlcNAc) core. The glycan name abbreviations are indicative of the total number of mannose residues in the structure. For example, high mannose glycan Man_5 contains five mannose residues, and Man_9 contains nine mannose residues [133].

Hybrid glycans are characterized as containing both unsubstituted terminal mannose residues (as are present in high-mannose glycans) and substituted mannose residues with N-acetylglucosamine linkage (as are present in complex glycans). These GlcNAc sequences added to the N-linked glycan core in hybrid and complex N-glycans are called "antennae." A biantennary glycan has two GlcNAc branches linked to the core. A triantennary glycan has three GlcNAc branches linked to the core [133].

Complex N-linked glycans differ from the high-mannose and hybrid glycans by having added GlcNAc residues at both the α-3 and α-6 mannose sites. Unlike high mannose glycans, complex glycans do not contain mannose residues apart from the core structure. Additional monosaccharides may occur in repeating lactosamine (GlcNAc-β(1$\rightarrow$4)Gal) units. Complex glycans exist in bi-, tri-, and tetraantennary forms and make up the majority of cell surface and secreted N-glycans. Complex glycans commonly terminate with sialic acid residues [133].
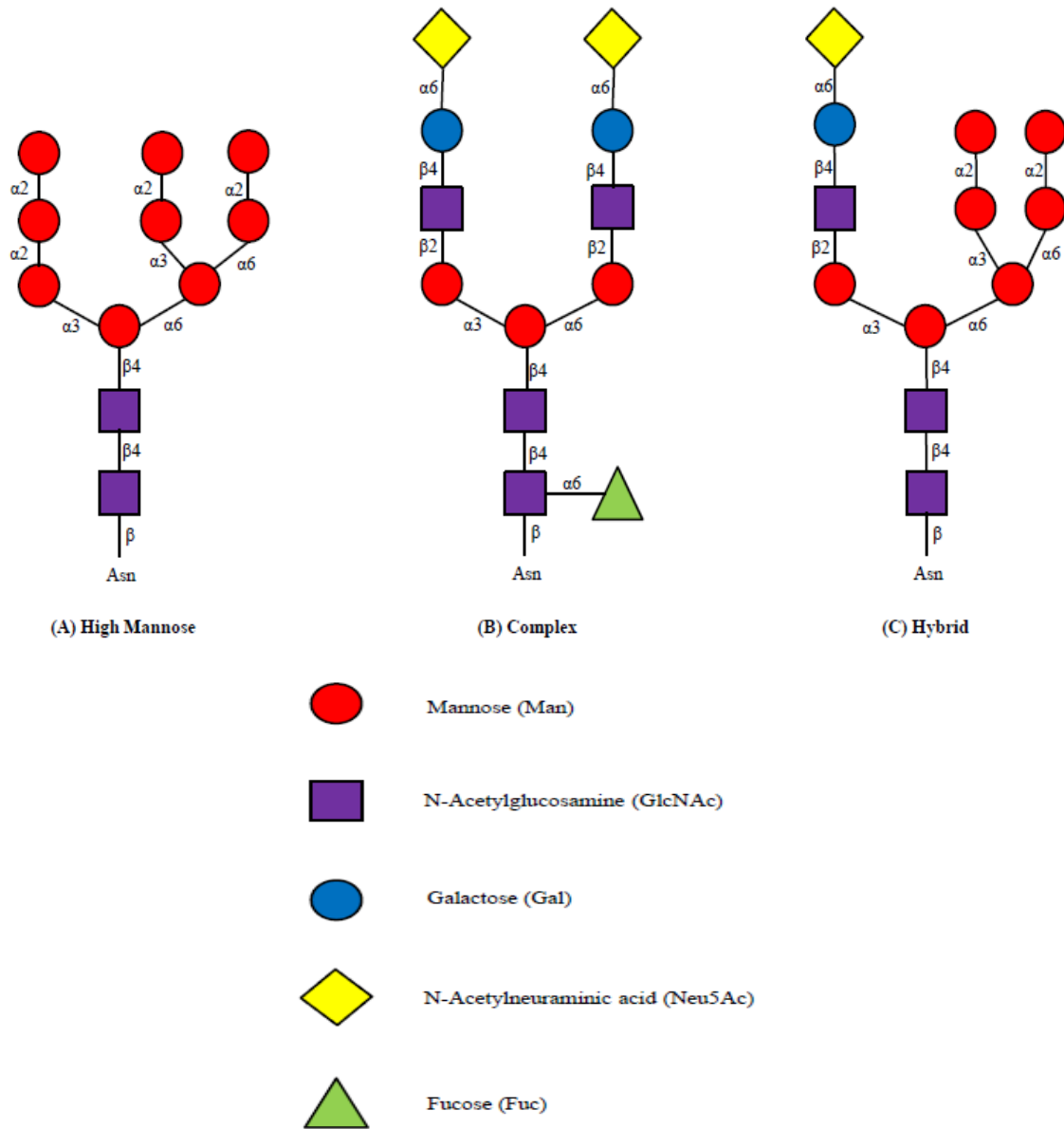
Figure 34. Types of N-glycans.
Each N-glycan contains the common core Man3GlcNAc2.

Sources of mucus secretions include the goblet cells within the columnar epithelium of the endocervix and the Bartholin's glands. The squamous epithelium of the vagina and ectocervix does not play a role in mucus production. The physical characteristics, composition, and volume of mucus secretions of the endocervical epithelium are dependent on the menstrual

cycle, which deems this secretion production as estrogen dependent. At the time of ovulation, the amount of cervical secretions increases, resulting in an increase in the overall volume of vaginal fluid. An increase in pH and mucin content and a decrease in viscosity are also evident. Cervical mucus has a complex net-like structure resembling interlacing microfibers. Orientation and pore size are influenced by circulating hormones. Under estrogen stimulation (during ovulation), mucus pore size is larger than when under progesterone stimulation. Additionally, during ovulation mucus fibers run parallel to each other and are long and thick, making the mucus less viscoelastic. Such organization is suitable for ease of sperm entry. Conversely, the nonovulatory mucus structure becomes dense and compact, which is unfavorable for sperm entry. Mucus-penetrating nanoparticles (MPP) have been utilized to assess the pore size of human cervicovaginal mucus. This assessment found the average pore size to be 340 ± 70 nm [48]. These dynamic changes in fluid volume and physical makeup can modify the drug release profiles from intravaginal formulations and alter drug targeting *in vivo*. Changes in mucus network pore size also have the ability to affect drug targeting.

Mucus has the ability to maintain an unstirred layer adjacent to epithelial surfaces despite the shearing action that occurs during vaginal intercourse. The depth of the unstirred layer is determined by the balance between the rate of secretion and the rate of shedding/degradation. Drugs delivered vaginally which have targets within the mucosa must move through and penetrate the unstirred layer before it is shed or degraded [49].

Within the vagina, cervical mucus can act as lubrication and a natural defense barrier to pathogens, of both bacterial and viral origin [134-137]. Glycosylation of proteins in cervicovaginal secretions has been shown to play an important anti-microbial role in this

environment [138-139]. Vaginal microflora may impact the glycosylation of proteins in human cervicovaginal secretions.

4.1.2 Bacterial Vaginosis

One pathogenic condition of the vagina that alters the microflora is bacterial vaginosis (BV). BV represents a common and complex change in the vaginal microflora that is characterized by a decrease in hydrogen peroxide producing *Lactobacillus* sp. and an increase in other organisms, especially anaerobic and facultative bacteria. BV-associated bacteria include *Gardnerella vaginalis*, *Prevotella* sp., *Porphyromonas* sp., *Bacteroides* sp., *Peptostreptococcus* sp., *Mycoplasma hominis*, *Ureaplasma urealyticum*, *Mobiluncus* sp., *Fusobacterium* sp., and *Atopobium vaginae* [140]. In clinical practice, BV is diagnosed using Amsel criteria [141-142]. For Amsel criteria, at least three of the following criteria must be present: (1) homogeneous, thin, grayish-white discharge that smoothly coats the vaginal walls; (2) vaginal pH > 4.5; (3) positive whiff-amine test, defined as the presence of a fishy odor when a drop of 10% potassium hydroxide is added to a vaginal discharge sample; (4) clue cells on saline wet mount. Clue cells are vaginal epithelial cells covered with coccobacilli that often obscure the margins of the cell. For a positive BV result, at least 20% of the epithelial cells on wet mount should be clue cells. The presence of clue cells is the single most reliable predictor of BV [143]. In clinical research studies, BV is diagnosed using Nugent criteria/score. Gram stain of vaginal discharge is the gold standard for BV diagnosis, but it is mainly performed during research studies because it requires more time, resources, and expertise than Amsel criteria. The Gram-stained vaginal discharge smear is evaluated using Nugent criteria/score. Nugent criteria is a scoring system based on

bacteria morphotypes present [144]. A total score of 7 to 10 is indicative of BV infection, 4 to 6 is intermediate/indeterminate, and 0 to 3 is normal (lactobacilli dominant).

Bacterial vaginosis has significant effects on human CVL viscosity, pH, glycan profile, and lectin binding. Chappell et al. found that CVL pH was highest among women with BV (4.9) as compared to women with intermediate (4.6) or normal (4.5) flora, which supports diagnostic criteria. Further, this group showed an inverse linear relationship between CVL viscosity and pH: women with BV (increased pH) had decreased CVL viscosity. However, the total protein content of normal versus BV CVL was found not to differ [145], indicating that the decreased viscosity of BV CVL was more likely due to mucin-degrading (glycan-cleaving) enzymes and not due to decreased protein content. Decreased CVL viscosity, caused by BV-associated enzymes, and decreased pH, caused by decreased presence of *Lactobacillus* sp., can make the cervical mucus to be more permissible to pathogens, like HIV. These physiochemical changes noted with BV CVL provide insight into the mechanism by which HIV acquisition and transmission are increased during BV [146-147].

Chappell et al. showed that CVLs from women with BV were associated with increased glycolytic enzymes and decreased lectin binding to both high mannose (GRFT) and a-2,6 sialic acid Sambucus nigra agglutinin (SNA) [130]. Glycomic profiling, utilizing a lectin microarray system [148-149], showed that CVLs from women with BV contained lower levels high-mannose glycans. This group observed a loss of the binding of GRFT in BV CVL. GRFT binds specifically to the Man_7-Man_9 high mannose glycan structures. This group did not observe a binding difference in the CVL (normal versus BV) of four other mannose-binding lectins. These four lectins can also bind Man_5-Man_6, which proposes that high mannose glycan loss is limited to Man_7-Man_9 [150].

4.1.3   Mucins and Their Interaction with Griffithsin

GRFT exists as a protein homodimer, with each monomer containing three binding sites for HIV gp120 [103] . In order for GRFT to effectively act upon gp120 within the cervicovaginal lumen, its binding pockets must be clear of unwanted substrates. Given information known about GRFT and cervicovaginal secretions, GRFT-gp120 binding may be negatively impacted in this target administration site: (1) GRFT is a lectin and binds to N-linked high mannose glycans; (2) N-linked glycans are present in human cervicovaginal secretion, with normal secretions containing more than BV secretions; and (3) decreased GRFT binding is found in BV cervicovaginal secretions in comparison to normal secretions. With all points considered collectively, we hypothesize that GRFT-gp120 binding will be negatively impacted in the presence of human cervicovaginal secretions. Specifically, normal human cervicovaginal secretions will inhibit GRFT-gp120 binding more than BV secretions because BV secretions contain less N-linked high mannose glycans.

The potential impact of high-mannose glycoproteins found in human cervicovaginal secretion on *in vitro* markers related to GRFT bioactivity needs to be investigated before moving forward with *in vivo* studies. The objective of this study was to investigate the GRFT-gp120 binding profile (gp120 binding ELISA) in the presence of CVL, both normal and with BV. Utilizing human CVL samples, the best ratio of GRFT protein to CVL protein for this gp120 binding enzyme-linked immunosorbent assay (ELISA) was determined. The amount of protein in each human CVL has previously been determined [145]. Further, gp120 binding ELISA results (OD values, % maximum binding values) of GRFT binding curves in normal CVL samples and CVL samples with BV were compared. Overall, the results generated will be important to gain

knowledge of potential barriers to successful *in vivo* bioactivity when GRFT is vaginally administered.

4.2   MATERIALS AND METHODS

Recombinant Griffithsin drug substance was manufactured by Kentucky Bioprocessing LLC (KBP; Owensboro, KY) and provided to our laboratory by Dr. Kenneth Palmer of the University of Louisville School of Medicine. The protein was supplied in a solution of phosphate buffered saline.

4.2.1   Human Cervicovaginal Lavages

Human cervicovaginal lavages (CVLs) were obtained at the University of Pittsburgh under IRB Protocol PRO14040250. This protocol was a subprotocol (use of archive samples) of the original University of Pittsburgh IRB protocol PRO10080337. The purpose of the protocol was to characterize the physical properties of human cervicovaginal secretions [145]. Informed consent was obtained from healthy, HIV-negative women who were either between 18-46 (pre-menopausal) or over 50 years of age (post-menopausal). Enrolled premenopausal women fit into five categories based on self-reported hormonal contraceptive use: (1) no hormonal contraception, days 1-14 of menstrual cycle (follicular phase); (2) no hormonal contraception, days15-28 of menstrual cycle (luteal phase); (3) using combined oral contraceptives for a minimum of six months; (4) using depot medroxyprogesterone acetate injections for a minimum of six months; (5) using levonorgestrel intrauterine device for at least one month. The menopause definition used was 50 years of age or older without vaginal bleeding for one year.

Women were excluded from study participation if they had been pregnant or breastfeeding within the past three months, had vaginal symptoms or vaginitis upon clinical examination, reported use of vaginal products in past week, reported use of antibiotics in past two weeks, had a hysterectomy, or had a positive rapid HIV test. Postmenopausal women who reported exogenous estrogen use were also excluded [145]. Vaginal swab for pH, wet mount microscopy, and Gram stain was collected for all participants. Nugent criteria was used to evaluate Gram stains [144].

