BIOPHARMACEUTICAL MICROBICIDES FOR TOPICAL HIV PREVENTION: PRE-CLINICAL EVALUATIONS AND FORMULATION DEVELOPMENT

by

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Over 60 million people have been infected with HIV since the beginning of the epidemic. Currently available methods for prevention have not been sufficient to stop the progression of this pandemic. Considering that many women are unable to negotiate condom use with their partners and are more susceptible to HIV, strategies to prevent heterosexual transmission of HIV must include female-controlled methods. A promising strategy is the development of a topical microbicide to prophylactically inhibit transmission of sexually transmitted infections, including HIV.

The work presented makes significant contributions to the microbicide research field, focusing on product development, preformulation strategies, stability in biological fluids, and drug targeting. We investigated two biomolecule (protein/peptide) microbicide candidates: PSC-RANTES, a chemokine analog of RANTES; and RC-101, a circular θ-defensin analog.

We hypothesized that the use of a drug delivery system will protect the microbicide candidate against degradation before administration, and in biological fluids after administration, while maintaining drug activity. Further, the interaction of the microbicide candidates with human vaginal fluids can result in chemical modification of the drug.

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Identification of degradation pathways for PSC-RANTES and RC-101 was conducted by performing preformulation studies under selected conditions of temperature, pH, and oxidative conditions. Analytical methods used included HPLC, MALDI-TOF MS, CD, and SDS-PAGE. Chemical modifications of RC-101 were evaluated in the presence of human vaginal fluid collected from healthy female volunteers and detected by LC-MS/MS. RC-101 was formulated in a quick-dissolving vaginal film and showed short term stability, efficacy *in vitro* and safety *in vivo* in an animal model. Tissue localization of RC-101 was evaluated using excised human (ectocervical and endometrium) and monkey (vaginal and endometrium) tissues.

Major findings from this work show that: RC-101 formulated in a film drug delivery system protected the peptide from degradation prior to administration; anti-HIV activity of RC-101 was maintained in the formulation; RC-101 was stable at least for 48 h in the presence of human vaginal fluid; and penetration of RC-101 into epithelial tissue was demonstrated. These results contribute to the development of RC-101 into a successful microbicide product and provide a systematic tool for the development of other microbicide molecules.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

There are not enough words to acknowledge all the professional, scientific and personal support I have received over the years during my Ph.D. Since, I moved to the United States, I have been blessed to meet wonderful people that treated me like family.

I have been very lucky to encounter Dr. Lisa Rohan and to have her as my mentor, friend, and advisor throughout this project and so many other aspects of my career and personal life. She has shown me the way and has believed in my potential since my initial days as a volunteer in her laboratory. Her scientific input, guidance, and enthusiasm for research were essential for the development of my passion to this project and the concretization of my ideas. Like a mother, however she is too young to be my mother, so I have to say, like an older sister, she was always pushing me to do better because she knew I could, and at the same time, was always there for me in case I needed it. Lisa, thank you very much for believing in my potential and always giving me the support to become an independent scientist. I would like to extend my thanks to Dr. Rohan's family who have always made me feel very welcome.

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So many thanks to all the lab members (past and present) at Magee-Womens Research Institute that have always been a source of knowledge, fun, friendship, and support throughout my time there. I will miss you all a lot! Special thanks to Marilyn Cost who has followed my growth in the lab since my first days here as a volunteer.

This work would not have been possible without the help of so many people that collaborated in this research. Specific acknowledgments to contributions made to each project have been presented in each chapter.

I am very grateful to my friends and family who have been very patient with my lack of time for them, and had always been a constant source of great moments. Many thanks to my family, my brother Roberto, my sister-in-law Rita and the sweetest niece ever: Julia! My brother was the one that instigated me as a scientist and the main reason I ended up in Pittsburgh. All of you make my life more colorful! Special thanks to my very proud parents who have to deal with the long distance. Thanks to both their eternal love and care. My achievements are the product of the education I received from my parents. From my mom, I learned values, respect, patience, and the ability to deal with different personalities. From my dad, I learned to persevere for my goals and to always give my best to any task that came to me.

Very special thanks to the love of my life Jan H. Beumer. He is the best thing that could have happen during my Ph.D work. His patience, love, great sense of humor, endless confidence in my abilities, and admiration to my work has made a vital driving force for the last final stretch of this process. Thank you very much for being this special person that loves me so much.

DEDICATION

To all the women that are in a constant battle against HIV.

LIST OF ABBREVIATIONS

AIDS	Acquired ImmunoDeficiency Syndrome
APC	Antigen Presenting Cell
amu	Atomic Mass Unit
BV	Bacterial vaginosis
CCR5	C-C motif receptor 5
CCR5Δ32	Mutation on allele 32 of CCR5
CD	Circular Dichroism
CD4	Cluster of Differentiation 4
CDC	Center for Disease and Control
CID	Collision Induced Dissociation
CONRAD	Contraceptive Research and Development
CV	Coefficient of variation
CXCR4	C-X-C motif receptor 4
DC	Dendritic Cell
DIC	Differential Interference Contrast
DMEM	Dulbecco's Modification of Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ESI	Electrospray ionization
FITC	Fluorescein isothiocyanate
GM	Growth Medium
gp	Glycoprotein
H & E	Hematoxylin and Eosin
HIV-1	Human Immunodeficiency Virus type 1
HPMC	Hydroxypropylmethylcellulose
HVF	Human Vaginal Fluid
IC_{50}	Inhibitory concentration that inhibits 50%
IC_{90}	Inhibitory concentration that inhibits 90%
IRB	Institutional Review Board

IUD	Intra Uterine Device
k	Retention factor for HPLC
LC	Langerhans cells
LC-MS/MS	liquid chromatography coupled online with tandem mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MALDI-TOF MS	Matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS)
MTT	3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N	Number of theoretical plates
N-9	Nonoxynol-9
NIAID	National Institute of Allergy and Infectious Diseases
OC	Oral Contraceptives
PBS	Phosphate Buffered Saline
PET	Polyethylene terephthalate
PSC-RANTES	PSC- Regulated upon Activation, Normal T Expressed and Secreted
PVA	Polyvinyl Alcohol
R^2	Coefficient of correlation
RC-101	Retrocyclin -101
RH	Relative Humidity
RNA	Ribonucleic acid
Rs	Resolution
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SHIV	HIV-1/SIV chimeric virus
SIV	Simian Immunodeficiency Virus
SRM	Selected Reaction Monitoring
STDs	Sexually Transmitted Diseases
STIs	Sexually Transmitted Infections
T	Tailing factor

TBS	Tris-Buffered saline
UV	Ultraviolet
Vitamin E TPGS	Vitamin E Tocopheryl Polyethylene Glycol Succinate
WHO	World Health Organization

1.0 INTRODUCTION AND PROJECT SPECIFIC AIMS

1.1 HIV INFECTION – A MAJOR HEALTH PROBLEM WORLDWIDE

In the summer of 1981, in California, young men began falling ill with opportunistic infections, especially pneumonia caused by *Pneumocystis pneumonia* (1). Upon more detailed examination, doctors discover that these men lack $CD4^+$ T-cells, a sub-group of lymphocytes that play an important role in the immune system. Several similar cases emerge in other parts of the country with the additional diagnosis of a rare and aggressive form of cancer called "Kaposi's sarcoma" (1-5). The only identified common link for all these patients is that they are either homosexuals or intravenous drug users. Suddenly, this mysterious disease receives the stigma of "gay plague".

These first cases were noted in 1981, but evidence has shown that the earliest case of Human Immunodeficiency Virus (HIV) positive infection had appeared a couple of decades before. Plasma samples taken in 1959 from a man living in Leopoldville, Belgian Congo (now Democratic Republic of Congo) that had died from an undiagnosed disease, were analyzed after HIV testing was developed. The blood sample from 1959 was confirmed later on as reactive with HIV-1 by established assays (6, 7).

In the early 1980s, the number of infected people started to increase rapidly. At that time, the Center for Disease Control (CDC) began to receive a number of reports of this mysterious disease, which resulted in a recommendation from the CDC to give the name of Acquired Immunodeficiency Syndrome (AIDS) in 1982 (1, 8). Later that year, indications were found that AIDS was an infectious disease, and it was transmitted by contact with body fluids

including contaminated blood (8). At that point, it was still unclear what caused the development of AIDS.

From being stigmatized as a homosexual and drug user disease, AIDS was only gradually seen as a more global epidemic when the first documented AIDS case by heterosexual transmission appeared in 1983 (9). Several researchers were investigating this new virus; however, the identification of HIV occurred only in 1984 when Robert Gallo at the National Institute of Health (NIH) and Jay Levy at the University of California San Francisco (UCSF) isolated the virus (3-5). After the discovery of HIV, the development of AIDS was more understood. AIDS emerges from the progression of an infection caused by HIV. In that year, the United States secretary of Health and Human Services, Margaret M. Heckler, optimistically predicted that a vaccine against HIV would be available within 2 years. Unfortunately, that prediction proved to be wrong.

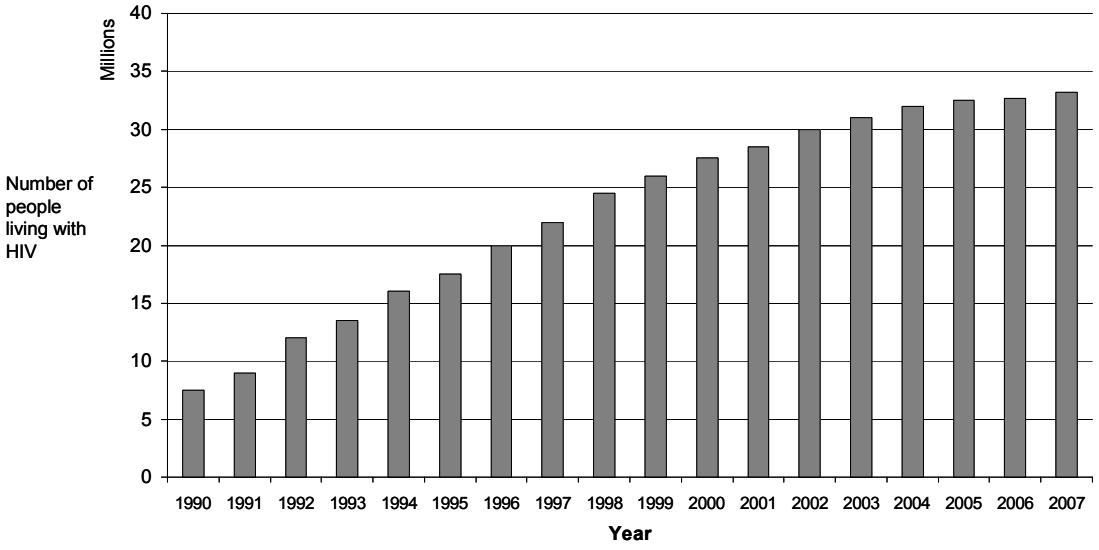


Figure 1-1. Estimated number of people (in millions) living with HIV worldwide from 1990 to 2007. Adapted from the World Health Organization (WHO) 2008 Annual AIDS Report (10).

From a few isolated cases, HIV quickly spread and became a pandemic (Figure 1-1). Twenty seven years have passed since the first few cases of HIV, and AIDS has become a significant medical problem and global concern. Over 60 million people have been infected with HIV since the beginning of the epidemic, and more than 25 million people have died of AIDS (11). Unfortunately, no cure or vaccine is available so far (12-14), and the traditional methods of prevention have not been sufficient as can be concluded from the alarming number of HIV infected people. In 2007, the number of people living with HIV was estimated at 33 million, with a total of 2.5 million new infections (Table 1-1), while the underdeveloped regions of the globe are affected the most by the AIDS pandemic. Sub-Saharan Africa accounts for more than 70% of the HIV infected people worldwide.

Table 1-1. Global summary of the AIDS epidemic, December 2007.

People living with HIV in 2007

Total	**33.2 million [30.6 – 36.1 million]**
Adults	30.8 million [28.2 – 33.6 million]
Women	15.4 million [13.9 – 16.6 million]
Children under 15 years	2.5 million [2.2 – 2.6 million]

People newly infected with HIV in 2007

Total	**2.5 million [1.8 – 4.1 million]**
Adults	2.1 million [1.4 – 3.6 million]
Children under 15 years	420,000 [350,000 – 540,000]

Adapted from the World Health Organization (WHO) 2008 Annual AIDS Report (11).

Recently, a feminization of the epidemic has been observed. In sub-Saharan Africa, almost 61% of adults living with HIV in 2007 were women, and in the Caribbean, that

percentage was 43% in 2007 compared with 37% in 2001. According to recent statistics, more than 80% of new infections are spread through unprotected sex (11), where heterosexual intercourse represents the primary source of infection for women (15).

Efforts have been made to develop a vaccine to prevent acquisition of HIV. However, limited success in animal models has been achieved thus far, highlighting the need to develop an alternative strategy to reduce or prevent the spread of the disease.

1.2 TOPICAL MICROBICIDES – A PROMISING APPROACH FOR HIV PREVENTION

Prevention of HIV has not been very successful, as evidenced by the increasing number of HIV infections every year (11, 16, 17). Prevention strategies currently being investigated include not only the development of a vaccine but also pre-exposure prophylaxis, behavior change, male circumcision, and female condom use.

Vaccines to prevent HIV have been studied in the past few years, but no successful vaccine has been developed yet (13, 14, 18). HIV has an extraordinary degree of diversity and rapid mutation rate, which makes the development of a vaccine a strenuous challenge (14). The strongest evidence of this challenge appeared in September 2007, when an HIV vaccine being developed by Merck failed, causing the company to stop the progress of a large and once-promising clinical trial. The vaccine was unable to decrease HIV infections in the group receiving the vaccine when compared to control groups, and did not reduce the amount of HIV in the bloodstream of those infected (19).

Pre-exposure malaria prophylaxis is a standard practice in travelers to malaria-endemic countries. The same idea of a prophylactic drug has been investigated for HIV infection, leading to the hypothesis that transmission could be decreased if treatment is delivered before exposure to the virus. Studies have demonstrated that pre-exposure to antiretroviral drugs in primates are efficacious against HIV (20-22). The use of prophylaxis therapy in humans has raised concerns due to the consequences of extended use of antiretroviral therapy in healthy people.

Circumcision is associated with reduced risk of HIV acquisition by virtue of decreasing the large surface area of inner foreskin mucosal epithelium, which is a favorable environment for viral sequestration. Several clinical trials have been conducted to evaluate the benefits of circumcision as a prophylactic tool for HIV prevention. Results have indicated that the risk of HIV acquisition decreases when circumcision is performed. However, a number of issues have been raised (religious, cost, effectiveness, hospital conditions) when applying this practice over an entire population (23, 24).

Prevention strategies available so far do not specifically address the increasing percent of infected women. The only female-initiated HIV prevention currently available is the female condom. They have been introduced in many countries; however, female condoms tend to be more expensive than male condoms, and their use is still not a common practice.

Considering that many women are unable to negotiate condom use with their partners and are more susceptible to HIV, strategies to prevent heterosexual transmission of HIV must include female-controlled methods (25). A promising current strategy is the development of a **topical microbicide** to prophylactically inhibit transmission of sexually transmitted infections (STIs), including HIV (26-28). The product would be applied intravaginally before intercourse by women and would inactivate pathogens deposited into the genital tract.

The microbicide movement was formed when Dr. Zena Stein *et al.* (29) identified the need for a method for women that would protect them against heterosexual infection with HIV. The term initially used was a "virucide", but it was decided that if the product also protects against other STDs then "microbicide" would be a more appropriate term. Nowadays, the term microbicide has been expanded to include not only the development of vaginally applied products but also rectal and penile microbicides (30-32). This dissertation work focuses on the development of a topical vaginal microbicide product.

Recently, a surprising setback occurred in the vaginal microbicides field. In January 2007, the Contraceptive Research and Development Program (CONRAD) announced that two Phase 3 clinical trials of the candidate microbicide cellulose sulfate, a poly-anion, were stopped (33). Cellulose sulfate use was associated with a trend towards an increase in HIV infectivity. Unfortunately, this was not the first product in the microbicides field to fail. Nonoxynol-9, a nonionic surfactant, was initially tested for anti-HIV activity in clinical trials after being used in the market for over 25 years as a contraceptive. Studies concluded that the therapeutic window for N-9 was very narrow, and toxicity included induction of inflammatory responses and cytotoxicity to epithelial cells (34-36). In February 2008 another unexpected setback occurred with a second poly-anionic compound derived from seaweed, Carraguard. The completion of a Carraguard Phase 3 clinical trial showed that no significant protection against HIV was observed when the product was compared to a placebo; however, Carraguard product showed to be safe for use in humans, and is still being investigated as a vehicle for other microbicide candidates (37, 38).

Lessons learned from those trials are being carefully evaluated to improve preclinical studies, animal models used, and the design of clinical trials. Investigations in the microbicide

field are still addressing several questions about HIV mucosal transmission, infectivity rate, and how to assess products *in vitro*. Ongoing research will contribute to the development of safe, efficacious, affordable, and acceptable microbicide products. The microbicide field has extensively discussed and inquired women about the ideal characteristics of a microbicide product (39-42). Some of the most important desired characteristics are compiled below:

- Potent activity against most of the HIV strains.
- Preferably broad activity against other sexually transmitted diseases.
- As effective as the (male) condom.
- Compounds must retain activity in the presence of vaginal fluids and semen, and over a broad pH range, ideally for several hours.
- Not disruptive to the normal vaginal flora.
- Compatible for use with condoms.
- Absence of local toxic effects.
- Low cost.
- Long shelf life.
- Easy to use.
- Products must not be too messy, smelly, easily detectable, strong tasting, or interfere with sexual pleasure in other ways.
- Noncarcinogenic.
- Not irritable to vaginal mucosa.
- No adverse effects on reproductive health.
- Acceptable to all sexual partners.

The long-term goal of our project is the development of a successful and safe topical vaginal microbicide product, keeping in mind the characteristics of an ideal microbicide product.

1.3 VAGINAL ANATOMY, PHYSIOLOGY, AND IMMUNOLOGY

The lower genital tract in women involves four distinct anatomical regions (Figure 1-2): 1) the introitus, which is covered by a keratinized, stratified squamous epithelium resembling skin, 2) the vaginal epithelium, which is covered by a nonkeratinized stratified squamous epithelium, 3) the ectocervix, which is covered by a mucosal layer histologically similar to that of the vagina, and 4) the endocervix, which consists of a simple columnar epithelium with numerous glands (43-45).

Normal cervical stratified squamous epithelium consists of several layers of epithelial cells. The layers can be subdivided into four different classes according to the stage of maturation: basal, parabasal, intermediate, and superficial. Basal cells are separated from the stroma by the basal lamina, layer of collagen fibers. They have the largest nuclear-to-cytoplasm ratio of the normal squamous cells. During maturation, cells move from the basal layer to the superficial layer, becoming flatter, with smaller nucleus, and a larger volume of the cell. Tight junctions are predominant in the superficial layers closer to the lumen. The thickness of cervical squamous epithelium varies; the most values are in the range of 0.2 and 0.5 mm. Age is one of the main parameters to determine epithelium thickness. After menopause the volume of the cytoplasm decreases, and the epithelium becomes atrophic, consisting only of a few layers (46).

The vagina is a thin-walled, collapsed fibromuscular tube (7 to 10 cm), extending from the body exterior to the uterus. The inner mucosal layer is subdivided into the epithelium and

lamina propria. The most external epithelium layer consists of squamous stratified epithelium, similar to the ectocervical epithelium. During perimenopausal (3 to 5 years preceding menopause), there is a reduction in the estrogen levels, which contributes to a reduction of epithelium thickness.

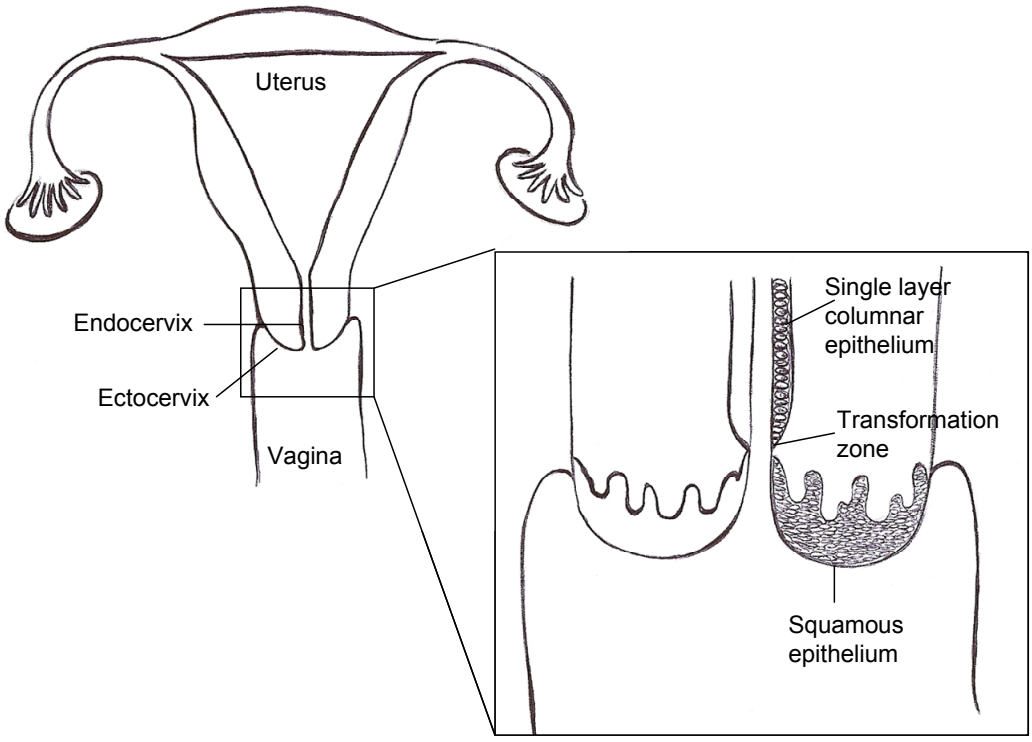


Figure 1-2. Schematic representation of the female reproductive tract.
In detail, the three areas of the cervical tissue: endocervix (single layer of columnar epithelium), transformation zone, and ectocervix (squamous epithelium).
Illustration by Rosaria Rita Siervo Sassi.

The vagina is kept moist predominantly by the transudation of fluid through the vaginal epithelium and by cervical secretion. The amount, consistency, and characteristics of the fluid that accumulates in the vagina are difficult to describe because these parameters change with the menstrual cycle and with reproductive age (47, 48). Vaginal fluid may include contributions from vaginal transudate, Bartholin's and Skenes's glands, exfoliated epithelial cells, residual

urine, and fluids from the upper reproductive tract such as cervical mucus and endometrial and tubal fluids (49, 50). The contribution of cervical mucus to the vaginal fluid has never been fully quantified. The major component of cervical mucus is water, but it also contains mucin, glycoproteins, plasma proteins, enzymes, amino acids, cholesterol, lipids and a range of inorganic ions (51-53). Vaginal fluids play a dual role in preventing HIV infection. On one hand, the vaginal fluids inhibit HIV transmission by acting as a natural mechanism of protection. Several antimicrobial peptides have been detected in the reproductive tissues and cervico-vaginal fluids, including lysozyme in cervical mucus (54), β-defensins in the endometrium (55, 56), defensins in endocervix and endometrium tissues (57), and secretory leukocyte peptidase inhibitor in cervical mucus (58) and in endometrium (59). On the other hand, enzymatic activity in the vaginal fluid has been identified as a major barrier for the delivery and absorption of proteins and peptide drugs from mucosal sites. Enzymatic levels in human cervical mucus is higher during the ovulation period (48). Proteins when administered intravaginally might suffer degradation by two major mechanisms, which include proteases enzymes present in the vagina, and oxidation by hydrogen peroxide produced from Lactobacillus, which are a major constituent of the normal vaginal flora. The identification and quantification of enzymes present in the vagina is not very well elucidated, but it is known that lysozyme and antitrypsin are present in human cervical mucus (60). Other enzymes such as aminopeptidases are present in both vaginal and cervical secretions and their activity may also vary with the menstrual cycle (61, 62). Certainly, enzymatic activity of vaginal fluid in combination with hydrogen peroxide has the potential to reduce biological activity of microbicide candidates. Of specific concern is vaginal administration of proteins and peptides (63).

The mucosal surface is the portal of entry for STIs, being the interface between host and environment. The immune system of the genital tract is part of the larger mucosal immune system that lines the mucosal membranes of the human body (64). The human female genital tract which includes the vaginal fluids present and vaginal mucosa, contains all the essential elements for an effective immune response against genital pathogens (65). Studies using immunohistochemistry and flow cytometry have shown that T cells and antigen-presenting cells (APC) are present throughout the human cervical and vaginal mucosa (66-69); however, the precise localization and quantity of these cells is still under investigation (70). It has been estimated that leucocytes represent 6 to 20% of the total number of cells in fallopian tubes, endometrium, cervix and vaginal mucosa. T cells account for about 50% of the leucocytes, with $CD8^+$ cells predominant over $CD4^+$ cells. A study conducted by Johansson *et al.* showed a band of $CD4^+$ and $CD8^+$ cells under the epithelium of vaginal and ectocervical tissues (64, 65). In addition, Langerhans cells (LC) have been shown to be present within the epithelial mucosa (71-73). Considering this complex immunological machinery, one might wonder how HIV can still be transmitted through the mucosa and proliferate. Surprisingly, it can. It is proven that the female genital tract is the primary route of heterosexual transmission of HIV (10, 29, 74).

1.4 HIV VIROLOGY

Currently, two types of HIV exist: HIV-1 and HIV-2. Both are transmitted through the same routes; however, HIV-2 is much more difficult to transmit and the development of HIV-2 into AIDS is more prolonged. The predominant virus type is HIV-1, and it can be divided in three groups: the major (M), the outlier (O), and the new group (N). More than 90% of HIV-1

infections belong to group M. Each group is subdivided into subtypes that are genetically distinct. Throughout this dissertation work, the abbreviation HIV will refer to HIV-1.

HIV-1 has a spherical morphology of 100 to 120 nm in diameter and consists of a lipid bilayer membrane that surrounds a dense core which contains the ribonucleic acid (RNA), and enzymes such as viral protease, reverse transcriptase, and integrase, and other cellular factors. The membrane contains approximately 72 spikes of the viral envelope (Env) glycoproteins (gp120 and gp41) (75).

1.5 HIV MUCOSAL TRANSMISSION

Two main chemokines, CCR5 and CXCR4, mediate the attachment of HIV to the host cell acting as a co-receptor. The virus that uses the co-receptor CCR5 has been named as R5 HIV, and the virus that uses the co-receptor CXCR4 has been named as X4 HIV. Other chemokine receptors can be used, but CCR5 has been shown to be the major co-receptor for sexual HIV-1 transmission, and most common in the early stages of the disease. CXCR4 co-receptor usage occurs more in the late stages of the disease and it has been associated with more rapid progression of AIDS (76, 77). A few rare HIV-1 isolates can also use other co-receptors (CCR1, CCR2b, CCR3, CXCR6), as shown in *in vitro* studies; although it is still uncertain if HIV-1 would use such co-receptors *in vivo* (78).

During male to female transmission, virus in semen will penetrate the stratified squamous epithelium of the vagina and ectocervix or the columnar epithelium of the endocervix to infect target immune cells (target cells: macrophages, T cells, and dendritic cells (DCs)) within and below the epithelium (79, 80). Macrophages, T cells, and DCs can be infected with HIV-1. The

majority of these cells are located in the subepithelial layers of vaginal and cervical mucosa. Studies have confirmed the presence of Langerhans cells (LCs) in the epithelial mucosa (72, 81). However, it is still uncertain if the virus must cross the epithelium or if the LCs will reach out for the virus. It is known that the majority of the target cells are located in the subepithelial layers of vaginal and cervical mucosa, but studies have also shown the presence of LCs in the epithelial mucosa (72, 81). Literature suggests that HIV-1 could directly infect epithelial cells (82, 83); however, studies conducted by Dezzutti *et al*. (66), and Wu *et al.* (84) have shown that HIV-1 was unable to infect epithelial cell lines, due to the lack of CD4 receptors in epithelial cells. Hladik *et al*. have shown that HIV-1 rapidly penetrates both intra-epithelial vaginal Langerhans and $CD4^+$ T cells in an *ex vivo* human organ culture system (85).

Several mechanisms have been proposed, however it is still uncertain if these mechanisms fully explain the HIV-1 transmission *in vivo*. Suggested mechanisms are illustrated in Figure 1-3. It has been suggested that transmission may occur by:

- direct infection to epithelial cells
- transcytosis through epithelial cells,
- epithelial transmigration,
- uptake by intra-epithelial Langerhans cells,
- circumvention of the epithelial barrier through physical breaches, which will both expose target cells to HIV-1 and trigger subsequent migration (82).

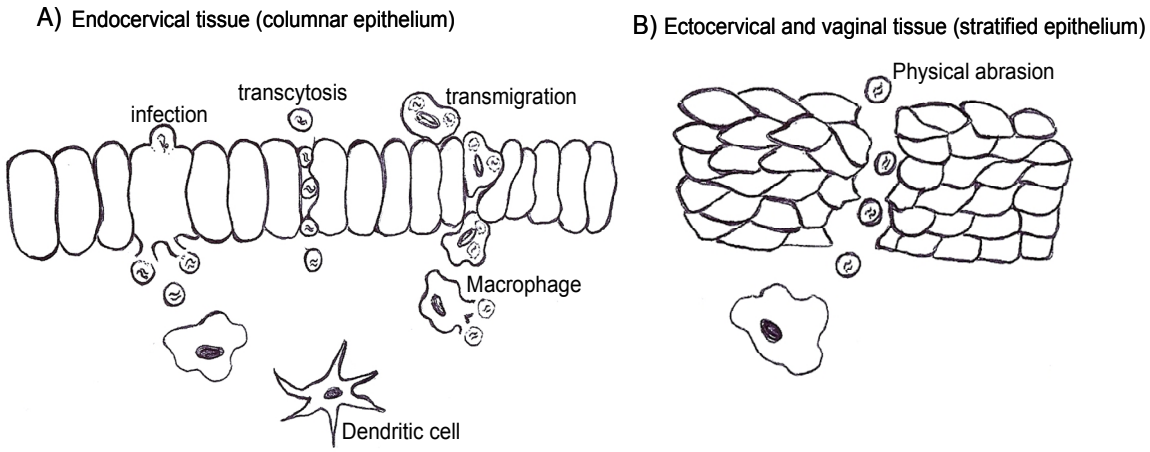


Figure 1-3. Schematic representation of HIV-1 mechanisms for mucosal transmission in female reproductive tract. A) Represents endocervical tissue (columnar epithelium), and B) represents ectocervical and vaginal tissue (stratified epithelium).
Illustration by Rosaria Rita Siervo Sassi.

The HIV-1 envelope is composed of a surface glycoprotein, gp120, and a transmembrane subunit, gp41. These glycoproteins are responsible for the fusion of the cellular and the viral membranes. HIV-1 infection of target cells requires fusion of the virus envelope and cell membrane by sequential interaction of the virus envelope with the cell surface $CD4^+$ and one of the two chemokine co-receptor molecules (CCR5 or CXCR4). The fusion of the virus into the host cell occurs through the formation of a six-helix bundle formation of the two heptad repeats (HRs): HR1 and HR2 domains in three gp41 subunits. After fusion of viral and host cell membrane, the viral RNA genome enters the cell, and undergoes reverse transcription, followed by integration of the pro-viral DNA into the host chromosome. After translation, the viral proteins assemble at the cell membrane, and the immature viral particle containing the RNA genome and viral enzymes egresses the cell. After virion budding, a structural rearrangement of the virion generates a mature viral particle that is ready to infect other cells and proliferate (86, 87), as illustrated in Figure 1-4. The targets in the HIV life cycle for a microbicide candidate are

fusion inhibitors, reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors (15, 82, 86).