CVLs were collected with 10 mL of normal saline. The saline was inserted into the vagina with a syringe. A wash/lavage was performed in the cervicovaginal vault for one minute. The CVL was placed into a 15 mL conical tube and stored on ice for transportation to laboratory within one hour. In the lab, 100uL of protease inhibitor was added to the CVL, and the CVL was aliquoted (1 mL aliquots) into 2 mL cryovials and stored at -80°C until thawed for immediate testing. The protein concentrations of the CVL samples were determined using a Lowry assay [151]. The subprotocol was allotted ten sample aliquots from the original protocol.

4.2.2 gp120 Binding ELISA

An Enzyme-linked Immunosorbent Assay (ELISA) was employed to evaluate the gp120 binding activity of select GRFT in the presence of human CVL. Briefly, gp120 is bound to the wells of a 96-well plate overnight at 4°C. HIV-1 gp120 CM was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Cat# 2968). Nunc Maxisorp 96-well plates were used for this experiment. The MaxiSorp surface is a hydrophilic/hydrophobic mix that binds to a wide variety of biomolecules. The solution of gp120 was removed after overnight incubation, and a blocking solution was applied for two hours at room temperature. The wells were then washed

and incubated for one hour with various dilutions of GRFT samples. gp120 binding was visualized by goat anti-GRFT primary antibody (one hour incubation) and HRP-labeled rabbit anti-goat secondary antibody (one hour incubation). TMB substrate was applied to the wells after washing of the secondary antibody. Wells were allowed to develop (blue color) for approximately three minutes before the application of sulfuric acid to stop the reaction (yellow color). gp120 binding was measured by optical density at 450nm on a plate reader. The OD values for the experimental samples will be divided by the OD values of the maximum of the standard curve. OD values are then converted into % maximum binding values using the following equation:

$$(OD_{\text{sample value}}/OD_{\text{GRFT250ng/mL}})*100 = \% \text{ maximum binding value}$$

The diluent was normal saline since normal saline was used as the wash for human CVL collection. Optical density (OD) values for the normal saline provided the background subtraction level for the experiment. GRFT combined with the normal saline provided standards for comparison of experimental samples. GRFT combined with CVL served at the experimental samples. These experimental results were compared to those of GRFT in normal saline to determine the degree to which CVL (both normal and BV) negatively affects GRFT binding to gp120.

4.2.3   Statistical Analyses

OD values were converted to % maximum binding values as described in Section 4.2.2 of this chapter. % maximum binding was compared using repeated-measures analysis of variance. Groups were compared using global (type III) tests to compare % binding across the entire GRFT curve as well as pointwise comparisons along the GRFT curve at prespecified

concentrations. Analyses were performed using SAS version 9.4 (SAS Institute, Cary, NC). The GRFT curves represent means ± standard deviations. EC_{50} values were determined using GraphPad Prism 6 software. P values of ≤ 0.05 will be considered statistically significant.

4.3   RESULTS

4.3.1   Human Cervicovaginal Lavages

The small human CVL sample set (n = 10) used for gp120 ELISA comparing normal versus BV CVL effects on GRFT-gp120 binding is of the larger sample size (n = 165) characterized in previously studies [130, 145, 152]. For the comparative gp120 binding ELISA experiments, interest lies in the microflora (normal versus BV) status and the total protein concentration of each sample aliquot. Table 10 displays the total protein content values (mean ± standard deviation) for the ten CVL sample aliquots used in the comparative gp120 binding ELISA experiment. As can be seen, these results for total protein content are not consistent with those of the larger group [145]. These results are reflective of mg/mL values converted into mg by multiplying mg/mL by mL (exact volume that was provided for this experiment; between 0.700mL-1mL). The results of the larger sample set would have taken into account the entire volume (10 mL or more) of each CVL and a larger sample size of CVLs. Thereby, variability was reduced. Also, of important note, is that of the ten CVL sample aliquots used in this study, three women provided two samples each (six samples total from three separate women, two

samples each). For these three women, at one sample collection their CVLs tested normal and at the other sample collection their CVLs tested positive for BV.

Table 10. Total Protein Content of Human Cervicovaginal Lavages

Variable	Total Protein, mg
Normal CVL, n = 5	1.936 ± 1.129
BV CVL, n = 5	0.559 ± 0.390
p value	*0.03*

4.3.2   gp120 Binding ELISA

Developmental Processes

Prior to performing the comparative gp120 ELISA with the normal flora and BV CVLs, it was necessary to perform some developmental work with the ELISA. In order to perform and analyze the comparative experiment, total protein concentration across samples must be controlled. To control this variable, an acceptable CVL total protein concentration to use with all secretion samples to be tested on the ELISA needed to be determined. This determination was achieved by using pooled CVL sample aliquots and additional normal flora and BV CVL sample aliquots. None of the sample aliquots used for the comparative gp120 ELISA was used during the development process. The protein concentrations of all of CVLs used during this development process were determined with the same Lowry assay, as previously described [151]. Table 11 represents all of the CVL total protein level dilutions that were tested during development for the gp120 ELISA comparative experiment. Two levels (250µg/mL and 500µg/mL) were the most

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helpful in determining the CVL total protein level to be used in the comparative normal versus

BV CVL gp120 binding ELISA experiment.

Table 11. Tested Dilutions of CVL Total Protein Concentrations

µg/mL levels
0.250
0.500
1
2
4
8
16
32
64
100
150
200
250
375
500
1000

Figure 35. GRFT-gp120 binding curves for GRFT in cervicovaginal lavages - DEVELOPMENT.
GRFT was combined with CVL containing normal flora at two total protein concentrations, 250ug/mL and 500ug/mL. GRFT was combined with CVL with BV at two total protein concentrations, 250ug/mL and 500ug/mL. GRFT concentrations in normal saline were used as a standard curve.

Table 12. GRFT-gp120 Binding Curves for GRFT in Cervicovaginal Lavages – DEVELOPMENT (*p* values)

	GLOBAL TEST	GRFT (ng/mL)					
		250	*125*	*62.5*	*31.25*	*7.8125*	*3.90635*
BV 250µg/mL vs. Standard	0.099	0.514	0.861	0.183	0.079	0.029	0.003
BV 500µg/mL vs. Standard	0.110	0.694	0.419	0.402	0.098	0.019	0.008
Normal 250µg/mL vs. Standard	0.025	0.017	0.023	0.027	0.019	0.141	0.019
Normal 500µg/mL vs. Standard	<.001	0.009	0.004	0.002	0.003	0.052	0.019

Figure 35 and Table 12 display the complete set of meaningful results obtained from the developmental work. Taken together, CVL containing normal flora, at both protein levels of 250 and 500µg/mL, produced a significantly different GRFT-gp120 binding curve in comparison to the standard curve (GRFT in normal saline). CVL with BV, at neither total protein dilution level, did statistically affect the overall GRFT-gp120 binding curve in comparison to the standard curve. However, pointwise comparisons along the GRFT curve at the lower end of the curve do show statistical significance.

In order to finalize the development process, statistical difference between the CVL containing normal flora at both total protein levels of 250 and 500µg/mL was determined. Figure 36 pictorially displays these two GRFT-gp120 binding curves. Table 13 includes the statistical results for these two curves. There is no statistical difference between these two curves (*p* = 0.250).
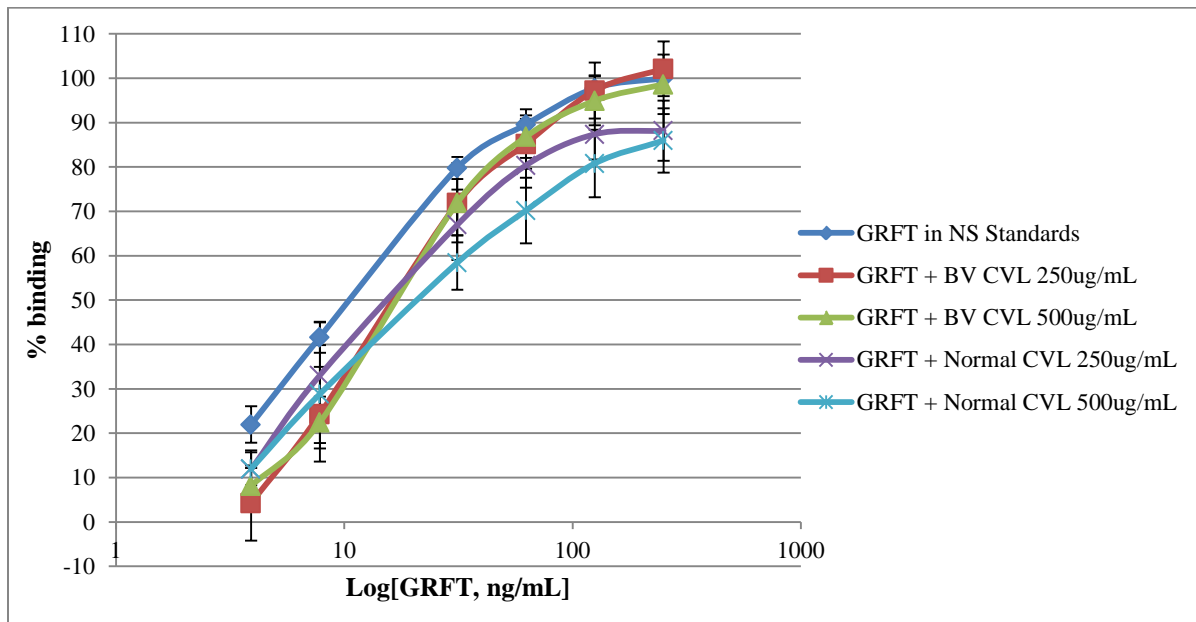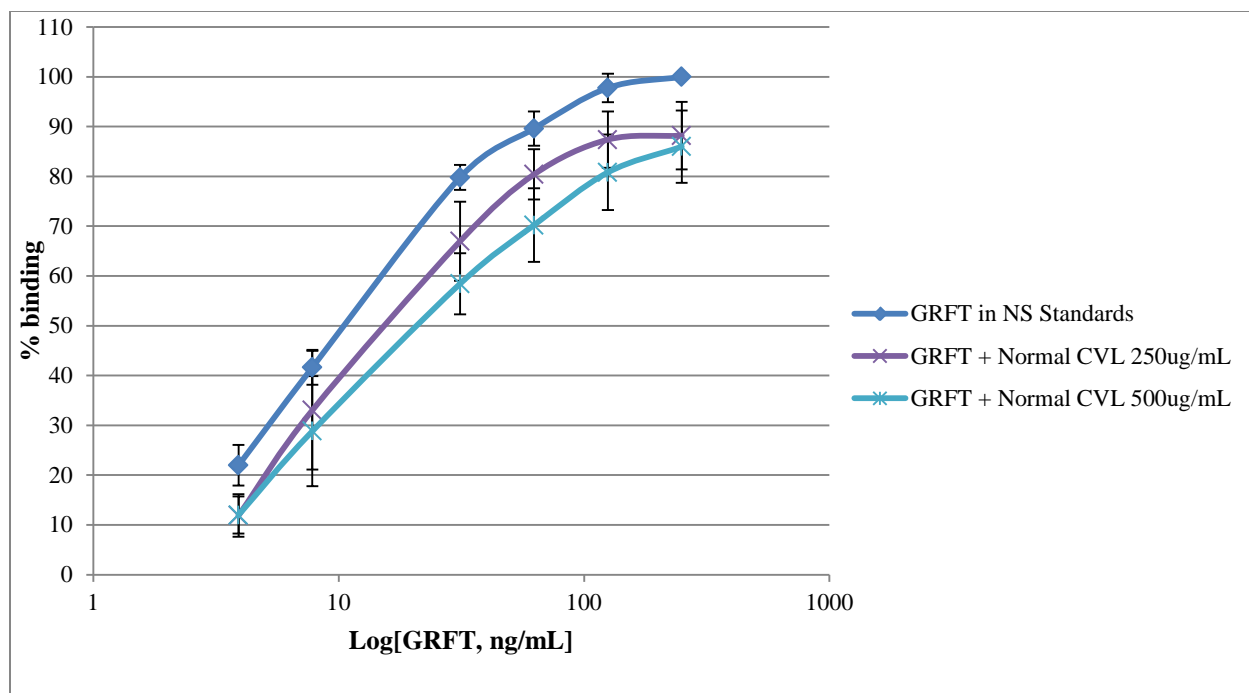
Figure 36. GRFT-gp120 binding curves for GRFT in cervicovaginal lavages with normal flora - DEVELOPMENT.
GRFT was combined with CVL containing normal flora at two total protein concentrations, 250μg/mL and 500μg/mL. GRFT concentrations in normal saline were used as a standard curve.

Table 13. GRFT-gp120 Binding Curves for GRFT in Cervicovaginal Lavages with Normal Flora – DEVELOPMENT (*p* values)

	GLOBAL TEST	GRFT (ng/mL)					
		250	*125*	*62.5*	*31.25*	*7.8125*	*3.90635*
Normal 250μg/mL vs. Normal 500μg/mL	0.250	0.499	0.086	0.020	0.065	0.420	0.971

From these results, the total protein concentration dilution to use for the comparative normal versus BV CVL GRFT-gp120 binding ELISA was determined: 250μg/mL total CVL protein concentration. Also, a practical perspective factored into this decision. To use a total protein concentration of 500μg/mL would use more volume of the already limited volume of the

CVL sample aliquots. Therefore, given the limited volume per aliquot (0.700 mL – 1 mL), utilizing a CVL total protein concentration of 250µg/mL was further preferred.

Normal versus BV CVL

The effects of CVL, normal microflora versus BV, on GRFT-gp120 binding were investigated with a gp120 binding ELISA. Ten CVL sample aliquots were tested for their effects on GRFT-gp120 binding: five CVL samples containing normal flora and five with BV. The total protein concentration of all CVL samples was normalized to a concentration of 250µg/mL, prior to the addition of GRFT. As shown in Figure 37 and Table 14, the GRFT binding curves in BV CVL were statistically different than GRFT standard binding curves ($p = 0.016$), and the GRFT curves in Normal CVL were statistically different than GRFT standards ($p = <0.001$), with normal CVL having a much stronger effect. However, no statistical difference was found between the GRFT curves of normal versus BV CVL ($p = 0.303$). This result is due to three pointwise comparisons along the GRFT curve carrying a stronger weight of non-significance than the three significant pointwise comparisons. Refer to Table 14. Additionally, the half-maximal effective concentration (EC_{50}) of GRFT-gp120 binding of GRFT in standards, GRFT in CVL with normal flora, and GRFT in CVL with BV was determined. The EC_{50} represents the GRFT concentration which produces a response (% GRFT binding to gp120) that is halfway between the baseline of the curve (bottom of the curve) and the maximal response of the curve (top of the curve). The EC_{50} values of GRFT in GRFT standards, GRFT in CVL with normal flora, and GRFT in CVL with BV were all similar at 0.4016 nM (10.23 ng/mL), 0.4210 nM (10.72 ng/mL), and 0.4198 nM (10.69 ng/mL), respectively, with no statistical difference present (p = 0.076).
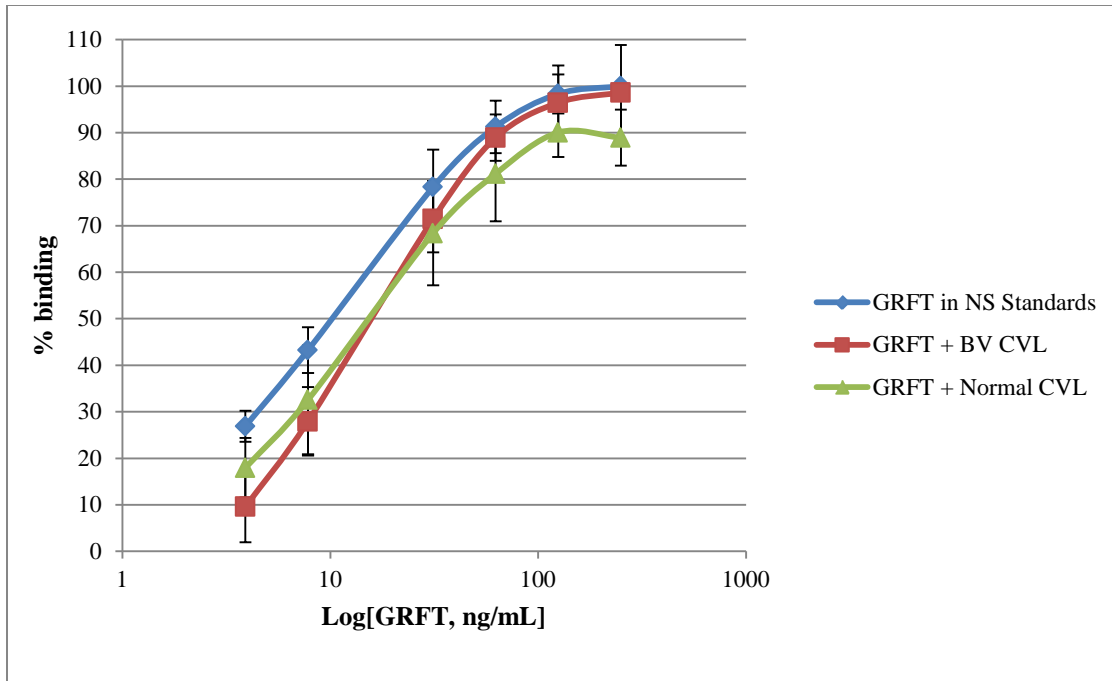
Figure 37. GRFT-gp120 binding curves for GRFT in Cervicovaginal Lavages with both Normal Flora and BV – COMPARATIVE STUDY.
GRFT was combined with CVL containing normal flora at a total protein concentration of 250μg/mL. GRFT was combined with BV CVL at a total protein concentration of 250μg/mL. GRFT concentrations in normal saline were used as a standard curve.

Table 14. GRFT-gp120 Binding Curves for GRFT in Cervicovaginal Lavages – COMPARATIVE STUDY (*p* values)

	GLOBAL TEST	GRFT (ng/mL)					
		250	125	62.5	31.25	7.8125	3.90635
BV CVL vs. Standard	0.016	0.518	0.158	0.286	0.022	0.003	<0.001
Normal CVL vs. Standard	<0.001	<0.001	<0.001	0.004	0.002	0.002	<0.001
BV CVL vs. Normal	0.303	0.001	0.006	0.086	0.424	0.211	0.012

Additionally, of the ten CVL sample aliquots used in this gp120 binding analysis, three women provided two samples each (six samples total from three separate women, two samples each). For these three participants, at one sample collection their CVLs tested normal and at the other sample collection their CVLs tested positive for BV. It was not possible to statistically

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analyze these 'converters'. There were missing data points on their GRFT-BV CVL curves due to volume constraints in BV CVL sample aliquots. Only one of these three participant's full binding curves for both conditions could be visually represented. Refer to Figure 38. The half maximal effective concentration (EC_{50}) of GRFT-gp120 binding during these two conditions from a single participant was also determined. The EC_{50} value for GRFT in CVL with normal flora from this participant was 1.460 nM (37.18 ng/mL), which is approximately 4X that of GRFT in CVL with BV from the same participant (0.3759 nM; 9.57 ng/mL).
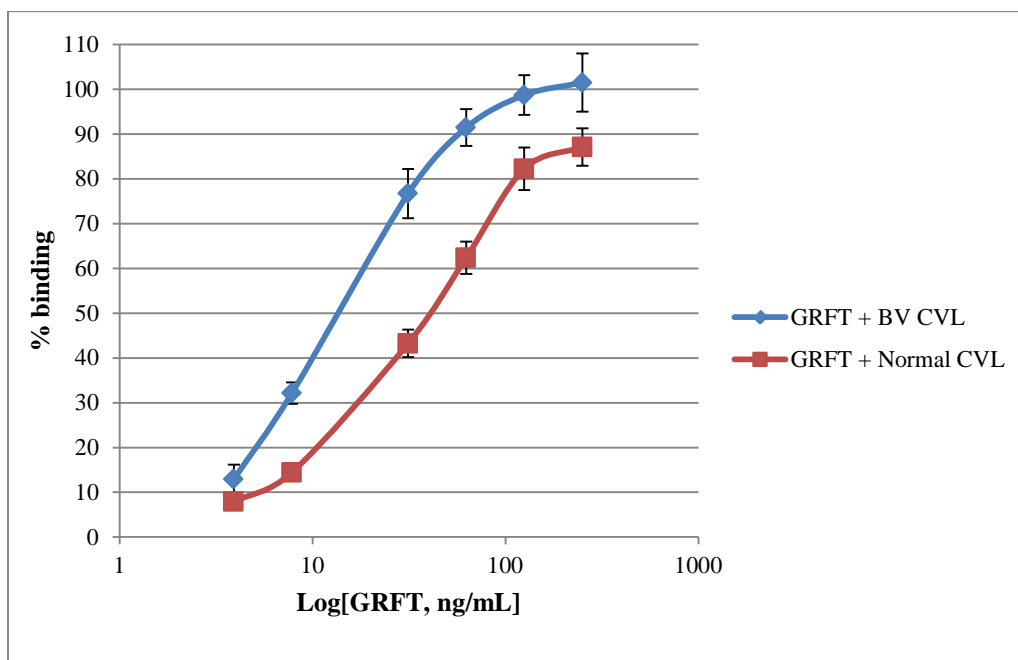
Figure 38. GRFT-gp120 binding curves for GRFT in cervicovaginal lavages with both normal flora and BV from a single participant (two separate collection visits).
This data is from the COMPARATIVE STUDY. GRFT was combined with CVL at a total protein concentration of 250μg/mL.

4.4   DISCUSSION AND CONCLUSIONS

GRFT is a lectin, a carbohydrate binding protein, which binds to N-linked high mannose glycans on glycoproteins [106-107]. GRFT elicits its main anti-HIV activity by binding to gp120 on the

virus and preventing viral attachment to the host cell [96]. The six binding pockets of GRFT must be clear of off-target substrates in order for GRFT to be effective.

Recent studies have revealed interesting and relevant data pertaining to human cervicovaginal secretions, N-linked glycans, and GRFT. N-linked high mannose glycans are present in human cervicovaginal secretions, with secretions containing normal microflora having more N-linked glycans than BV secretions [152]. Also, decreased GRFT lectin binding was found in BV cervicovaginal secretions in comparison to secretions with normal microflora [130]. These data taken together cause concern for GRFT vaginal delivery and bioactivity within this compartment. Due to these results, we hypothesized that GRFT-gp120 binding would be inhibited in cervicovaginal secretions. This inhibition would be more pronounced in cervicovaginal secretions with normal flora as compared to cervicovaginal secretions with BV, since (1) less N-linked glycans exist in BV secretions, and (2) GRFT binding in BV secretions is less. This hypothesis was tested utilizing a gp120 binding ELISA and a subset of well characterized human CVL sample aliquots. Overall, results showed that human cervicovaginal secretions containing normal flora do negatively impact (decrease) GRFT-gp120 binding. Human cervicovaginal secretions with BV did not negatively impact GRFT-gp120 binding. Conversely, normal flora in the vagina decreases HIV infection, whereas the presence of BV increases the likelihood of HIV acquisition and transmission [146-147].

These results are supportive of those of the larger study [130, 145, 152] and represent the first set of experiments performed to investigate the effects of human cervicovaginal secretions on GRFT-gp120 binding. Of important note is that a cervicovaginal lavage is a dilution of pure cervicovaginal secretions. Additionally, cervicovaginal lavage samples had to be further diluted for use with the gp120 binding ELISA in these experiments. The total protein concentration

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(250µg/mL) used for the comparative experiment between normal and BV CVL was an approximate 200-fold dilution of the total protein concentration found in a pure, undiluted cervicovaginal secretion sample. Due to the high viscosity of pure cervicovaginal secretions and volume constraints of CVL, neither pure secretions nor an increased CVL total protein concentration could be used in these experiments. Therefore, effects on GRFT bioactivity may be more pronounced *in vivo*. Biologically relevant assessments, like this one, deserved further attention.

ACKNOWLEDGEMENTS

5.0   HUMAN CERVICAL TISSUE PERMEABILITY AND TISSUE LOCALIZATION OF GRIFFITHSIN

5.1   INTRODUCTION

The epithelial tissue environment within the human vaginal cavity consists of vaginal epithelium, ectocervical epithelium, and endocervical epithelium. The vagina and ectocervix, the portion of the cervix that is exposed to the vaginal environment, are lined by a nonkeratinized, stratified squamous epithelium (Figure 39 [34]). The vaginal mucosal layer is composed of multiple rugal folds, increasing the surface area. The squamous epithelium is constantly renewed and desquamated during the premenopausal years and contains three cellular zones: (1) germinal or basal cell layer; (2) midzone or stratum spinosum (intermediate), the dominant portion of the epithelium; and (3) superficial zone, consisting of the most mature cell population [40]. The superficial cells help to protect the underlying epithelial cells and subepithelial vasculature from trauma and infection. The squamous cells feature gap junction nexuses that represent an open channel system between adjacent cells through which certain molecules and electrolytes can transverse [40].

The endocervix is lined by a single layer of columnar epithelium and invaginates the underlying stroma (Figure 39 [34]). The transformation zone is located on the exposed portion of the cervix and is found in most women of reproductive age [40]. This area is the region where the columnar endocervical epithelium meets the squamous epithelium of the ectocervix. The

transformation zone is thought to be a highly immunologically active region important in HIV infection.

The connective tissue of the cervical stroma may be divided into two zones. One zone has a superficial, subepithelial location and is rich in interstitial fluid. The second is a deep, dense collagen layer. The superficial endocervical stroma also contains many capillaries, particularly abundant beneath the endocervical epithelial lining. These blood vessels do not have direct contact with the overlying squamous or columnar epithelial membranes. Therefore, metabolic exchanges including oxygen supply of the cervical epithelium must occur via diffusion [40].

The thickness of the vaginal epithelium varies as a result of changes in estrogen levels during the menstrual cycle. This estrogenization has important consequences for drug permeation through the tissue and may influence targeting and the pharmacokinetics (PK) of vaginally delivered drugs [42, 46].

Figure 39. Female Genital Tract Anatomy.
(A) Represents a side view of the gross anatomy of the female genital tract with respect to other organs. (B) Represents tissue types and areas of the upper vagina and cervix. (C) Display of cells composing the vaginal and ectocervical tissue and display of cells composing the endocervical tissue.

GRFT has two mechanisms of action against HIV infection. This protein's main mode of anti-HIV activity consists of binding to viral gp120 and preventing attachment of HIV to host cells [96]. When vaginally delivered, this mechanism would be carried out within the vaginal lumen. This compound can also inhibit HIV binding to the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-Integrin (DC-SIGN) receptor and transfer to CD4 cells [97]. This secondary mechanism of action would require GRFT to permeate the cervicovaginal epithelial tissue in order to reach DCs beneath the epithelium. Given GRFT's proteinaceous

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nature, we hypothesize that it will not permeate the cervicovaginal epithelium in order to elicit its secondary mechanism of action against HIV infection.

The objective of this study is to investigate human cervical tissue permeability of GRFT. Utilizing excised human cervical tissue and the Franz cell system, tissue permeability and localization will be determined. Overall, the results generated will be important to determine the cervical permeability profile of GRFT, which is of importance for a vaginally delivered compound which must reach below the epithelium in order to elicit anti-HIV activity. This is the first study to investigate cervical tissue permeability and localization of GRFT.

5.2 MATERIALS AND METHODS

Recombinant Griffithsin drug substance was supplied by Kentucky Bioprocessing LLC (KBP; Owensboro, KY). The protein was supplied in a solution of phosphate buffered saline. For fluorescence experiments, GRFT was tagged using an Alexa Fluor® 488 Protein Labeling Kit from Invitrogen. GRFT 0.1% gel was compounded and supplied for permeability experiments as well. Phosphate buffered saline 10X molecular biology grade was purchased from Mediatech, Inc (Manassa, VA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were obtained from Fisher Scientific (Pittsburgh, PA).

5.2.1 Human Cervical Tissue Procurement

Human excised cervical tissue was obtained from the University of Pittsburgh Health Sciences Tissue Bank under IRB Protocol PRO09110431. Tissue specimens were collected from healthy volunteers undergoing hysterectomy for non-cervical issues.