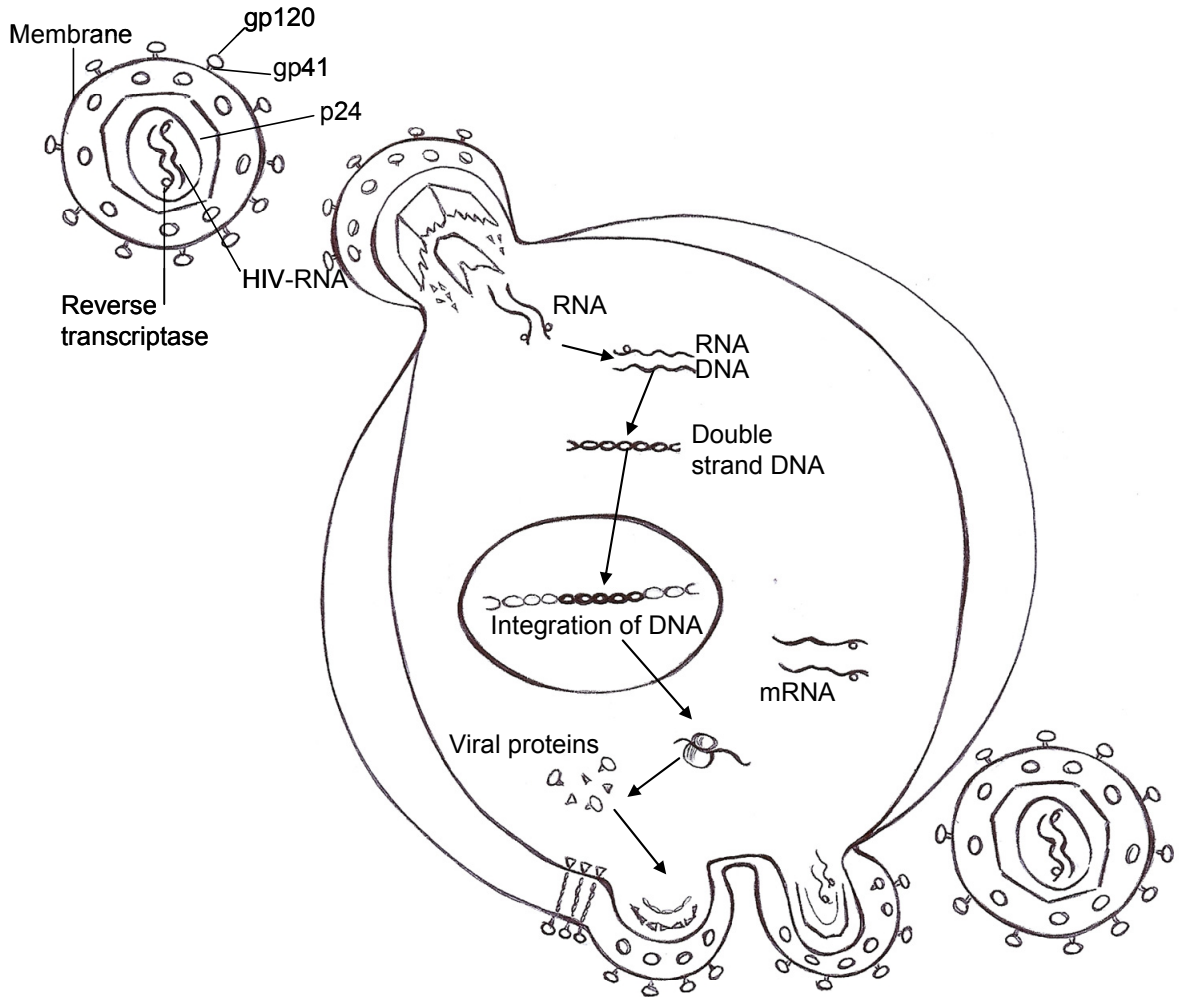


Figure 1-4. Schematic representation of HIV-1 life cycle.
Illustration by Rosaria Rita Siervo Sassi.

1.6 MICROBICIDE CANDIDATES UNDER INVESTIGATION

Several microbicide agents have been identified for and showed activity against HIV-1 *in vitro* by targeting the virus in different stages of its life cycle (27, 88-93). The mechanism of action of each microbicide agent is one property that will define the appropriate vaginal drug delivery system for those products. If a microbicide agent acts by blocking the receptor or co-receptor in the target host cells (macrophages, T cells, and dendritic cells), it is imperative that the delivery system is able to deliver the drug to the site of action, i.e. it must penetrate the epithelial barrier. Conversely, a microbicide agent that disrupts the viral membrane before attachment of the virus to the host cell can be delivered to the vaginal lumen without deeper penetration in the vaginal mucosa.

Microbicide candidates currently being investigated target the virus via different mechanisms of action and are classified as virucides, entry/fusion inhibitors, reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors. A complete list of microbicides in preclinical and drug discovery phase is provided in Appendix A.

1.7 PROTEIN AND PEPTIDE MICROBICIDE AGENTS AND THEIR MECHANISM OF ACTION

1.7.1 PSC-RANTES

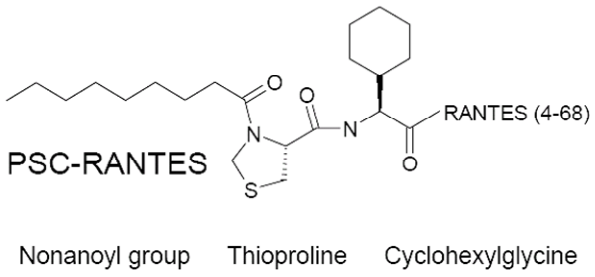
RANTES (Regulated upon Activation, Normal T Expressed and Secreted), CCL5, is a natural chemokine member of the interleukin-8 super family of cytokines. RANTES consists of 68

amino acid residues including four cysteines, the first two being located adjacently to form the sequence unique to the CC-chemokine family. RANTES is a selective protein attractant for memory T lymphocytes and monocytes. It has been shown *in vitro* that RANTES inhibits R5 tropic HIV by binding to the co-receptor CCR5 and competitively inhibiting fusion of the virus (81, 94, 95). Consequently, several analogs of chemokine ligands of CCR5 with enhanced anti-HIV activity have been investigated (96). The first was AOP-RANTES, the aminooxypentane oxime of [glyoxylyl1] RANTES. This molecule was designed to incorporate a more hydrophobic and nearly isosteric replacement for the side chain of the N-terminal methionine of Met- RANTES, a known CCR5 antagonist. All of the analogs have hydrophobic extensions to the N-terminal region, and most do not act as simple receptor antagonists. Instead, their inhibitory mechanism has the additional potential to induce intracellular sequestration of CCR5 (96, 97).

The rationale to use CCR5 inhibitors as a microbicide product is the low toxicity from this type of molecules. Some healthy individuals possess a mutated allele (Δ32) of the CCR5 gene, making these individuals unable to express the co-receptor CCR5 on cell surfaces. CCR5Δ32 homozygotes appear to be resistant to R5 virus infection, whereas CCR5Δ32 heterozygotes show a much slower progression of the disease when infected (98, 99).

PSC-RANTES, (N-α-(nonanoyl)-des-Ser1-[L-thioproline2, L-α-cyclohexyl-glycine3] RANTES), molecular mass of 7,876.63 Da, is chemically identical to native RANTES except for the substitution of a nonanoyl moiety, a thioproline, and a cyclohexyl glycine for the first three N-terminal amino acids of the native protein (Figure 1-5) (91, 96).

A)



B)

S S D T T P C C F A Y I A R P L P R A H
I K E Y F Y T S G K C S N P A V V F V T
R K N R Q V C A N P E K K W V R E Y I N
S L E M S

Figure 1-5. PSC-RANTES structure and aminoacid sequence.
A) Represents PSC-RANTES and the three groups that replaced the first three amino acids of RANTES. B) Represents the amino acid sequence of RANTES (4 – 68).

The mechanism of action of PSC-RANTES occurs by binding of the N-terminal portion of PSC-RANTES to the co-receptor CCR5 causing internalization of the co-receptor. In addition, PSC-RANTES inhibits expression of CCR5 promoting a more prolonged sequestration of the co-receptor when compared to RANTES and other RANTES analogs (91). PSC-RANTES exhibits *in vitro* antiviral activity, against some HIV-1 isolates in the picomolar range (97). However, when challenged with HIV-1/SIV chimeric virus (SHIV) the PSC-RANTES concentration required for *in vivo* protection from infection (1 mM) in Rhesus macaques was 10^6-fold greater than that needed *in vitro* (91).

1.7.2 RC-101

Defensins are a component of host defense against bacterial infections (100). They are cysteine-rich cationic antimicrobial peptides expressed by the leucocytes and epithelial cells of birds and mammals (100, 101). There are three main subfamilies of defensins: α- and β-defensins, and circular θ-defensins, depending on the cysteine pairing and the length of the peptide fragments between the cysteines (100, 102). It has been shown that α-defensins expressed by human $CD8^+$

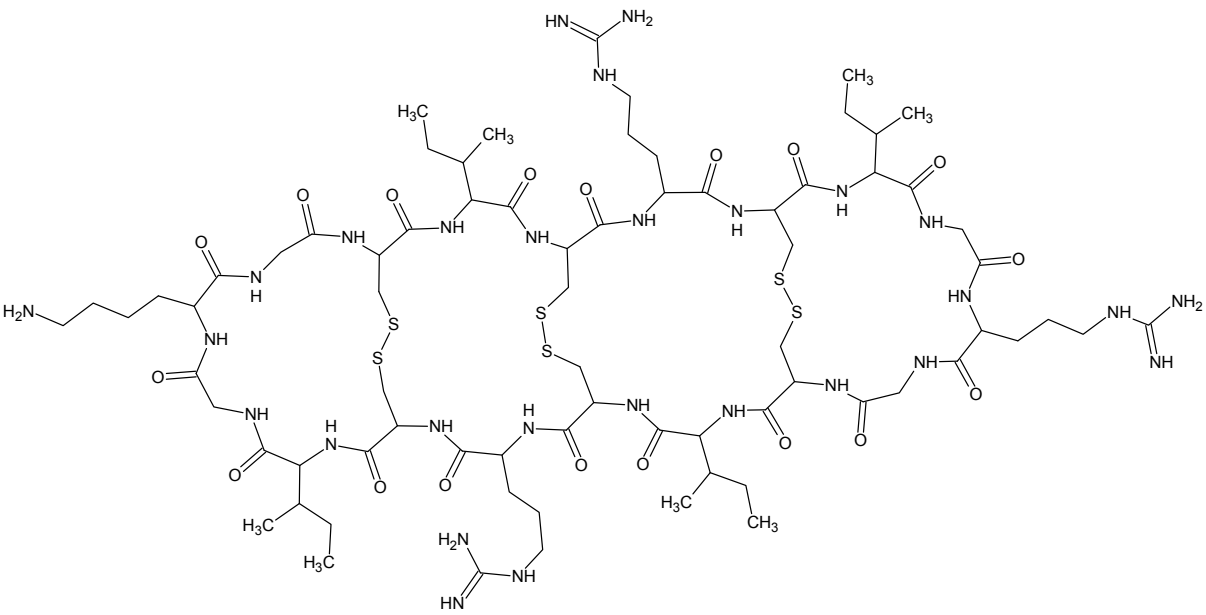
T cells may prevent the development of AIDS. Human α-defensins were shown to protect cells from *in vitro* HIV-1 infection (102). Human β-defensins are highly expressed in the oral mucosa and are induced by bacterial products, and by pro-inflammatory cytokines normally produced in response to infection (103, 104). In addition, human β-defensins-1 isolated from the epithelial layers of the vagina, cervix, uterus, and fallopian tubes, suggest that defensins play a role in microbial host defense in the female reproductive tract (105). β-defensins have shown to possess a high antiviral activity, inhibiting both X4 and R5 strains of HIV-1 (104).

The third subfamily of defensins is the circular θ-defensins, and are only found in non-human primates (100). θ-defensins are peptides that contain 18 residues, tetracyclic with three cysteine disulfide bonds. Retrocyclin, a θ-defensin peptide, has the ability to competitively bind with high affinity to CD4, galactosylceramide (alternative cell surface receptor for $CD4^-$ cells), and gp120; and thereby confers protection against HIV-1 (106). Several analogs of retrocyclin have been investigated for their bioactivity against HIV. One of the analogs, RC-101 (Figure 1-6), possesses a single amino acid substitution from an arginine (R) to a lysine (K) on one of the β turns, conferring stronger bioactivity. The binding site of RC-101 to the gp120 and CD4 is still not completely elucidates; and smaller fractions of RC-101 have not shown to be as effective as the complete circular sequence of RC-101 (personal communication – Dr. Alexander Cole, University of Central Florida).

The binding of retrocyclin to CD4, galactosylceramide and gp120 is a reversible process, and the mechanism of protection against HIV occurs by a competitive inhibition. It has been shown that RC-101 has a higher affinity to gp120 than the retrocyclin. In addition, the affinity of RC-101 to the glycoprotein is 6- to 25- fold higher than the affinity of HIV isolates (107).

It has been shown that RC-101 is non-hemolytic for human red blood cells and non-cytotoxic against several human cell lines at maximum concentrations of 500 μg/mL. RC-101 operates by preventing six-helix bundle formation of gp41 (a 41,000 MW glycoprotein), suggesting a promising antiretroviral drug. The advantage of developing a gp41 target molecule as a microbicide is that the drug will be effective against both R5 and X4 virus types. Furthermore, gp41 sequence is more conservative than gp120, which has been shown to generate a lower incidence of mutation in that region and consequently lowering the chance for viral resistance development based on gp41 mutations (108). RC-101 can inhibit infection *in vitro* by over two dozen primary isolates of HIV-1 representing most known viral clades and subtypes (107, 109). In addition, RC-101 effectively inhibits HIV-1 infection of explant human cervicovaginal tissue (109).

A)



B)

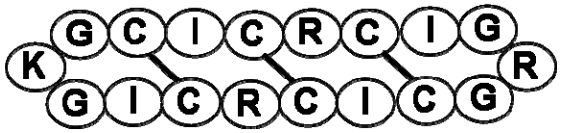


Figure 1-6. RC-101 3D predicted structure and amino acid sequence.
A) Represents RC-101 predicted structure using ChemDraw Ultra, version 11.0.1, Cambridgesoft Corp. (Cambridge, MA). B) Amino acid sequence of RC-101

1.8 VAGINAL DOSAGE FORMS AS MICROBICIDE PRODUCTS

1.8.1 Vaginal drug delivery

Vaginal drug delivery has been used for a number of therapeutic agents, mostly for local delivery of drugs such as antibacterial, antifungal, spermicidal, and steroids (110, 111). There are unique challenges to vaginal drug delivery that must be considered when developing a dosage form.

The vaginal pH is normally between 3.5 and 5.0, but it can change under disease state and in the presence of semen (50). In addition to pH, other physiological factors may impact the absorption and efficacy of vaginally delivered drugs. Cyclical changes, associated to age, phase of the menstrual cycle, and pregnancy, are known to modify the epithelial thickness and composition of vaginal fluids, changing absorption of drugs (112-118).

The most common vaginal dosage forms utilized are also being currently investigated as microbicides. Most of the vaginal microbicides being developed are formulated as gels (119-121). This semi-solid dosage form is largely associated with messiness and leakage of the product which represents a large disadvantage. More recently, new dosage forms have been investigated such as vaginal rings, foams, vaginal tablets, ovules, and polymeric films (122-125). The choice of dosage form is directly dependent on the physical and chemical characteristics of the agent to be delivered as well as patient acceptability. Vaginal rings currently available in the US market as contraceptive products are being investigated as controlled release products for vaginal microbicide application. These microbicide vaginal ring products would provide a long-term release of the drug which results in less frequent need for application and, in the end, improved patient compliance. Vaginal films have the potential advantages of easier application, lower product cost, increased patient acceptability, increased retention time, and increased drug stability.

Delivery of biopharmaceuticals (proteins or peptides) has additional challenges. The simplest way to formulate a protein is in a lyophilized powder to be reconstituted in a solution prior to administration. However, liquid dosage forms will unlikely have the appropriate environment to guarantee protein stability, and would not be suitable for a vaginal delivery of the drug. When applied vaginally, a solution would be prone to leakiness, causing a poor retention

of the drug in the vaginal lumen (26, 110, 111, 122). In addition, the use of a traditional dosage form (hydrogel) for vaginal delivery of proteins or peptides will compromise the stability of the drug due to the high water content in these formulations. As an alternative, formulation of a non-aqueous based semi-solid might eliminate the water content problem, but will decrease patient acceptability and interaction of the product with vaginal fluids present in the vaginal lumen. A novel delivery system should be used to avoid degradation of the protein in the vehicle and maintain the ideal characteristics of the microbicide product.

1.8.2 Quick-dissolving polymeric films

With the advances in the field of polymer science, there is an increasing interest in the development of drug delivery systems that utilize newly available polymeric materials. Polymeric films have recently become a widely accepted dosage form, and have been utilized in the oral delivery of vitamins, minerals, herbal remedies, supplements, cold remedies, pain medications, gastric disturbance medications, treatment of halitosis, antimicrobial agents, and for topical applications (126, 127). Polymeric films form a thick, bioadhesive solution upon contact with cervicovaginal fluids, resulting in rapid release of the drug from the delivery system. Bioadhesive properties of films can increase the retention time of the drug at the target tissue.

The use of vaginal films has several advantages: convenience for women to use without the use of an applicator, lower cost per dose, portability, absence of messiness relative to other current gel vaginal products, increased stability of the drug, and increased retention time of drug in the vagina.

To ensure successful use as female-controlled products, vaginal microbicides need to be designed for women's convenience. In recent years, women's preferences regarding the vaginal

formulations have been researched (128-130). The consumer's perspectives and their choice of formulation vary depending on the individual, partners, cultural norms, age, and economical, social and climatic conditions of the specific geographical region. In many parts of the world, vaginal films are preferred over other dosage forms due to their unique advantages of portability, ease of application, ease of storage and handling. An acceptability study conducted at the University of Alabama and at the University of Zambia (130) has shown that film formulations are more likely to be accepted by women than other vaginal formulations, such as gels, foams, and suppositories. A more detailed discussion of the dosage form chosen for this dissertation work has been included in Chapter 5.

1.9 HYPOTHESIS AND SPECIFIC AIMS

Strategies to prevent infection with human immunodeficiency virus (HIV) have largely been limited to behavior change and condom use. New strategies are being investigated and include the development of topical microbicides to prophylactically inhibit transmission of sexually transmitted infections (STIs), including HIV (15, 17, 74). The product is designed to be applied intravaginally by women before sexual intercourse. HIV-1 infection of target cells requires fusion of the virus membrane and cell membrane by sequential interaction of the virus envelope glycoproteins with the cell surface CD4 receptor and one of the two chemokine co-receptor molecules (CCR5 or CXCR4) (40, 131). Several microbicide agents that target the virus in different stages of the virus life cycle have been developed.

We are investigating two biopharmaceutical (peptide/protein) microbicide candidates: 1) PSC-RANTES, a chemokine analog of RANTES, which binds to co-receptor CCR5, inhibiting

virus fusion; and 2) RC-101, a circular θ-defensin analog peptide, which prevents six-helix bundle formation of gp41, conferring strong protection against HIV-1.

The delivery of topical microbicide agents represents several challenges. The product must overcome physical and chemical barriers such as the presence of mucus and enzymatic degradation in addition to effectively delivering the active pharmaceutical compound to the site of action**.** Furthermore, it is well known that formulation and delivery of biopharmaceuticals (proteins/peptides) can be difficult due to degradation. Stability of the biopharmaceutical seems to be the first obstacle for development of a microbicide product. Protection of the protein or peptide in the formulation may prevent degradation and consequently preserve the activity of the formulation during its shelf life or in use. Our research focused on the interaction of proteins and peptides with biological fluids, the development of a novel vaginal delivery system for proteins and peptides, and the investigation of the efficacy of the drug delivery system in delivering the active substance to the site of action.

Hypothesis

We hypothesize that the use of a drug delivery system will protect a biopharmaceutical (protein/peptide) microbicide candidate against degradation before administration, and in biological fluids after administration, while maintaining drug activity. We further hypothesize that the interaction of biopharmaceutical microbicides with human vaginal fluids can result in chemical modification of the drug.

Specific aim 1. Identify degradation pathways for biopharmaceutical microbicide candidates (PSC-RANTES and RC-101) by performing preformulation studies under selected conditions of temperature, pH, and oxidative conditions. (Chapters 2 and 3)

Specific aim 2. Evaluate chemical modifications to RC-101 in the presence of human vaginal fluid collected from female volunteers. (Chapter 4)

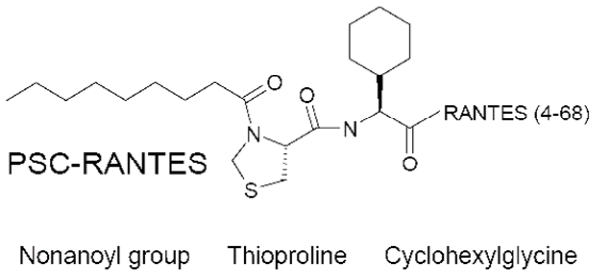
Specific aim 3. Develop a formulation for RC-101 that delivers the peptide and protects it from degradation prior to administration as well as in the presence of biological fluids, maintaining bioactivity. (Chapters 5 and 6)

2.0 PREFORMULATION EVALUATION OF PSC-RANTES

2.1 INTRODUCTION

Regulated upon Activation, Normal T Expressed and Secreted (RANTES, CCL5) is a natural chemokine, that attracts memory T lymphocytes and monocytes (132). RANTES consists of 68 amino acid residues including four cysteine residues, the first two being located adjacently to form the sequence unique to the CC-chemokine family (132). It has been shown *in vitro* that RANTES inhibits R5 tropic HIV-1 by blocking the CCR5 co-receptor, thus preventing HIV infection (94, 95). HIV-1 infection requires fusion of the virus membrane and cell membrane by sequential interaction of the virus envelope glycoproteins with the cell surface receptor CD4 and one of the two chemokine coreceptor molecules (CXCR4 or CCR5). Several analogs of RANTES have been developed to improve the affinity of the drug to the co-receptor CCR5 (95). These analogs, unlike the natural chemokine, have the additional capacity to inhibit the recycling of internalized CCR5, resulting in more profound and prolonged sequestration of CCR5 (133). PSC-RANTES, (N-α-(nonanoyl)-des-Ser1-[L-thioproline2, L-α-cyclohexyl-glycine3] RANTES), is chemically identical to native RANTES except for the substitution of a nonanoyl moiety, a thioproline, and a cyclohexyl glycine for the first three N-terminal amino acids of the native protein (96). The sequence of PSC-RANTES is shown in Figure 2-1.

A)



B)

S S D T T P C C F A Y I A R P L P R A H
I K E Y F Y T S G K C S N P A V V F V T
R K N R Q V C A N P E K K W V R E Y I N
S L E M S

Figure 2-1. PSC-RANTES structure and RANTES amino acid sequence.
A) Represents PSC-RANTES and the three groups that replaced the first three amino acids of RANTES. B) Represents the amino acid sequence of RANTES (4 – 68).

PSC-RANTES exhibits antiviral activity *in vitro* for some HIV-1 isolates in the picomolar range (97). In studies conducted in rhesus monkeys peripheral blood mononuclear cells (PBMCs), PSC-RANTES inhibited propagation of the SHIV SF162 R5 tropic virus and completely blocked SHIV F162 replication, with median inhibitory concentration (IC_{50}) values in the picomolar range (91). However, when challenged with SHIV vaginal transmission in rhesus macaques, the PSC-RANTES concentration necessary for *in vivo* activity (1 mM) was 10^6-fold greater than that needed *in vitro* (91).

Reduced potency observed *in vivo* may reflect: 1) protein modification due to conformational changes, including aggregation; 2) protein modification due to covalent chemical changes; 3) proteolytic inactivation in the vaginal lumen, 4) low penetration of the drug into the mucosal tissue, and/ or 5) drug binding (134). The purpose of this study was to understand how potential formulation conditions as well as the vaginal environment could impact both physical and chemical stability of PSC-RANTES. This data will be required in the formulation of an eventual microbicide product. This study evaluated selected conditions of temperature, pH, ionic strength, presence of hydrogen peroxide, and presence of enzymes to establish rate and extent of degradation, and identify potential degradation pathways of PSC-RANTES. Protective effects of

excipients against oxidation were also investigated. Chemical and physical stability were evaluated by high performance liquid chromatography (HPLC), circular dichroism (CD), UV-spectroscopy, SDS-PAGE, and MALDI-TOF MS. In addition, the anti-microbiological activity of PSC-RANTES to normal vaginal flora and possible pathogens present in the vaginal lumen was determined. Overall, these results will be crucial to establish a preformulation data set to expedite formulation development of a suitable dosage form for PSC-RANTES as a microbicide product.

2.2 MATERIALS AND METHODS

2.2.1 Materials

PSC-RANTES was synthesized by the Peptide Synthesis Facility at the University of Pittsburgh (Pittsburgh, PA), and the molecular mass confirmed by mass spectrometry. Acetonitrile (HPLC grade), trifluoroacetic acid (TFA), sodium phosphate dibasic, sodium phosphate monobasic, phosphoric acid 85%, sodium acetate, formic acid, sodium hydroxide, hydrochloric acid, ß-mercaptoethanol, urea, and glacial acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ). Vitamin E tocopheryl polyethylene glycol succinate (TPGS) was purchased from Eastman Chemical Company (Kingsport, TN). Aprotinin and insulin were purchased from MP Biomedicals (Solon, OH). Polypeptide SDS-PAGE molecular mass standards, 30% acrylamide/bisacrylamide solution (29:1), tricine sample buffer, silver staining kit, and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals were purchased from Sigma (St. Louis, MO). De-ionized

water was prepared from house-distilled water with a Milli Q (Millipore, Milford, MA) water system operating at 18.2 MΩ.cm.

2.2.2 High performance liquid chromatography

A high performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA) was used for the analysis of PSC-RANTES. The HPLC system was equipped with an autoinjector model 717 (Waters), a quaternary pump model 600 (Waters), and an ultraviolet (UV) detector model 2487 (Waters) operated at 280 nm. Separation of the PSC-RANTES peak from degradant products was achieved using a Phenomenex Jupiter Proteo 4 µm 90 Å (4.6 x 250 mm) column (Phenomenex, Torrance, CA) protected by a Jupiter C12 (4.0 x 3 mm) guard cartridge (Phenomenex). The gradient consisted of mobile phase A (0.1% TFA in water (v/v)), and mobile phase B (0.1% TFA in acetonitrile (v/v)) pumped at a flow rate of 1.0 mL/min. Elution conditions started at A:B (70:30) and linearly changed to A:B (57:43) over the first 25 min, followed by a linear decrease to A:B (70:30) in 5 min, and maintaining isocratic condition at A:B (70:30) for 10 min to allow equilibration of the system prior to injection of the next sample. After completion of each sample set, a cleaning step was performed with 90% mobile phase B to guarantee that the more hydrophobic impurities would be eliminated from the column. The retention time of PSC-RANTES was 18 min and the total run time was 40 min. Empower Pro 2 software (Waters Corporation) was used to control the HPLC system. To ensure that the method was suitable for the intended purposes of quantitation and separation of degradants, the accuracy, precision, repeatability, Limit of Quantification (LOQ), and Limit of Detection (LOD) were determined in addition to other suitability parameters defined by the United States Pharmacopeia (USP) (135). Forced degradation studies at extreme conditions of

temperature, pH and oxidative potential were initially conducted to confirm that the HPLC analysis was suitable as a stability-indicating assay to separate and detect potential degradant products.

2.2.3 SDS-PAGE

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed for PSC-RANTES samples. PSC-RANTES was analyzed under reduced and non-reduced conditions on a Tris/Tricine gel with two layers: a stacking layer (3.10% T, 3.25% C) and a separating layer (14.75% T, 0.34% C), where T is the total percentage of acrylamide and bisacrylamide in the gel, and C is the bisacrylamide concentration with respect to T. The reduced samples of PSC-RANTES were prepared from 5 µl of PSC-RANTES solution and 95 µl of SDS reducing buffer (Tricine sample buffer:β-mercaptoethanol (95:5)). The sample was heated for 5 min at 95 °C and cooled to room temperature, followed by transferring 15 µl of the sample to the well. The non-reduced sample of PSC-RANTES was prepared with 5 µl of PSC-RANTES solution and 95 µl of water. Standards in their reduced form were prepared from Polypeptide SDS-PAGE molecular mass standards (Bio-Rad). Detection was performed by silver staining procedure. Gel images were collected with a Geldoc Imaging system (Bio-Rad).

2.2.4 Circular Dichroism Spectroscopy

Circular Dichroism (CD) was used to monitor conformational changes in the secondary structure of the proteins. CD measurements were performed on an AVIV Circular dichroism spectrophotometer model 202 (AVIV Biomedical, Lakewood, NJ) equipped with a 0.1 cm path

length quartz cell. Spectra were recorded between 198 and 300 nm at 25 °C from a 500 μg/mL protein solution in 10 mM phosphate buffer at pH 3.0, 7.0, and 12.0. Three scans were averaged and subtracted from buffer background over the same wavelength range.

2.2.5 MALDI-TOF MS

Matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometric (MS) experiments were performed in the positive-ion linear mode on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA). Samples were prepared by pipetting 1 μL of sample and 1 μL of matrix on an MS stainless steel sample probe. Matrix was prepared by dissolving sinapinic acid in a solution of 0.5% trifluoroacetic acid in acetonitrile:water (1:1 v/v). The probe containing the sample was left to dry completely in air. The spectra were calibrated using external calibration with Insulin B chain oxidized (Sigma) and Aprotinin (Sigma), and Sequazime Peptide Mass Standard calibration mixture 3 (Applied Biosystems). The following parameters were used: accelerating voltage 20 kV with delayed extraction, mass range of 1,000–20,000 Da, and 400 laser shots spectrum.

2.2.6 UV-spectroscopy

PSC-RANTES in concentrations of 0.125 to 2.5 mg/mL was prepared in buffer solutions and UV scans were performed by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). Scans were performed from 220 to 700 nm. NanoDrop software was used to control the spectrophotometer.

2.2.7 Stability of PSC-RANTES

For all stability studies described below, triplicate solutions of PSC-RANTES were prepared by adding PSC-RANTES at a concentration of 500 μg/mL (63.3 μM) in either water or an aqueous buffer solution. The pH of each solution was measured after solubilization of PSC-RANTES in the vehicle. All samples were used within 5 min of preparation, and analyzed by HPLC using the method described in Section 2.2.2. Additional analytical techniques (MALDI-TOF MS, UV-spectroscopy, CD, and SDS-PAGE) were used to confirm results obtained by HPLC.

Effect of temperature

PSC-RANTES solutions in water were placed in incubation chambers for thermal degradation studies conducted at 25, 40, and 65 °C for a minimum period of 1 week. Samples were removed from each chamber 30 min prior to analysis by HPLC to allow the samples to equilibrate to ambient temperature. Thermal degradation samples were also evaluated by MALDI-TOF MS.

Effect of pH

The effect of pH on the stability of PSC-RANTES was evaluated over the pH range from 3 to 12 using 10 mM phosphate buffer solutions. All buffers were prepared using sodium phosphate to avoid interference of other salts in the solution. Knowing that phosphate buffer is not a strong buffer at certain pHs, the pH values were confirmed at time 0 and at the end of each experiment. PSC-RANTES samples over the pH range from 3 to 12 were also evaluated by MALDI-TOF MS, SDS-PAGE, CD, and UV spectroscopy.

Effect of ionic strength

PSC-RANTES solutions at concentrations ranging from 0.125 to 2.5 mg/mL were prepared in water and phosphate buffers (pH 4, 7, and 12) at low (50 mmol/kg) and high (500 mmol/kg) ionic strength to evaluate the effect of ionic strength on PSC-RANTES stability. Additionally, PSC-RANTES was prepared in phosphate buffered saline (PBS) (pH 7.4, 290 mmol/kg). Effect of high ionic strength was evaluated by UV-spectroscopy analysis to determine the formation of insoluble aggregates.

Effect of hydrogen peroxide

The stability profile of PSC-RANTES under oxidative conditions was evaluated by exposing PSC-RANTES solutions to concentrations of 3.0, 0.80, 0.02, and 0.002% (v/v) hydrogen peroxide (H_2O_2). A minimum of five time points were taken from each triplicate solution over a 5-hour period and analyzed by HPLC. Oxidative PSC-RANTES samples were also evaluated by MALDI-TOF MS.

Protective effect of antioxidants

Solutions of PSC-RANTES were prepared followed by the addition of antioxidants commonly used in pharmaceutical products. These solutions were then exposed to 0.8% or 0.02% hydrogen peroxide. The antioxidants used in this study were: sodium metabisulfite, ascorbic acid, methionine, cysteine, glutathione, and vitamin E TPGS. Samples were analyzed by HPLC at times 0, 40 and 80 min after exposure to 0.8% H_2O_2, and at specified times for a 6-hour period after exposure to 0.02% H_2O_2. The concentrations of antioxidants used in the final solution were: 500 μg/mL of sodium metabisulfite, 1000 μg/mL of ascorbic acid, 92.7 μg/mL or

463.5 μg/mL of DL-methionine, 92.7 μg/mL of DL-cysteine, 90.9 μg/mL of L-glutathione, or 90.9 μg/mL of Vitamin E TPGS. Concentration of sodium metabisulfite, ascorbic acid, and vitamin E TPGS were selected based on the inactive ingredient list for FDA drug approved products (http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm - last accessed on 10/22/2008) and the Handbook of Pharmaceutical Excipients (136). Concentration of methionine, cysteine, and glutathione were based on literature reviews on the use of amino acids as competitive inhibitors of oxidation (137, 138).