5.2.2 Human Cervical Tissue Permeability

In preparation for permeability experiments, a section of each tissue was retained for histological evaluation (pre-tissue). Excess stromal tissue was removed using a Thomas-Stadie Riggs tissue slicer by a longitudinal slice through the specimen. Tissue thickness was measured by placing the tissue between two pre-measured slides and measuring with a hand-held digital caliper. Tissue permeability studies were performed in a Franz cell system (Figure 40). Tissue sections were placed between the donor and receptor compartments of the Franz cell with the epithelial side of the tissue oriented toward the donor compartment, and the stromal side of the tissue oriented toward the receptor compartment (Figure 41). The tissue-loaded Franz cells were maintained in an environment at 37°C throughout the experiment via a heated water bath. The receptor was filled with PBS1X. 450µL of GRFT or GRFT-AlexaFluor488 drug substance in PBS1X, at a concentration of 2.25mg/mL for a total dose of approximately 1mg GRFT, was loaded into the donor compartment for drug substance experiments. 500µL of GRFT 0.1% gel (loading dose of 500µg) or GRFT drug substance (1.125mg/mL; loading dose of approximately 500µg) was loaded into the donor compartment for gel formulation permeability experiments. The 500µg level was used due to the volume restriction (500µL) of the Franz cell donor compartment. At time zero of the permeability experiment, 50µL was removed from the donor compartment. At predetermined time points, 200µL was removed from the receptor chamber and replaced with the same volume of fresh PBS1X. At the end of the experiment, a sample of the receptor medium and the remaining volume of donor medium were removed. Samples were held at 2-8°C until analyzed by HPLC, gp120 binding ELISA, and SDS PAGE (as indicated).
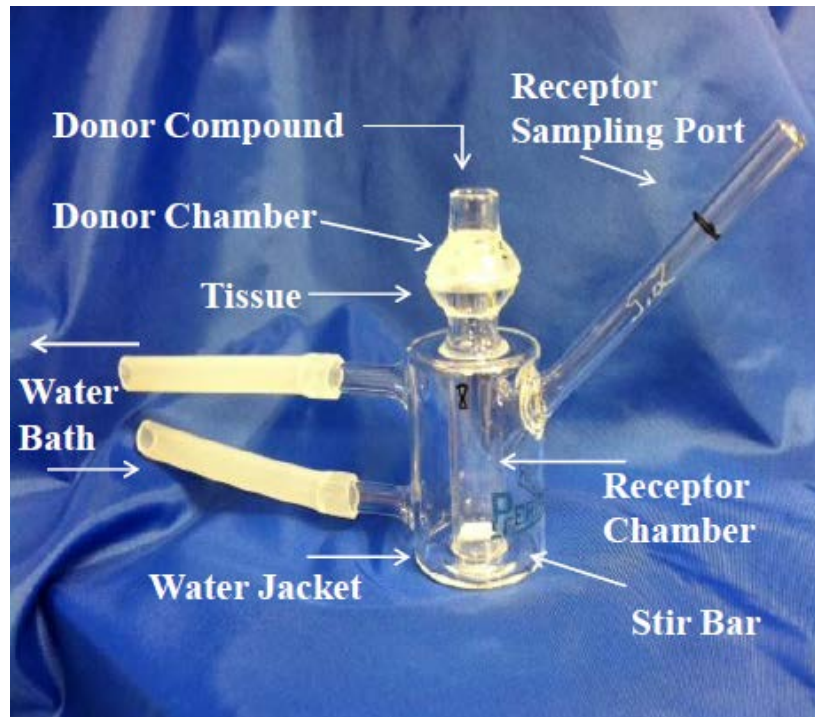
Figure 40. One Franz Cell of the Franz Cell System.
The Franz cell system is used for the GRFT-human cervical tissue permeability study. Image courtesy Marilyn Cost.
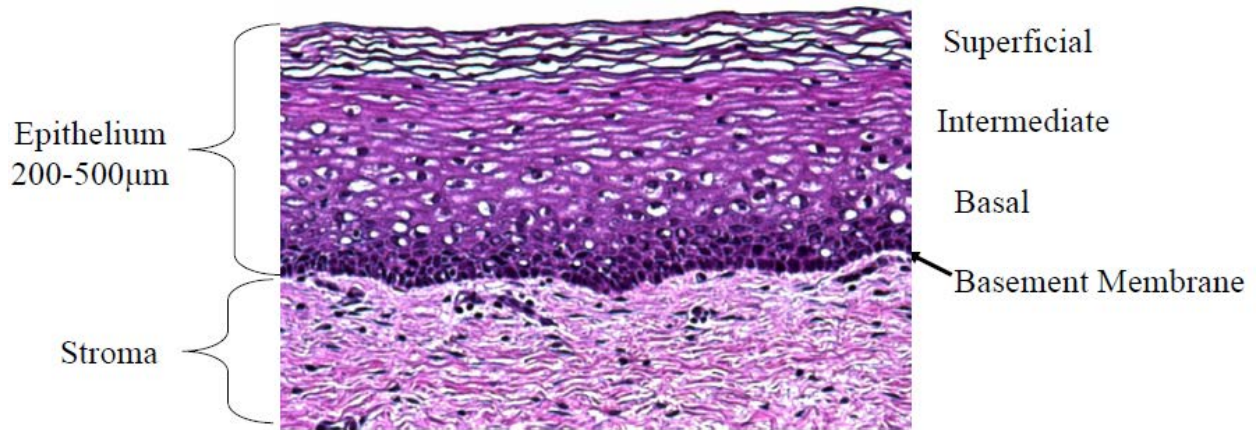
Figure 41. Section of Human Cervical Tissue, Representative H&E Staining for Tissue Orientation.
In the Franz cell, the cervical epithelium will face up toward the donor compartment (apical), and the cervical stroma will be oriented toward the receptor compartment (basal). The human cervical epithelium thickness can range from 200-500 microns, on average. Micrograph taken with a 20X objective.

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5.2.3 Tissue Processing

Prior to all permeability experiments, one section of the cervical tissue specimen was retained for histological evaluation (denoted as pre-tissue). All tissues were processed for paraffin sectioning, as follows. At the end of the permeability experiment, tissues were placed in biopsy processing/embedding cassettes (one tissue section in each cassette) and treated in the following order: formalin for 12-24 hours; 70% ethanol (tissue can remain in 70% ethanol until further processing occurs), 95% ethanol for one hour, 100% ethanol for one hour, xylene for two hours, and paraffin for three hours (three changes X one hour per each change). The tissue sections were embedded into paraffin blocks using the Leica EG 1160 embedding station. Tissues were then sectioned at five microns (five µm) with the Olympus CUT 4060 microtome and placed on slides for further processing or staining procedures.

5.2.4 Fluorescence Detection Method in/on Tissue

GRFT was tagged using an Alexa Fluor® 488 Protein Labeling Kit from Invitrogen. GRFT-AlexaFluor488 has been previously used in tissue and cellular studies [108]. Slides containing tissue sections were developed to determine GRFT tissue localization. Slides were heated at 60°C for ten minutes. The tissue sections on the slides were deparaffinised in the following manner: xylene for five minutes; xylene for five minutes; 100% ethanol for one minute; 100% ethanol for one minute; 95% ethanol for one minute; 70% ethanol for one minute; and water for five minutes. Then 4',6-diamidino-2-phenylindole (DAPI; VECTASHIELD HardSet Mounting Medium with DAPI) was used for fluorescence visualization of cellular nuclei (tissue orientation

aid) and to seal the cover slip. Areas on the tissue containing GRFT-AlexaFluor488 will appear bright green. Nuclei of epithelial cells will appear blue/purple.

5.2.5   Antibody Detection Method in/on Tissue

Slides containing tissue sections were developed to determine GRFT tissue localization for the permeability experiments performed with unlabeled GRFT drug substance and GRFT gel. Unlabeled GRFT was used as within this secondary tissue detection method because the AlexaFluor488 dye compound has a molecular weight of approximately 900 and may impact GRFT permeability due to increased size or conformation of test compound GRFT-AlexaFluor488. Slides were heated at 60°C for ten minutes. The tissue sections on the slides were deparaffinised in the following manner: xylene for 5 minutes; xylene for 5 minutes; 100% ethanol for 1 minute; 100% ethanol for 1 minute; 95% ethanol for 1 minute; 70% ethanol for 1 minute; and water for 5 minutes.

The following antibodies were used in this staining protocol: polyclonal primary goat anti-GRFT and biotinylated rabbit anti-goat IgG secondary antibody. With these two antibodies, a tissue staining procedure was developed and used to detect GRFT in human cervical tissue. Both the development and use of this protocol will be presented in the results section.

5.2.6   Hematoxylin and Eosin Staining for Human Cervical Tissue Sections

To determine the tissue safety (any gross morphological changes) of GRFT [drug substance (labelled and unlabeled) and gel] application on human cervical epithelial tissue, hematoxylin

and eosin (H&E) were used. Hematoxylin stains the nuclei of cells purple. Eosin stains the other structures of the tissue section red/pink.

5.2.7 High Performance Liquid Chromatography

The High Performance Liquid Chromatography (HPLC) system (Waters Corporation; Milford, CA) was equipped with an autosampler model 717plus, a quaternary pump controller model 600, and a fluorescence detector model 2487, using an excitation wavelength of 273 nm and an emission wavelength of 303 nm. Separation of GRFT from degradants was achieved using a Phenomenex Jupiter C18 5µ 300Å (4.6x250 mm) column (Phenomenex; Torrance, CA) protected by a Gemini C18 (4x3 mm) guard cartridge (Phenomenex; Torrance, CA), both maintained at ambient temperature. The mobile phase gradient consisted of mobile phase A (0.1% TFA in water (v/v)), and mobile phase B (0.05% TFA in acetonitrile (v/v)) pumped at a flow rate of 1.0 mL/min. The gradient was proportioned as follows (minute, %B): 0, 12; 15, 20; 16, 50; and 20, 12. Linearity of the detector response curve was over the range of 10 µg/mL to 500 µg/mL. Empower software was used to control the HPLC system.

5.2.8 gp120 Binding ELISA

An Enzyme-linked Immunosorbent Assay (ELISA) was employed to evaluate the gp120 binding activity of select GRFT donor and receptor samples from permeability experiments. Briefly, gp120 is bound to the wells of a 96-well plate overnight at 4°C. HIV-1 gp120 CM was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Cat# 2968). Nunc MaxiSorp 96-well plates were used for this experiment. The MaxiSorp surface is a

hydrophilic/hydrophobic mix that binds to a wide variety of biomolecules. The solution of gp120 was removed after overnight incubation, and a blocking solution was applied for two hours at room temperature. The wells were then washed and incubated for one hour with various dilutions of GRFT samples. gp120 binding was visualized by goat anti-GRFT primary antibody (one hour incubation) and HRP-labeled rabbit anti-goat secondary antibody (one hour incubation). TMB substrate was applied to the wells after washing of the secondary antibody. Wells were allowed to develop (blue color) for approximately three minutes before the application of sulfuric acid to stop the reaction (yellow color). gp120 binding was measured by optical density at 450nm.

5.2.9   SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for select GRFT donor and receptor samples from permeability experiments. An SDS-PAGE SOP was developed by Dr. Kenneth Palmer's laboratory and utilized for these experiments. In brief, BioRad precast Mini-PROTEAN® TGX Any kD gels, BioRad Precision Plus Protein™ Kaleidoscope™ Standards, and 1X Tris/Glycine/SDS Running buffer were used. GRFT samples were diluted in specified solutions to a concentration of 1-8µg protein (0.033mg/mL – 0.267 mg/mL for 30 µL well size). 30ul of sample was then combined with 30uL Laemmli sample loading buffer and boiled for 5 minutes. Samples were cooled on ice. 15uL of sample was loaded into a single well. At the end of the run, protein band detection was performed by Coomassie staining (Bio-Safe Coomassie Stain). Gel images were collected with a Gel DocTM EZ System (Bio-Rad).

5.2.10 Microscopy

All microscopy was performed with a Zeiss Axioskop 40 Microscope equipped with a Prior Scientific Lumen 200 Fluorescence Illumination System (for fluorescence imaging). Micrographs were obtained with an AxioCam MRc 5 color camera and AxioVision software. For fluorescence detection, the FITC filter (AlexaFluor488 and FITC have comparable excitation and emission wavelengths.) and the DAPI filter were used, and micrographs using these two fluorescence filters overlaid. No filter was used for GRFT drug substance (unlabeled) and GRFT gel. No filter was used for H&E micrographs. All micrographs were taken with a 20X objective.