Effect of light exposure

PSC-RANTES solution (500 μg/mL) prepared in 50 mM acetate buffer pH 4 was placed in an environmental control chamber Caron 6010 (Caron Inc., Marietta, OH) at 40 °C. A D65 light source provided continuous irradiation over the course of one week at an overall illumination of not less than 1.2 x 10^6 lux.h and an integrated near-ultraviolet energy of not less than 200 watt.h/m^2. Samples were removed from the chamber 30 min prior to HPLC analysis to allow equilibration to ambient conditions.

Effect of freeze-thaw cycles

PSC-RANTES solutions (500 μg/mL) prepared in water were stored at -80 °C and allowed to freeze for at least 1 h. Samples were thawed immediately prior to HPLC analysis. Six freeze-thaw cycles were used for this study. After completion of six freeze-thaw cycles, PSC-RANTES solutions were analyzed by CD.

Effect of enzymatic degradation

PSC-RANTES solutions (500 µg/mL) in water were prepared followed by the addition of enzymes commonly present in human vaginal fluid. The enzymes used in this study were: aminopeptidase, lysozyme, and proteinase K. Samples were tested by MALDI-TOF MS at time 0 and after 1 h exposure at 37 °C.

2.2.8 Microbiological activity

Both reference strains and fresh clinical isolates of bacteria were used in microbiological evaluations of PSC-RANTES. *Lactobacillus jensenii* (ATCC 25258), *Lactobacillus crispatus* (ATCC 20225 and ATCC 33197), *Lactobacillus iners* (BCRC 1202 and BCRC 2146), *Gardenerella Vaginalis* (ATCC 14018), and *Neisseria gonorrhoeae* (ATCC 19424, and ATCC 49226) were tested. PSC-RANTES solutions in water were tested as reported previously (139). Briefly, bacteria were cultured on an appropriate medium overnight at 37 °C in air containing 5% CO_2. Isolated colonies were suspended in 0.5 mM N-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer pH 7.0 to a density of 0.5 McFarland units. Samples were diluted 1:10 in the same buffer and 10 µl of cells were added to 90 µl of the test solutions and incubated as described above for 30 min. Samples were taken (25 µl) and placed on the appropriate medium and spread using an inoculation loop.

2.2.9 Statistical analysis

HPLC data obtained from the preformulation studies were expressed as the average percentage (%) of the peak area from time 0 ± standard deviation, n = 3. Results were analyzed by one-way

analysis of variance (ANOVA), with multiple comparisons of individual time points by using post hoc Bonferroni correction to detect significant differences under different conditions. P-values ≤ 0.05 were considered to be statistically significant, unless specified otherwise.

2.3 RESULTS

High Performance Liquid Chromatography (HPLC) assay

Linearity of response was shown by analyzing serial dilutions of a standard aqueous PSC-RANTES solution ranging from 10 to 500 μg/mL (1.27 to 63.3 μM). Results for the assay qualification are shown in Table 2-1. The coefficient of regression (R^2) and the residual (%) from the observed values to the back-calculated standard curve were determined. Accuracy was determined by the mean of the response areas from ten injections of PSC-RANTES 10 μg/mL solution. Precision was determined from the coefficient of variation (CV) of the response areas of those ten injections. The Limit of Detection (LOD) was calculated as three times the ratio of area standard deviation to the slope of the calibration curve (140). The Limit of Quantitation (LOQ) was defined as 10 times the ratio of the area standard deviation to the slope of the calibration curve (140) Repeatibility was demonstrated by analysis of calibration curves on different days, and reproducibility was measured by analyzing calibration curves on different instruments by different analysts. The results demonstrate that the method is suitable for the intended purpose.

Table 2-1. Qualification of HPLC method for PSC-RANTES

Factor	Criteria	Results
k	$2 < k < 10$	9.1
N	> 4000	14228
Rs	> 2	3.3
T	< 1.5	1.3
R^2	> 0.999	0.99973
Residual	< 10 %	3.45%
CV	< 1%	0.76%
LOD		0.24 µg/ mL (30.3 pM)
LOQ		0.79 µg/ mL (100.7 pM)

Where k = retention factor, N = number of theoretical plates, Rs = resolution, T = tailing factor, R^2 = coefficient of correlation, Residual = the average difference between the observed values for the standard and the calculated standard curve, CV = coefficient of variation of the response of 10 injections at 10 µg/mL PSC-RANTES concentration, LOD = Limit of Detection, and LOQ = Limit of Quantification.

A blank (water) and a PSC-RANTES (500 µg/mL in water) control solution chromatograms are shown in Figure 2-2 and Figure 2-3, respectively. PSC-RANTES chromatogram elutes two peaks: a main peak (PSC-RANTES) at 18.3 min and a secondary peak pertinent to PSC-RANTES (peak 1) at 17.5 min.

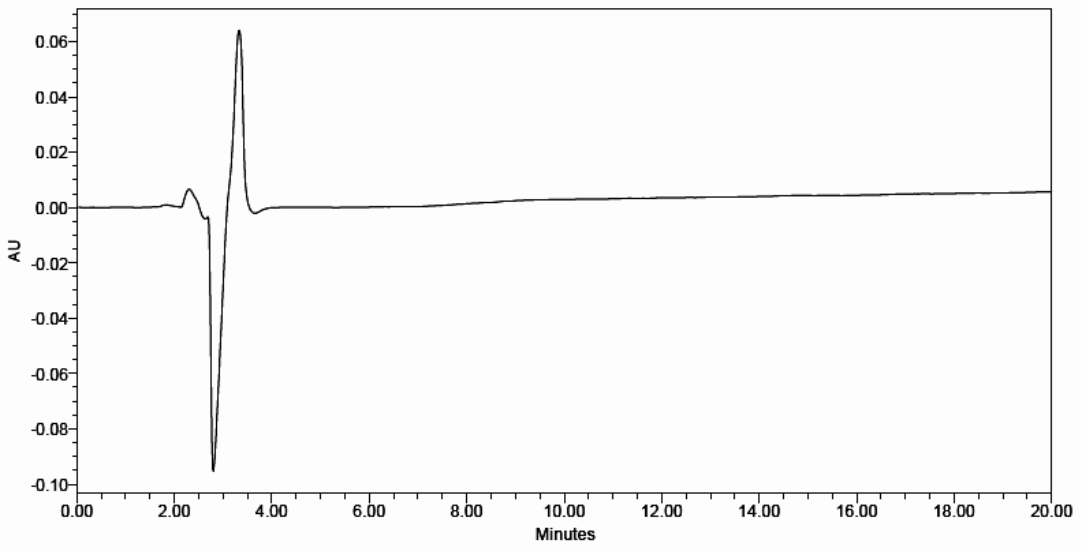


Figure 2-2. Representative HPLC chromatogram of a blank solution.

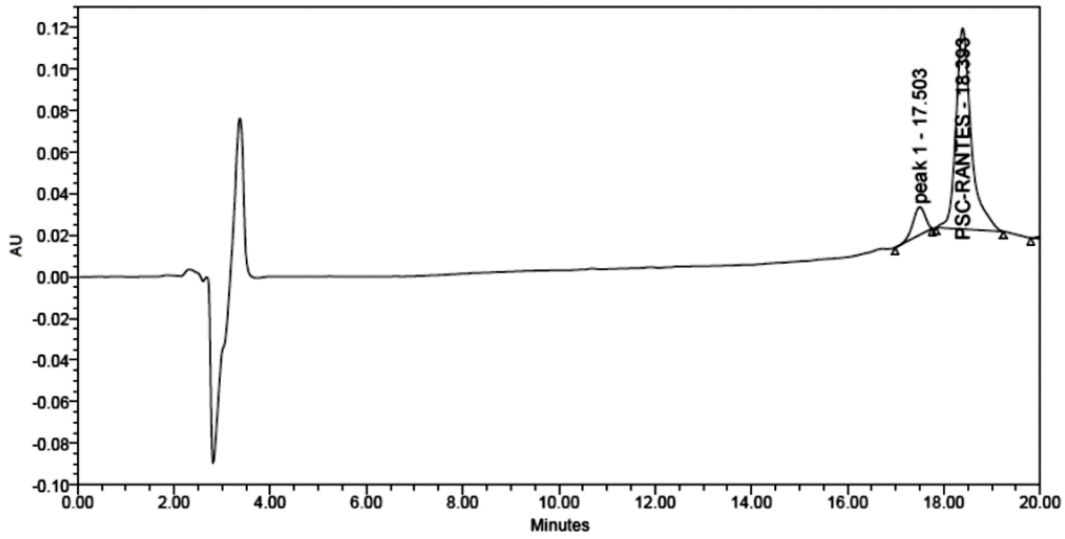


Figure 2-3. Representative HPLC chromatogram of PSC-RANTES (500 µg/mL) in water.

SDS-PAGE assay qualification

The range of detection, specificity and repeatability of the SDS-PAGE method for PSC-RANTES was evaluated. The intensities of the bands increased with amount of protein loaded 0.06 µg (0.51 µM) to 1.2 µg (10.26 µM), as shown in Figure 2-4, wells 2 to 6. The log-

molecular masses of the standards were plotted against their mobility. The mobility of each protein was calculated by dividing the distance the protein traveled from the well, by the distance between the marker and the well, and the molecular mass was calculated. Specificity was confirmed by visual inspection of the bands. PSC-RANTES control samples had a calculated average molecular mass (n = 4) of 7,134 ± 411 Daltons (mean ± SD).

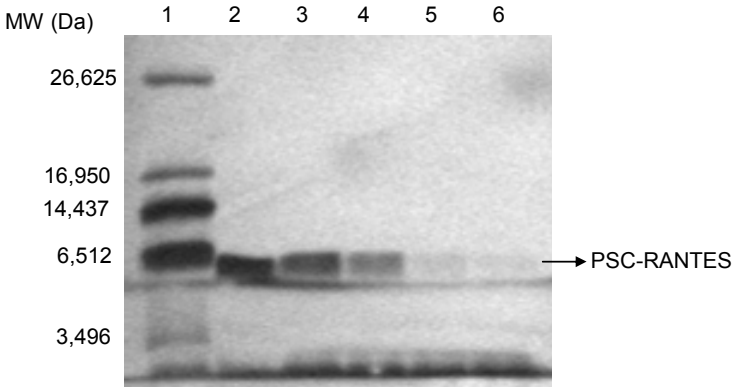


Figure 2-4. Detection limit of reduced PSC-RANTES on SDS-PAGE.
PSC-RANTES detected by silver staining on a SDS-PAGE gel. From left to right: 1) Polypeptide SDS-PAGE Standards, 2 to 6) PSC-RANTES 1.2, 0.6, 0.36, 0.12, and 0.06 µg, respectively.

Stability – Effect of temperature

The effect of temperature on the stability of PSC-RANTES solutions (500 µg/mL in water, pH 4.5) is shown in Figure 2-5. The graph represents the amount of PSC-RANTES (% relative to the amount at time 0). The decrease of PSC-RANTES observed over a period of 1 week (168 h) at room temperature was not statistically significant (ANOVA, $p > 0.38$). PSC-RANTES was stable at 40 °C (97.6 % PSC-RANTES remaining in the aqueous solution) for the first 24 h; however, statistically significant protein loss was observed after one week ($p < 0.001$). PSC-RANTES was stable for the first 2 h of exposure at 65 °C (97.5% PSC-RANTES remaining in

the aqueous solution). Post hoc, Bonferroni correction for multiple comparisons were conducted for the 24 and 168 h time points, and statistically significant differences were observed among room temperature, 40 °C, and 65 °C ($p < 0.05$).

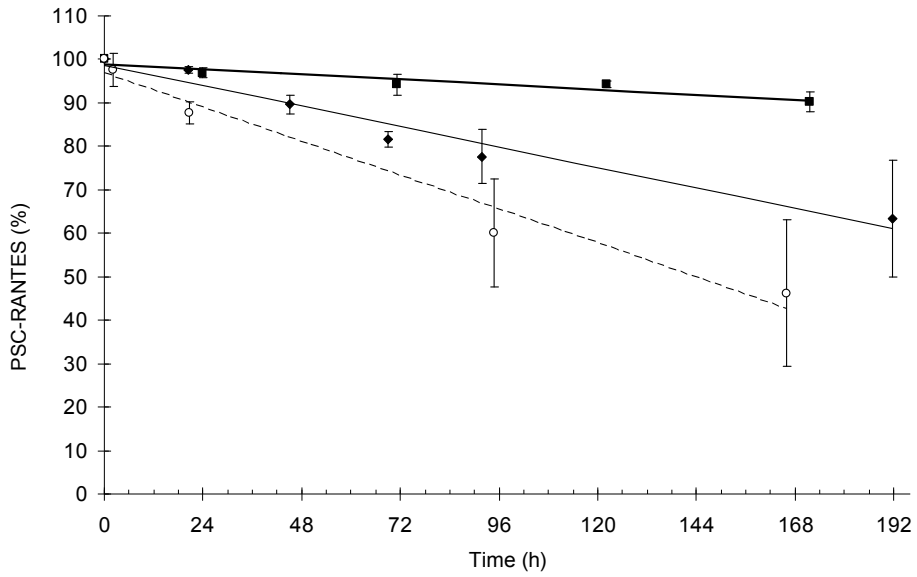


Figure 2-5. Effect of temperature on PSC-RANTES.
PSC-RANTES (500 µg/mL) in water stored over time at 25 °C (■), 40 °C (●), and 65 °C (○), analyzed by HPLC (n=3)

The log concentration versus time was plotted for each temperature condition (Figure 2-6). The slope of each curve was calculated to confirm the statistical results obtained from Figure 2-5, and to estimate the rate of degradation (rate constant, k) under each condition. Chemical stability is generally expressed by rate constant to estimate rate of drug degradation. Assuming first order kinetics, rate constant (k) can be calculated by the slope of the curve from a plot of log concentration versus time.

Slope = -k / 2.303

The calculated rate constant is used to predict stability for a longer time period. Results show that at 25 °C, the slope of the curve was not statistically significant different than zero; and the rate constant was zero, indicating no degradation over time. At 40 and 65 °C, the rate constants were 0.005 h^{-1} and 0.014 h^{-1} respectively.

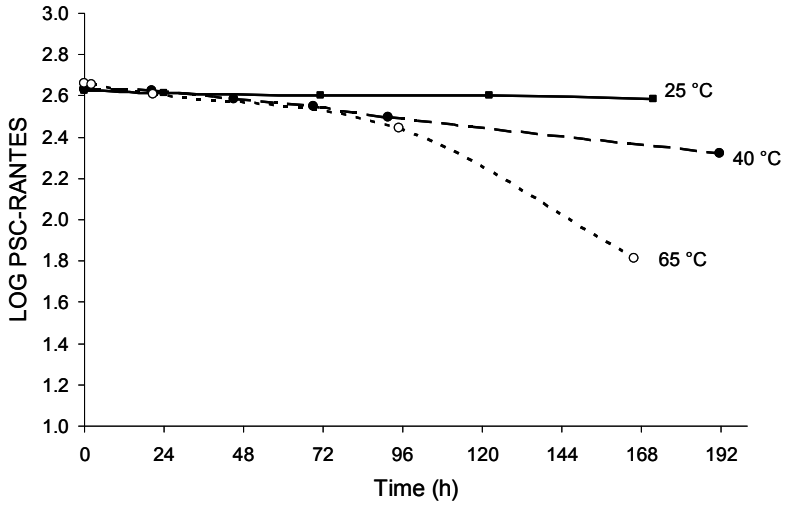


Figure 2-6. Effect of temperature on PSC-RANTES, log concentration versus time (h). Obtained from Figure 2-5 for each temperature condition 25 °C (■), 40 °C (●), and 65 °C (○), analyzed by HPLC (n=3)

The chemical degradation of PSC-RANTES observed by HPLC was confirmed by MALDI-TOF MS. Figure 2-7 shows the spectrum of sinapinic acid, blank matrix used in the analysis of PSC-RANTES. No peaks were detected in the blank sample that could interfere with the PSC-RANTES samples.

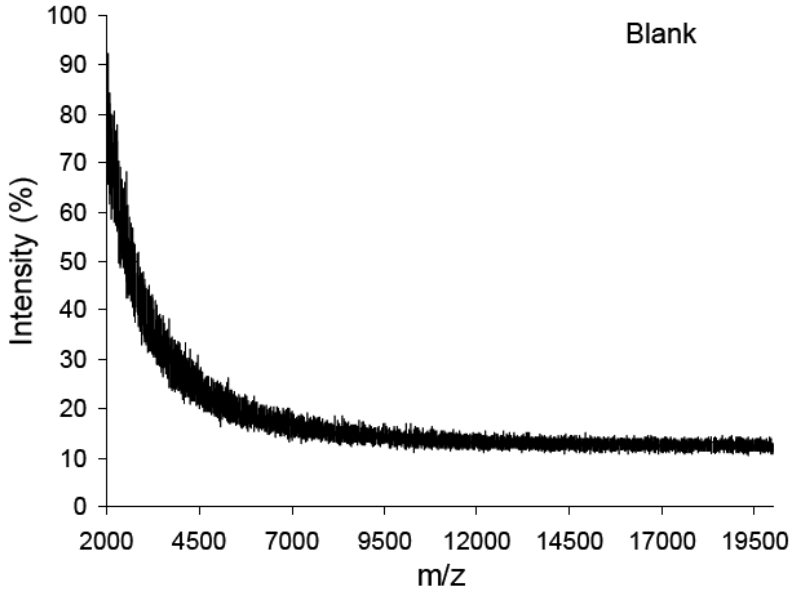


Figure 2-7. MALDI-TOF MS spectrum of blank, sinapinic acid matrix. 100% intensity = 1752 counts.

The spectra of PSC-RANTES after 7 days at 25 °C (Figure 2-8), 40 °C (Figure 2-9), and 65 °C (Figure 2-10) show the intensity (%) of the *m/z* fragments of PSC-RANTES. Samples at 25 and 40 °C showed similar fragments, including the presence of a dimer (15,789 *m/z* for 25 °C, and 15,938 *m/z* for 40 °C), and a smaller fragment around 3,900 *m/z* $[M + H]^{2+}$.

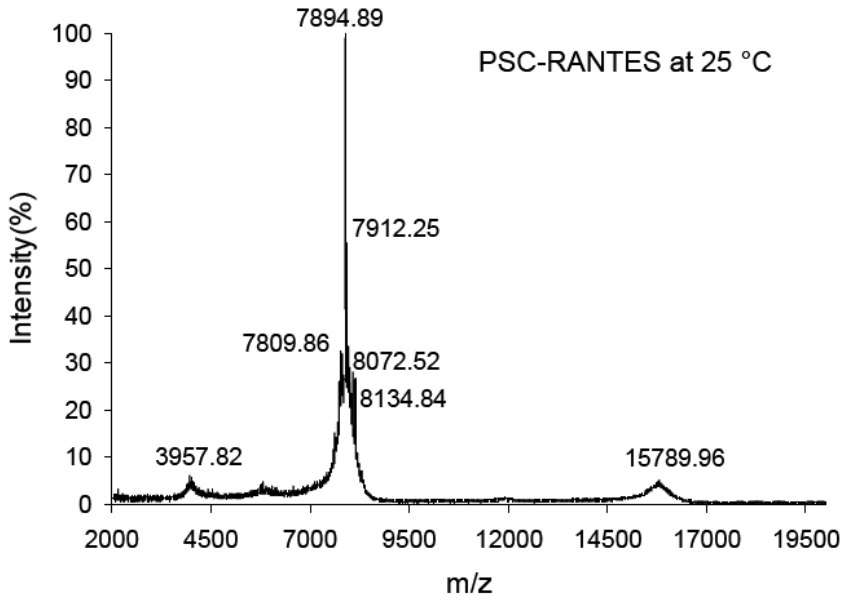


Figure 2-8. MALDI-TOF MS spectrum of PSC-RANTES (500 µg/mL), 25 °C for 7 days. 100% intensity = 8375 counts.

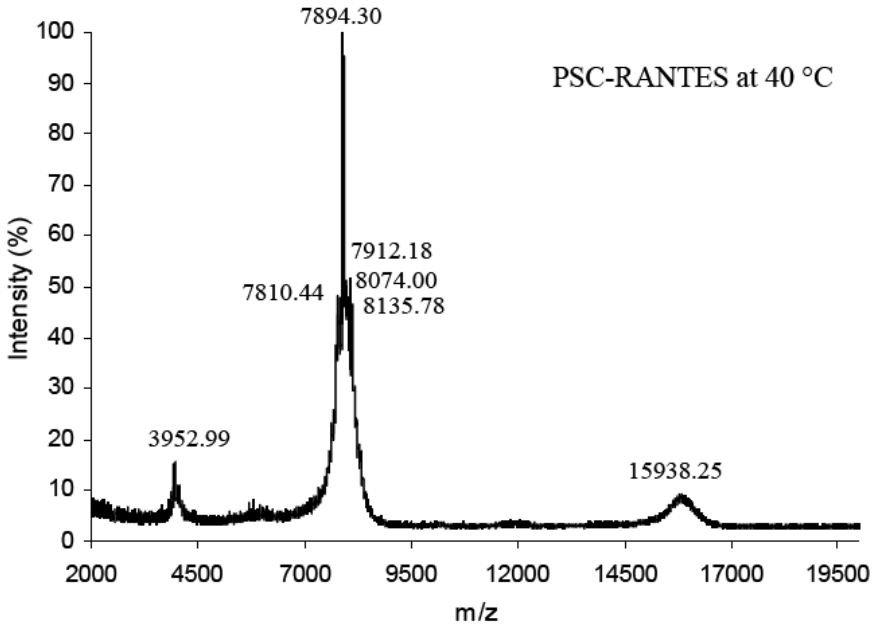


Figure 2-9. MALDI-TOF MS spectrum of PSC-RANTES (500 µg/mL), 40 °C for 7 days. 100% intensity = 8034 counts.

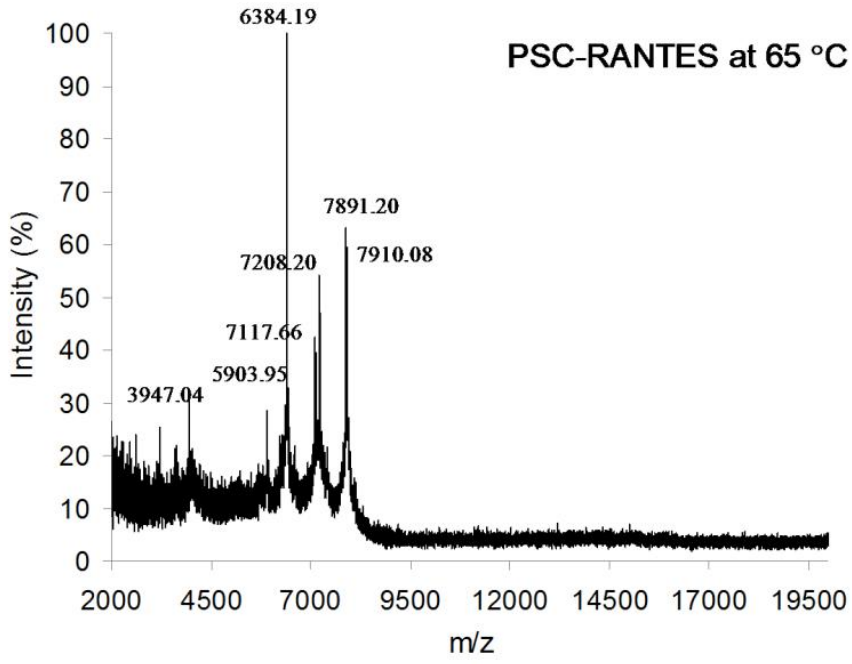


Figure 2-10. MALDI-TOF MS spectrum of PSC-RANTES (500 µg/mL), 65 °C for 7 days. 100% intensity = 2069 counts.

Stability – Effect of pH

Solutions of PSC-RANTES were made at a concentration of 500 µg/mL in phosphate buffer, and the pH was confirmed before starting incubation at room temperature and at the end of the experiment. The pH for each buffer solution after addition of PSC-RANTES was no more than 0.5 units different than the initial buffer pH. All solutions were stored in a glass container, as it was shown that no absorption to the container occurred during the period of time of the study. Samples at pH 8 could not be analyzed by HPLC due to a precipitation observed in all samples prior to the 24 h sampling point. A decrease in the amount of PSC-RANTES remaining in the solution was observed at pH 3, 4, 5, and 7 over a period of 168 h, indicating loss of stability over time. It was also observed that the initial concentration of PSC-RANTES at pH 7 was

consistently 20% lower than expected (Figure 2-11). No additional peaks were detected by HPLC in any of the pH conditions.

The log concentration at each pH condition (3 to 7) was plotted against time (Figure 2-12), for the calculation of the rate constant. The rate constant for each pH value (Table 2-2) was not significantly different from zero ($p < 0.001$), suggesting that the degradation observed at pH 7 is immediate and not dependent on time.

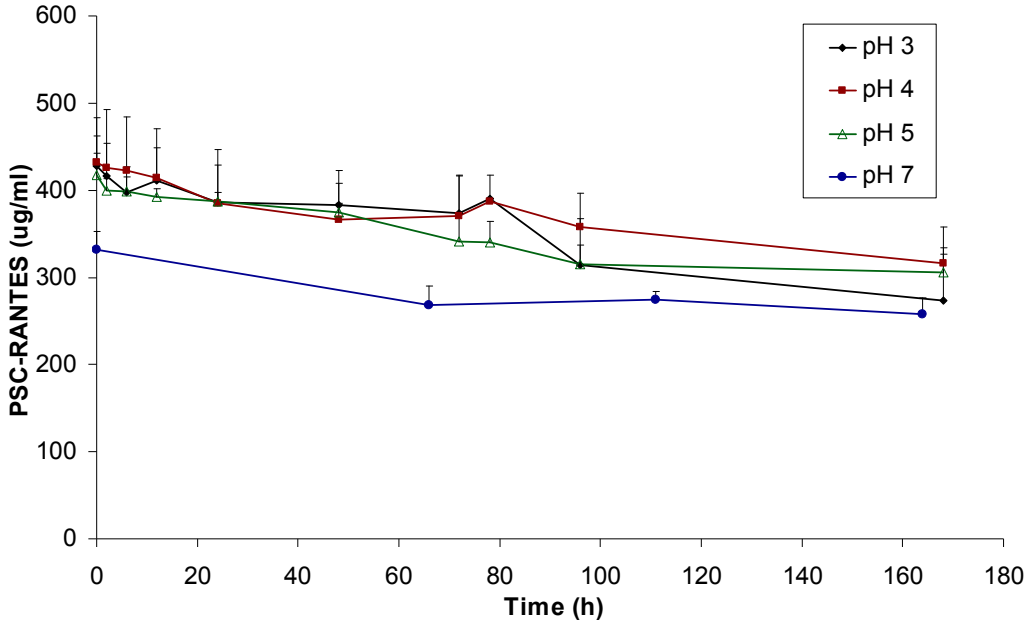


Figure 2-11. pH profile of PSC-RANTES. Concentration (µg/mL) versus time (h).
PSC-RANTES (500 µg/mL) prepared in 10 mM phosphate buffers pH 3, 4, 5 and 7. Analyzed by HPLC (n = 3).

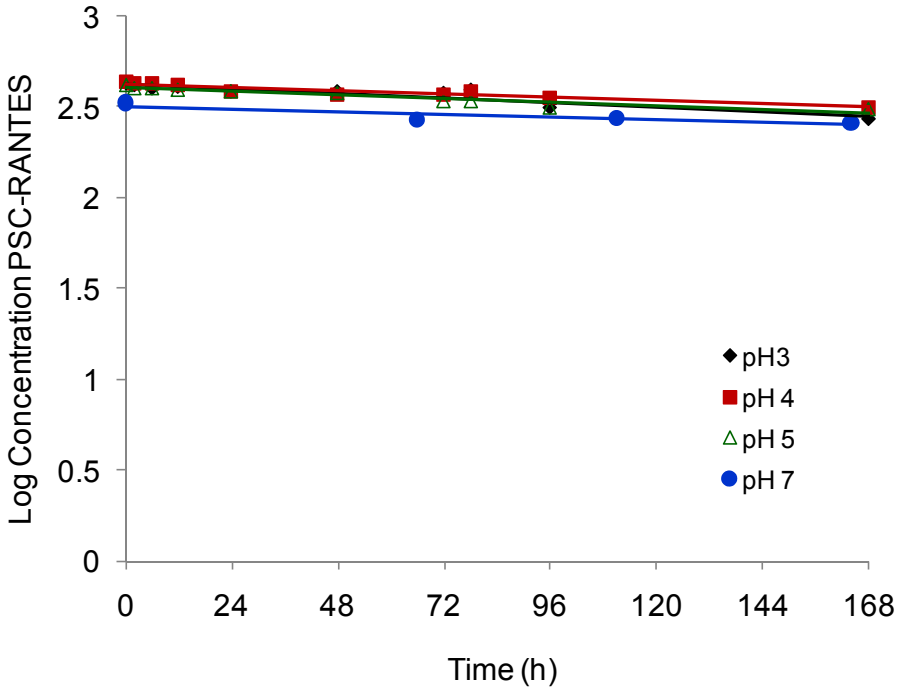


Figure 2-12. pH profile of PSC-RANTES. Log concentration versus time (h). Obtained from Figure 2-11 for each pH buffer condition.

Table 2-2. Rate constant for PSC-RANTES exposed to pH buffer.

PSC-RANTES in buffer	Slope	Rate constant
pH 3	-0.001	2.3×10^{-3}
pH 4	-0.0007	1.6×10^{-3}
pH 5	-0.0008	1.8×10^{-3}
pH 7	-0.0006	1.4×10^{-3}

Slopes were calculated from the Log concentration of PSC-RANTES over time (Figure 2-12) for each pH buffer. Rate constant (h^{-1}) were calculated from the slope of the curve.

Further investigations with SDS-PAGE, CD, MALDI-TOF, and UV spectroscopy were conducted to determine if there was any immediate degradation of PSC-RANTES at pH 7. PSC-

RANTES solutions at pH 3, 4, 5, 7, and 12 were prepared following the same procedure as described in Section 2.2.7. Samples were analyzed by SDS-PAGE under reducing conditions (Figure 2-13), at time 0 and after 168 h to observe changes in molecular mass or the appearance of any additional bands. No additional bands were observed for any of the PSC-RANTES samples studied. The mobility of the PSC-RANTES band was calculated, resulting in a molecular mass of 7,625 Daltons at time 0 and 7,844 Daltons after 1 week exposure.

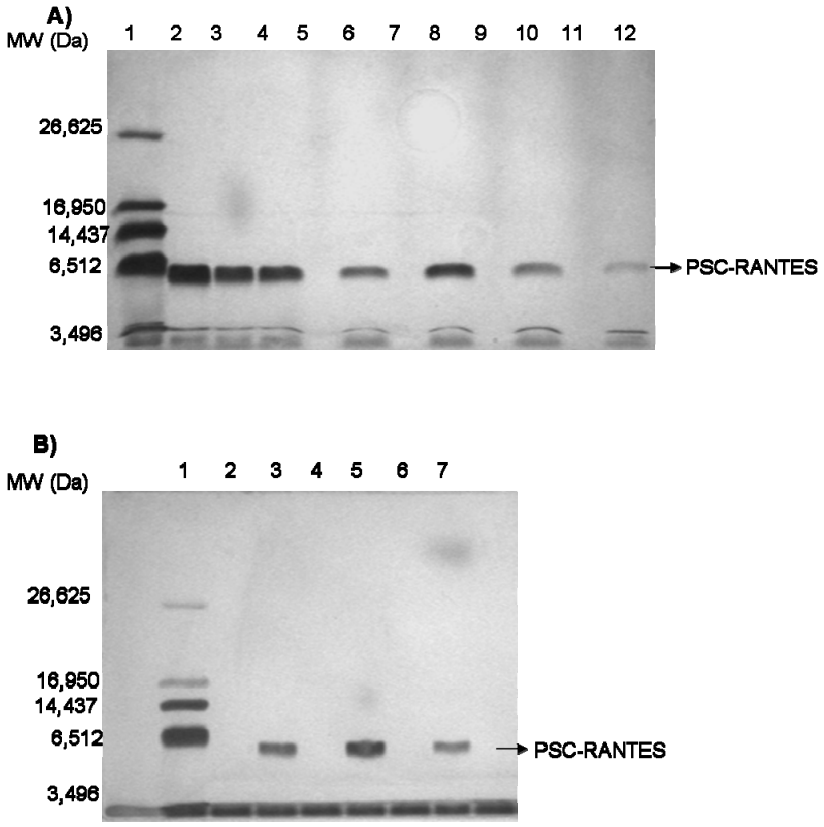


Figure 2-13. pH profile of PSC-RANTES on SDS-PAGE.