5.3   RESULTS

5.3.1   Human Cervical Tissue Permeability – GRFT-AlexaFluor488

Prior to performing any experiments with GRFT-AlexaFluor488 drug substance, it was necessary to confirm that this tag/label did not impact HPLC chromatographic profile in order to properly quantitate Franz cell permeability donor and receptor samples. As shown in Figure 42, tagging GRFT with this fluorescence label did not negatively impact the HPLC chromatographic profile of GRFT. The GRFT peak remains at the retention time of approximately 16.5 minutes, and the drug substance impurity peak (denoted at OX-1 in the chromatograms) has a relative retention time (RRT) of approximately 0.900 minutes.
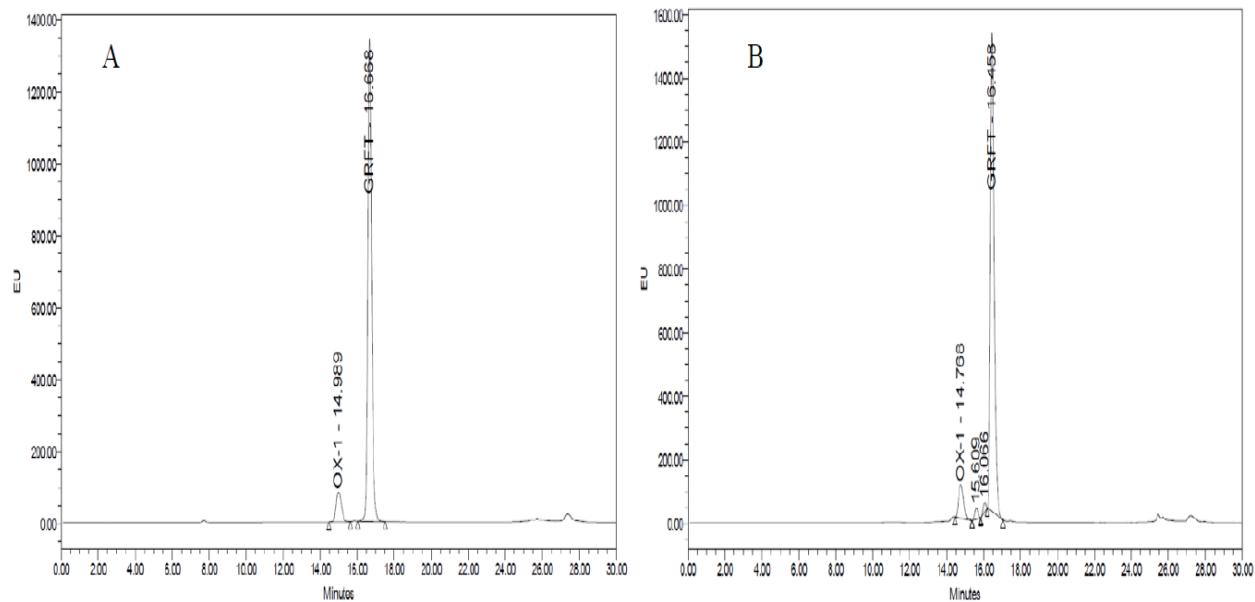
Figure 42. HPLC-F Chromatograms of GRFT and GRFT-AlexaFluor488.
(A) HPLC chromatogram of GRFT 250μg/mL in PBS1X; (B) HPLC chromatogram of GRFT-AlexaFluor488 250μg/mL in PBS1X.

GRFT-AlexaFluor488 permeability was performed on six human cervical tissue specimens. Female human donors were in the age range of 40-50 years old. Three of the specimens were used fresh (same day as excision; within 6 hours of surgery), and three were used after being flash frozen and held at -80°C; no differences in any results were noted. Therefore, most of the permeability studies were performed with frozen tissue specimens due to difficulty obtaining fresh tissue is a timely manner and ease of scheduling studies with frozen tissue.

Mass balance for GRFT was conducted using data obtained from HPLC drug quantification in donor and receptor solutions. The following equation was used:

GRFT in $Donor_{end}$/GRFT in $Donor_{time0}$ * 100 = % GRFT remaining in Donor (Mass Balance)

Table 15 displays the average % remaining in donor values for each of the six specimens. GRFT recovery from the donor compartment at the end of the experiment ranged from 94.1% to 103.2% per HPLC-F analysis, across the six tissue specimens. GRFT was not quantifiable with HPLC-F in the receptor samples at any time point for any of the specimens. ELISA results were unremarkable and confirmed HPLC results.

Table 15. GRFT-AlexaFluor488 Cervical Tissue Permeability - % GRFT Remaining in Donor

(n = 6; time = 6 hours)	
Tissue	*% GRFT (mean ± SD)*
1	97.8% ± 2.6%
2	95.4% ± 0.4%
3	94.0% ± 5.0%
4	103.4% ± 3.5%
5	96.9% ± 6.2%
6	99.4% ± 1.5%
Overall	**98.0% ± 4.5%**

GRFT-AlexaFluor488 has been previously used and has been shown to adhere to the surface of Ect1/E6E7 cells, a human ectocervical cell line [108]. In the cervical tissue permeability studies performed with GRFT-AlexaFluor488 drug substance, GRFT-AlexaFluor488 was found to adhere to the surface (superficial layer) of the cervical epithelium. Refer to Figure 43.
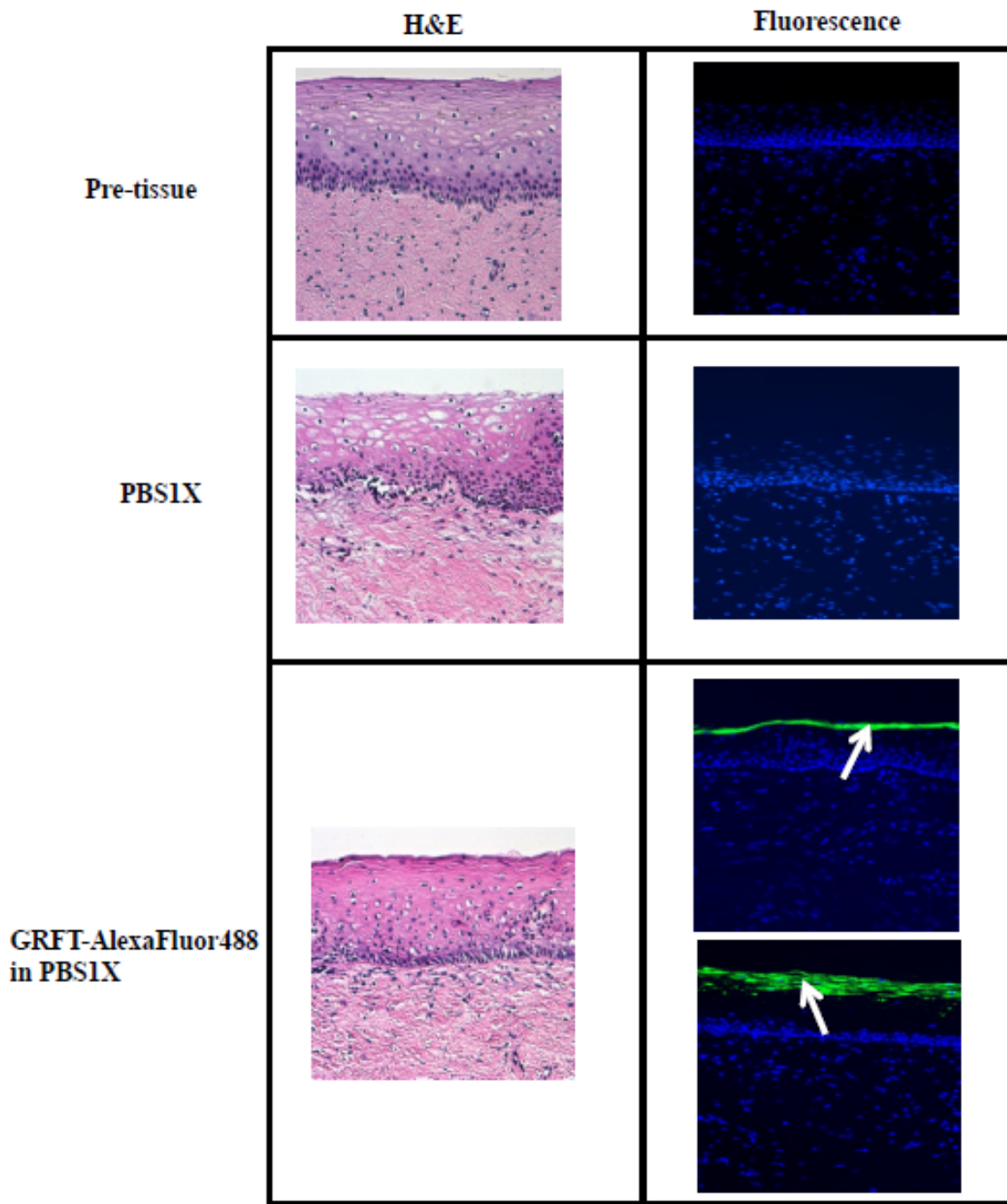
Figure 43. Micrographs from GRFT-AlexaFluor488 Cervical Tissue Permeability Experiments.
Representative H&E (first column) and fluorescence (FITC and DAPI filter overlay; second column)
micrographs for pre-tissue (first row), PBS1X (second row), and GRFT-AlexFluor488 (third row; two
micrographs of fluorescence imaging) exposed tissues are shown. GRFT tissue localization is represented by
green fluorescence, as indicated by the white arrows. Micrographs were taken with a 20X objective.

No gross morphological changes were observed upon H&E staining for pre-tissue in comparison to post-tissue (GRFT-AlexaFluor488 exposed). All of these results show that GRFT-AlexaFluor488 remained in the donor compartment and adhered to the top of the cervical epithelium. These results indicate that GRFT will remain in the vaginal lumen while forming a protective barrier on the surface of intact cervical epithelium.

5.3.2   Human Cervical Tissue Permeability – GRFT and Antibodies

To confirm findings obtained from GRFT-AlexaFluor488 experiments, GRFT (1mg loading) permeability was performed on five human cervical tissue specimens. Female human donors were in the age range of 30-50 years old. One specimen was used fresh (same day as excision; within 6 hours of surgery), and four were used after being flash frozen and held at -80°C; no differences were found.

GRFT (500µg loading) and GRFT 0.1% gel (500µL for a load of 500µg GRFT) permeability were performed on four human cervical tissue specimens. Female human donors were in the age range of 30-55 years old. All specimens were used after being flash frozen and held at -80°C. The GRFT 1% vaginal gel contains carbopol 974p, glycerin, methylparaben, propylparaben, disodium EDTA, sodium hydroxide 18%, and water and was manufactured as previously described [153].

Mass balance for GRFT was conducted using data obtained from HPLC drug quantification in donor and receptor solutions. To calculate % GRFT remaining in donor (mass balance), the equation found in section 5.3.1 was used. Table 16 displays the average % remaining in donor values for GRFT API 1mg, GRFT API 500µg, and GRFT gel 500µg.

Table 16. GRFT API and GRFT Gel Cervical Tissue Permeability - % GRFT Remaining in Donor

(time = 6 hours)	
Article	*% GRFT (mean ± SD)*
GRFT API, 1mg	101.3% ± 6.8%
GRFT API, 500µg	96.1% ± 4.8%
GRFT gel, 500µg	97.5% ± 3.5%

GRFT recovery from the donor compartment at the end of these experiments ranged from 96.1% to 101.3% per HPLC-F analysis, across the nine tissue specimens for GRFT API and gel. GRFT was not quantifiable with HPLC-F in the receptor samples at any time point for any of the specimens. ELISA results were unremarkable and confirmed HPLC results.

As a confirmatory tissue detection method, unlabeled GRFT was used in this set of permeability experiments. GRFT tissue detection employed antibodies. During the development of this staining protocol, multiple steps were taken to achieve an effective procedure that minimized/eliminated non-specific binding of the antibodies: (1) endogenous peroxidise, pseudoperoxidase, and/or alkaline phosphatise activity in the cervical tissue was ruled out (2) endogenous biotin activity in the cervical tissue was ruled out; (3) a protein blocking solution was utilized within the antibody diluents (1% bovin serum albumin, BSA); and (4) the primary antibody (goat anti-GRFT) dilution was optimized (1:200,000). The combination of the protein blocking reagent and primary antibody dilution significantly decreased non-specific reactions with the tissue and the staining protocol. The resulting staining protocol is below.

After deparaffinization and hydration of the tissue sections on the slides, primary antibody was added (goat anti-GRFT 1:200,000 in 1% BSA in PBS1X with 0.05% Tween-20). Incubation of primary antibody occurred for one hour at room temperature (RT). After primary

antibody was removed, secondary antibody (biotinylated rabbit anti-goat IgG; from VectaStain Elite ABC kit PK-6105, Vector Labs) was added to the tissue sections and incubated for 30 minutes at RT. This secondary antibody was diluted with 1% BSA in PBS1X with 0.05% Tween-20. After the secondary antibody was removed, Avidin-HRP (from VectaStain Elite ABC kit PK-6105) was added and incubated for 30 minutes at RT. Tissue sections were then rinsed of the of Avidin-HRP and DAB (3,3'-diaminobenzidine; ImmPACT DAB HRP Substrate, Vector Labs) was applied and incubated for one minute at RT. Tissues sections were rinsed with milliQ water until the water ran clear. DAB HRP substrate produces a brown reaction color product on the tissue section where GRFT is present. Tissue sections were counterstained with hematoxylin to stain nuclei for improved over tissue visualization and orientation. Finally, tissue sections on the slides were dehydrated and cover slips were sealed with CytoSeal 60.

In the cervical tissue permeability experiments performed with GRFT(unlabeled) drug substance and GRFT gel, GRFT was found to adhere to the surface (superficial layer) of the cervical epithelium, as evidenced by brown staining on the cervical epithelial surface. Refer to Figure 44. No gross morphological changes were observed upon H&E staining for pre-tissue in comparison to post-tissue (both GRFT API and gel exposed). All of these results show that GRFT-AlexaFluor488 remained in the donor compartment and adhered to the top of the cervical epithelium. These results are confirmatory to those of GRFT-AlexaFluor488 and indicate that GRFT will remain in the vaginal lumen while coating the surface of intact cervical epithelium.

	H&E	Antibody/DAB
Pre-tissue		
GRFT, 1mg		
GRFT, 500μg		
Placebo gel		
GRFT gel, 500μg of GRFT		
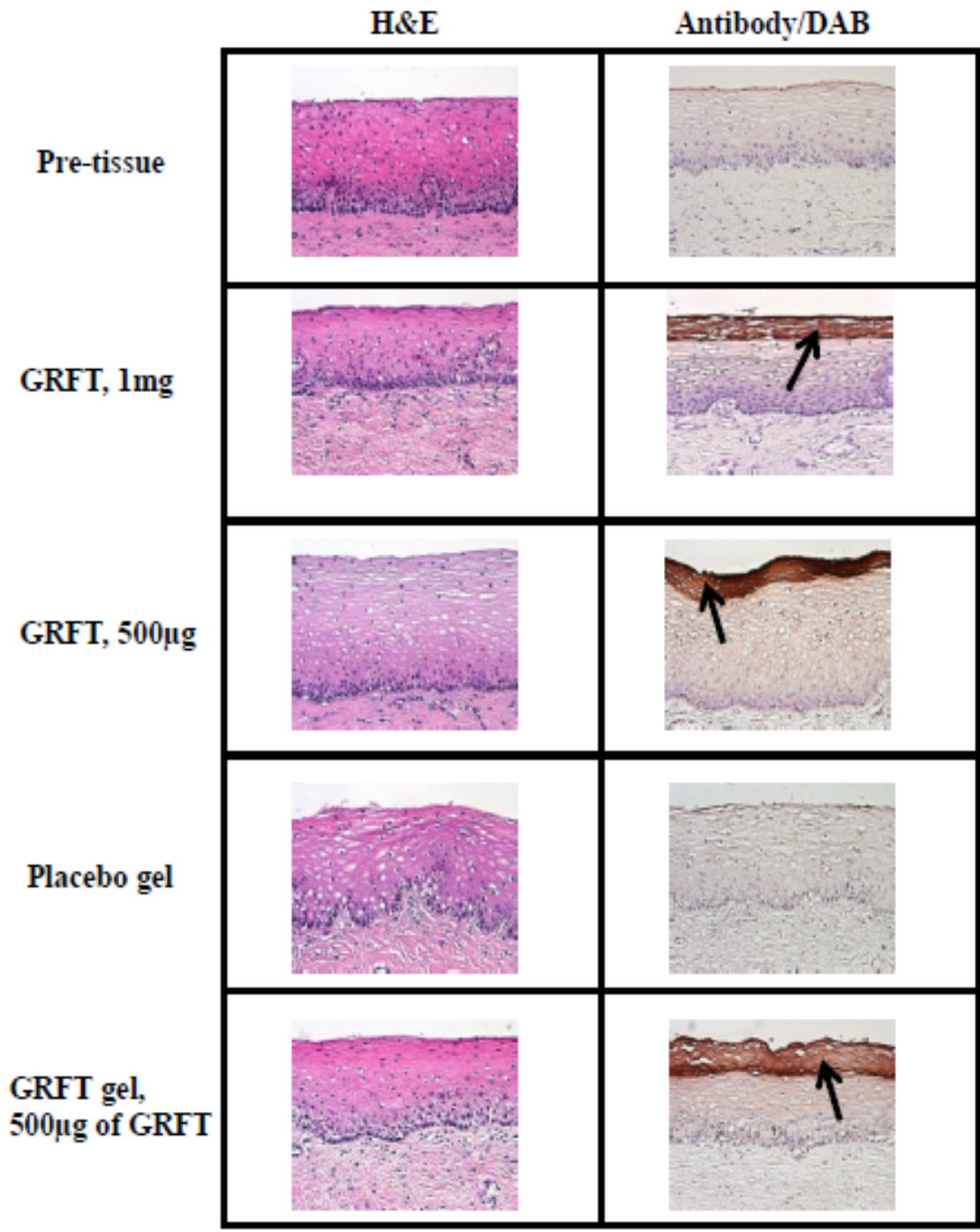
Figure 44. Micrographs from GRFT (unlabeled API and gel) Cervical Tissue Permeability Experiments.
Representative H&E (first column) and Antibody/DAB staining (second column) micrographs for pre-tissue (first row), placebo gel (fourth row), and GRFT exposed (API and gel; second, third, and fifth rows) tissues are shown. Tissue localization of GRFT is represented by reddish-brown coloring, as indicated by the black arrows. Micrographs were taken with a 20X objective.

5.3.3 SDS-PAGE of Franz Cell Donor and Receptor Solutions

SDS-PAGE was also used to investigate the protein content of the GRFT (GRFT labeled and unlabeled drug substance and gel) donor and receptor solutions from the permeability experiments. In the following pages, three SDS PAGE gels will be presented. These SDS PAGE gel results are from one tissue specimen (four Franz cells were conducted for this one human cervical tissue specimen; three containing GRFT 1mg in donor (cells 1, 2, 3) and one containing PBS1X in donor (cell 4)). However, these results also representative of the other permeability experiments (GRFT-AlexaFluor488 and GRFT gel).

Lanes 2-5 of SDS-PAGE gel #1 (Figure 45) indicate that there are proteins present within the equilibration solution. During the initial set up of the Franz cell system, there is an equilibration step. This step occurs directly after the tissue is clamped between the donor and receptor compartment of the Franz cell. PBS1x is placed within the donor compartment and remains there for approximately ten minutes. This allows the user to determine if the tissue is properly place or has any wholes, tears, etc (the level of the PBS1X would decrease). The equilibration solution is then removed and investigational compound is added. Lanes 2-5 show that proteins from the tissue/cells of the cervical epithelium washed off into the equilibration solution. Lanes 7-9 and Lanes 12-14 indicate that GRFT was present in the donor compartments at the beginning of the experiment and at the end of the experiment and the dilution was comparable to control (Lane 6). Lanes 10 and 14 confirmed that no GRFT was present in the donor compartment, at the start and end of the experiment, of the Franz cell that contained PBS1X only.

Figures 46 and 47 represent protein bands within the receptor medium (PBS1X) of all four Franz cells at the beginning of the experiment (time zero) and throughout the experiment.

Protein bands of molecular masses 50 and 250 kD are present in receptor samples. The molecular mass of 50kD could represent a dimer of a GRFT protein dimer (12.7 X 2 = 50.8kD). However, this cannot be true for multiple reasons: (1) These protein bands are present at time zero in all Franz cells, even PBS1X only; (2) These protein bands are present in the receptor samples of the PBS1X only cell (cell 4; Lanes 10 and 11 of Figure 47); and (3) HPLC data indicated that no GRFT was found in any receptor samples. The bands found in these receptor samples represent proteins from the cervical epithelial and stroma tissue.

Lane	Description
1	MW Markers
2	Donor cell 1, equilibration solution
3	Donor cell 2, equilibration solution
4	Donor cell 3, equilibration solution
5	Donor cell 4, equilibration solution
6	GRFT 125µg/mL in PBS1X control
7	Donor cell 1, 0H; diluted to ~125µg/mL
8	Donor cell 2, 0H; diluted to ~125µg/mL
9	Donor cell 3, 0H; diluted to ~125µg/mL
10	Donor cell 4, 0H; diluted in same fashion
11	MW Markers
12	Donor cell 1, 6H; diluted to ~125µg/mL
13	Donor cell 2, 6H; diluted to ~125µg/mL
14	Donor cell 3, 6H; diluted to ~125µg/mL
15	Donor cell 4, 6H; diluted in same fashion

Figure 45. SDS-PAGE Gel #1 from GRFT Cervical Tissue Permeability Experiment.
Detection by Coomassie blue staining on a BioRad precast Mini-PROTEAN® TGX Any kD gel. All samples are from a single specimen. Franz cell 1, 2, and 3 donor compartments were loaded with 450µL of GRFT drug substance in PBS1X, at a concentration of 2.25mg/mL for a total dose of approximately 1mg GRFT. Franz cell 4 donor compartment was loaded with PBS1X. Donor samples were taken at time zero and at the end of the experiment (six hours).
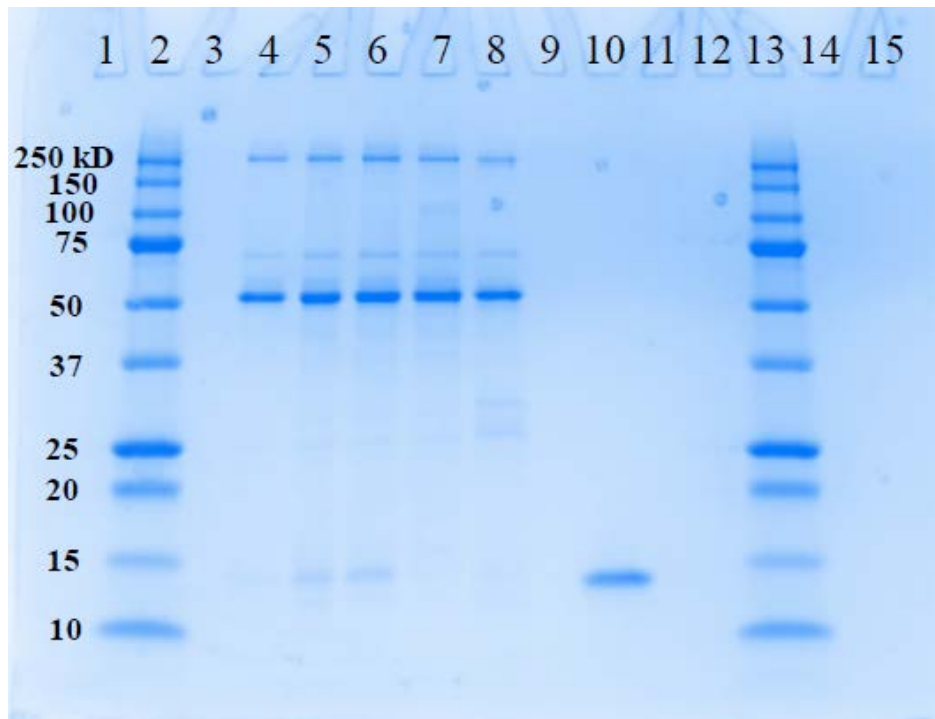
Lane	Description
1	Blank
2	MW Markers
3	Blank
4	Receptor cell 1, 0H
5	Receptor cell 1, 5H
6	Receptor cell 1, 6H
7	Receptor cell 2, 0H
8	Receptor cell 2, 6H
9	Blank
10	GRFT 125µg/mL in PBS1X control
11	Blank
12	Blank
13	MW Markers
14	Blank
15	Blank

Figure 46. SDS-PAGE Gel #2 from GRFT Cervical Tissue Permeability Experiment. Detection by Coomassie blue staining on a BioRad precast Mini-PROTEAN® TGX Any kD gel. All samples are from a single specimen. Franz cell 1, 2, and 3 donor compartments were loaded with 450µL of GRFT drug substance in PBS1X, at a concentration of 2.25mg/mL for a total dose of approximately 1mg GRFT. Franz cell 4 donor compartment was loaded with PBS1X. Receptor samples were taken at time zero and over time up until the end of the experiment (six hours).
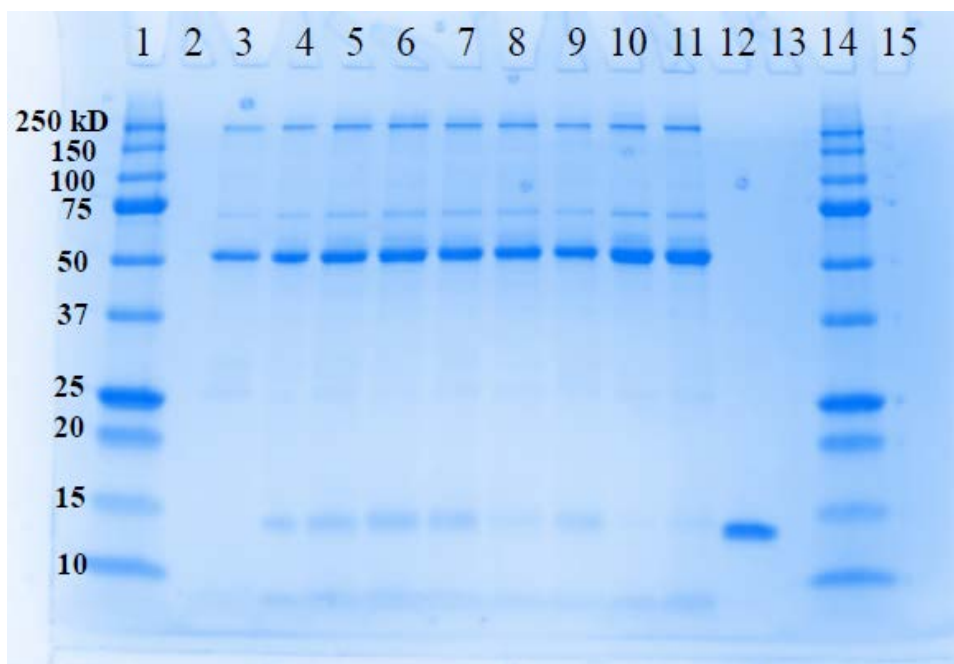
Lane	Description
1	MW Markers
2	Blank
3	Receptor cell 3, 0H
4	Receptor cell 3, 1H
5	Receptor cell 3, 2H
6	Receptor cell 3, 3H
7	Receptor cell 3, 4H
8	Receptor cell 3, 5H
9	Receptor cell 3, 6H
10	Receptor cell 4, 0H
11	Receptor cell 4, 6H
12	GRFT 125µg/mL in PBS1X control
13	Blank
14	MW Markers
15	Blank

Figure 47. SDS-PAGE Gel #3 from GRFT Cervical Tissue Permeability Experiment.
Detection by Coomassie blue staining on a BioRad precast Mini-PROTEAN® TGX Any kD gel. All samples are from a single specimen. Franz cell 1, 2, and 3 donor compartments were loaded with 450µL of GRFT drug substance in PBS1X, at a concentration of 2.25mg/mL for a total dose of approximately 1mg GRFT. Franz cell 4 donor compartment was loaded with PBS1X. Receptor samples were taken at time zero and over time up until the end of the experiment (six hours).

5.4   DISCUSSION AND CONCLUSIONS

GRFT has two mechanisms of action against HIV infection. This antiviral protein can bind to gp120 and prevent attachment of HIV to host cells [96]. When vaginally delivered, this primary mechanism of action would be fulfilled within the vaginal lumen. GRFT also has the ability to inhibit HIV binding to the DC-SIGN receptor and viral transfer to CD4 cells [97]. This secondary mechanism of action would require GRFT to permeate the cervicovaginal epithelial tissue in order to reach DCs beneath the epithelium.

The cervical tissue permeability and localization of GRFT, upon cervical epithelium application, were investigated utilizing excised human cervical tissue and the Franz cell system. Test compounds included GRFT 1mg drug substance solution, GRFT 500ug drug substance solution (to match gel loading), GRFT gel (500µL of GRFT 0.1% gel; loading dose of 500µg GRFT), and GRFT-AlexaFluor488 1mg drug substance solution. Unlabeled GRFT was evaluated to confirm that labelling would not have an effect on permeability findings. HPLC-F analyses of donor and receptor solutions showed >94% of the loading dose of GRFT (drug and gel) remained in the donor compartment of the Franz cell. No GRFT was found in the receptor. ELISA and SDS-PAGE data were in agreement with HPLC data. These results suggest that upon human vaginal delivery, GRFT will remain in the vaginal lumen.