Separation of polypeptide standards and PSC-RANTES on SDS-PAGE, detected by silver staining. A) From left to right: 1) Polypeptide SDS-PAGE Standards, 2) PSC-RANTES control 1.2μg; 3) PSC-RANTES pH 3; 4) PSC-RANTES pH 4; 6) PSC-RANTES pH 5; 8) PSC-RANTES pH 7; 10) PSC-RANTES pH 7.4 PBS; 12) PSC-RANTES pH12. Note: wells 5, 7, 9, and 11 were blank.

B) PSC-RANTES pH profile after 1 week. Separation of polypeptide standards and PSC-RANTES on SDS-PAGE, detected by silver staining. From left to right: 1) Polypeptide SDS-PAGE Standards, 3) PSC-RANTES pH 4; 5) PSC-RANTES pH 7; 7) PSC-RANTES pH 12. Note: wells 2, 4, and 6 were blank.

CD was conducted on PSC-RANTES (500 μg/mL) solutions at pH 3 and pH 7 phosphate buffers. Under both conditions, the protein showed a random conformation, with a maximum lower absorbance at 200 nm for pH 3 and 202 nm for pH 7 (Figure 2-14). The shift in maximum wavelength (λ_{max}) as well as the decrease observed in the absolute signal for the sample at pH 7 suggests conformational changes.

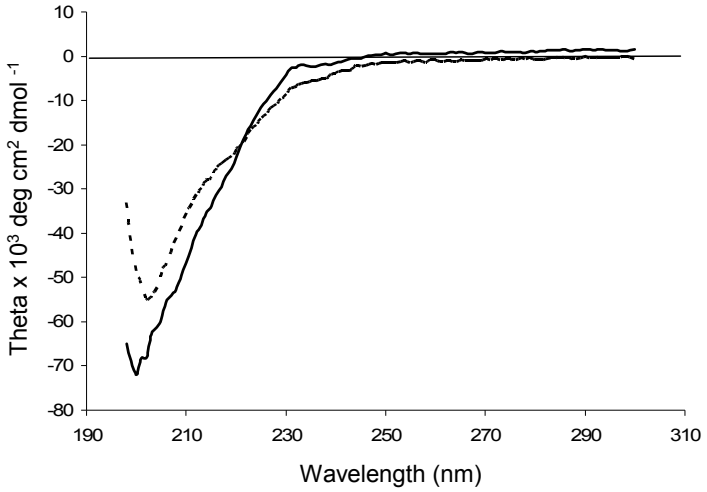


Figure 2-14. CD spectra demonstrating the effect of pH on PSC-RANTES (500 μg/mL) in solution. Average of 3 individual spectra after subtraction of background control (pH 3 or pH 7 phosphate buffer). (—) PSC-RANTES in pH 3 buffer; (- - -) PSC-RANTES in pH 7 buffer.

MALDI-TOF MS was employed to analyze PSC-RANTES samples in water (Figure 2-15), pH 4 (Figure 2-16), and pH 7 (Figure 2-17). The highest intensity peaks observed for PSC-RANTES samples in water and pH 4 represent the *m/z* $[M + H]^{1+}$ of PSC-RANTES. A dimer and a smaller fragment are also observed in those conditions. However, MALDI-TOF mass spectrometry showed degradation, as evidenced by completed fragmentation, of PSC-RANTES to lower molecular mass peptides after 7 days exposure at pH 7 (Figure 2-17).

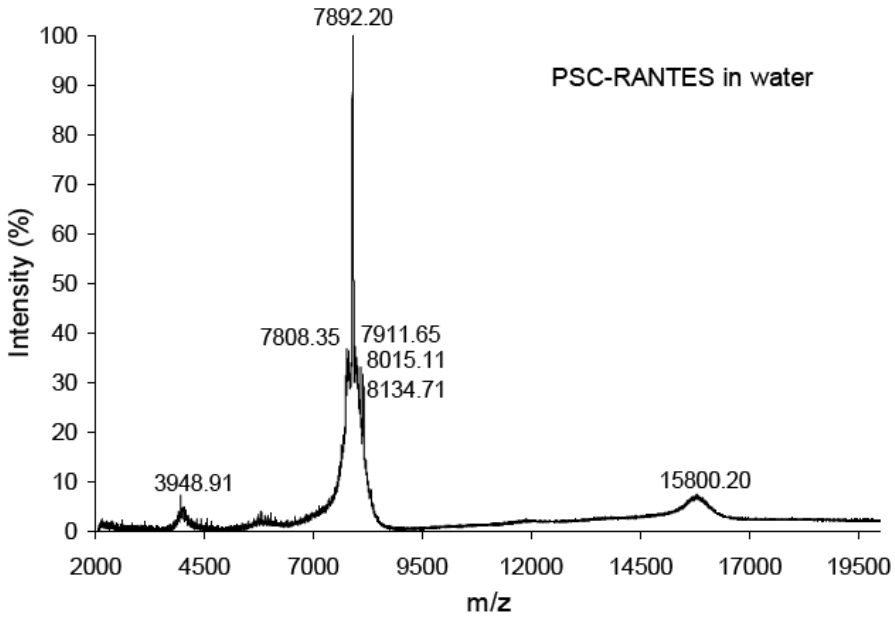


Figure 2-15. Representative MALDI-TOF MS spectrum of PSC-RANTES in water. 100% intensity = 12574 counts.

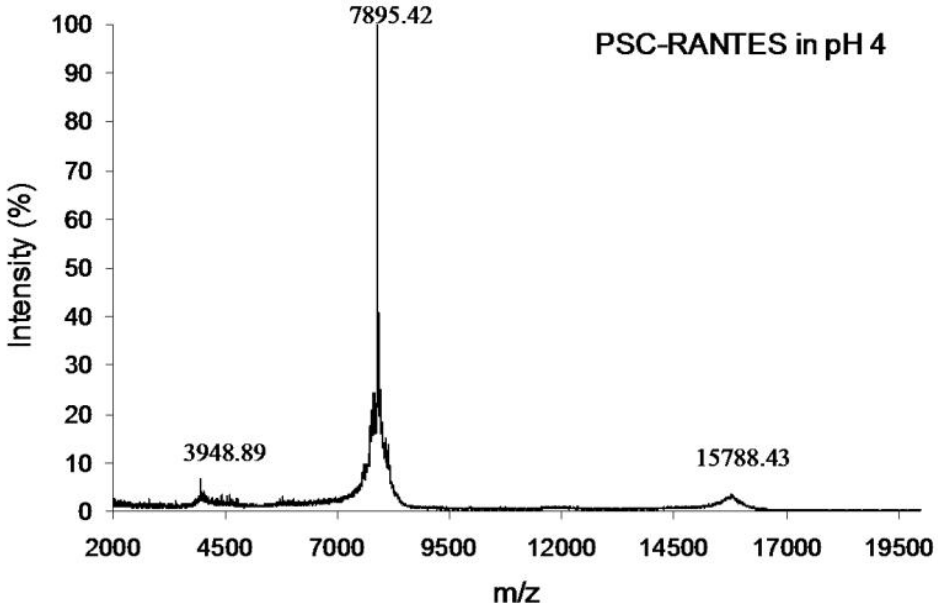


Figure 2-16. MALDI-TOF MS spectrum of PSC-RANTES in pH 4 10mM phosphate buffer, after 7 days. 100% Intensity = 18175 counts.

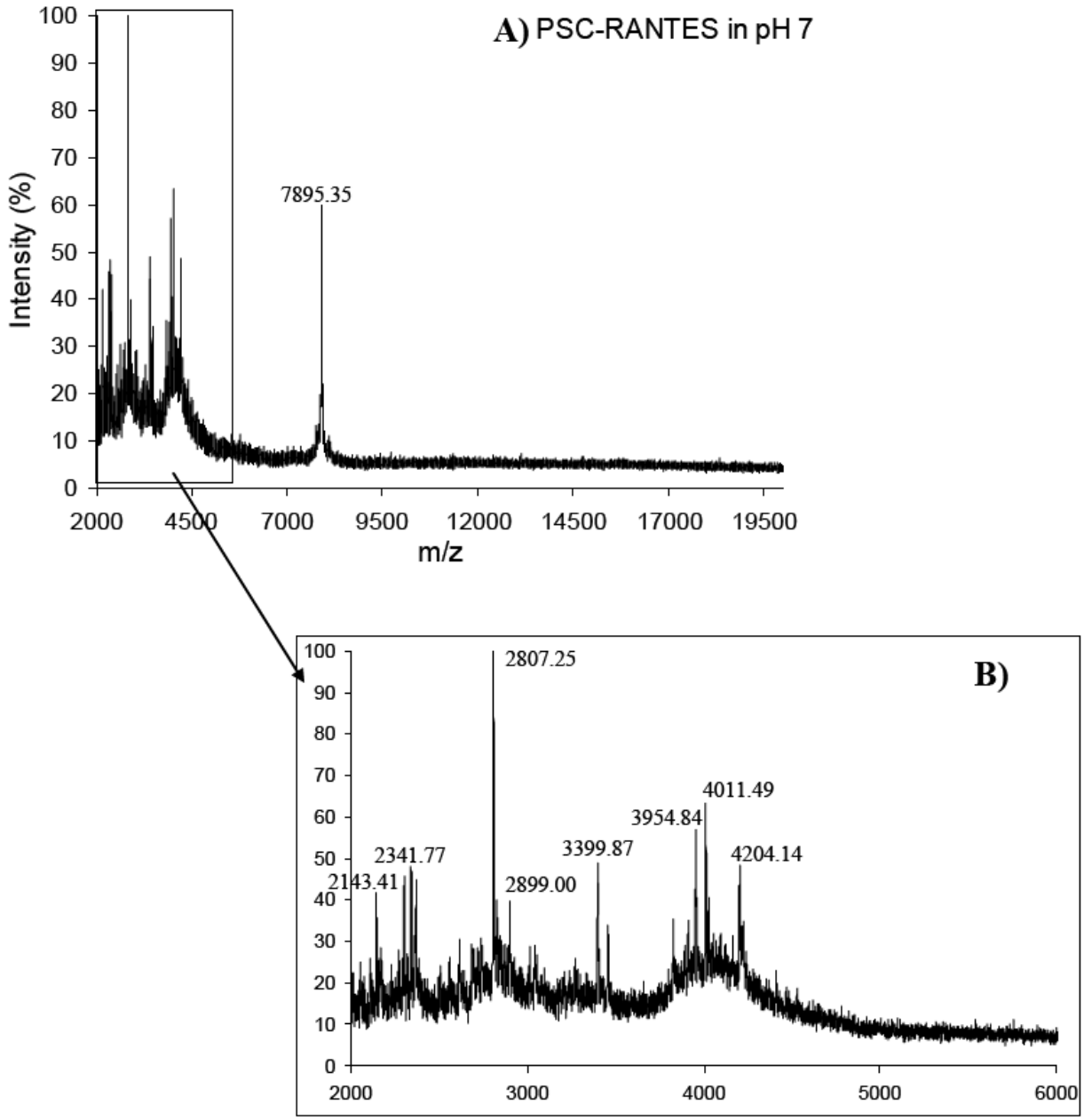


Figure 2-17. MALDI-TOF MS spectrum of PSC-RANTES in pH 7

A) PSC-RANTES after 7 days exposure to 10mM phosphate buffer pH 7, spectrum from 2000 to 20000 *m/z*. B) Expanded spectrum A, region 2000 to 6000 *m/z*, to facilitate visualization of peaks. 100% Intensity = 2156 counts

Further studies were conducted to verify if the fragments observed in MALDI-TOF MS under pH 7 buffer condition corresponded to the potential sites of degradation of PSC-RANTES.

Figure 2-18 shows the amino acid sequence of PSC-RANTES and the amino acids highlighted that are more susceptible to degradation by hydrolysis and oxidation degradation pathways. With this information, the predicted fragments were calculated and presented in Table 2-3. Based on the high number of fragments obtained from MALDI-TOF MS for PSC-RANTES samples at pH 7 buffer, it was difficult to predict the sites of degradation. However, the several analytical methods utilized (HPLC, CD, UV, and MALDI-TOF MS) have clearly shown that PSC-RANTES is unstable in pH 7 buffer.

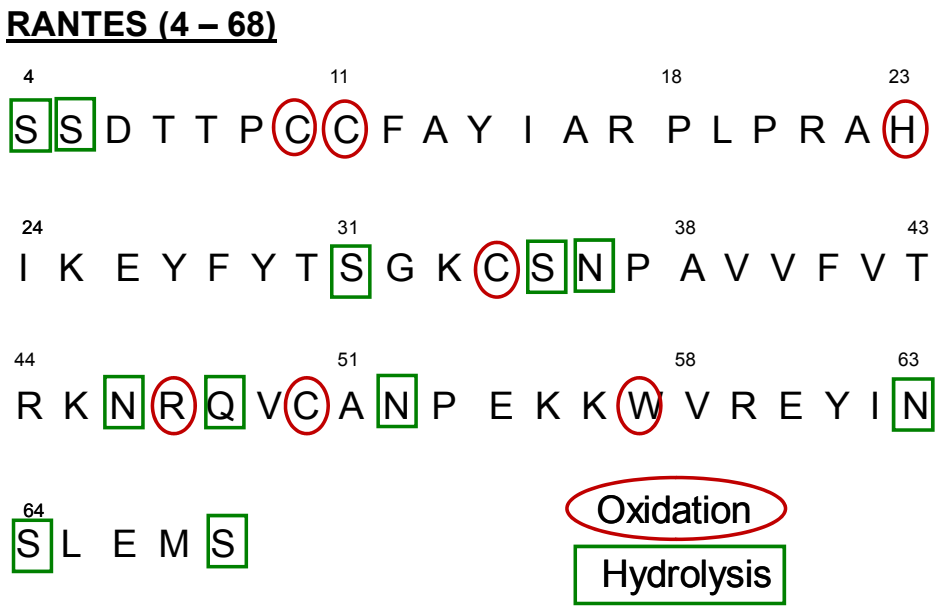


Figure 2-18. PSC-RANTES amino acid sequence and potential sites of degradation

Table 2-3. Predicted fragments for PSC-RANTES after degradation

Position	Potential mechanism	Fragments (*m/z*)
Ser4	Hydrolysis	482.14 7394.49
Ser5	Hydrolysis	569.22 7307.41
Cys10	Oxidation	1085.77 6790.86
Cys11	Oxidation	1187.91 6688.72
His23	Oxidation	2581.58 5295.05
Ser31	Hydrolysis	3613.72 4262.91
Cys34	Oxidation	3901.08 3975.55
Ser35	Hydrolysis	3988.16 3888.47
Asn36	Hydrolysis	4102.26 3774.37
Asn46	Hydrolysis	5214.59 2662.04
Arg47	Oxidation	5370.78 2505.85
Gln48	Hydrolysis	5498.91 2377.72
Cys50	Oxidation	5771.26 2105.37
Asn52	Hydrolysis	5982.48 1894.15
Trp57	Oxidation	6554.14 1322.48
Asn63	Hydrolysis	7329.00 547.63

Studies of the stability of PSC-RANTES at pH 12 were conducted as a forced degradation study. Results showed no detectable PSC-RANTES by HPLC analysis in two out of three samples after 2 h exposure to phosphate buffer pH 12. One PSC-RANTES sample was undetectable after a 6-hour period. In all three PSC-RANTES samples, fast degradation under high pH conditions was observed. CD analyses were conducted to verify changes in secondary structure at this pH. PSC-RANTES in pH 12 buffer after 2 and 24 h exposure (Figure 2-19)

revealed a decrease in absorbance, indicating conformational changes of the protein. MALDI-TOF MS spectrum confirmed the degradation observed in HPLC and CD. The spectrum showed a complete fragmentation of PSC-RANTES into smaller fragments (Figure 2-20).

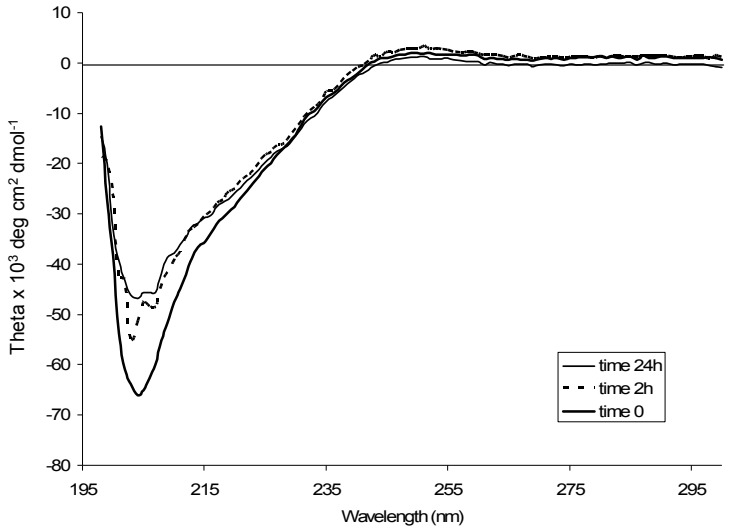


Figure 2-19. CD spectra of PSC-RANTES (500 µg/mL) in phosphate buffer pH 12.
Average of 3 individual spectra after subtraction of background control (pH 12 phosphate buffer)

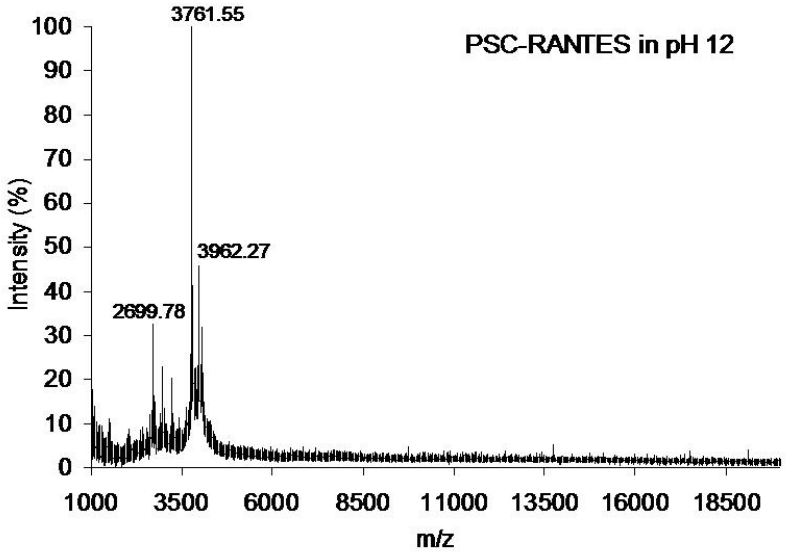


Figure 2-20. MALDI-TOF MS spectrum of PSC-RANTES in pH 12
10mM phosphate buffer after 24 h exposure. 100% Intensity = 3230 counts.

Stability - Effect of ionic strength

The effect of ionic strength on the stability of PSC-RANTES was evaluated by UV-spectroscopy and the results for time 0 are shown in Figure 2-21 (low ionic strength and high ionic strength). UV scans of PSC-RANTES in water, and in pH 4 and pH 12 phosphate buffers at low ionic strength (50 mmol/kg) showed similar profiles for all concentrations tested. However, UV scans for PSC-RANTES in pH 7 phosphate buffer solutions (at low ionic strength) showed a higher absorbance from 300 to 600 nm, indicating the presence of insoluble aggregates.

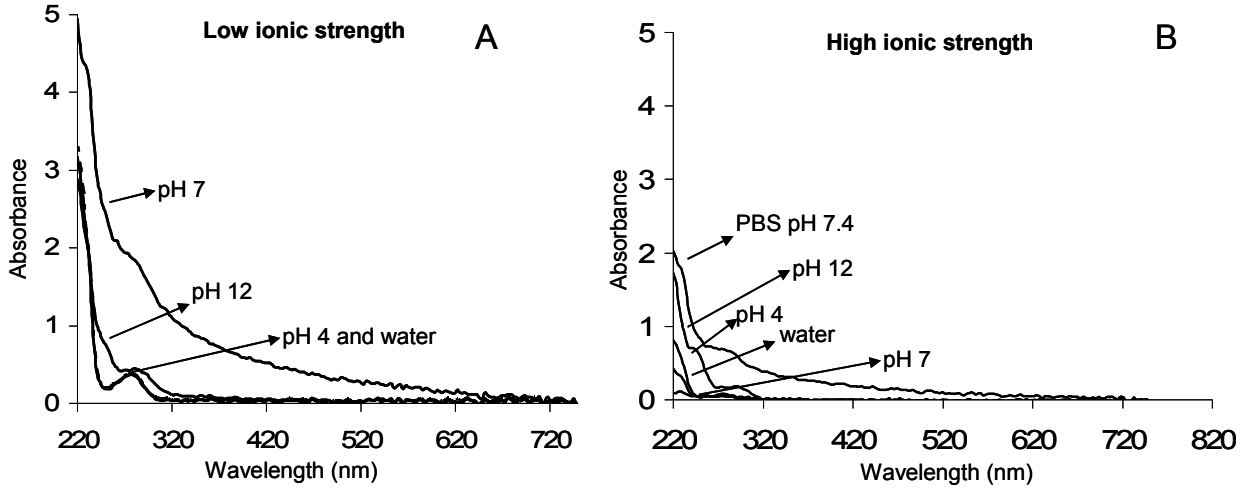


Figure 2-21. Effect of pH and ionic strength on PSC-RANTES analyzed by UV-spectroscopy. PSC-RANTES 500 µg/mL solutions in water, and in phosphate buffer solutions at pH 4, 7, and 12. A) UV scans for low ionic strength buffer, 50 mmol/kg. B) UV scans for high ionic strength buffer, 500 mmol/kg. On graph A, PSC-RANTES in water and in pH 4 buffer are superimposed.

At high ionic strength buffers (500 mmol/kg), UV-scans of PSC-RANTES in phosphate buffer pH 7 did not show the same increase in absorbance from 300 to 600 nm as observed for low ionic strength pH 7 buffer. Additional studies in phosphate buffered saline (PBS, 290 mmol/Kg, pH 7.4) were conducted, which showed evidence of aggregation by the increase in

absorbance observed from 300 to 600 nm (Figure 2-21). When PSC-RANTES was prepared in PBS pH 7.4 in different concentrations, solutions were only visually soluble at concentrations equal to or lower than 0.5 mg/mL.

Effect of hydrogen peroxide

After exposure to 3.0 or 0.8% (v/v) H_2O_2, no PSC-RANTES could be detected by HPLC at the initial time point, indicating instantaneous degradation of the protein. Results of PSC-RANTES exposed to 0.02 and 0.002% H_2O_2 are shown in Figure 2-22. PSC-RANTES was quickly degraded, and after a 5-hour period, only 10% of PSC-RANTES could be detected by HPLC when exposed to 0.02% H_2O_2. The degradation was slower when in the presence of 0.002% H_2O_2, about 50% of PSC-RANTES remained after 5 h exposed to hydrogen peroxide. Log of the concentration of PSC-RANTES was plotted against time to obtain the rate constant (Figure 2-23). The calculated rate constant was 0.19 h^{-1} for 0.002%, and 0.48 h^{-1} for 0.02% H2O2, indicating degradation over time.

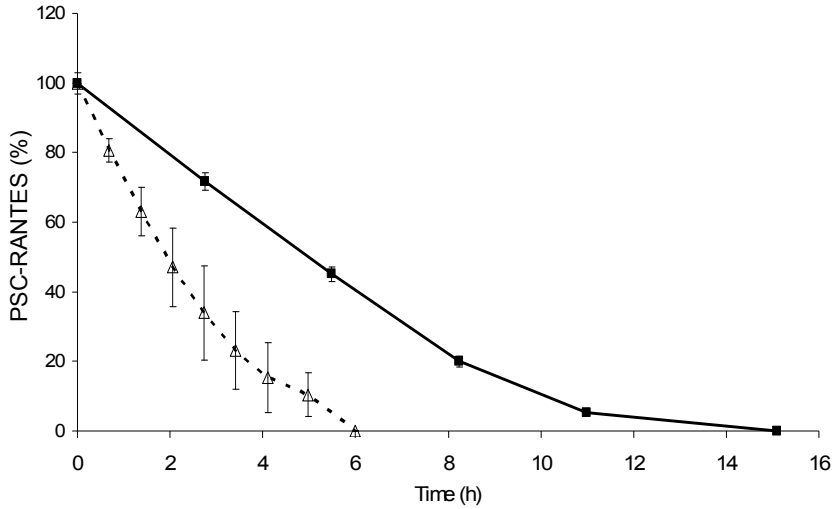


Figure 2-22. PSC-RANTES (500 µg/mL) after exposure to H_2O_2 over time (h). (- - -) 0.02% and (—) 0.002% H_2O_2. Analyzed by HPLC, n = 3.

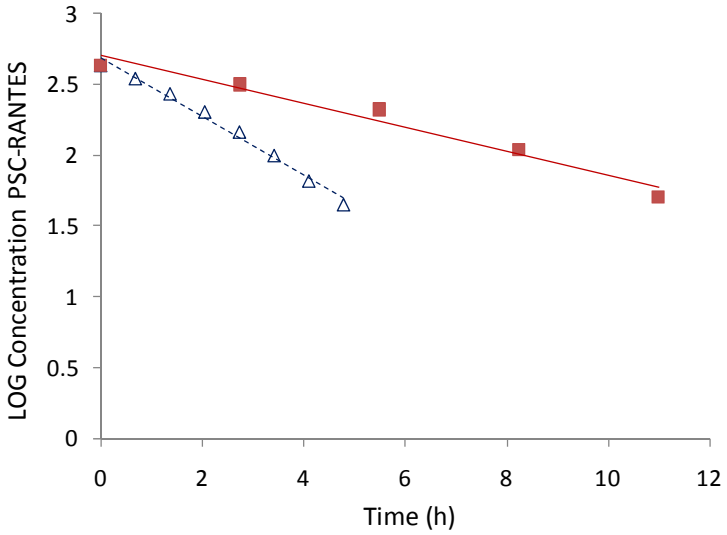


Figure 2-23. Log concentration versus time (h) of PSC-RANTES exposed to H_2O_2. (- - -) 0.02% and (—) 0.002% H_2O_2. Obtained from Figure 2-22 for each hydrogen peroxide condition.

In HPLC analysis, additional peaks were observed after incubation of PSC-RANTES with 0.8% hydrogen peroxide and at lower concentrations. Figure 2-24 and Figure 2-25 show the HPLC chromatograms for PSC-RANTES in the presence of 0.002% hydrogen peroxide at time 0 and after 12 h.

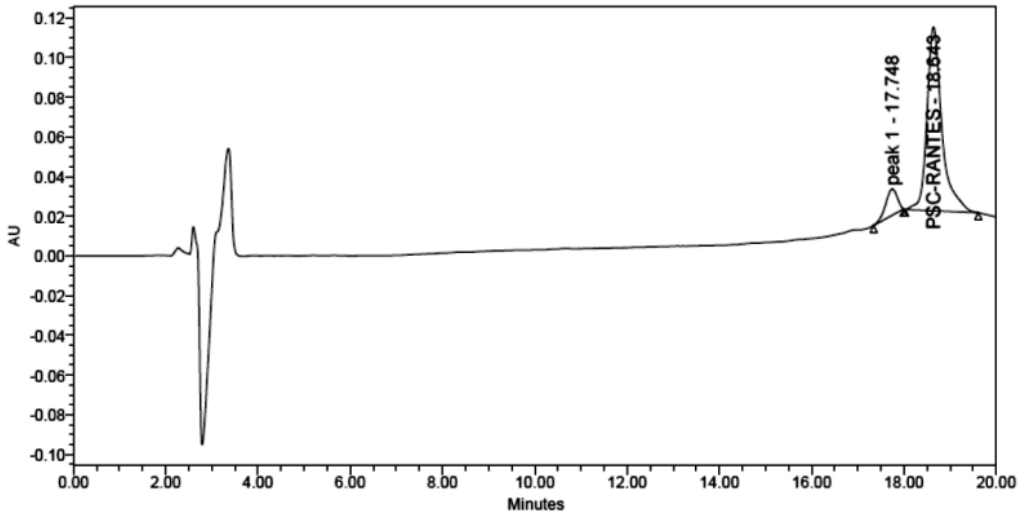


Figure 2-24. HPLC chromatogram of a PSC-RANTES (500 µg/mL) in 0.002% H_2O_2 at time 0.

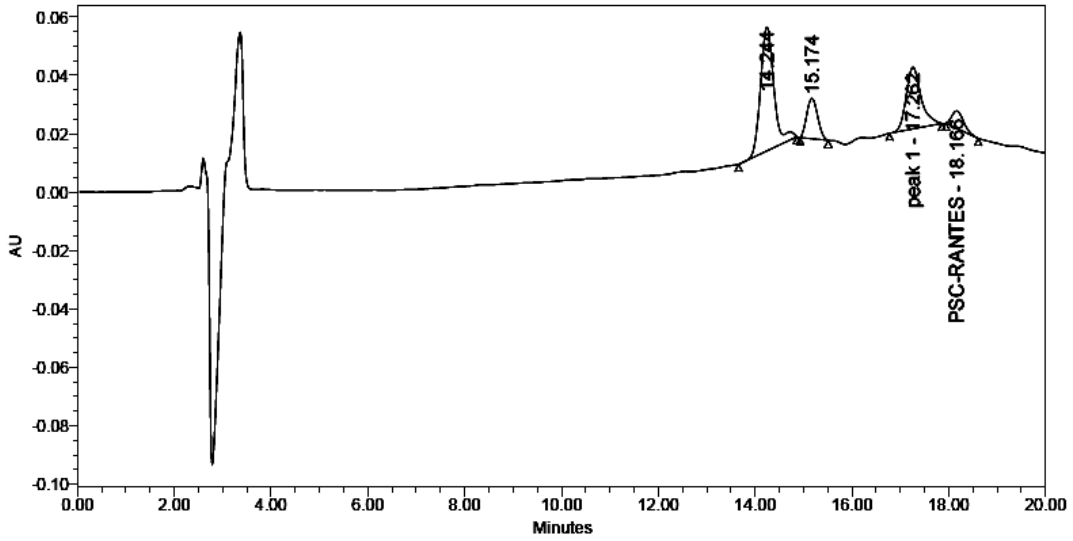


Figure 2-25. HPLC chromatogram of a PSC-RANTES (500 µg/mL) in 0.002% H_2O_2, after 12 h exposure.

MALDI-TOF MS spectra of PSC-RANTES after exposure to hydrogen peroxide were conducted to indicate the presence of oxidative products generated by the exposure H_2O_2. Figure 2-26 shows the spectrum of PSC-RANTES after 15 h exposure to 0.8% hydrogen peroxide. A higher *m/z* fragment (7927.97) was observed than the results obtained for PSC-RANTES in water (7892.20). The difference between the two major peaks (higher intensity) is about 36, which indicates the incorporation of oxygen in the protein. This oxidation can be occurring in one of the cysteines, methionine, or the thioproline present in PSC-RANTES.

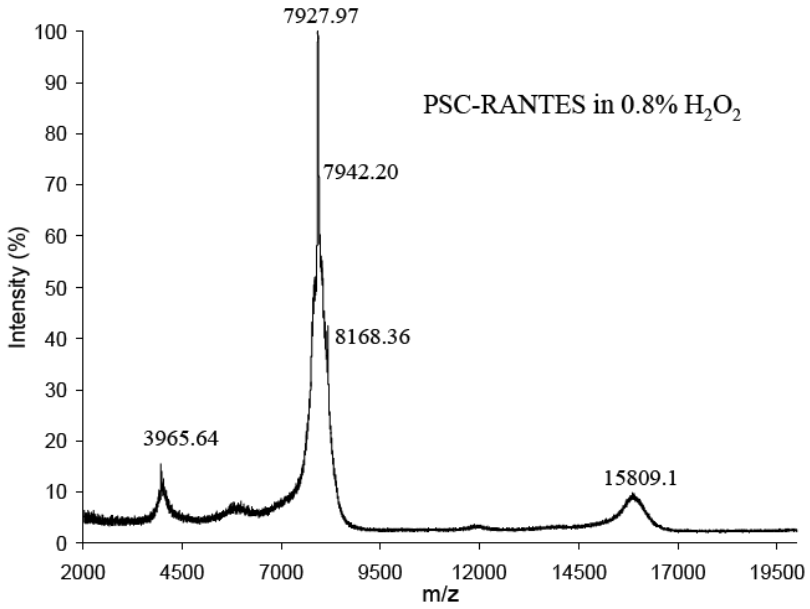


Figure 2-26. MALDI-TOF MS spectrum of PSC-RANTES (500 µg/mL) in 0.8% H_2O_2 after 15 h exposure. 100% intensity = 11254 counts.