Further, for both tissue detection methods and for both drug substance and gel formulation, GRFT adhered to the superficial cells of the human cervical epithelium. GRFT did not permeate past this superficial portion of the cervical epithelium; it did not reach the intermediate epithelium, basal epithelium, or stromal tissue. Additionally, no gross morphological changes were evident for GRFT application to human cervical tissue over six hours of exposure, in comparison to non-exposure tissues (pre-tissue). These results show that

GRFT adheres to the superficial layer of the cervical tissue, and it is safe toward that tissue. However, GRFT does not penetrate past the initial layer of this type of intact tissue, due to its large protein size. Therefore, when vaginally delivered to an intact tissue, GRFT will most likely not reach its target for its secondary mode of action. In the presence of epithelium abrasion or tears that can be present due to injury, or STIs, GRFT could find access to a tissue breach in order to pass through and reach sub-epithelium components, including DC-SIGN on DCs.

This is the first set of experiments to investigate cervical tissue permeability and localization of GRFT API and formulation. The cervical tissue permeability results generated here support experimental results of other groups working with GRFT. Kouokam et al. showed that GRFT has the ability to bind to cells within a cross section of human cervical epithelium tissue and adhere to the surface of Ect1/E6E7 cells, a human ectocervical cell line. Further, this adherence was dependent on the amino acid aspartic acid in GRFT's three binding pockets [108]. Nixon et al. vaginally administered GRFT gel to mice and found no systemic uptake of GRFT [154]. Taken together, when GRFT is delivered locally to the vagina, it will not penetrate the tissue but has the ability to bind to oligosaccharides on the cells of the superficial cervicovaginal epithelium.

In conclusion, these permeability experiments established the human cervical permeability and localization profile of GRFT, which is of importance for a vaginally delivered compound. When vaginally administered, GRFT will remain within the cervicovaginal luminal environment and has the ability to form a protective barrier layer on the human cervical epithelial tissues. By remaining in this site, GRFT will elicit its potent antiviral activity by binding to viral gp120 [96, 105]. However, given what has been determined about GRFT in the presence of cervicovaginal secretions (Chapter 4), a potential major limitation to these

experiments may be present. These experiments did not include cervicovaginal secretions in the donor compartment of the Franz cell, which is to represent the cervicovaginal lumen. In the presence of cervicovaginal secretions and GRFT non-specific binding within these secretions, GRFT may not adhere to the cervicovaginal superficial epithelium. Further studies are warranted to investigate this situation.

6.0   MAJOR FINDINGS AND FUTURE DIRECTIONS

6.1   INTRODUCTION

HIV/AIDS persists as a global pandemic, with the overwhelming majority of the infections caused by sexual transmission. Physiological susceptibility and socioeconomical disadvantages make women more likely to become infected than men [3].The lack of effective prevention modalities for women has prompted research and development of new approaches to prevent disease spread. In the early 1990s, vaginal microbicide development emerged in the field of HIV prevention research. The work presented within this dissertation project contributes to vaginal microbicide research, focusing on elucidation of potential challenges to the optimal formulation and vaginal delivery of Griffithsin.

In this dissertation project, we tested three hypotheses related to potential formulation and delivery challenges of GRFT as a vaginal microbicide: (1) We hypothesized that GRFT, given its proteinaceous nature, will undergo physical and chemical instabilities that will affect its successful formulation and vaginal delivery (2) We hypothesized that GRFT, due to its lectin property, will undergo binding interactions with human cervicovaginal secretion (glycoproteins) that will negatively impact GRFT-gp120 binding; and (3) We hypothesized that GRFT, given its proteinaceous nature, will not permeate into human cervical subepithelial tissue in order to reach areas containing dendritic cells and CD4 T cells. Therefore, if HIV reaches the subepithelium but GRFT does not, GRFT will not inhibit HIV binding to the DC-SIGN receptor and subsequent

viral transfer to CD4 cells. Our hypotheses were addressed by three specific aims that identified degradation pathways of GRFT (Chapters 2 and 3), evaluated GRFT chemical modifications and GRFT non-specific binding within human cervicovaginal secretions (Chapters 3 and 4), and assessed GRFT human cervical tissue permeability (Chapter 5). The experiments conducted throughout this dissertation project also fulfilled some of those necessary to investigate GRFT's potential to be a leading vaginal microbicide candidate. Specific ideal vaginal microbicide drug candidate characteristics explored were #1, 2, 4, and 5, as outlined in Table 6.

The purpose of this final dissertation chapter, Chapter 6, is to present a summary of major findings, including implications and limitations. Additionally, the contributions that this work makes to the microbicide field and directions for further development of GRFT as a vaginal microbicide candidate will be also described

6.2   SUMMARY OF MAJOR FINDINGS AND IMPLICATIONS

6.2.1   Griffithsin Oxidation

Preformulation studies are essential in pharmaceutical R&D. Within these assessments, the prospective API is exposed to a variety of environments in order to determine its susceptibility to instabilities or potential obstacles for formulation, administration, and targeting. These experiments are important in preparing a drug formulation by providing knowledge of processing, storage, and usage conditions necessary to avoid or against which to protect. It is important for pharmaceutical agents to maintain chemical and physical stability in their dosage

forms and during their specified delivery. Therefore, both standard preformulation assessments and vaginal delivery-specific preformulation assessments were investigated for GRFT.

Preformulation stability assessments were conducted for GRFT drug substance under the following conditions: temperature variations, presence of light, freeze/thaw cycling, agitation, various ionic strength solutions, oxidation, in vaginal fluid simulant, and with cervicovaginal enzymes. Additionally, the compatibility of GRFT with commensal vaginal bacteria was tested. During these evaluations, GRFT proved to be a robust protein and compatible with the commensal vagina bacteria, which supports the continued development of a vaginally-applied GRFT microbicide product. However, one chemical instability emerged – GRFT was highly prone to oxidation. This chemical degradation pathway is of major concern for the vaginal delivery of a GRFT product, due to the potential for oxidation in formulation from impurities found in the excipients [155-156], and during vaginal delivery, due to hydrogen peroxide-producing *Lactobacillus* sp. and enzymes [50, 125-129].

Therefore, it was a rational step to further explore GRFT oxidation, including GRFT stability in human cervicovaginal secretions and to identify the oxidized amino acids. These analyses included: (1) determining the stability of GRFT in human cervicovaginal secretions, and (2) identifying the amino acid(s) of GRFT that are prone to oxidization. To evaluate the impact of cervicovaginal secretions on the chemical stability of GRFT, this microbicide candidate was exposed to human cervicovaginal lavage (CVL) samples. Upon GRFT exposure to human CVL, GRFT displayed a similar degradation pattern as when exposed to exogenous hydrogen peroxide. To further investigate this degradation pathway(s), intact mass analysis was performed on GRFT and GRFT oxidation products after exposure to both hydrogen peroxide and human CVL, separately. These two conditions produced the same chemical modification – a

single amino acid oxidation (16 Da addition to GRFT monomer molecular mass), as determined by LC-MS intact mass analyses of HPLC-F GRFT protein eluent fractions (denoted as HPLC Peak A, Peak B, and/or Peak C throughout Chapter 3).

Prior to this analysis, the amino acid sequence of recombinant GRFT had not been confirmed. The amino acid sequence of recombinant GRFT was determined to be the same as the published sequence of GRFT isolated from red alga *Griffithsia* sp. [96]. To determine which amino acid was oxidized in this protein, peptide sequencing was performed on parent GRFT and one hydrogen peroxide oxidative product (Peak C; both monomers of the dimer containing one oxidized amino acid). Peptide sequencing of Peak C indicated an oxidized methionine at position 78 (single oxidation; methionine sulfoxide).

These results support our hypothesis that GRFT is chemically unstable – it oxidizes in the presence of hydrogen peroxide and upon exposure to human cervicovaginal secretions, which are both of concern for successful vaginal formulation and delivery. However, oxidation via hydrogen peroxide exposure does not affect GRFT-gp120 binding. These outcomes bring about several points or approaches to take into consideration when addressing the issue of GRFT oxidation:

1. Antioxidants could be added to the formulation for the prevention of GRFT oxidation. In a separate set of studies, the capacity of several antioxidant excipients to protect GRFT from oxidation was evaluated. Tested antioxidant excipients included: methionine (0.09 and 0.25%), cysteine (0.09 and 0.25%), glutathione (0.09 and 0.25%), vitamin E TPGS (0.009%), disodium EDTA (0.05%), ascorbic acid (0.1%). None of these antioxidants afforded any significant protection from oxidation to this protein.

2. GRFT drug substance and GRFT drug product must maintain impurity and/or degradation product levels (e.g., oxidation products) the specified threshold [157-158]. For example, if investigating a dose of 1mg with GRFT drug substance that contains 92% GRFT homodimer and 8% GRFT heterodimer (920μg GRFT homodimer and 80μg heterodimer with one monomer containing an oxidized methionine), the level of impurity exceeds the qualification threshold. Manufacturers can work to decrease the level of impurity to below the threshold value. However, in GRFT's case, this impurity is also part of a degradation pathway; therefore, decreasing the starting impurity level will not change the overall outcome of oxidative degradation. On a positive note, the oxidative products of GRFT retain gp120 binding activity, so this conversion process is not likely to affect bioactivity of GRFT.

3. GRFT could be made available in its fully oxidized state (both monomers containing one oxidized methionine at position 78), since the oxidative product maintains gp120 binding activity. This protein would be considered a new API since it is chemically different than GRFT. This new protein would need to be fully characterized based on stability, compatibility, activity, and safety.

4. Another approach could be the elimination of methionine at position 78 of the GRFT amino acid sequence. This mutation/variation should completely eradicate any impurity issues with stock material and oxidation concerns. Efforts are being made by Dr. Kenneth Palmer at the University of Louisville and Dr. Barry O'Keefe at NCI to eliminate this oxidizable amino acid of GRFT. GRFT variants without methionine at position 78 could be manufactured and characterized.

5. Utilizing a suitable drug delivery strategy to protect GRFT from oxidation might also prove advantageous. Encapsulating GRFT, in nanoparticles for example, might protect GRFT against oxidation in the formulated state and within the vaginal lumen.

6.2.2 Evaluation of the Effect of Human Cervicovaginal Secretions on Griffithsin Binding to gp120

Given that GRFT will encounter human cervicovaginal secretions upon vaginal administration, it is not only important to investigate this protein's stability within this biological fluid but to also evaluate the effect of this biological fluid on GRFT binding to its anti-HIV target, gp120. GRFT is a lectin and binds to N-linked high mannose glycans on glycoproteins [96, 106-107]. Therefore, the potential exists for GRFT to bind, non-specifically to glycoproteins within the cervicovaginal environment, which could negatively impact anti-HIV activity [130-131].

Due to these observations from the GRFT-oxidation experiments (For intact mass analyses, CVL samples containing GRFT needed to be concentrated. Further, the GRFT oxidative products from CVL exposure displayed decreased gp120 binding in comparison to their hydrogen peroxide exposed counterparts.) and with the knowledge of non-specific binding of GRFT [108, 130-131], analyses were conducted to explore the effects of human cervicovaginal secretions on GRFT binding to gp120. We hypothesized that GRFT-gp120 binding would be inhibited in cervicovaginal secretions. This microbicide candidate was exposed to human cervicovaginal lavage (CVL) samples from women with normal vaginal flora or from women with bacterial vaginosis. Binding to gp120 was assessed utilizing an ELISA. These two categories of CVL were tested because: (1) N-linked high mannose glycans are present in human

cervicovaginal secretions, with normal microflora secretions containing more N-linked glycans than BV secretions [131], and (2) Decreased GRFT binding was found in BV cervicovaginal secretions in comparison to secretions with normal microflora [130]. Therefore, we predicted that the GRFT-N-linked glycan binding would be more pronounced (more inhibition of GRFT-gp120 binding) in cervicovaginal secretions with normal flora as compared to cervicovaginal secretions with BV. These results are supportive of those of the larger studies [130-131, 145] with these human CVLs and represent the first set of experiments performed to investigate the effects of human cervicovaginal secretions on GRFT-gp120 binding. Overall, the human cervicovaginal secretions containing normal flora negatively impacted (decreased) GRFT-gp120 binding. Human cervicovaginal secretions with BV did not negatively impact GRFT-gp120 binding. The results of this experiment bring attention to several important factors to be kept in mind while performing GRFT nonspecific binding within cervicovaginal secretions:

1. GRFT is a lectin; it is a 'sticky' macromolecule. If it is binding to 'off-target' biological constituents, then it is less likely to bind to its intended target, HIV-1 gp120. Therefore, the loading dose may need to be increased in order to compensate for these interactions. In order to determine a dose that takes into consideration GRFT-nonspecific binding, one could take several approaches which could include: (1) Comparison of EC_{50} or EC_{90} (conservative approach for HIV prevention) between GRFT control and GRFT exposed to CVL containing normal microflora; and (2) Comparison of gp120 binding values between GRFT control and GRFT exposed to CVL containing normal flora and the dose contained within the current gel and film dosages [153], which is 1mg per dose. If using approach #2: GRFT-gp120 binding was decreased by 10% when exposed to CVL from women with normal microflora,

so the new dose to account for this binding must be 110% of 1mg, which is 1.1mg/dose. In order to move forward with increased loading dose, *in vitro* and *in vivo* (animal) safety would need to be performed and confirmed.

2. Normal vaginal flora is associated with decreased HIV infection, whereas the presence of BV increases the likelihood of HIV acquisition and transmission [146-147]. Normal vaginal flora is associated with decreased GRFT-gp120 binding, whereas the presence of BV does not affect GRFT-gp120 binding. Given these opposing circumstances, it would be interesting to investigated GRFT *in vitro* efficacy under these conditions.

3. Utilizing a drug delivery strategy might also prove advantageous. Encapsulating GRFT, in nanoparticles for example, might protect GRFT from non-specific binding within the vagina lumen. This may however add to the cost and require additional physical and chemical stability testing.