Protective effect of antioxidants

In the previous sections, it has been identified that oxidation and hydrolysis are the major degradation pathways for PSC-RANTES. Considering that this protein will be administered vaginally in an environment that contains hydrogen peroxide, protection against oxidation is necessary. A range of antioxidants from different pharmaceutical classes have been tested. Most of the antioxidants studied did not show a significant protective effect against oxidation in the presence of hydrogen peroxide. When analyzed by HPLC, oxidation degradation of PSC-RANTES was faster in the presence of antioxidants than without the addition of antioxidants selected in this study.

Effect of light exposure

Aqueous PSC-RANTES samples (500 μg/mL) stored in a photostability chamber were compared to control samples kept in the dark over a one week period. No statistically significant difference was observed between the amounts of PSC-RANTES detected by HPLC in the samples stored in the dark when compared to the samples stored under light over a one week period ($p > 0.1$), indicating that PSC-RANTES is stable under light exposure.

Effect of freeze-thaw cycles

Solutions of PSC-RANTES (500 μg/mL) in water showed a 30% loss in the PSC-RANTES peak area after 6 freeze-thaw cycles analyzed by HPLC (Figure 2-27). No additional bands were identified in the SDS-PAGE, and no changes in the CD spectra were observed for the PSC-RANTES samples after 6 freeze-thaw cycles (Figure 2-28). Results indicate that changes in the protein occur after freezing-thaw process and should be considered when utilizing samples stored in the freezer.

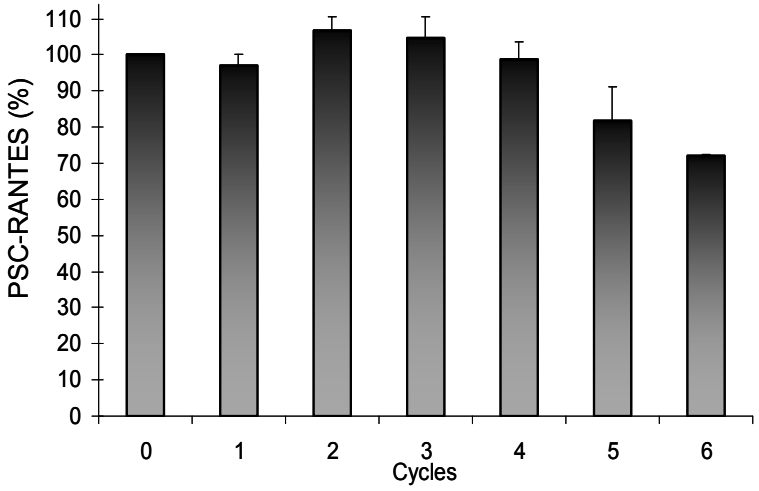


Figure 2-27. Effect of freeze-thaw cycles stability on PSC-RANTES (500 μg/mL). Analyzed by HPLC, n = 3.

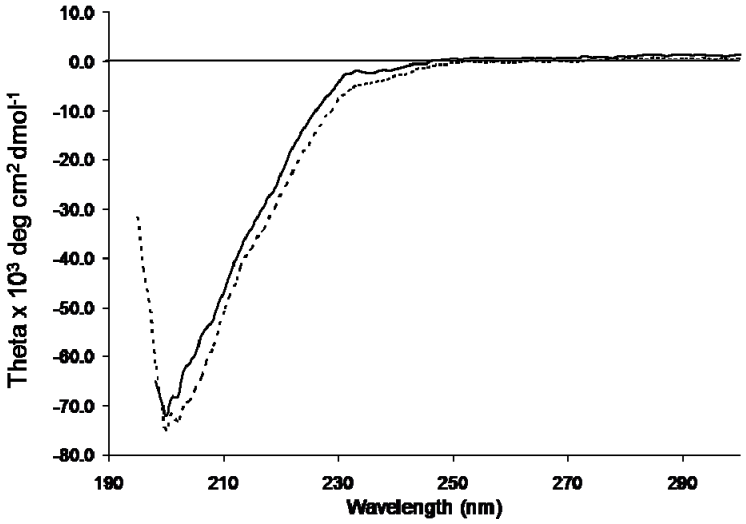


Figure 2-28. CD spectra of PSC-RANTES (500 µg/mL) in water after freezing-thaw cycles. PSC-RANTES solution before (—) and after freezing (- - -). Average of 3 individual spectra after subtraction of background control (water).

Effect of enzymatic activity

PSC-RANTES was evaluated in the presence of enzymes commonly encountered in the vaginal fluid and cervical mucus. PSC-RANTES was exposed to aminopeptidase, lysozyme, and proteinase K at concentrations of 100 units/mL, 100 units/mL, and 100,000 units/mL concentrations respectively. After exposure, samples were analyzed by MALDI-TOF MS. Fragments observed in MALDI-TOF MS were compared to the predicted sites of degradation (from Figure 2-18 and Table 2-3). No changes were observed for PSC-RANTES when exposed to aminopeptidase or lysozyme after a period of 1 h at 37 °C. When PSC-RANTES was exposed to proteinase K under the same conditions, a complete fragmentation of the protein was observed using MALDI-TOF MS (Figure 2-29). The fragment 11,181.55 (*m/z*) in Figure 2-29 corresponds to the proteinase K (Figure 2-30); other fragments are originated by degradation of PSC-RANTES.

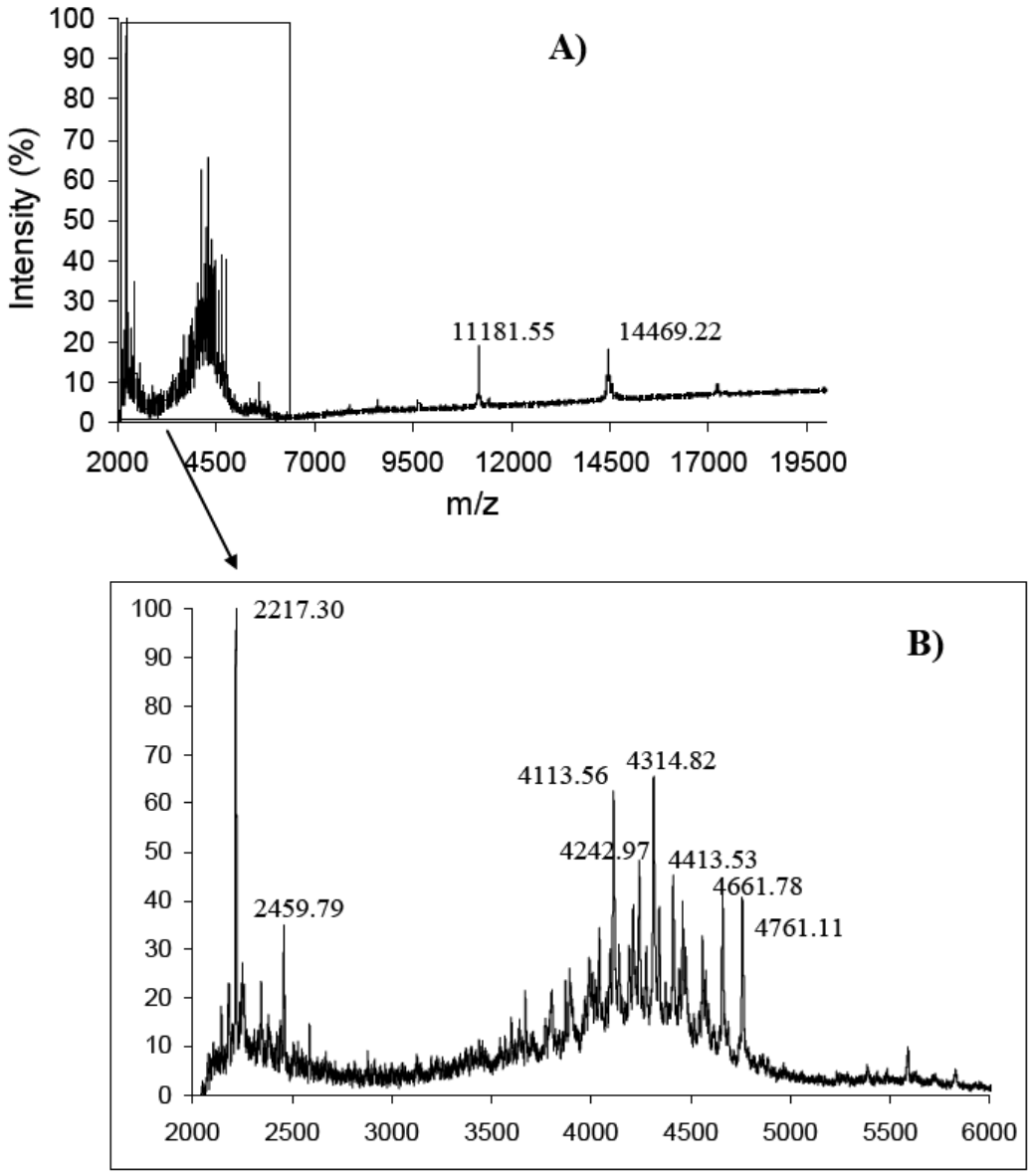


Figure 2-29. MALDI-TOF MS spectrum of PSC-RANTES after 1 h exposure to proteinase K.

A) PSC-RANTES after 1h exposure to proteinase K at 37 °C. 100% intensity = 4062 counts.

B) Expanded spectrum A to facilitate visualization of peaks.

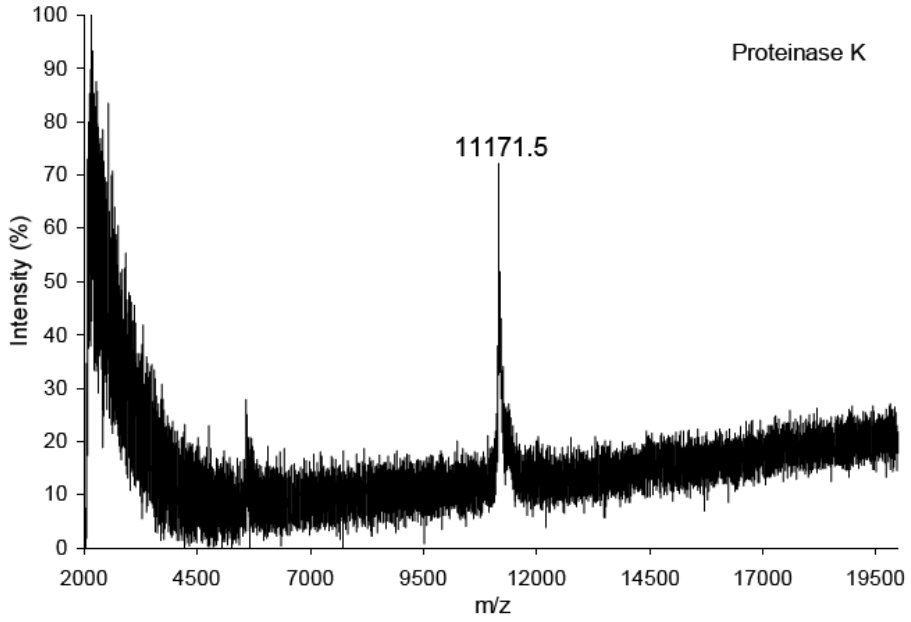


Figure 2-30. MALDI-TOF MS spectrum of Proteinase K.
100% intensity = 372 counts.

Table 2-4. Potential cleavage sites of PSC-RANTES in the presence of enzymes.

Name of enzyme	Number of cleavages	Positions of cleavage sites
Asp-N endopeptidase	1	5
Lysozyme C	5	25 33 45 55 56
Proteinase K	28	7 8 12 13 14 15 16 19 22 24 27 28 29 30 38 39 40 41 42 43 49 51 57 58 61 62 65

Calculated using Peptide Cutter software program (http://us.expasy.org)

Potential cleavage sites on PSC-RANTES in the presence of the enzymes studied have been calculated using Peptide Cutter software program (http://us.expasy.org) an enzymatic activity analysis tool to predict the sites more predisposed to cleavage, and are presented in Table

2-4. Due to the high number of fragments observed after exposure to enzymatic degradation (proteinase K), it was not possible to predict the sites of hydrolysis of the protein. It is clear that PSC-RANTES is highly susceptible to degradation in the presence of proteinase K.

Microbiological activity

The effects of PSC-RANTES on normal vaginal flora were investigated (Table 2-5). The lactobacilli strains selected are those most commonly present in the vaginal environment. PSC-RANTES did not kill or inhibit the growth of *Lactobacillus crispatus* or *L. vaginalis*; however, in 2 of 10 strains of *L. jensenii* and in 4 of 5 strains of *L. iners* PSC-RANTES caused a 99.99% kill or 4 log reduction in viability after a 30 min exposure. The viability of *N. gonorrhoeae* (7 strains), *L. gasseri* (1 strain), *L. johnsonii* (1 strain), *L. acidophilus* (2 strains), and *G. vaginallis* (5 strains) were not affected by PSC-RANTES.

Table 2-5. Microbiological activity of PSC-RANTES (300μg/mL) in water.

Organism	*L. crispatus*	*L. iners*	*L. jensenii*	*G. vaginalis*	*N. gonorrhoeae*
PSC-RANTES No. killed / no. tested	0/ 9	4/ 5	2/ 10	0/ 5	0/ 7

2.4 DISCUSSION AND CONCLUSIONS

It is well known that the formulation and the delivery of proteins can be difficult due to alteration of the protein during the manufacturing process, during the shelf-life of the product, and after the protein enters the biological system (141, 142). Topically delivered protein microbicide products will encounter the same obstacles. Stability of the active protein is essential for the successful

development of a protein microbicide product. Protection of the protein in a drug delivery system will prevent the protein microbicide from being modified before administration, and in biological fluids after administration, preserving and prolonging drug activity.

According to the Alliance for Microbicide Development (143), several needs in microbicide formulation are considered to have a high priority. One of these priorities is to perform preformulation studies to accelerate product development. The current study addresses this issue by identifying and characterizing the microbicide PSC-RANTES to further understand its stability, which will impact formulation development and, eventually, efficacy of the product.

The PSC-RANTES amino acid sequence is comprised of 65 amino acids and 3 chemical groups attached to the N-terminal sequence: nonanoyl, thioproline, and cyclohexyl glycine groups (Figure 2-1) (91). The estimated pI for PSC-RANTES is 9.27 (http://ca.expasy.org/tools/protparam.html). PSC-RANTES, an analog of RANTES, acts by inhibiting HIV virus fusion, through blocking the CCR5 co-receptor. This antiviral mechanism makes PSC-RANTES a very attractive microbicide candidate, due to the specificity of the molecule and low toxicity. Previous studies using a modified severe combined immunodeficient (SCID) mouse model showed that an intra-peritoneal single injection of PSC-RANTES (500 µg or 150 µg) protected all of the challenged mice against HIV-1 infection (96). In a macaque model, topical application of PSC-RANTES demonstrated that, like other microbicide candidates, much more material was required to give protection than the amount expected from *in vitro* potency data (91). The ability to protect against infection in the macaque studies was assessed by intravaginally delivering 4 mL of PSC-RANTES in PBS pH 7.4. The need for such large amounts could be explained by poor stability of the drug in the delivery vehicle, as well as poor retention of the drug in the vaginal lumen. The purpose of this study was to characterize

the stability of PSC-RANTES as a function of temperature, pH, ionic strength and the presence of hydrogen peroxide. This preformulation study will contribute to the development of a suitable drug delivery system for a PSC-RANTES microbicide product.

An HPLC and a SDS-PAGE method were developed for the quantitation and detection, respectively, of PSC-RANTES and its degradants. PSC-RANTES proved to be stable in water at 25 °C over a period of 1 week, but not at 40 °C. Temperatures of 40 and 65 °C are accelerated conditions used to force the degradation of the protein, and to verify analytical method's capability to indentify degradant peaks. PSC-RANTES was quickly modified at 65 °C. Evaluating protein stability at increased temperatures (40 and 65 °C) is important for several reasons. It facilitates an understanding of how the drug will be affected through the manufacturing process of the final product, during storage and transport, and ultimately in the body. These studies demonstrated that increased manufacturing process temperatures may result in degradation of PSC-RANTES.

The development of a successful microbicide product is dependent on the ability to prevent the oxidative effects of H_2O_2, generated in the vaginal lumen by Lactobacillus. Forced degradation studies to evaluate oxidative effects are commonly conducted by exposing the molecule of interest to a solution of 3.0% H_2O_2 (144). Initial studies with PSC-RANTES using 3.0% H_2O_2, resulted in complete degradation of PSC-RANTES by HPLC immediately after exposure. To evaluate the effect of hydrogen peroxide on the stability of PSC-RANTES, we selected three other concentrations of H_2O_2: 0.8, 0.02, and 0.002% (145, 146). The concentrations of H_2O_2 were based on reports that determined the production of H_2O_2 by Lactobacillus present and calculations of concentrations Lactobacillus present in the normal vaginal flora (146). The concentration of 0.8% H_2O_2 represents 10 times the highest estimation

of H_2O_2, and it was used for forced degradation studies. The concentrations of 0.02%, and 0.002% H_2O_2 were used to simulate biological conditions. Oxidative studies (exposure to H_2O_2) resulted in degradation of PSC-RANTES at all concentrations tested. MALDI-TOF MS spectra confirmed the results obtained by HPLC by showing higher *m/z* fragments when compared to PSC-RANTES not exposed to hydrogen peroxide. Increase in *m/z* can be attributed to chemical reactions to the molecule, such as incorporation of oxygen. However, the analytical methods used were not able to determine the mechanism of the modifications observed. We can suggest that formulation development of PSC-RANTES will require the addition of an antioxidant to protect it against these oxidative effects. The PSC-RANTES amino acid sequence contains four cysteines (Cys10, Cys11, Cys34, and Cys50) and one methionine (Met67) which are prone to oxidation. In addition, the thioproline group in position two of the sequence might also be subjected to oxidation. However, the binding of PSC-RANTES to CCR5 co-receptor is not associated with the C-terminal region of the protein (97) (40), which suggests that the oxidation of Met67 may not strongly affect binding. It is likely that oxidation of thioproline will significantly affect binding to the target and therefore affect potency. It has been shown that oxidation of PSC-RANTES results in a 10 log fold decrease in bioactivity when compared to PSC-RANTES before oxidation (personal communication – Oliver Hartley, Ph.D. University of Geneva). Protection against oxidation was studied by evaluating the protective effects of a series of antioxidants. None of the antioxidants selected were successful in protecting PSC-RANTES. The effects and mechanisms of several antioxidants will result in different oxidation or protection profiles of the protein. Antioxidants were selected in this study to cover all classes of antioxidants commonly used in pharmaceutical formulations: “true” antioxidants (or phenolic compounds), reducing agents, and chelating agents (138). Methionine, ascorbic acid, cysteine,

glutathione, and sodium metabisulfite are reducing agents and act as oxygen scavengers as they oxidize in preference of the protein. Based on their mechanism of action, it was believed that these antioxidants would be the most suitable excipients to protect against oxidation of the cysteine, thioproline, and methionine in PSC-RANTES. However, a faster oxidation was observed by HPLC when in the presence of antioxidants. It has been shown that some antioxidants, such as ascorbic acid, can also act as a prooxidant due to the strong electron donor capability, which explains the observed faster oxidation profile in the presence of antioxidants (147, 148). Further studies should be conducted with chelating agents to verify if removing trace metal ions will decrease initiation of oxidation.

Freeze-thaw cycle stability of PSC-RANTES solutions was determined at -80 °C, and suggested that there was a 30% loss of PSC-RANTES after 6 cycles. This loss indicates that control solutions for the study should be prepared fresh, or used frozen for no more than 3 freezing-thaw cycles, after which no significant loss was observed.

Presence of insoluble aggregates at pH 7 was observed at low ionic strength and high ionic strength, by UV-spectroscopy methods. Changes in the absorbance baseline of the UV spectrum in non-absorbing regions (> 300 nm), indicates the presence of aggregates. This method is only qualitative, but indicates aggregation formation (141). Aggregates can result in an increase in immunogenicity, and a decrease in bioactivity (138, 149), presenting a challenge for formulation development. 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 (149). Some external factors such as temperature, pH and protein concentration may induce the

aggregation formation. Results presented in this chapter have shown that PSC-RANTES forms insoluble aggregates at concentrations higher than 0.5 mg/mL at pH 7. This finding will impact decisions made on maximum concentration to be used in a formulation, as well as the pH and ionic strength of the formulation, limiting the options for formulation development.

HPLC results for PSC-RANTES in phosphate buffers showed that PSC-RANTES was more stable at pH 4 than at pH 7 buffer. This loss of stability at pH 7 was confirmed by MALDI-TOF MS and circular dichroism, suggesting that hydrolysis may occur at this pH level. As mentioned in Section 2.1, potency studies of PSC-RANTES evaluated in a macaque model resulted in a 10^6-fold loss in potency for PSC-RANTES as compared with *in vitro* study results. The *in vivo* animal studies were conducted using a 30 mg/mL concentration of PSC-RANTES in PBS pH 7.4. Our studies demonstrate that the observed loss of *in vivo* potency is partially due to its instability, lack of solubility, and aggregate formation under these conditions.

Exposure of PSC-RANTES to a pH 12 phosphate buffer was conducted to force degradation by deamidation. It resulted in loss of the peak area observed by HPLC over the course of 2 h. Investigation using UV-spectroscopy, MALDI-TOF, and CD was conducted and confirmed degradation of the protein over time in the presence of pH 12 phosphate buffer. Non-enzymatic deamidation is a very common hydrolytic reaction responsible for degradation of peptides and proteins. The amide group of asparagine (Asn) and glutamine (Gln) residues are hydrolyzed to free carboxylic acid groups: aspartic acid (Asp) and glutamic acid (Glu). PSC-RANTES contains four Asn residues and one Gln. In the presence of pH 12 buffer, we hypothesize that those groups will change the primary amino acid sequence, potentially affecting protein stability and its biological activity. HPLC chromatograms of PSC-RANTES showed the presence of secondary peaks in the void volume of the column. Optimization of the method was

attempted to separate the degradant peaks at pH 12 from the void volume of the column, but it was not successful.

In addition to chemical degradation, PSC-RANTES when administered vaginally, will encounter enzymes present in vaginal fluids that will potentially degrade the protein. The degradation of PSC-RANTES in the presence of the three most common enzymes in vaginal fluid (aminopeptidase, lysozyme, and proteinase K) has been investigated in this study. Degradation was only observed when PSC-RANTES was exposed to proteinase K, a broad spectrum serine protease. The predominant site of cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. Based on an enzymatic activity analysis performed to predict the sites more predisposed to cleavage using Peptide Cutter software program (http://us.expasy.org), the possible cleavage sites for proteinase K were established. This software provides the query sequence with the possible cleavage sites mapped on it and a table of cleavage site positions. The position of potential cleavage sites for the amino acid sequence of PSC-RANTES is shown in Table 2-4, and confirms the results obtained for PSC-RANTES by MALDI-TOF MS analysis when in the presence of Proteinase K, with a high number of fragments observed.

Investigation of a potential microbicide agent must take into consideration the toxicity of the agent to the normal vaginal flora. The ability of PSC-RANTES to preserve the normal vaginal flora was investigated. The killing of *Lactobacillus* species was unexpected considering the mechanism of action for this drug. Hydrogen peroxide producing Lactobacilli and streptococci are considered essential for a healthy vaginal microflora. The hydrogen peroxide is protective, and the lactic acid produced by these organisms is primarily responsible for maintaining the low vaginal pH which provides a native mechanism of protection against some

pathogens. The results from this study suggest a potential incompatibility of PSC-RANTES with certain strains of normal vaginal flora. The most common species of Lactobacillus present in the normal vaginal flora in the United States population are *L. crispatus* and *L. jensenii*. Both are hydrogen peroxide producing Lactobacillus, which are beneficial to the normal vaginal microflora. PSC-RANTES showed incompatibility with *L. iners*, which is a non-hydrogen peroxide producing strain and less beneficial to the normal flora. This finding is similar to the results obtained from other microbicide proteins recently studied and represents a low concern in the development of a microbicide product (personal communication - Bernard Moncla, Ph.D., Magee-Womens Research Institute).

This study has characterized the degradation of PSC-RANTES under various conditions, which is essential for the development of an effective microbicide product, and provided several significant findings relevant to formulation and drug delivery. The physical instability of PSC-RANTES observed at pH 7 suggests that any aqueous based pharmaceutical product should be formulated at a more acidic pH. The identified potential for oxidation raises the need to evaluate protective strategies such as antioxidants for final delivery system design.

It is essential that a microbicide product overcome physical and chemical barriers such as the presence of mucus and enzymatic degradation in addition to the delivery of the active pharmaceutical compound to the site of action. A formulation of PSC-RANTES must include better protective strategies to ensure the stability of the protein. This protection can be obtained by encapsulation of PSC-RANTES into nanoparticulated drug delivery systems, which will facilitate distribution of the microbicide agent and preserve the stability of the drug as recently reported (150). However, development of such a delivery system is outside the scope of this dissertation, but is currently being pursued in our laboratory.

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3.0 PREFORMULATION EVALUATION OF RC-101

3.1 INTRODUCTION

Defensins are cysteine-rich cationic antimicrobial peptides expressed by the leucocytes and epithelial cells of mammals. It has been shown that α-defensins expressed by human T cells may prevent the development of clinical Acquired Immunodeficiency Syndrome (AIDS) (151). Experiments conducted showed that defensins protect cells from *in vitro* infection by human immunodeficiency virus (HIV-1). β-defensins have shown to possess a high antiviral activity, inhibiting both X4 and R5 strains of HIV-1 (104). θ-defensins have the ability to bind with high affinity to CD4, galactosylceramide (alternative cell surface receptor for $CD4^-$ cells), and gp120; and thereby confer protection against HIV-1 (106). Retrocyclins (θ-defensins) are the evolutionary descendants of α-defensin genes. Retrocyclins are circular 18-residue, tetracyclic peptides with three cysteine disulfides bonds. RC-101 (GICRCICGKGICRCICGR) (Figure 1-6), a cationic retrocyclin analog, synthesized by solid phase peptide synthesis, has shown activity against X4 and R5 strains of HIV-1 *in vitro* (102). The mechanism occurs by preventing six-helix bundle formation of gp41 (a 41,000 MW glycoprotein), conferring a strong mechanism of protection against HIV-1 (109). The strong net negative charge of heptad repeat 2 (HR2) and the net positive charge of RC-101 create a strong electrostatic attraction that promotes binding. In addition, ability of HIV to generate escape mutants against RC-101 is limited. As a result, RC-101 has been identified as a potential microbicide candidate to prevent mucosal transmission of HIV-1 (102, 106).

A microbicide product is defined as a female controlled method, applied intravaginally to prevent transmission and acquisition of sexually transmitted infections, mainly HIV. Biopharmaceutical molecules (proteins and peptides) have demonstrated advantages over small molecule microbicides. However, it is a challenge to formulate a protein or peptide into a microbicide product. The product must overcome *in vivo* barriers that will affect efficacy of the product. Changes in efficacy can be related to: 1) protein modification, mostly due to conformational changes; 2) chemical degradation in the drug delivery vehicle or biological environment; 3) proteolytic inactivation in the vaginal lumen, and/or 4) low penetration of the drug into the mucosal tissue (45, 134). It is crucial to understand the potential degradation pathways through a complete pre-formulation study. The preformulation study will expedite formulation of a successful microbicide product.

The purpose of this research was to perform a preformulation study to characterize the peptide RC-101 to a sufficient extent that it can be further used in the development of a microbicide product. This study evaluated selected conditions of temperature, pH, and the presence of hydrogen peroxide to establish rate and extent of degradation, and identify potential degradation pathways of RC-101. Protective effects of excipients against oxidation were also investigated. Samples were analyzed by high performance liquid chromatography (HPLC), circular dichroism (CD), MALDI-TOF MS, and UV-spectroscopy.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Retrocyclin-1 (RC-101) was synthesized by the Peptide Synthesis Facility at the University of Pittsburgh (Pittsburgh, PA). Mass spectrophotometry was conducted using Electrospray Mass Spectroscopy (Quattro II, Fisons Inc., Valencia, CA) to confirm the molecular weight of the compound. Acetonitrile (HPLC grade), trifluoroacetic acid (TFA), sodium phosphate dibasic, phosphoric acid 85%, sodium acetate, and acetic acid glacial were obtained from Fisher Scientific (Fair Lawn, NJ). Vitamin E tocopheryl polyethylene glycol succinate (TPGS) was purchased from Eastman Chemical Company (Kingsport, TN). Edetate disodium (EDTA) was obtained from Spectrum Chemicals (Gardena, CA). All other materials were obtained from Sigma (St. Louis, MO). De-ionized water was prepared from house-distilled water with a Milli Q (Millipore, Milford, MA) water system operating at 18.2 MΩ.cm.

3.2.2 High performance liquid chromatography

A high performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA) was used for the analysis of RC-101. The HPLC system was equipped with an autoinjector model 717 (Waters), a quaternary pump model 600 (Waters), and an ultraviolet (UV) detector model 2487 (Waters). Separation of RC-101 from degradant products was achieved using a Phenomenex Jupiter 5 μ C5 300 Å (4.6 x 250 mm) column (Phenomenex, Torrance, CA) protected by a Widepore C5 (4 x 3.0 mm) guard cartridge (Phenomenex). The gradient consisted of mobile phase A (0.1% TFA in water (v/v)), and mobile phase B (0.07% TFA in acetonitrile

(v/v)) pumped at a flow rate of 1.0 mL/min. Elution conditions started at A:B (80:20) and linearly changed to A:B (60:40) for the first 12 min, returned to the A:B (80:20) condition in 1 min, followed by a 13 min isocratic condition at A:B (80:20) to allow equilibration of the system before the next sample was injected. Retention time of RC-101 was around 10 min and the total run time was 26 min. Empower PRO, Empower 2 software (Waters Corporation) was used to control the HPLC system. To ensure that the method was suitable for the intended purposes of quantification and separation of degradants, the accuracy, precision, repeatability, Limit of Quantification (LOQ), and Limit of Detection (LOD) were determined in addition to other suitability parameters defined by the United States Pharmacopeia (135). Forced degradation studies at extreme conditions of temperature, pH and oxidative potential were initially conducted to confirm that the HPLC system was suitable as a stability-indicating assay.

3.2.3 Circular Dichroism Spectroscopy

Circular dichroism (CD) was used to monitor conformational changes in the secondary structure of RC-101. CD measurements were performed on an AVIV Circular Dichroism spectrophotometer model 202 (AVIV Biomedical, Lakewood, NJ) equipped with a 0.1 cm path length quartz cell. Spectra were recorded between 198 and 300 nm at 25 °C from a 500 μg/mL RC-101 solution in 10 mM phosphate buffer pH 3.0, 7.0, or 12.0. Three scans were averaged and subtracted from buffer background over the same wavelength range.

3.2.4 MALDI-TOF MS

Matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometric (MS) experiments were performed in the positive-ion linear mode on a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA). Samples were prepared by pipetting 1 μL of sample and 1 μL of matrix on an MS stainless steel sample probe (Applied Biosystems). The matrix was prepared by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in 0.5% trifluoroacetic acid in acetonitrile:water (1:1 v/v). The probe containing the sample was left to dry completely in air. The spectra were calibrated using external calibration Sequazime Peptide Mass Standards calibration mix 1 (Applied Biosystems). The following parameters were used: accelerating voltage 20 kV with delayed extraction, mass range of 500–5,000 Da, and 400 laser shots spectrum.

3.2.5 UV-spectroscopy

RC-101 solutions at concentrations of 0.125 to 2.5 mg/mL were prepared in phosphate buffer solutions and UV scans were performed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). Scans were performed from 220 to 700 nm. NanoDrop software was used to control the spectrophotometer.

3.2.6 Stability of RC-101

For all stability studies described below, triplicate solutions of RC-101 were prepared by adding RC-101 at a concentration of 500 μg/mL (26.4 μM) in either water or aqueous phosphate buffer

solutions. The pH of each solution was measured after solubilization of RC-101 in the vehicle and at the end of the experiment. All samples were used within 5 min of preparation, and analyzed by HPLC using the method described in section 3.2.2. Additional analytical techniques (MALDI-TOF MS, UV-spectroscopy, and CD) were used to confirm results obtained by HPLC.