6.2.3   Human Cervical Tissue Permeability and Tissue Localization of Griffithsin

When delivered vaginally, GRFT must enter the cervicovaginal epithelium in order to elicit its secondary mechanism of anti-HIV activity. To determine the cervical tissue permeability and localization of GRFT, excised human ectocervical tissue and the Franz cell diffusion system were used. Test compounds evaluated include GRFT drug substance solution, GRFT gel, and GRFT-AlexaFluor488 drug substance solution. Two tissue detection methods were used for cervical tissue localization of GRFT. GRFT labeled with the green fluorescence tag AlexaFluor488 was previously developed [108]. Immunohistochemical staining with GRFT and antibodies was developed and utilized in this dissertation project as a secondary detection

method. These permeability experiments were within the first study to investigate the human cervical tissue permeability and localization of GRFT. These results established the human cervical permeability profile of GRFT, which is of importance for a vaginally delivered compound. In both tissue detection methods and for drug substance and gel formulation, GRFT adhered to the superficial cells of the human ectocervical epithelium. GRFT did not permeate through the cervical tissue; no GRFT was found in the receptor chambers of the Franz cells throughout the duration of the experiment (six hours). Additionally, no gross morphological changes were evident after GRFT application, as a drug solution or in gel formulation, in human cervical tissue over six hours of exposure, in comparison to non-exposed control tissues. These results create several implications for consideration:

1. Since GRFT does not permeate into or past the superficial ectocervical epithelium:

    a. No systemic absorption should be expected. Therefore, the production of off-target adverse side effects is unlikely.

    b. GRFT will remain in the lumen, so it will bind to HIV gp120 in the vaginal lumen and prevent HIV infection.

    c. GRFT will not localize to areas of DCs and CD4 cells [159]. If HIV reaches here and GRFT is not present, then HIV infection may occur.

    d. GRFT will bind to the cells of the human superficial ectocervical epithelium. It would be interesting to determine if this binding provides a barrier to infection and/or if it prevents GRFT-gp120 binding, since GRFT-cell surface binding is also oligosaccharide dependent [108]. Additionally, it would be interesting to determine how long GRFT stays on/within the superficial epithelium considering the cells that compromise this layer undergo desquamation.

e. In the presence of epithelium abrasions or tears that can be present due to injury or STIs, GRFT could pass through and reach sub-superficial epithelium components.

2. The epithelium of the endocervix is composed of a single layer of columnar cells; therefore, the permeability/localization profile of GRFT may differ than that of the ectocervix (multi-layered, stratified squamous epithelial tissue).

3. *In vivo* GRFT-gp120 binding will be affected by both GRFT-glycoprotein binding and GRFT binding to cells (bound and sloughed) within the vagina.

These results support those of other groups working with GRFT. Kouokam et al. showed that GRFT can bind to a cross section of human cervical epithelium tissue and adhere to the surface of Ect1/E6E7 cells, a human ectocervical cell line. Further, this adherence was dependent on the amino acid aspartic acid in GRFT's three binding pockets [108]. When GRFT is delivered locally to the vagina, it will not penetrate the tissue but has the ability to bind to oligosaccharides on the cells of the superficial cervicovaginal epithelium. Systemic uptake is unlikely, in the presence of intact mucosal tissue. Lack of systemic uptake is beneficial to decrease the potential for GRFT off-target actions that could result in adverse side effects.

6.3   SUMMARY OF LIMITATIONS

Although this dissertation has made several contributions toward the development of GRFT as a vaginal microbicide, there are some limitations:

1 – The human CVL samples used for the oxidation experiments contained normal flora. It would be interesting to compare the oxidation profile of GRFT in cervicovaginal secretions from women with normal vaginal flora versus those with BV. Secretions from women with BV

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contain less hydrogen peroxide producing Lactobacillus sp.; therefore, a decreased oxidative potential in BV secretion is anticipated.

2 – The gp120 binding ELISA is not a marker of *in vitro* bioactivity. This *in vitro* biochemistry assay only involves drug compound (GRFT) binding to target molecule (gp120); no virus is involved. Therefore, investigating GRFT bioactivity in a cell-based model, like the TZM-bl cell culture, and in an *ex vivo* cervical tissue explant challenge model would provide efficacy data to either support or refute data from gp120 binding ELISA experiments.

3 – Human cervicovaginal lavage samples were used to expose GRFT to cervicovaginal secretions. CVLs were used because they are easier to handle (overall larger volume and acceptable viscosity for sample processing) than undiluted secretions [145], and these specific samples were intensively characterized [130-131, 145]. Of important note is that a cervicovaginal lavage is a dilution of pure cervicovaginal secretions. Additionally, cervicovaginal lavage samples had to be further diluted for use with the gp120 binding ELISA (Chapter 4). The total protein concentration (250µg/mL) used in the comparative experiment between normal and BV CVL was an approximate 200-fold dilution compared to an undiluted cervicovaginal secretion. Due to the high viscosity of pure cervicovaginal secretions and volume constraints of CVL, neither pure secretions nor an increased CVL total protein concentration could be used in these experiments. Therefore, effects on GRFT gp120 binding and bioactivity may be more pronounced *in vivo*. Biologically relevant assessments, like this one, deserved further attention.

4 – No clinically relevant target loading dose has been identified for the vaginal administration of GRFT. Thus, the human cervical tissue permeability and localization research conducted was guided by available literature/research findings, models for testing, and

limitations of GRFT detection. The average surface area of the human vaginal vault is 87.46 cm^2 [44]. Current GRFT vaginal gel and vaginal film under development are loaded with 1 mg of GRFT per dose [153]. The orifice donor area of the 7mm Franz cell is 0.38cm^2. Therefore, the appropriate loading amount of GRFT for this Franz cell area is 4µg. However, the HPLC method of GRFT detection is not be able to detect this low drug load or possible lower concentrations within the receptor compartment. Consequently, loading doses of 1mg and 500µg of GRFT were used for the permeability experiments.

5 – Excised human ectocervical tissue was used for permeability experiments with the Franz cell diffusion system. The ischemia process in excised tissues begins at the moment when they are removed from their organ, due to lack of blood and oxygen supply.

The cervical tissue permeability experiments in this dissertation project were performed over six hours. Studies from our laboratory have shown that human cervical tissue possesses viability in the Franz cell diffusion system for this time frame. Additionally, both fresh and frozen tissues were used. Previously no impact of freezing and thawing on the diffusion pathway was found [160-161]. However, due to the lack of blood and oxygen supply, no guarantee exists that permeability will be the same as *in vivo*. Further, viability does not assure that physiological functions are present.

6.4   CONTRIBUTIONS TO THE MICROBICIDE FIELD

In vaginal microbicide research, the spotlight has shifted from non-specific microbicide candidates to drug candidates with specific activity against HIV. Vaginal microbicide drug candidates under preclinical and clinical investigation span small molecules to peptides to proteins including monoclonal antibodies [57-58, 92]. Mechanisms of action for microbicide

candidates that act specifically on the HIV life cycle include antagonists against the CD4 receptor, CXCR4 and CCR5 co-receptors, or C-type lectin receptor; gp120 and gp41 binding agents; reverse transcriptase inhibitors; integrase inhibitors; and protease inhibitors. Multiple preclinical evaluations of GRFT, a biopharmaceutical microbicide, were investigated in this dissertation project. The work presented in this dissertation contributes to the advancement of knowledge within the research field of vaginal microbicides. Additionally, these results are paramount to take into consideration for the further development of a GRFT-based microbicide product and will contribute significantly to the Chemistry, Manufacturing, and Controls (CMC) section of the GRFT-based drug substance Investigational New Drug Application (IND).

Standard preformulation evaluations were adapted from ICH (International Conference on Harmonization) guidelines. Vaginal delivery-specific assessments were developed and initiated in our laboratory. This type of preformulation approach can be used as a systematic tool or guide for the development of other vaginally delivered drug candidates.

Specific to further development of GRFT as a microbicide for HIV prevention, several novel observations were made in this dissertation project:

(1) *GRFT Oxidation*: GRFT oxidized upon exposure to hydrogen peroxide and upon exposure to human cervicovaginal secretions. Prior to this report, the amino acid sequence of recombinant GRFT had not been established. Through further investigation into the oxidative profile of GRFT, the amino acid amino acid sequence of recombinant GRFT was confirmed, and the methionine at position 78 was the amino acid prone to oxidation, producing methionine sulfoxide. However, this oxidation profile did not affect gp120 binding. These GRFT findings do not support one of the characteristics of an ideal vaginal microbicide drug candidate – the environment(s)/event(s) involved in male-to-female sexual transmission of HIV should not

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negatively impact the API (refer to Chapter 1). These details of GRFT oxidization are being used as guidance for the researchers who are creating GRFT mutants/variants that are immune to oxidization.

(2) *Evaluation of the Effect of Human Cervicovaginal Secretions on GRFT Binding to gp120*: This set of data represents the first experiments performed to investigate the effects of human cervicovaginal secretions on GRFT-gp120 binding. This type of experimental approach highlights the importance of performing biologically-relevant assessments, *in vitro* or *ex vivo*, prior to performing expensive clinical trials. Performing this type of novel compatibility/interaction assessment with biological secretions should be employed as a preclinical tool during microbicide product development. However, these GRFT results do not support one of the characteristics of an ideal vaginal microbicide drug candidate – the environment(s)/event(s) involved in male-to-female sexual transmission of HIV should not negatively impact the API.

(3) *Human Cervical Tissue Permeability and Localization of GRFT*: When vaginally administered, GRFT will remain within the cervicovaginal luminal environment to elicit its anti-HIV potential and has the ability to adhere to the superficial ectocervical epithelium. These experiments were the first to establish the human cervical permeability and localization profile for GRFT, which is of importance for a vaginally delivered compound. Given this profile, GRFT remains exposed to it anti-HIV target, and off-target systemic side effects are unlikely to occur *in vivo*. These results are in support of an ideal drug candidate characteristic for a vaginal microbicide – If the API acts on viral gp120 or gp41, then the API should be active within the vaginal lumen. Tissue permeability is not necessary for GRFT bioactivity.

Overall, this dissertation project has addressed gaps in our knowledge about a drug candidate in the microbicide field and will guide further development of GRFT. Further, the methodologies implemented throughout this dissertation project can be used or adapted as part of a strategy to preclinically evaluate other vaginal microbicide candidates.

6.5   FURTHER DEVELOPMENT OF GRIFFITHSIN AS A MICROBICIDE

Findings from this dissertation project show that: (1) GRFT is prone to oxidation with the amino acid methionine at position 78 becoming oxidized to methionine sulfoxide; (2) Human cervicovaginal secretions containing normal flora inhibit GRFT binding to gp120; and (3) GRFT does not permeate through human ectocervical epithelial tissue, but can adhere to the cells of the superficial ectocervical epithelium. This dissertation project and the characteristics of an ideal vaginal microbicide drug candidate (Table 6 of Chapter 1) have identified several future studies which can further contribute to the development of GRFT. Below is a list of proposed studies which can enhance the efforts toward developing GRFT as a microbicide candidate:

1 – Longer term drug substance stability should be performed in order to confirm the data generated in this dissertation project. Storage at 25°C/60%RH or 30°C/65%RH for 12 months, and storage at 40°C/75% RH for six months should be evaluated. Assessments during this time frame should include evaluations of chemical and physical stability, gp120 binding, bioactivity (TZM-bl cells), and safety (*Lactobacillus* sp. and TZM-bl cells).

2 – Due to the known GRFT oxidation vulnerability and the oxidative potential of impurities in formulation excipients [155-156], excipient compatibility studies should be carried out with GRFT and a panel of commonly used excipients for vaginal formulations [162]. These results will efficiently guide excipient selection for future vaginal dosage forms.

3 – During sexual intercourse between a male and female, GRFT will not only come in contact with cervicovaginal secretions, but also with semen, which consists of seminal fluid and sperm. The surface of sperm contains both O-and N-linked glycans [163]. Consequently, the potential exist for binding interactions between GRFT and the glycocalyx of the sperm surface that may interfere with GRFT bioactivity. GRFT-gp120 binding and GRFT bioactivity in a cell based model in the presence of semen should be investigated to confirm or refute this potential concern. Also, characterization of the chemical and physical stability of GRFT within semen would be beneficial.

4 – Testing the safety and efficacy of GRFT and GRFT formulation(s) in the presence and absence of cervicovaginal secretions in TZM-bl cell culture and *ex vivo* cervical tissue explant model would provide further confirmation on anti-HIV activity to support the GRFT/CVL-gp120 binding findings. It is important to correlate bioactivity with the findings from tissue exposure experiments and the cervicovaginal secretion experiments to better understand if GRFT oxidation, binding, and non-penetration are associated with any alterations in efficacy.

5 – It would be valuable to confirm the findings of the human cervical tissue exposure studies (GRFT localization and safety at the superficial cervical epithelium) conducted *ex vivo* with *in vivo* experiments from an animal model, such as macaques or mice. In an animal model, intact blood and oxygen supplies to the tissues would be present, unlike with the excised human ectocervical tissues. These studies could be conducted by vaginally administering GRFT drug substance or GRFT formulation, waiting for a specified duration of time, and then sacrificing the animals for necroscopy. Cervicovaginal lavage could be obtained for analyses. GRFT potentially bound to tissue could be visualized by IHC and microscopy.

6 – GRFT nanoparticle encapsulation has the potential to protect GRFT from the chemical instability of oxidation and from non-specific binding. Further, since nanoparticles undergo cellular endocytosis [164], GRFT nanoparticle encapsulation could be used in order to co-localize GRFT with DC-SIGN within the cervical tissue. For example, nanoparticles that are able to specifically target DCs exist [165]. Nanoparticle encapsulation of GRFT is currently being developed and evaluated in our laboratory.

7 – Unfortunately, no anti-oxidants protect GRFT from oxidative degradation [132]. An alternative approach to eliminate concern for GRFT oxidization within formulation prior to administration and *in vivo* would be to modify methionine 78 to a non-oxidizable amino acid during protein synthesis. GRFT variants are currently being developed with this point in mind. Given GRFT's current chemical instability (oxidation), it is not an ideal vaginal microbicide candidate. If a GRFT protein variant without the oxidizable methionine at position 78 can be successfully synthesized, can be manufactured in large supply (as with wild type GRFT [98]), maintains potent anti-HIV activity, and generates favorable results within the future study points above, then this improved protein variant would show great promise as a microbicide for HIV prevention.

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