Effect of temperature

Thermal degradation studies were conducted at 25, 37, and 65 °C for a minimum period of 1 week. RC-101 (500 µg/mL) samples were removed from each chamber 30 min prior to analysis by HPLC to allow the samples to equilibrate to ambient temperature. Thermal degradation samples were also evaluated by MALDI-TOF MS.

Effect of pH

The effect of pH on the stability of RC-101 (500 µg/mL) was evaluated over the pH range from 3 to 12 using 10 mM phosphate buffer solutions. All buffers were prepared using phosphate sodium to avoid interference of other salts in the solution. Knowing that phosphate buffer is not a strong buffer at certain pHs, the pH values were confirmed at time 0 and at the end of each experiment. Samples were analyzed by HPLC, MALDI-TOF MS, CD, and UV spectroscopy.

Effect of ionic strength

RC-101 solutions at concentrations ranging from 0.125 to 2.5 mg/mL were prepared in water and phosphate buffers (pH 4, 7, and 12) at low (50 mmol/kg) and high (500 mmol/kg) ionic strength to evaluate the effect of ionic strength on RC-101 stability. Effect of high ionic

strength was evaluated by UV-spectroscopy analysis to determine the formation of insoluble aggregates.

Effect of hydrogen peroxide

The stability profile of RC-101 (500 µg/mL) under oxidative conditions was evaluated by exposing a solution of RC-101 to hydrogen peroxide (H_2O_2) at concentrations of 3.0, 0.08, 0.02, and 0.002% (v/v). A minimum of five time points were taken from each of triplicate solutions for HPLC analysis. Oxidative RC-101 samples were also evaluated by MALDI-TOF MS.

Protective effect of antioxidants

Solutions of RC-101 (500 µg/mL) were prepared followed by the addition of antioxidants commonly used in pharmaceutical products. The antioxidants used in this study were: methionine (95.2 µg/mL or 250 µg/mL), cysteine (95.2 µg/mL), glutathione (90.9 µg/mL), vitamin E TPGS (90.9 µg/mL), ascorbic acid (1.0 mg/mL), sodium ascorbate (1.0 mg/mL), and EDTA (0.5 mg/mL). Explanation on the concentration of antioxidants used was described in Section 2.2.7. Briefly, the concentrations of sodium ascorbate, ascorbic acid, EDTA, and vitamin E TPGS were selected based on the inactive ingredient list for FDA drug approved products (http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm - last accessed on 10/22/2008) and the Handbook of Pharmaceutical Excipients (136). Concentrations of methionine, cysteine, and glutathione were based on literature reviews on the use of amino acids as competitive inhibitors of oxidation (137, 138). RC-101 solutions prepared with EDTA were analyzed by HPLC for a period of 30 days after exposure with 0.002% H_2O_2. All other solutions were analyzed by HPLC for a period of 48 h.

Effect of freeze-thaw cycles

RC-101 (500 μg/mL) solutions were stored at -80 °C and allowed to freeze for at least 1 h. Samples were thawed immediately prior to HPLC analysis. The test was repeated 5 times, resulting in 5 freeze-thaw cycles. After completion of five freeze-thaw cycles, RC-101 solutions were analyzed by CD.

Effect of enzymatic degradation

RC-101 (500 μg/mL) solutions in water were prepared followed by the addition of enzymes commonly present in human vaginal fluid. The enzymes used in this study were: aminopeptidase, lysozyme, and proteinase K. Samples were tested by MALDI-TOF MS at time 0 and after 1 h exposure at 37 °C.

3.2.7 Statistical analysis

HPLC data obtained from the preformulation studies were expressed as the average percentage of the peak area from time 0 ± standard deviation, n = 3. Results were analyzed by one-way analysis of variance (ANOVA) with multiple comparisons of individual time points by using post hoc Bonferroni correction to detect significant differences under different conditions. P-values ≤ 0.05 were considered to be statistically significant, unless specified otherwise.

3.3 RESULTS

High Performance Liquid Chromatography (HPLC) assay

Linearity of the response was shown by analyzing serial dilutions of a standard solution of RC-101 in water ranging from 10 to 500 μg/mL (0.53 to 26.4 μM). Results for the assay qualification are shown in Table 3-1. The coefficient of regression (R^2) and the residual (%) from the observed values to the calculated standard curve were calculated. Precision was determined from the standard deviation (CV) of the response areas from ten injections of a low concentration solution of RC-101 (10 μg/mL). The Limit of Detection (LOD) was determined as three times the ratio of area standard deviation to the slope of the calibration curve (140). Limit of Quantitation (LOQ) was evaluated as 10 times the area standard deviation to the slope of the calibration curve (140). Repeatability was demonstrated by analysis of calibration curves on different days, and reproducibility was measured by analyzing calibration curves on different instruments by different analysts. The results demonstrate that the method is suitable for the intended purposes.

Table 3-1. Qualification of HPLC method for RC-101

Factor	Criteria	Result
k	2<*k*<10	2.72
N	>4000	32501
R_s	>2	2.72
T	<1.5	0.986
R^2	>0.999	0.99973
Residual	< 10%	4.68%
CV	<1%	0.65%
LOD		2.4 µg/mL (1.25 nM)
LOQ		7.9 µg/mL (4.20 nM)

Where k = retention factor, N = number of theoretical plates, Rs = resolution, T = tailing factor, R^2 = coefficient of correlation, Residual = the average difference between the observed values for the standard and the calculated standard curve, CV = coefficient of variation of the response of 10 injections, LOD = Limit of Detection, and LOQ = Limit of Quantification.

A representative chromatogram of blank (water) and RC-101 (100 µg/mL) in water are shown in Figure 3-2 and Figure 3-1, respectively. RC-101 eluted in 10 minutes.

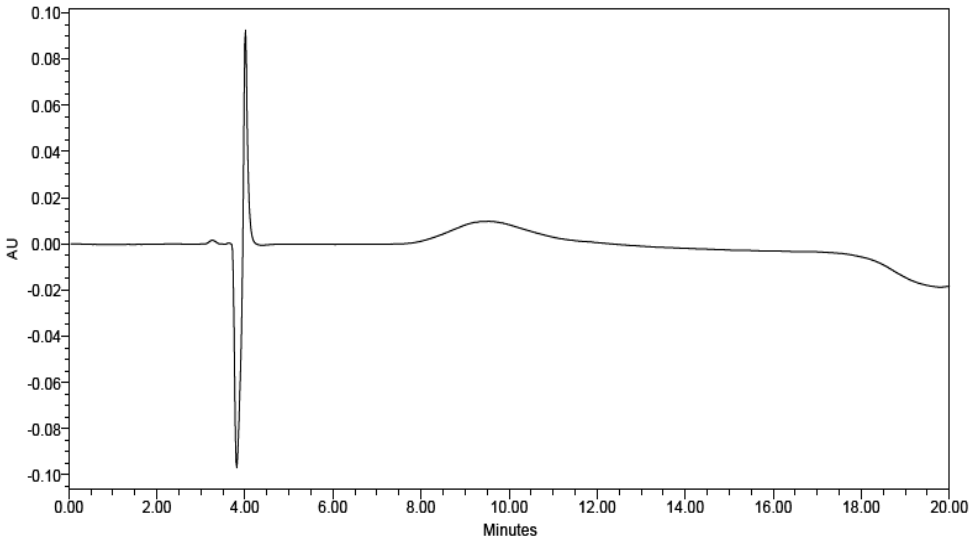

Figure 3-1. Representative HPLC chromatogram of blank sample (water).

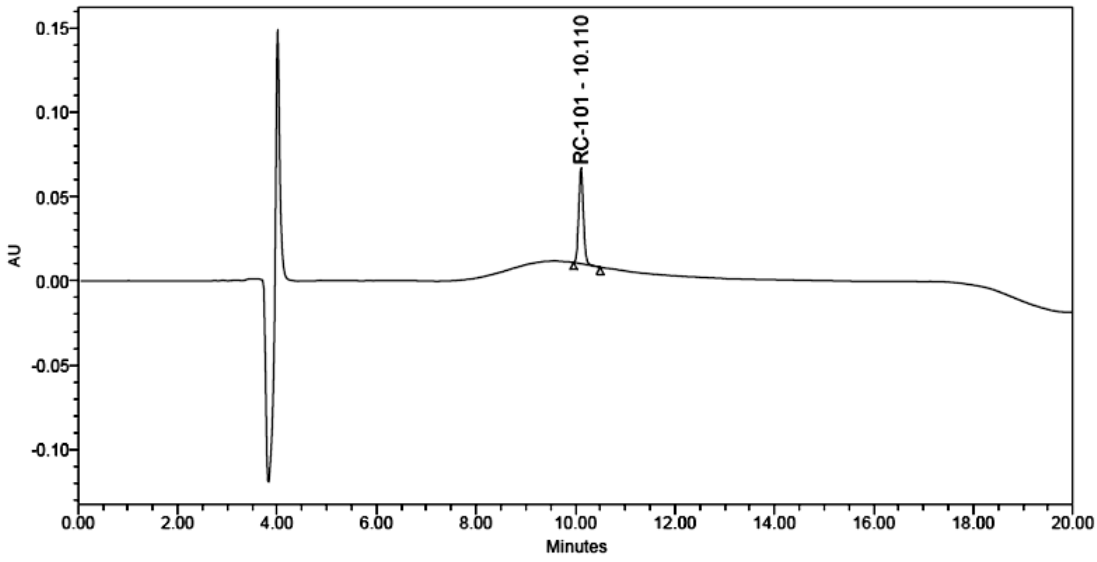

Figure 3-2. Representative HPLC chromatogram of RC-101 (100 µg/mL) in water at time 0.

Stability - Effect of temperature

The effect of temperature on the stability of RC-101 solutions (500 µg/mL in water, pH 5.7 ± 0.3) is shown in Figure 3-3. The graph represents the amount of RC-101 (% of the amount at time = 0) remaining at each time point. Post hoc Bonferroni correction for multiple comparisons was applied and no statistically significance difference was observed for RC-101 stored at 25 and 37 °C for a period of 13 days ($p > 0.5$). Samples stored at 65 °C showed a significant decrease in the amount of RC-101 remaining over time. However, at 24 h, the amount of RC-101 at 65 °C was not significantly lower than the amount observed at 25 °C ($p > 0.07$). For the subsequent time points, the amount of RC-101 was statistically significantly lower than the amount at 25 °C ($p < 0.04$).

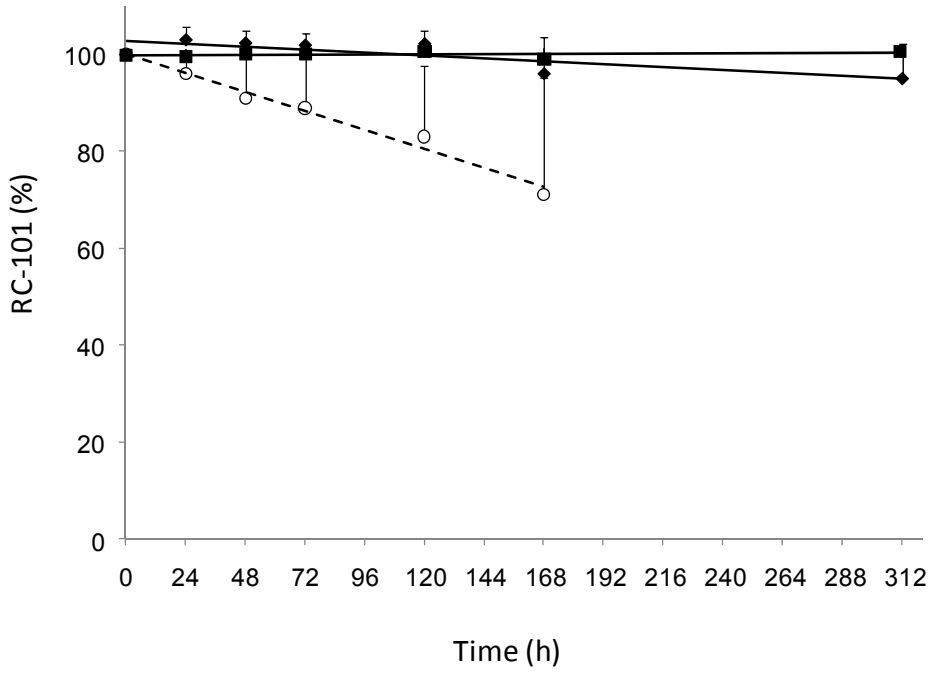

Figure 3-3. Effect of temperature on RC-101.
RC-101 (500 µg/mL) in water stored over time at 25 °C (■), 37 °C (♦), and 65 °C (○), analyzed by HPLC (n=3)

The log concentration versus time was plotted for each temperature condition (Figure 3-4). The slope of each curve was calculated to confirm the statistical results obtained from Figure 3-3, and to estimate the rate of degradation (rate constant, k) at each condition. Chemical stability is generally expressed by rate constant to estimate rate of drug degradation. Assuming first order kinetics, rate constant (k) can be calculated by the slope of the curve from a plot of log concentration versus time.

Slope = -k / 2.303

The calculated rate constant is used to predict stability for a longer time period. Results show that at 25 and 37 °C, the slope of the curve was not statistically significant different than zero ($p > 0.5$); and the rate constant was zero. This implies a stable molecule over time. At 65

°C, a forced degradation temperature, the slope was -0.001 and it was statistically significant different than zero ($p < 0.001$). The rate constant was 2.3×10^{-3} h^{-1}, indicating degradation over time.

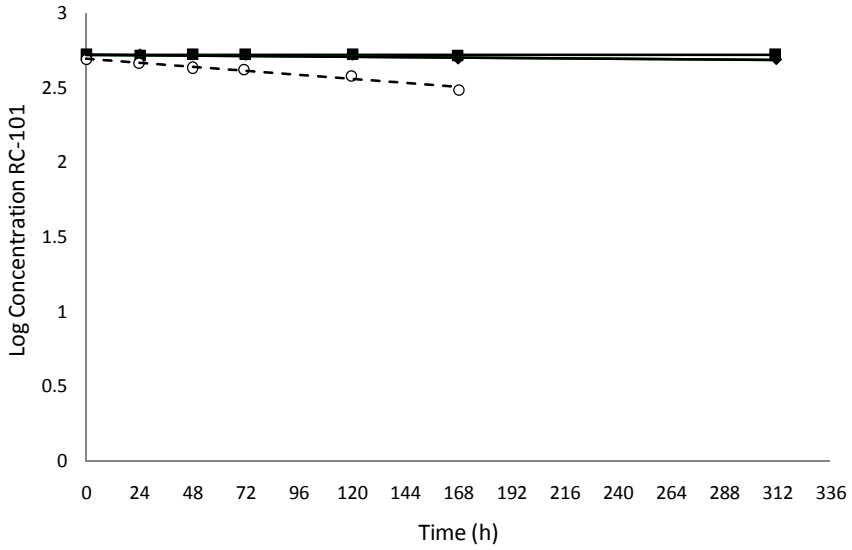

Figure 3-4. Log concentration of RC-101 versus time (h).
Obtained from Figure 2-5 for each temperature condition 25 °C (■), 37 °C (♦), and 65 °C (○), analyzed by HPLC (n=3)

To confirm the results obtained by HPLC, MALDI-TOF MS analysis were conducted with RC-101 (500 µg/mL) in water after 10 days incubation at room temperature, 37 °C, and 70 °C. The intensity (%) of the *m/z* fragments of RC-101 determined by MALDI-TOF MS are shown in Figure 3-5 for room temperature, Figure 3-6 for 37 °C, and Figure 3-7 for 70 °C. The spectra show a fragment around 1890 *m/z* correspondent to RC-101 $[M + H]^+$; a fragment at 948 *m/z* correspondent to $[M + H]^{2+}$, and the presence of a dimer (3,789.99). No additional fragments were observed when compared to RC-101 in water at time 0. The thermal degradation detected for RC-101 at 65 °C using HPLC assay (Figure 3-3) was not observed using MALDI-TOF MS. After incubation of RC-101 for 10 days at 70 °C, the spectrum did not contain any

additional fragments when compared with RC-101 incubated at room temperature. Two possible reasons may play a role in the inability of MALDI-TOF MS spectra to display the degradation products. First, if hydrolysis is occurring during thermal degradation, the molecule may lose that water during the ionization step of MALDI-TOF MS. In that way, the *m/z* fragment of the hydrolyzed compound would appear in the spectrum with the same *m/z* ratio as RC-101. The second explanation is the optimization of the method. MALDI-TOF MS parameters have been optimized to detect the RC-101 ion. Possibly, under thermal degradation, the degradation products are not detected because they are too small, or are ionized less efficiently than RC-101. The decrease in the parent peak (RC-101) observed by HPLC under thermal degradation suggests degradation of the peptide at higher temperatures (65 °C). However, no indication of the mechanism of degradation could be concluded by MALDI-TOF MS.

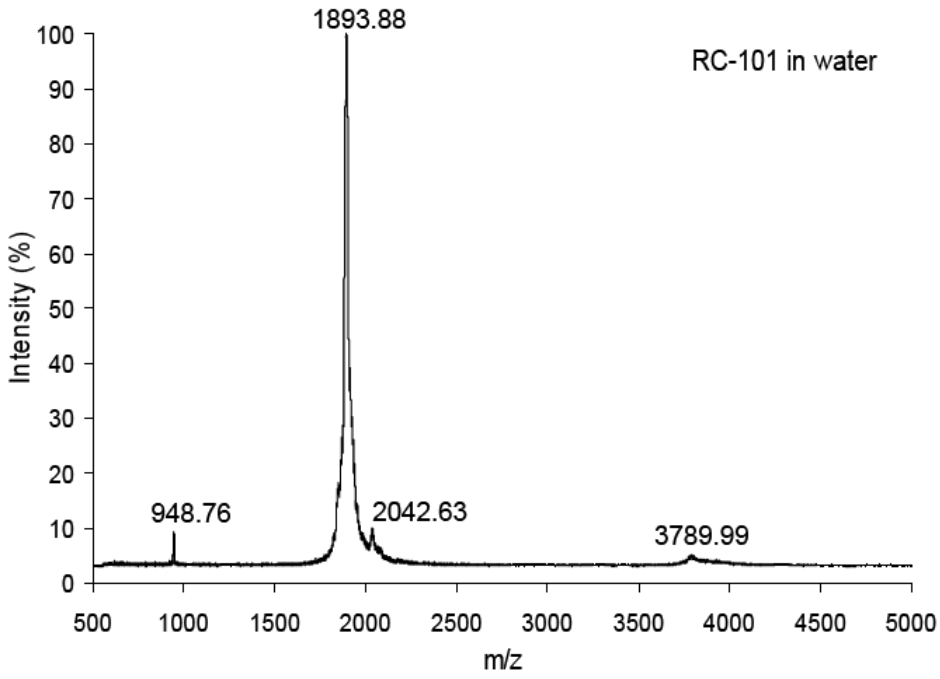

Figure 3-5. MALDI-TOF MS spectrum of RC-101 in water, exposed for 10 days at RT. 100% intensity = 38291 counts

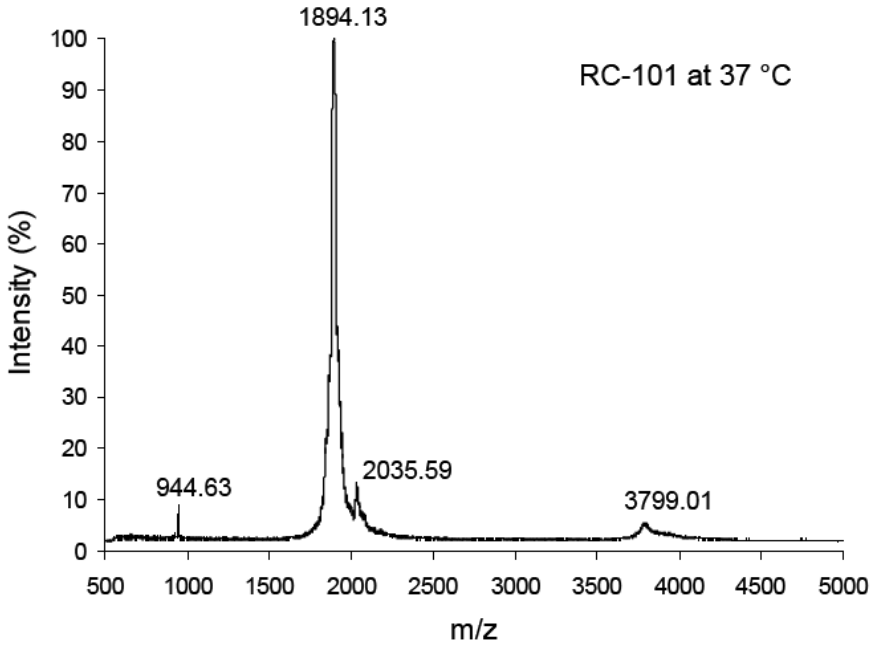

Figure 3-6. MALDI-TOF MS spectrum of RC-101 in water, exposed for 10 days at 37 °C. 100% intensity = 61027 counts

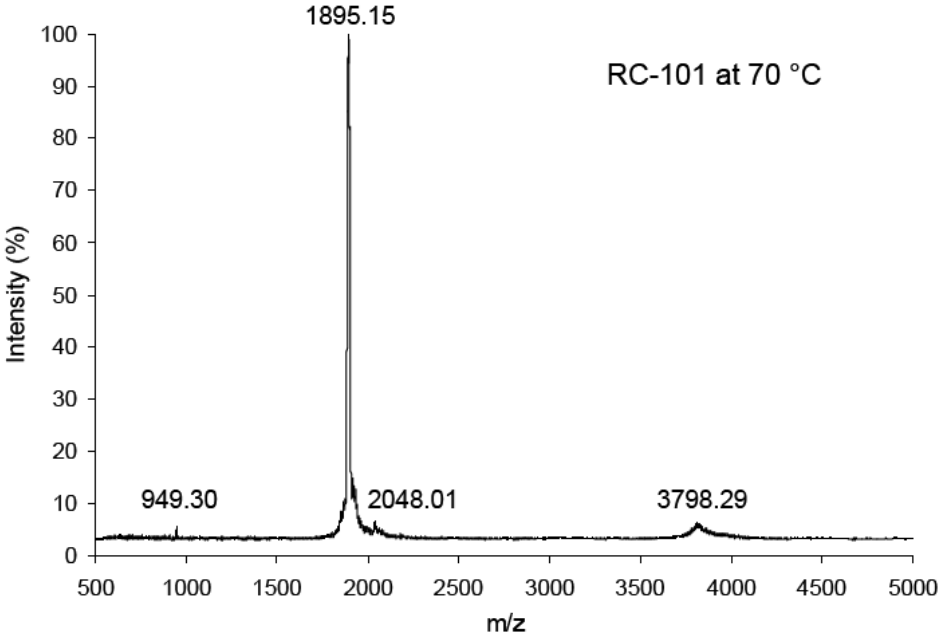

Figure 3-7. MALDI-TOF MS spectrum of RC-101 in water exposed for 10 days at 70 °C. 100% intensity = 39078 counts

Stability - Effect of pH

Solutions were made at a concentration of 500 µg/mL in phosphate buffers (pH 3 to 12) and analyzed over time by HPLC (Figure 3-8). The pH was confirmed before starting incubation at room temperature and at the end of the experiment. The pH for each buffer solution after addition of RC-101 was no more than 0.5 units different than the initial buffer pH. All solutions were stored in a glass container, as it was shown that no absorption to the container occurred during the period of time of the study. RC-101 was stable over the pH range of 3 to 7, indicated by no loss of the parent peak in the HPLC. Post hoc Bonferroni analysis for multiple comparisons was applied and no statistically significant decrease was observed over a period of 10 days for the samples at pH 3, 4, and 7 ($p > 0.83$). A significant decrease was observed for RC-101 at pH 12 phosphate buffer in the first 2 h (Figure 3-8), and degradation at this pH continued to occur over time. Representative chromatogram of RC-101 exposed to phosphate buffer pH 4 is shown in Figure 3-9. Loss of parent peak and generation of secondary peaks in the HPLC for RC-101 at pH 12 phosphate buffer were observed in the chromatograms (Figure 3-10).

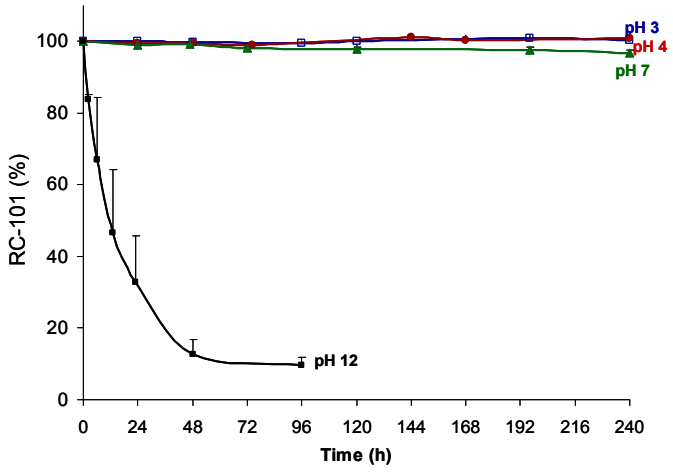

Figure 3-8. pH profile of RC-101 (500 µg/mL). Concentration (µg/mL) versus time (h). RC-101 prepared in mM phosphate buffers pH 3, 4, 7 and 12. Analyzed by HPLC (n = 3).

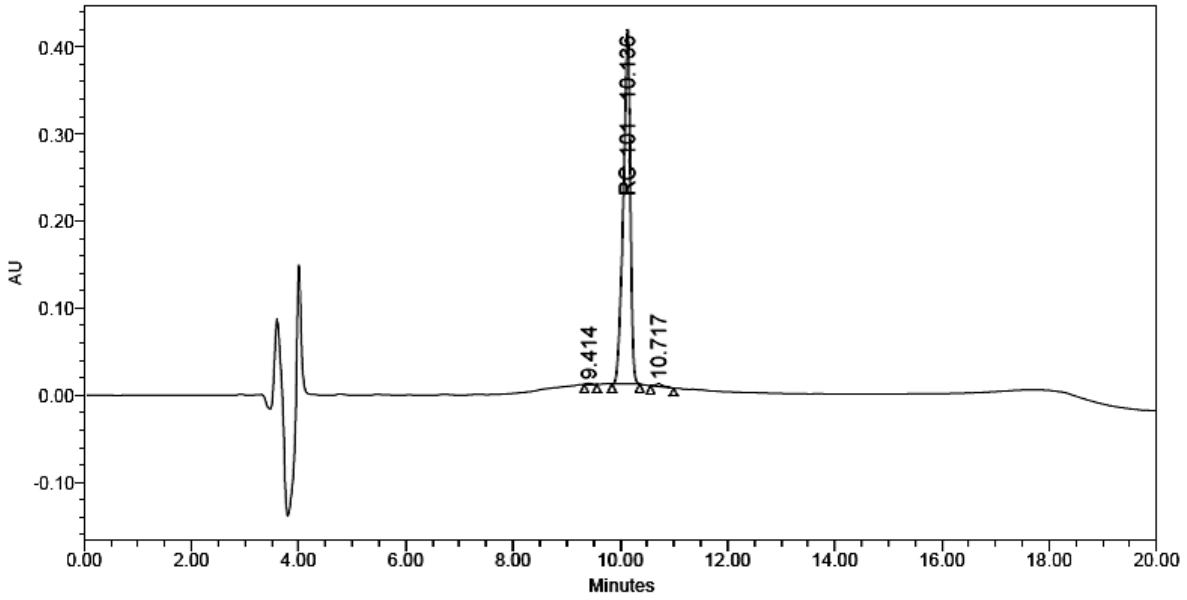

Figure 3-9. HPLC chromatogram of RC-101 (500 µg/mL) exposed for 10 days at pH 4 buffer.

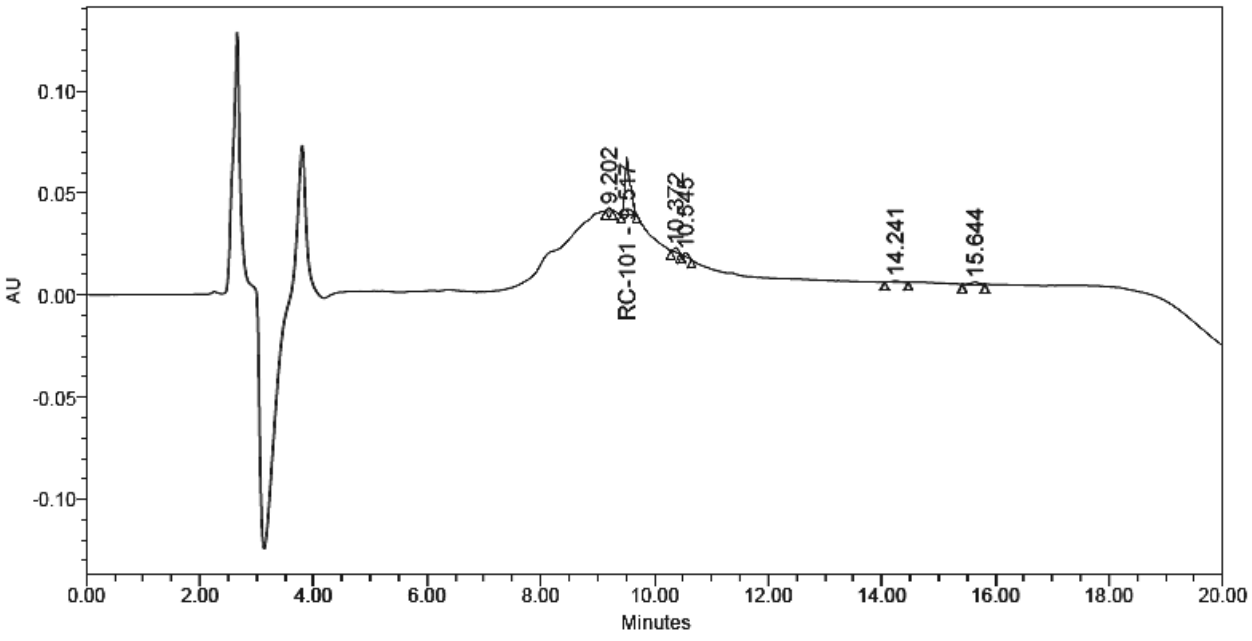

Figure 3-10. HPLC Chromatogram of RC-101 (500 µg/mL) exposed for 48 h at pH 12 buffer.

The log concentration at each pH condition was plotted against time (Figure 3-11) for the calculation of the rate constant (k), as described in Section 3.3 (Stability - Effect of temperature). The rate constant for each pH value was calculated by the slope of the linear regression of the concentration versus time plot. The results show that at pH 3, 4, and 7, the rate constant was not statistically significant different than zero ($p > 0.27$), indicating stability of RC-101 over this pH range. However, at pH 12, biphasic degradation, defined by two rate constants, was observed. The rate constant values were calculated as 0.076 h^{-1} from time 0 to 12 h, and 0.051 h^{-1} from time 24 to 96 h. Rate constant values at pH 12 indicate that RC-101 degrades over time at this pH condition.

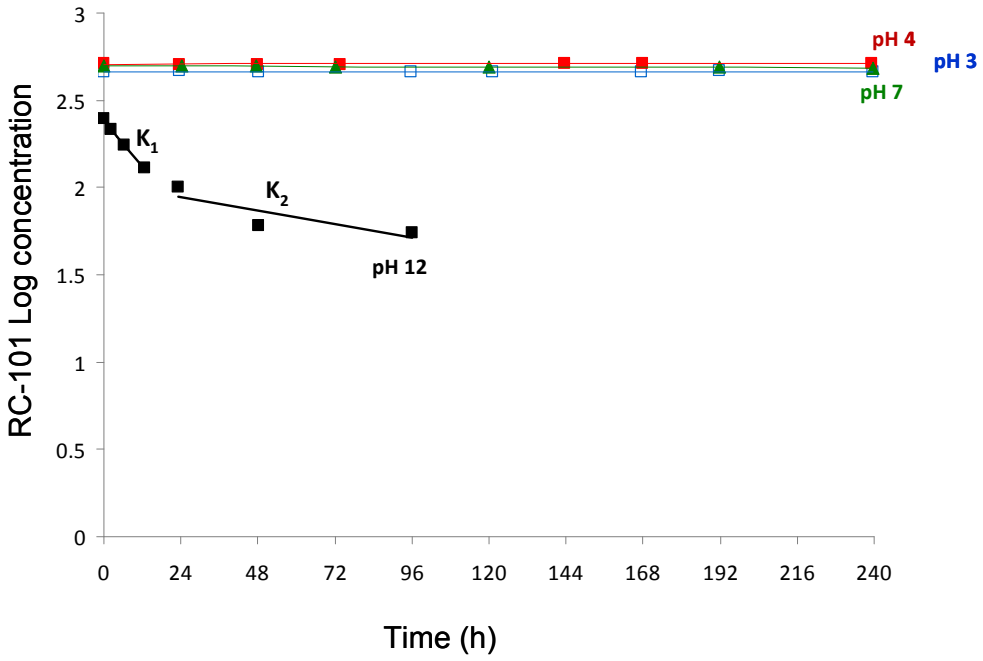

Figure 3-11. pH profile of RC-101. Log Concentration versus time (h).
Obtained from Figure 3-8 for each pH condition, analyzed by HPLC (n=3). K_1 and K_2 are the rate constants that defined the biphasic degradation of RC-101 in pH 12 phosphate buffer.

Circular Dichroism (CD) was conducted on phosphate buffer solutions of 500 µg/mL RC-101 at pH 3, pH 7, and pH 12 to verify changes in secondary structure. CD spectra for each

pH are shown in Figure 3-12. Under all conditions, the protein showed a random conformation, with a maximum absorbance at 230 nm and a minimum absorbance at 200 nm for pH 3, 205 nm for pH 7, and 210 nm for pH 12. The peak shift in the wavelength and the loss of absorbance for pH 7 and pH 12 samples indicate a change in folding of the protein under these pH conditions.

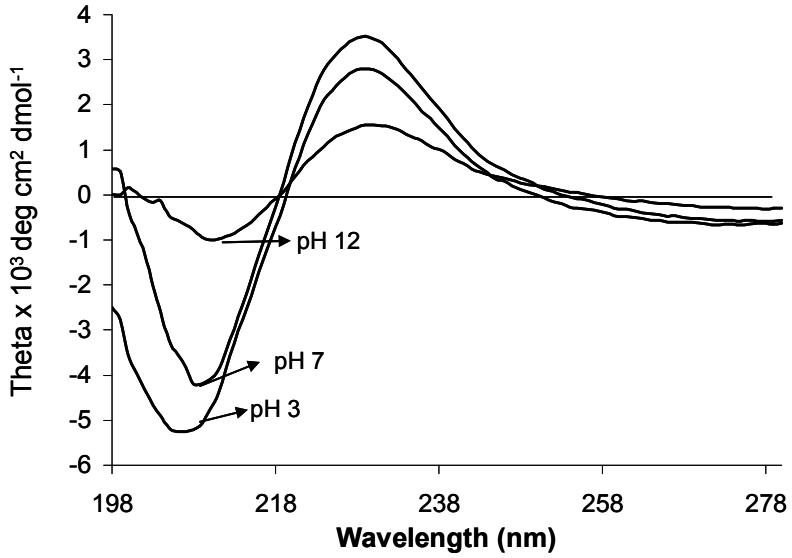

Figure 3-12. CD spectra of RC-101 (500 µg/mL) under different pH conditions.
Average of 3 individual spectra after subtraction of background control correspondent to each buffer solution (pH 3, 7, and 12 phosphate buffer)

In addition to HPLC and CD, MALDI-TOF MS was performed to confirm stability at pHs 3 to 7 and degradation at pH 12 observed in the other analytical techniques. Results for RC-101 (500 µg/mL) exposed to pH 4 (Figure 3-13), pH 7 (Figure 3-14) and pH 12 (Figure 3-15) phosphate buffer for 10 days are shown. An additional peak at 1915 *m/z* was observed at pH 4 and 7, which is likely to be a sodium adduct from the buffer, a single charged specie $[M + Na]^+$. Stability of RC-101 at pH 4 and 7 phosphate buffer was confirmed. The faster degradation

observed at pH 12 in the HPLC and the conformational changes in the CD assay have been confirmed in MALDI-TOF MS, with the appearance of several smaller fragments (Figure 3-15).

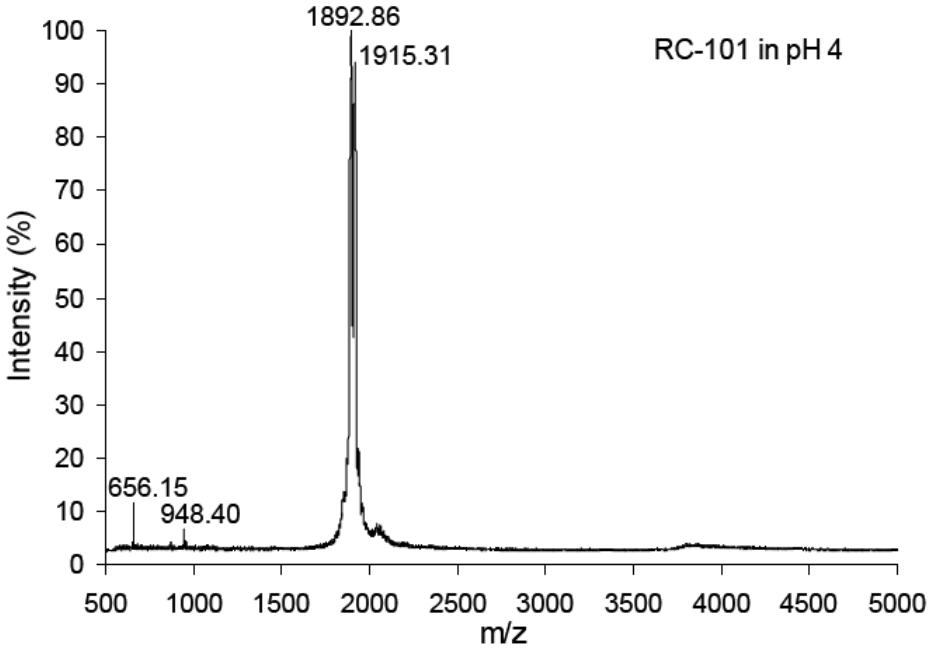

Figure 3-13. MALDI-TOF MS spectrum of RC-101 (500 µg/ml) in pH 4 (10 mM phosphate buffer), after 10 days. 100% intensity = 44735 counts

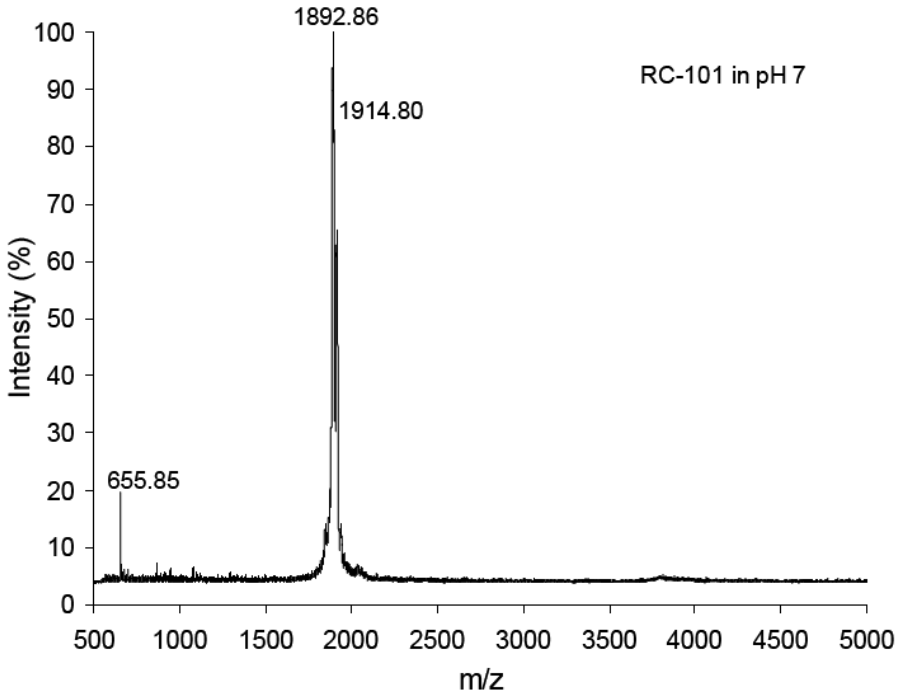

Figure 3-14. MALDI-TOF MS spectrum of RC-101(500 µg/ml) in pH 7 (10 mM phosphate buffer), after 10 days. 100% intensity = 30633 counts

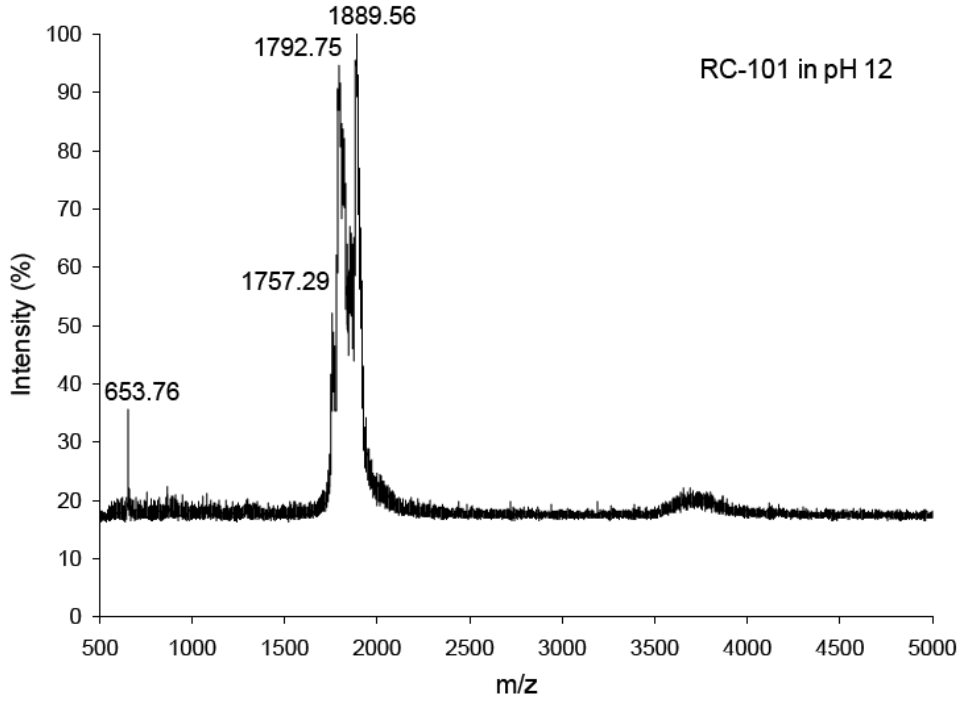

Figure 3-15. MALDI-TOF MS spectrum of RC-101(500 µg/ml) in pH 12 (10mM phosphate buffer), after 48h. 100% intensity = 7116 counts

UV spectroscopy was conducted to evaluate the formation of insoluble aggregates at different pH buffers. UV scans of RC-101 (500 µg/mL) in phosphate buffers pH 4, 7, and 12 were conducted (Figure 3-16). Similar scans were observed for RC-101 pH 4 and 7, indicating no formation of insoluble aggregates at these two pH buffers. However, a small increase in the absorbance at RC-101 pH 12 buffer was observed in the range of 300 to 600 nm, implying the presence of insoluble aggregates. No significant changes in the UV spectra were observed in all samples when analyzed after 1 week exposure to the buffers.

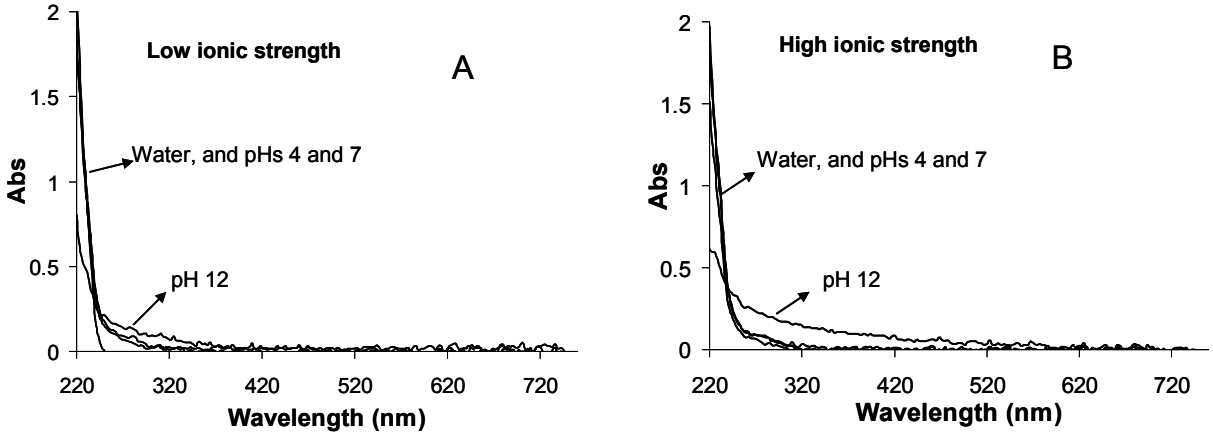

Figure 3-16. UV scans to determine the effect of pH and ionic strength.
RC-101 solutions in water, pH 4, pH 7, and pH 12 buffers were analyzed by UV-spectroscopy at time 0 to determine formation of insoluble aggregates. A) Represent RC-101 solutions prepared in low ionic strength buffer (50 mmol/kg); and B) represent RC-101 solutions prepared in high ionic strength buffers (500 mmol/kg).

Stability – Effect of ionic strength

The effect of ionic strength on the stability of RC-101 (500 µg/mL) was evaluated by UV-spectroscopy and results are shown in Figure 3-16. Similar UV scans were observed for the RC-101 solutions in high ionic strength buffers in water, pH 4 and pH 7. However, in phosphate

buffer pH 12, high ionic strength, an increase in the absorbance from 300 to 600 nm was observed, indicating the formation of insoluble aggregates.

Effect of hydrogen peroxide

Effect of hydrogen peroxide (H_2O_2) on the stability of RC-101 (500 μg/mL) was evaluated in several concentrations of H_2O_2 as discussed in Section 2.4. Briefly, forced degradation studies to evaluate oxidative effects are commonly conducted by exposing the molecule of interest to a solution of 3.0% H_2O_2 (144). For a more biologically relevant level of H_2O_2, the concentrations were based on reports that determined the production of H_2O_2 by Lactobacillus present and calculations of the number of Lactobacillus present in the normal vaginal flora (146). HPLC results of RC-101 (500 μg/mL) exposed to 0.002, 0.08 and 3.0% hydrogen peroxide are shown in Figure 3-17. RC-101 was quickly degraded (more than 20% loss) over a 4-hour period when exposed to a 3.0% H_2O_2, which is a stressed condition used for preformulation studies. When RC-101 was exposed to 0.002 and 0.08% H_2O_2, the loss detected by the HPLC was less than 10% over a period of 24 h for both concentrations. The data suggests that RC-101 is less susceptible to degradation by oxidative pathways than PSC-RANTES (Section 2.3).

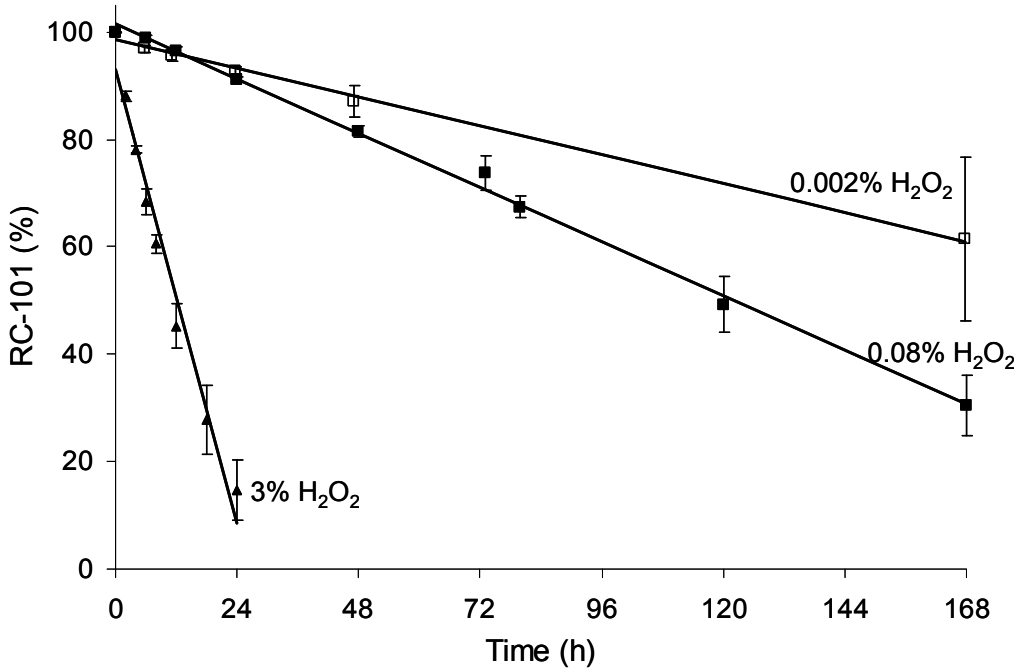

Figure 3-17. Effect of H_2O_2 on RC-101 (500 µg/mL). Concentration (%) versus time (h). RC-101 exposed to 0.002, 0.08, and 3% hydrogen peroxide, stored at room temperature and analyzed over time by HPLC (n = 3).

The log concentration at each oxidative condition was plotted against time (Figure 3-18) for the calculation of the rate constant (k). The rate constant for each concentration of hydrogen peroxide value was calculated and the results show that the rate constant was 0.066 h^{-1} for 3% H_2O_2, 0.007 h^{-1} for 0.08%, and 0.003 h^{-1} for 0.002% H_2O_2, indicating that degradation of RC-101 by oxidation occurs over time.

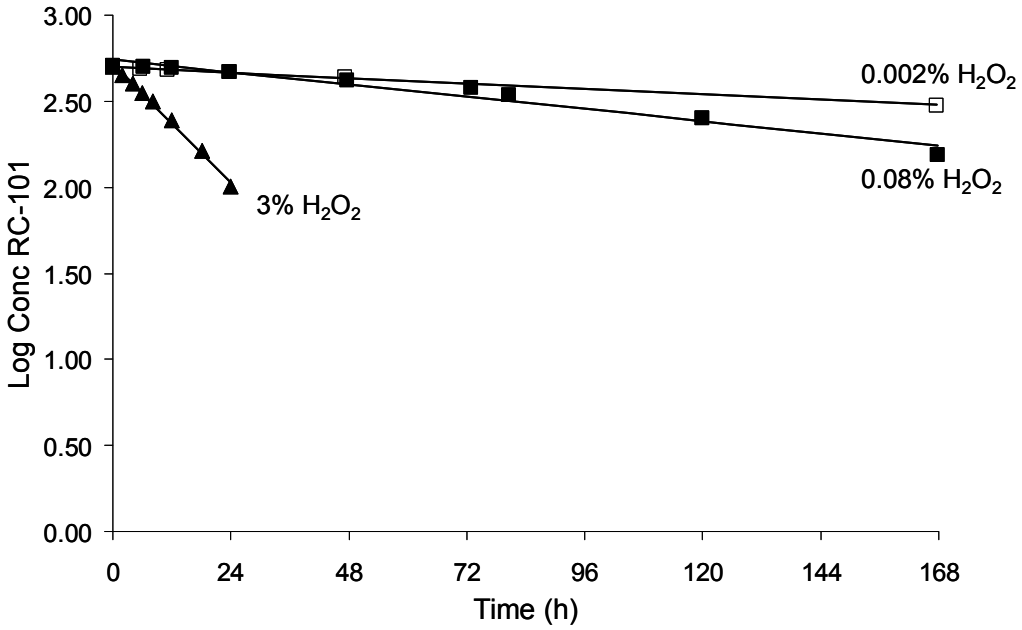

Figure 3-18. Effect of H_2O_2 on RC-101. Log Concentration versus time (h).
Obtained from Figure 3-17 for each concentration of hydrogen peroxide (0.002, 0.08, and 3%), analyzed by HPLC (n=3).

HPLC chromatogram of RC-101 in the presence of 0.08% hydrogen peroxide after 120 h exposure is shown in Figure 3-19. Loss of parent peak and the presence of additional peaks are observed and confirm the degradation by oxidation of RC-101.

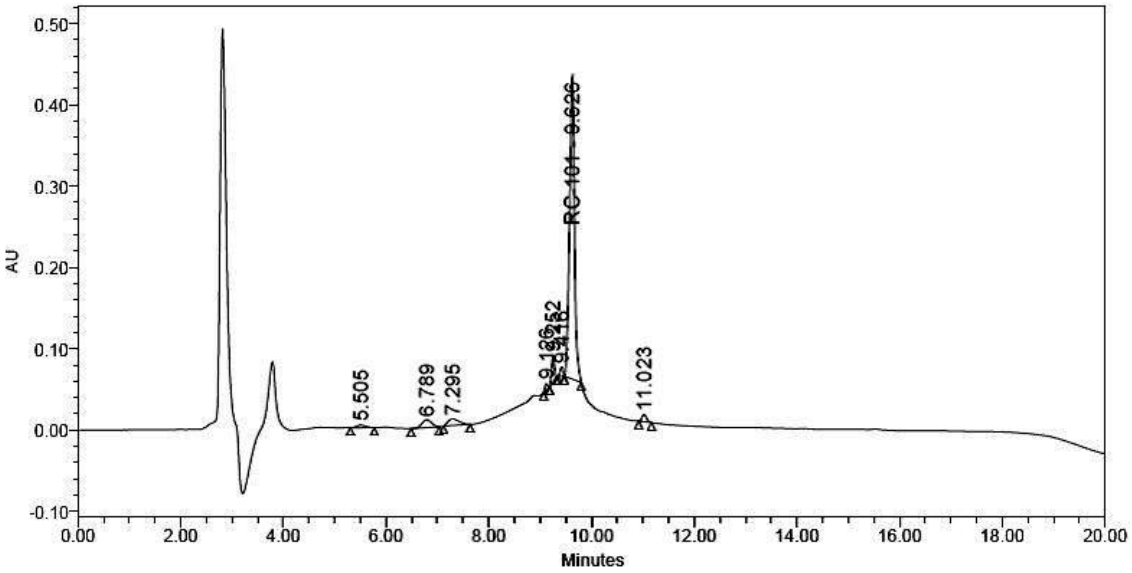

Figure 3-19. Representative HPLC chromatogram of RC-101 (500 µg/mL) in 0.08% H_2O_2 after 120 h exposure.

MALDI-TOF MS was performed for RC-101 exposed to all three conditions of H_2O_2 over time as an additional method to verify degradation pathways of RC-101. MALDI-TOF MS spectra for RC-101 in 3% H_2O_2 for 24 h is shown in Figure 3-20. MALDI-TOF MS spectra for RC-101 in 0.08% and 0.002% H_2O_2 for 168 h is shown in Figure 3-21 and Figure 3-22, respectively. In all studies, the main peak corresponding to RC-101 (*m/z* = 1892) is still present at a maximum intensity. However, the additional peak at *m/z* = 1988 is observed in all conditions tested and can be associated to oxidation degradation. The difference between the *m/z* of these two peaks is 96, which can be associated to the incorporation of six atoms of oxygen. The disulfide bridges (R – S – S – R) in the peptide RC-101 are prone to oxidation. It is possible that, in the presence of H_2O_2, the disulfide bridges in some of the RC-101 peptide underwent oxidation forming disulfide monoxide (R – SO – S – R), disulfide dioxide (R – SO_2 – S – R), or sulfonic acid (R – SO_3 – H).

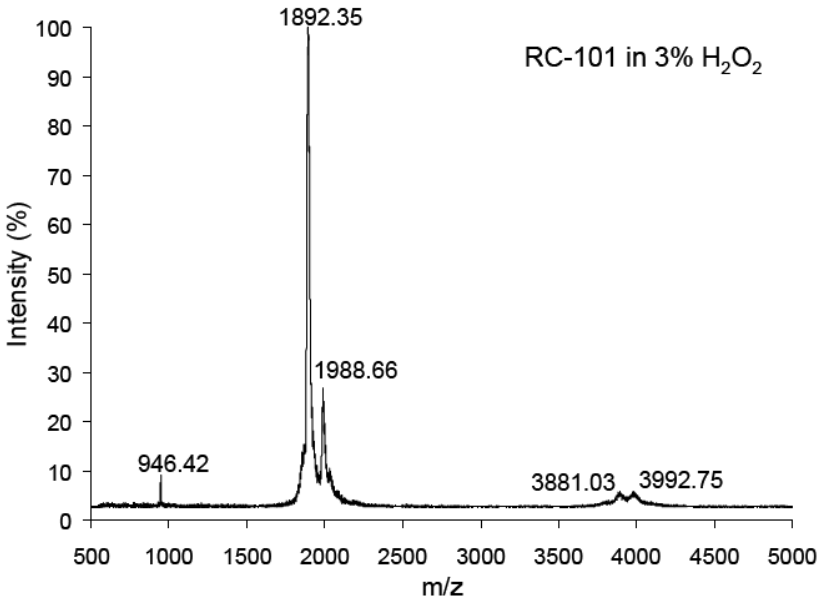

Figure 3-20. MALDI-TOF MS spectrum of RC-101 after 24 h exposure to 3% H_2O_2. 100% intensity = 46676 counts

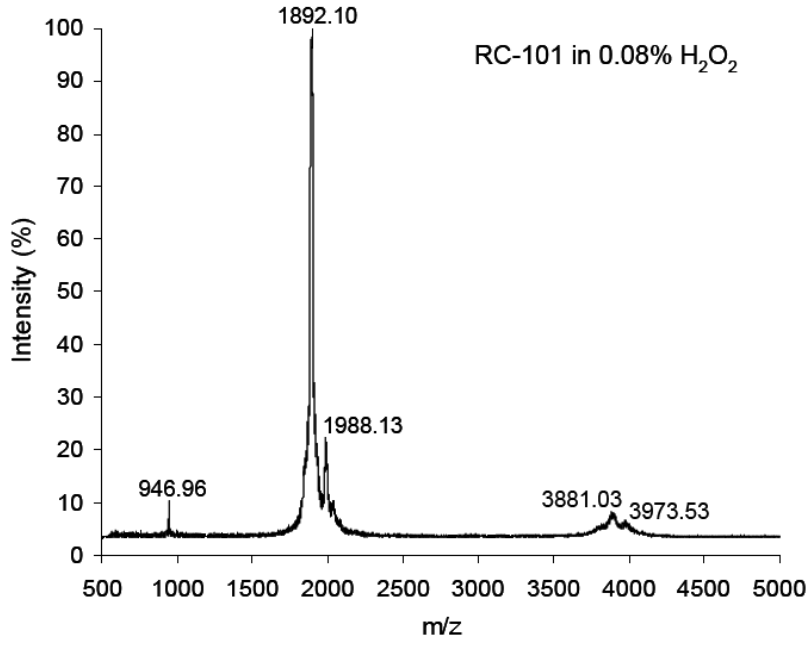

Figure 3-21. MALDI-TOF MS spectrum of RC-101 after 168 h exposure to 0.08% H_2O_2. 100% intensity = 34954 counts

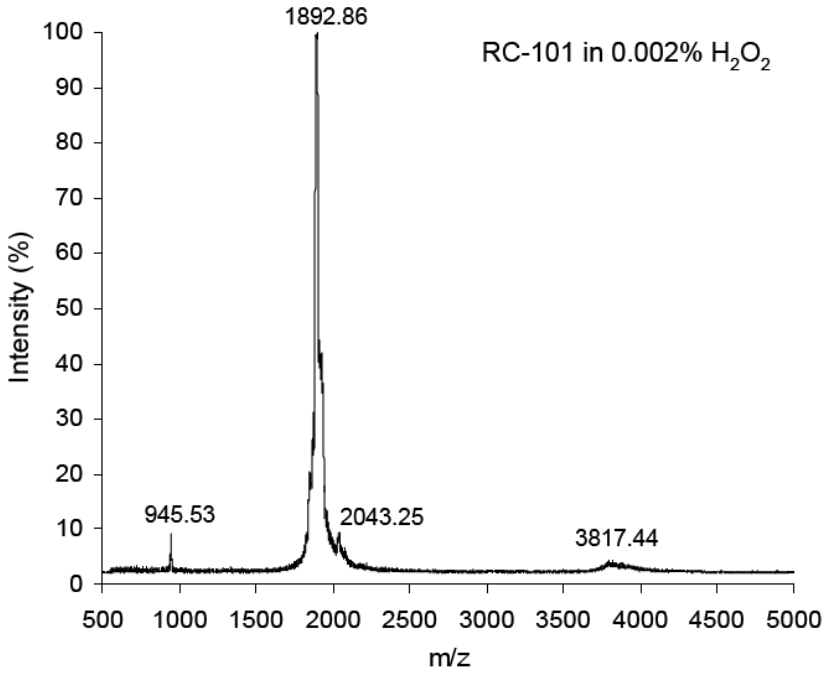

Figure 3-22. MALDI-TOF MS spectrum of RC-101 after 168 h exposure to 0.002% H_2O_2. 100% intensity = 56864 counts

Protective effect of excipients

In the previous sections, it has been identified that oxidation is the major degradation pathway for RC-101. Oxidation of RC-101 may disrupt the disulfide bridges, changing secondary structure of the peptide and affecting bioactivity. Considering that this peptide will be administered vaginally in an environment that contains hydrogen peroxide, protection against oxidation is necessary. A range of antioxidants from different pharmaceutical classes have been tested. Most of the antioxidants studied did not show a significant protective effect against oxidation in the presence of hydrogen peroxide. When analyzed by HPLC, oxidative degradation of RC-101 was faster in the presence of antioxidants than without them. EDTA was the only antioxidant investigated that showed protection of RC-101 against oxidation after exposure to H_2O_2 (Figure 3-23). Ethylenediaminetetraacetic acid (EDTA) is a widely used chelating agent, approved by the Food and Drug Administration (FDA) as a preservative for

pharmaceutical products. RC-101 in the presence of EDTA was exposed to 0.002% H_2O_2 and compared to RC-101 without EDTA in HPLC analysis. About 10% loss of RC-101 was observed after 30 days of exposure in the presence of EDTA, when compared to 40% loss of RC-101 after 7 days in the absence of EDTA.

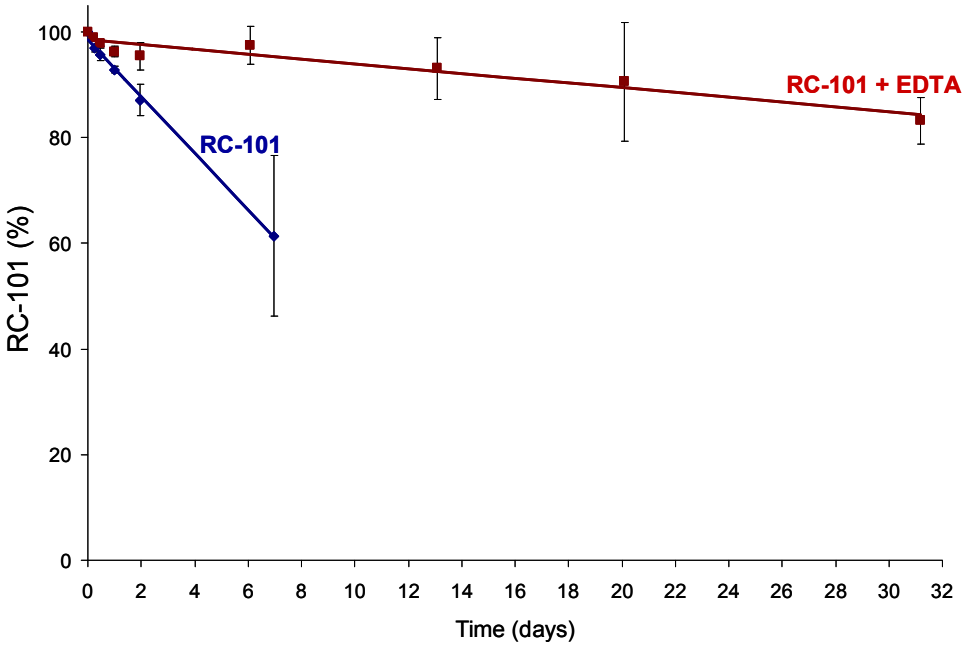

Figure 3-23. Effect of H_2O_2 in RC-101 stability in the presence and absence of EDTA. Concentration versus time (days). RC-101 (500 µg/mL) exposed to 0.002% H_2O_2 over a period of 30 days, analyzed by HPLC (n = 3).

Effect of enzymatic activity

Further studies with RC-101 were conducted to verify potential for degradation in the presence of the three most common enzymes present in the vaginal fluid. The same experimental conditions used for the effect of enzymatic activity in Chapter 2, Section 2.2.7 were used for RC-101. RC-101 was exposed to aminopeptidase (100 units/mL), lysozyme (100 units/mL), and proteinase K (100,000 units/mL), and results for MALDI-TOF MS experiments are shown on Figure 3-24, Figure 3-25, and Figure 3-26, respectively. No significant changes compared to the

MALDI-TOF MS spectrum of RC-101 in water prior to enzyme exposure have been found, indicating stability of the peptide in the presence of all enzymes tested.

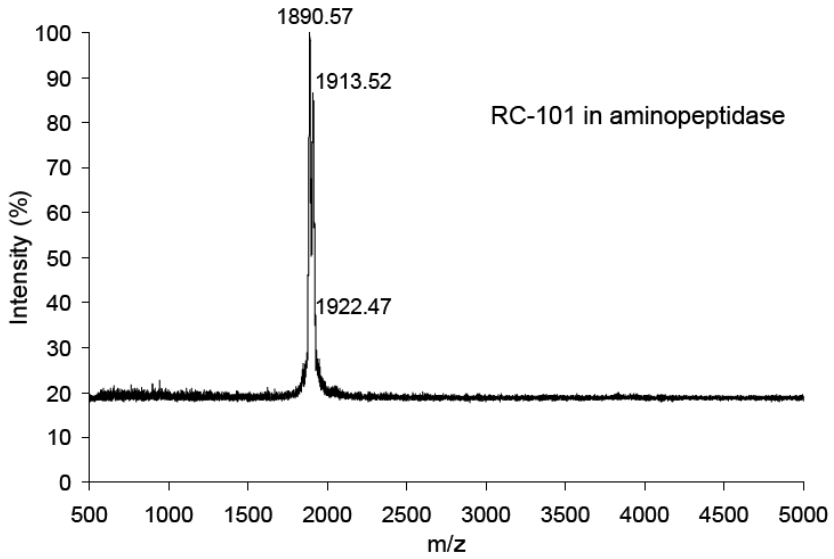

Figure 3-24. MALDI-TOF MS spectrum of RC-101 in the presence of aminopeptidase at 37 °C for 1 h. Intensity = 6536 counts.

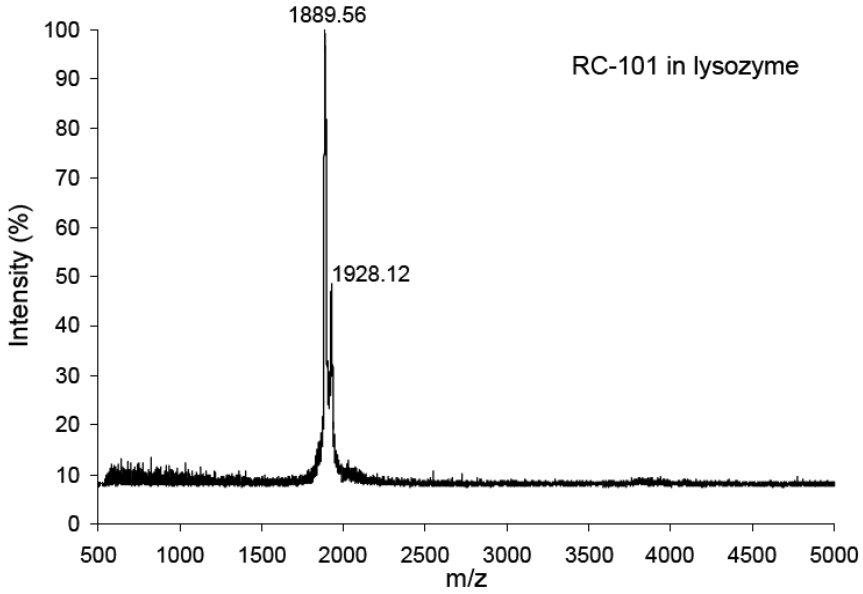

Figure 3-25. MALDI-TOF MS spectrum of RC-101 in the presence of lysozyme at 37 °C for 1 h. Intensity = 15272 counts

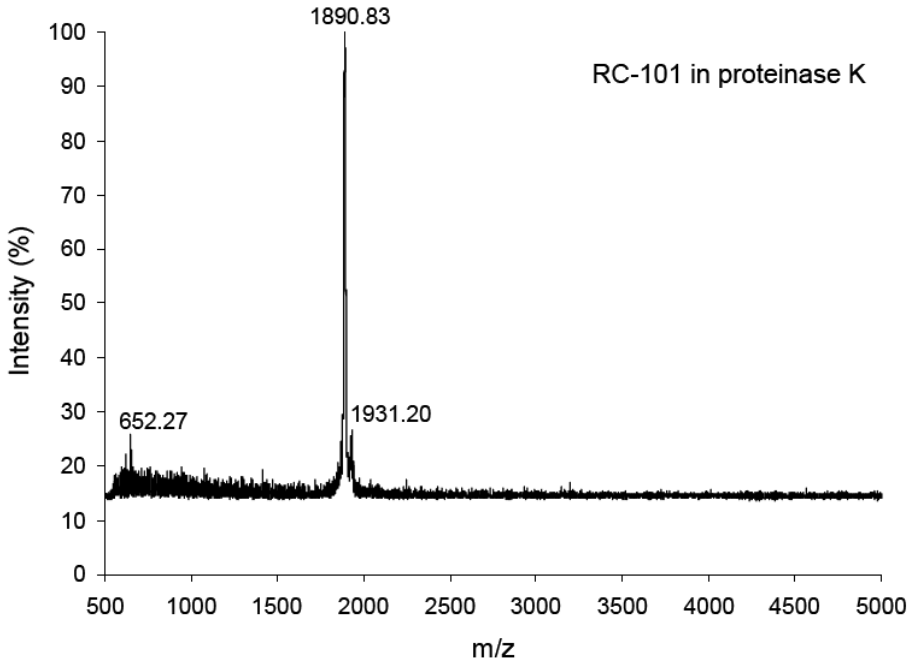

Figure 3-26. MALDI-TOF MS spectrum of RC-101 in the presence of proteinase K at 37 °C for 1 h. Intensity = 8437 counts

Effect of freeze-thaw cycles

RC-101 in solution was not affected by freeze-thaw after 5 cycles. Samples were analyzed after each cycle. Results of analysis by HPLC showed 97.7% of RC-101 remaining in solution after 5 cycles (Figure 3-27). This decrease was not statistically significant (ANOVA, $p > 0.18$). However, when the freeze-thaw samples were analyzed by Circular Dichroism, a slight shift in the wavelength and a change in the signal were observed in the CD spectra (Figure 3-28), suggesting that a change in the secondary structure may occur upon freezing. In the other hand, this slight change in secondary structure does not significantly affect bioactivity, as tested by Dr. Cole (personal communication – Alexander Cole, Ph.D., University of Central Florida).

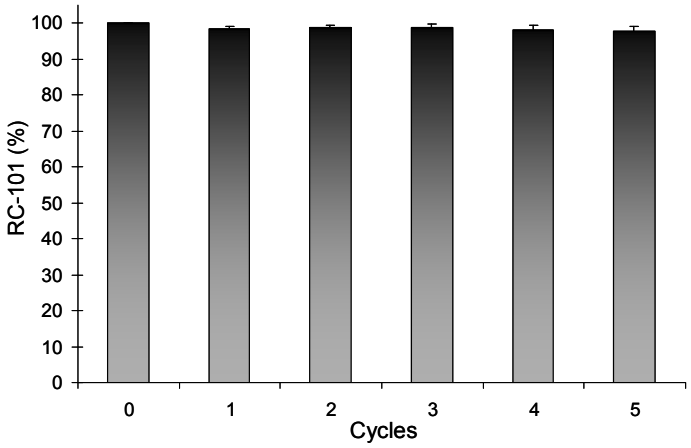

Figure 3-27. Effect of freezing-thaw cycles on RC-101 (500 µg/mL) in water analyzed by HPLC (n = 3).

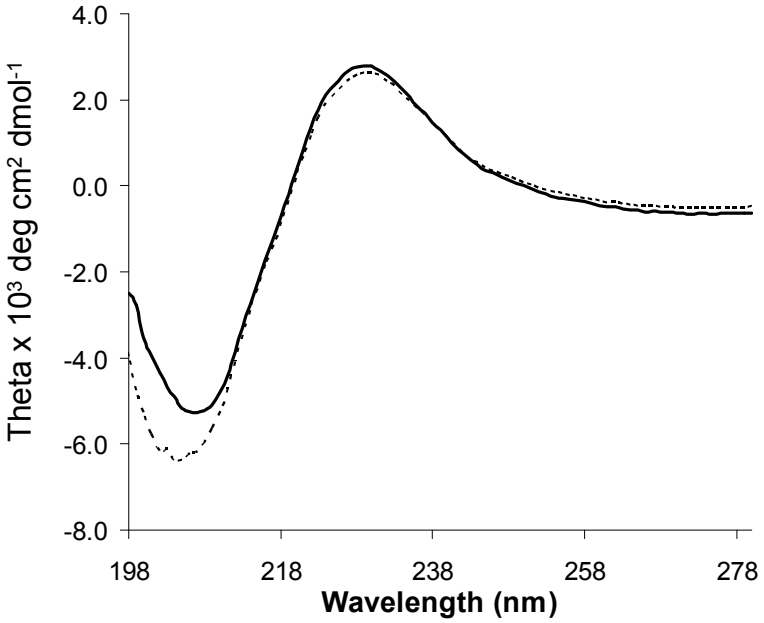

Figure 3-28. CD spectra of RC-101 (500 µg/mL) in water before and after freezing.
(—) before freeze-thaw cycles, (- - -) after freeze-thaw cycles. Average of 3 individual spectra after subtraction of background control (water).

3.4 DISCUSSION AND CONCLUSIONS

Recently, several biopharmaceuticals (proteins and peptides) have been investigated as potential microbicides for prevention of HIV (134, 152-154). However, formulation and delivery of biopharmaceuticals can be difficult due to degradation. A successful formulation will protect the peptide against degradation during the manufacturing process, during the shelf-life of the product, and after the protein enters the biological system (141, 142). According to the Alliance for Microbicide Development (143), several needs in microbicide formulation are considered to have a high priority, which includes preformulation evaluation. Preformulation studies will accelerate product development of a successful microbicide product. The current study addresses this issue by identifying and characterizing the microbicide RC-101 to further understand its stability, which will impact formulation development and, eventually, efficacy of the product.

RC-101 (MW = 1890.42) (GICRCICGKGICRCICGR) is a circular cationic 18-residue peptide (Figure 1-6), tetracyclic peptide with three cysteine disulfides bonds (107). The amino acid sequence comprises 10 polar amino acids, 4 hydrophobic residues, and 4 basic residues.

RC-101 proved to be stable in water, by HPLC analysis, at 25 °C over a period of 13 days, and 7 days at 37 °C, which suggests that the peptide will be stable at body temperature for a prolonged period of time. The stability of RC-101 at 25 and 37 °C has been confirmed by MALDI-TOF MS spectra showing the *m/z* peak correspondent to RC-101, and no presence of additional peaks. At higher temperatures (65 °C), the stability of RC-101 was compromised, being determined by loss of parent peak in the HPLC. However, MALDI-TOF MS technique was not able to detect fragments of thermal degradation, as explained in Section 3.3, suggesting

that the method was not suitable for detection of the degradation peaks. Protein stability at high temperatures should be considered not only to understand how the drug will be affected in the body, but also how the compound will behave during the manufacturing process for the final product, in case that high temperature is required for processing, and to extrapolate shelf-life predictions or enhance degradation rates. The data showing that RC-101 is susceptible to degradation at 65 °C indicates that the manufacturing process of a RC-101 microbicide product should avoid prolonged exposure of the drug to high temperatures. However, chemical stability of RC-101 under temperature conditions is superior to several other proteins studied that showed fast thermal degradation at temperatures higher than 40 °C (155-157).

The peptide RC-101 was shown to be stable in phosphate buffer solutions pHs 3, 4 and 7 using HPLC assay. Stability at different pHs is helpful in the development of a microbicide product. For instance, the stability of RC-101 in acidic pH will be important due to the drug being exposed to the acidic environment of the normal vaginal pH (3.5 to 5.0). In addition, since the peptide is stable from pH 3 to pH 7, it expands the pH range for formulation of the microbicide product, and upon exposure to semen. Stability over the pH range of 3 to 7 was confirmed by MALDI-TOF MS, where no additional fragments were observed. UV spectroscopy results for RC-101 with high ionic strength buffers did not show any significant differences in stability profiles, increasing the flexibility for formulation development. The secondary structure of RC-101 was also preserved in the pH range from 3 to 7, as observed by CD. The spectrum obtained by CD for RC-101 in pH 3 and 7 showed a random conformation; similar to the spectrum obtained for the natural defensin Retrocyclin, indicating stability of the peptide (102).

The development of a successful peptide microbicide product is primarily dependent on the ability to prevent the oxidative effects of H_2O_2, present in the vaginal lumen. The stability of RC-101 was investigated under different levels of hydrogen peroxide. Forced degradation studies to evaluate oxidative effects are commonly conducted by exposing the molecule of interest to a solution of 3.0% H_2O_2 (144). RC-101 quickly degraded in the presence of 3.0% H_2O_2 (20% loss in 4 h); however, the degradation was much slower when compared to the immediate degradation observed for PSC-RANTES (Chapter 2). In the presence of more biological relevant concentrations (0.002% and 0.08% H_2O_2), the degradation rate was slower. The amount of H_2O_2 selected was based on reported studies which determined the amount of hydrogen peroxide produced by Lactobacillus present in the normal vaginal flora, and estimated calculations based on concentrations of Lactobacillus present (145, 146). RC-101 amino acid sequence contains six cysteines which are prone to oxidation; however the cysteines are present in their oxidized form, decreasing the likelihood of oxidative degradation. The intramolecular disulfide bonds may further oxidize resulting in sulfenic acid. The oxidation of the cysteine residues is a metal-ion catalyzed oxidation reaction. In the presence of EDTA, this oxidative mechanism was decreased by chelation of EDTA with metal ions, eliminating the cysteine oxidation. Further formulation development may include the addition of EDTA. However, preliminary studies have shown that EDTA is toxic to human ectocervical tissue and normal vaginal microflora in concentrations of 1% or higher. Due to this fact, this preservative should be further characterized regarding its potential for toxicity *in vivo*.

Protection of RC-101 against oxidation may be necessary during the shelf-life of the final formulation and during the delivery in the vaginal lumen. The result from the addition of EDTA to the RC-101 solution is indicative of a method to protect RC-101 from oxidation during shelf-

life of the product. The use of the preformulation data in the development of a suitable microbicide formulation is further discussed in Chapter 5. In a biological environment, when the microbicide product is administered intravaginally, it will encounter the presence of vaginal fluids and cervical mucus that will not only dilute the microbicide agent, but also be a potential for degradation. The enzymatic activity present may initiate degradation of the peptide, in addition to the normal vaginal flora that produces hydrogen peroxide which will accelerate oxidation of RC-101. Our studies have shown that RC-101 is susceptible to oxidation, but in a very slow kinetic of degradation. Depending on the time for binding of RC-101 to receptors and glycoproteins, oxidation of RC-101 after 48 h may be an irrelevant degradation pathway and may not affect bioactivity. It is still unknown how long the drug should be active in the vaginal lumen, but it has been suggested that the virus stays in the vaginal lumen for a period of 48 h (66, 158, 159). If that is the case, short-term protection of RC-101 may be sufficient to overcome oxidative degradation pathways in the vaginal lumen and guarantee biological activity.

Another concern during preformulation studies for peptide microbicide product development is the presence of enzymatic activity in the vaginal lumen. As discussed in Chapter 2, proteins and peptides will be prone to degradation when in contact with enzymes present in vaginal fluids. The most common enzymes in vaginal fluid are aminopeptidases, proteinases, and lysozyme (54, 60-62). A study was conducted to verify the potential for degradation of RC-101 in the presence of these three enzymes. RC-101 was shown to be much less sensitive to enzymatic degradation than PSC-RANTES (Chapter 2), analyzed by MALDI-TOF MS. This indicates that RC-101 will be more stable when in the presence of human vaginal fluid. Further experiments exposing RC-101 to human vaginal fluid and cervical mucus collected from healthy female volunteers were conducted and are discussed in Chapter 4. These fluids were incubated

with RC-101 at 37 °C and analyzed by LC-MS/MS, to verify stability of RC-101 in biological fluids.

Overall, RC-101 was stable over a wide range of pH, temperature and presence of hydrogen peroxide. Some degradation pathways were identified, such as thermal degradation at high temperature (65 °C), hydrolysis at very alkaline conditions (pH 12), and oxidation at high concentrations of hydrogen peroxide (3%) or prolonged time with low concentrations of H_2O_2, which can be partially protected in the presence of antioxidant EDTA. Furthermore, enzymatic degradation was not observed when RC-101 was exposed to the three most significant enzymes with regards to vaginal delivery. This study has characterized the degradation pathways of RC-101 under various conditions, which is essential for the development of an effective microbicide product. The major degradation pathway identified is the oxidation of RC-101, and, as discussed above, it may not impact bioactivity, but still needs to be further characterized to verify its impact on shelf-life stability. Compared to other proteins studied (138, 141, 156, 157, 160), RC-101 is more stable at a larger range of pH and for a prolonged time over other stressed conditions. We conclude that RC-101 has the potential to become a microbicide product and the properties of RC-101 have been further investigated and are shown in Chapters 4, 5, and 6.

ACKNOWLEDGEMENTS

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I would like to thank Dr. Michael Cascio at the Molecular Genetics and Biochemistry Department at the University of Pittsburgh for the use of the Circular Dichroism spectrophotometer and the assistance provided with the experimental design. I would like to thank Dr. Billy W. Day and Dr. Manimalha Balasubramani at the Genomics and Proteomics Core Laboratories at the University of Pittsburgh for the assistance provided for the MALDI-TOF MS analysis. And many thanks to Phillip Graebing at the Magee-Womens Research Institute for the analytical help and support.

4.0 EVALUATION OF RC-101 STABILITY IN THE PRESENCE OF HUMAN VAGINAL FLUID

4.1 INTRODUCTION

Vaginal fluid covers the vaginal epithelium and is composed of secretions from the cervical vestibular glands, plasma transudate, and endometrial and oviductal fluids. The fluid covering the vaginal mucosa protects against entry of pathogens into deeper tissues. Periodic sloughing of mucus and underlying cells removes adherent microbes. Cervical mucus has similar functions, and in addition facilitates sperm penetration by changing its viscoelastic properties during ovulation. Properties of the mucus layer can either facilitate or impede the efficacy of a drug product. It is important to study the impact of vaginal fluids on vaginal microbicide product functionality. When a vaginal microbicide product is applied, its presence should not disrupt the natural protective mechanisms associated with the mucus layer, which also provides a target for bioadhesive products. In some cases, vaginal fluids may be disadvantageous. The presence of physiological fluids may alter the characteristics of a vaginal product, which can reduce the overall efficacy of the drug substance, increase leakage, and decrease drug residence time at the target tissue (161). More importantly, enzymatic activity and the presence of hydrogen peroxide produced by Lactobacillus will greatly affect the stability of microbicide agents, especially proteins and peptides. This enzymatic barrier in vaginal fluid has been identified as a major barrier to the delivery and absorption of microbicides and other drugs (63).

The interaction of microbicide drug candidates with human vaginal fluids can result in chemical modification of the drug by oxidation, hydrolysis, or proteolysis, thereby decreasing its potential for biological activity. In addition, dilution of the microbicide agent with vaginal fluids

may affect its efficacy. Biopharmaceuticals (proteins and peptides), as discussed in Sections 1.3 and 1.8.1, when administered intravaginally might suffer degradation by several mechanisms, which include action of protease enzymes present in the vagina, and oxidation by hydrogen peroxide produced from Lactobacillus which are a major constituent of the normal vaginal flora.

The peptide, RC-101, a potential microbicide drug, has been shown to prevent mucosal transmission of HIV-1 *in vitro* (102, 106). RC-101 is a strong candidate for use as a microbicide product because of its activity against HIV and its stability in preformulation studies shown in Chapter 3. In addition, previous studies have shown that RC-101 retained full activity in the presence of human vaginal fluid lavages (diluted 1:20) (109).

The purpose of this study was to investigate the potential for degradation of RC-101, alone and formulated, when exposed to human vaginal fluids collected from healthy premenopausal female volunteers. The abundance of RC-101 was monitored by liquid chromatography (LC) coupled online with tandem mass spectrometry (MS/MS) at specific time points after incubation with human vaginal fluids. Demographics of the volunteer population were collected by application of a questionnaire which included questions about age, smoking and drinking status, race, and gynecological history. All volunteers were tested for *Chlamydia. trachomatis*, *Neisseria gonorrhoeae*, and bacterial vaginosis.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Retrocyclin-1 (RC-101) was synthesized by the Peptide Synthesis Facility at the University of Pittsburgh (Pittsburgh, PA). Urea was purchased from Spectrum Laboratory Products Inc. (Gardena, CA). Polyvinyl alcohol (PVA) was obtained from Kuraray America Inc. (New York, NY). Hydroxypropylmethylcellulose (HPMC) was obtained from Sigma (St. Louis, MO). Glycerin was obtained from Dow Chemical Company (Midland, MI). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

4.2.2 Human vaginal fluid collection protocol

Human vaginal fluid (HVF) was collected from 79 healthy volunteer, premenopausal women, between the ages of 19 and 45 years old, mean (± S.D.) 31.3 (± 7.6), according to approved Institutional Review Board (IRB) protocol #07050142. After a phone screening (Appendix B.1), eligible volunteers were scheduled to participate in the research study. Volunteers were asked to abstain from sexual intercourse and not to use any vaginal products for a period of 48 h prior to fluid collection. After signing informed consent, volunteers were submitted to a urine pregnancy test using Sure-Vue® (Fisher Healthcare, Houston, TX). If the result was positive, the subject was informed and excluded from the study. If the result was negative, the subject proceeded with the study by answering a questionnaire. The subject then received the Instead Softcup® (Instead Inc., La Jolla, CA) device and instructions on how to use it. Instead Softcup® is a FDA approved device used to hold menstrual fluid during the menstrual period in replacement of a

tampon or pad. Volunteers inserted the cup and waited for 30 min. After this time period, a physician removed the cup, and placed it into a 50 mL conical centrifuge tube. Human vaginal fluid (HVF) collected from healthy volunteer women was stored at 4 °C until used. All samples were used within 4 h after collection. HVF collected contained approximately 40% of its weight in sloughed epithelial cells, which were separated from the supernatant after incubation period with RC-101.

After removing the Instead Softcup®, a speculum examination was performed. Swab specimens of the endocervix were obtained using the Mini-tip Culturette TN collection system (Becton Dickinson, Sparks, MD) according to the manufacturer's guidelines. Testing for *C. trachomatis* and *N. gonorrhoeae* was conducted using an amplified DNA assay based on the simultaneous amplification and detection of target DNA amplification primers and a fluorescent label detector probe (162). Bacterial vaginosis (BV) testing was conducted by using the Gram stain method and assessed by the Nugent score, where score results between 0 and 3 indicate a normal flora, between 4 and 6 indicates an intermediate state, and between 7 and 10 indicates bacterial vaginosis (163). Volunteers were notified by the physician of the test results by telephone within two weeks of collection, and directed to the Allegheny County Public Health Department (Pittsburgh, PA) for treatment and additional testing, if needed.

4.2.3 Method development

Preparation of RC-101 solution and film

RC-101 100 μg/mL solutions were prepared by dissolving RC-101 in Milli Q water. RC-101 and placebo films were prepared by the method described in Chapter 5. Briefly, a quick-dissolve vaginal film formulation containing RC-101 was prepared by casting a polymeric film solution

into an 8-well-plate (dimensions: 26.7 x 33.3 mm/well). A polymeric film solution was prepared by adding Milli Q water, PVA, and Hydroxypropylmethylcellulose (HPMC). Next, the solution was heated at 95 °C for 20 min for complete dissolution of the polymers. After cooling, glycerin and RC-101 were added. Film solution (2.4 g) containing RC-101 was poured into each well of the 8-well plate. The plate was placed into a vacuum oven at 30 ± 2 °C for 20 ± 4 h. All dried films were removed from the plates and stored at room temperature in PET/Aluminum foil pouches (Amcor Flexibles Healthcare Inc, Mundelein, IL) until further analysis. Placebo films were prepared in the same way without the addition of RC-101. Each RC-101 film contained 100 μg of RC-101. For analytical purposes, films (RC-101 and placebo) were dissolved in 1 mL of Milli Q water before addition to HVF.

Preparation of RC-101 + HVF sample

The Instead Softcup® containing HVF and the conical tube were centrifuged for 10 min at 5,000 rpm. HVF was then removed from the cup and the pH of each HVF sample was measured with Fisher Alkacid pH filter strips (Fisher Scientific). All samples collected on a specific day were pooled to be used for the research studies. If the pH of the individual samples was higher than 5, which is indicative of the presence of bacterial vaginosis (BV), the sample was not included in the pool but it was stored at –80 °C for separate analysis. If the sample contained blood, it was immediately discarded.

Samples were prepared as described in Table 4-1, and are detailed as follows. Samples A, control blank samples, were prepared by combining HVF with water. Samples B, RC-101 solution test, were prepared by combining RC-101 solution (100 μg/mL in water) with HVF. Samples C, RC-101 solution control, were prepared by combining RC-101 solution (100 μg/mL

in water) with water. Samples D, RC-101 film test, were prepared by dissolving RC-101 100 µg/film in 1 mL of water and then combining with HVF. Samples E, RC-101 100 µg/film control, were prepared by dissolving one film in 1 mL of water and then combining with water. Samples F, placebo film test, were prepared by dissolving one placebo film in 1 mL of water and then combining with HVF; however, due to the limited amount of HVF, this sample was not prepared. Samples G, placebo film control, were prepared by dissolving one placebo film in 1 mL of water and then combining with water. All samples (RC-101 solution, RC-101 film or placebo film) were combined with vehicle (HVF or water) in a ratio of 1:1. Because of its high viscosity, HVF was measured by weight and not by volume. All solutions were prepared fresh and incubated with HVF (or water) at 37 °C for specific periods of time (0, 2, 6, 12, 24, 48, and 72 h), unless specified otherwise.

To evaluate the influence of freezing the fluid prior to the analysis, the last pool of HVF was divided into two samples: one used fresh (at the time of collection), and the other one stored at -80 °C for a 3-month period. After that time period, HVF was thawed and processed for blank and RC-101 solution only, as described in Table 4-1. For control blank samples, frozen HVF was combined with water (Sample A_F). For RC-101 solution with frozen HVF, RC-101 (100 µg/mL in water) was combined with HVF frozen (Sample B_F). All solutions were prepared fresh and incubated with HVF (or water) at 37 °C for specific periods of time (0, 2, and 48 h).

HVF samples collected with a high pH value indicative of BV were stored at -80 °C as previously mentioned. After confirmation of BV on those fluid samples by Gram stain score, the fluid samples (HVF BV^+) were thawed, pooled, and processed as described in Table 4-1. For control blank samples, HVF BV^+ was combined with water (Sample A_{BV+}). For RC-101 solution with HVF BV^+, RC-101 (100 µg/mL in water) was combined with HVF BV^+ (Sample

B_{BV+}). All solutions were prepared fresh and incubated with HVF (or water) at 37 °C for specific periods of time (0, 2, 6, 24, and 48 h).

Table 4-1. Summary of RC-101, in solution and formulated, samples combined with HVF.

Sample Code	Sample Description	HVF	RC-101 (100 μg/mL) SOLUTION	Water	RC-101 100 μg/ FILM*	Placebo FILM*
A	Blank HVF	100 mg	-----	100 mg	-----	-----
B	RC-101 solution combined with HVF	100 mg	100 mg	-----	-----	-----
C	RC-101 solution control	-----	100 mg	100 mg	-----	-----
D	RC-101 film combined with HVF	100 mg	-----	-----	100 mg	-----
E	RC-101 film control	-----	-----	100 mg	100 mg	-----
F	Placebo film combined with HVF	Not performed	-----	-----	-----	-----
G	Placebo film control	-----	-----	100 mg	-----	100 mg
A_F	Blank HVF frozen	100 mg	-----	100 mg	-----	-----
B_F	RC-101 solution with frozen HVF	100 mg	100 mg	-----	-----	-----
A_{BV+}	Blank HVF BV positive	100 mg	-----	100 mg	-----	-----
B_{BV+}	RC-101 solution with HVF BV^+	100 mg	100 mg	-----	-----	-----

Amounts correspond to one time point.

***RC-101 film and placebo film were initially dissolved in 1 mL of water prior to addition to HVF. Amounts in the films columns correspond to the solution of the film in 1 mL of water.**

Sample processing

At each time point, the sample was removed from the incubation chamber and centrifuged for 10 min at 10,000 rpm, at 4 °C to separate the supernatant from cell pellet. Supernatant (100 µL) was added to microcentrifuge filters Ultracel YM-10 Microcon MWCO 10,000 (Millipore Corporation, Bedford, MA), which were pre-washed with Milli Q water to eliminate any trace of propylene glycol from the filters. Samples were centrifuged twice for 15 min at 8,500 rpm, at 4 °C. The filtrate was collected and frozen at –80 °C until further analysis. A solution of 3 M urea was added to the cell pellet (1:1 w/w) obtained from the first centrifugation, to lyse the cells. This mixture was vortexed three times for 30 sec, and then centrifuged for 10 min at 10,000 rpm, at 4 °C. The supernatant from the cell lysate was then added to microcentrifuge filters Ultracel YM-10 Microcon MWCO 10,000 pre-washed with Milli Q water. Samples were centrifuged twice for 15 min at 8,500 rpm, at 4 °C. The filtrate was collected and frozen at –80 °C until analysis. The peptide RC-101 has been shown to be stable in 3 M urea for at least 24 h.

Samples were thawed and added to PepClean^{TM} C-18 spin columns (Pierce Biotechnology Inc., Rockford, IL) for desalting, after column conditioning with acetonitrile:water (50:50) and equilibration with 0.1% trifluoroacetic acid. The column was washed three times with 0.1% trifluoroacetic acid, and RC-101 was eluted with acetonitrile:water (60:40) in 0.1% trifluoroacetic acid. Samples were dried in a speed vacuum CentriVap concentrator (LabConco Corp., Kansas City, MO) and resuspended with 200 µL of Milli Q water for LC-MS/MS analysis. Each sample described in Table 4-1 originated two sets of samples: one labeled as supernatant and the second one labeled as cells. LC-MS/MS chromatogram in the results (Section 4.3) show separated chromatograms for supernatant and cells.

4.2.4 Nanoflow LC Selected Reaction Monitoring Mass Spectrometry

Integrated electrospray ionization (ESI)-capillary reversed-phase columns (75 μm inner diameter x 360 μm outer diameter x 100 mm length) packed with 5 μm 300 Å pore size Jupiter C18 reversed-phase stationary phase (Phenomenex, Torrance, CA) were prepared, as previously described (164). Solvent flow was supplied by a nanoflow HPLC system (Ultimate 3000, Dionex Corporation, Sunnyvale, CA). Each sample (3 μL) was loaded onto the column through a 5 μL loop at a flow rate of 0.5 μL/min in 98:2 mobile phase A (0.1% formic acid in water, v/v) and mobile phase B (0.1% formic acid in acetonitrile, v/v) for 30 min. The step-wise linear gradient was delivered at 250 nL/min as follows: 2 to 40% mobile phase B over 40 min, followed by 40 to 98% mobile phase B over 30 min. High voltage contact for ESI was provided through a metal union connecting the microcapillary column to the pump. The RC-101 peptide abundance was measured by selected reaction monitoring (SRM) using a triple quadrupole MS (TSQ Quantum Ultra, Thermo Fisher Scientific Inc., San Jose, CA). While operating in SRM mode, Q1 and Q3 resolutions were set to 0.7 atomic mass unit (amu), and the collision induced dissociation (CID) gas pressure was 1.5 mTorr with a collision energy (CE) of 18 volts. Each SRM scan width was set to 0.002 *m/z* units and the scan rate was 0.020 sec. Data were analyzed by construction of mass chromatograms for each SRM transition separately, and peak areas were manually tabulated.

4.3 RESULTS

Demographics of the volunteers enrolled in the study

To this date, 117 volunteers have been screened over the phone (for phone screening script see Appendix B.1), and 83 volunteers have signed the consent form and were enrolled in the study. However, 4 participants withdrew from the study prior to sample collection. Information collected from these 4 volunteers was not used in the study.

A total of 79 human vaginal fluid collections were made from premenopausal women. Since the main objective of this study was to evaluate the stability of RC-101 in the presence of HVF, some of the volunteers gave more than one sample in different days, but always one sample only per day. All volunteers reported to be not pregnant at the date of the fluid collection, and this information was confirmed by urine pregnancy test. None of the participants were using a vaginal ring or Intra Uterine Device (IUD) as contraceptive. None of the volunteers tested positive for either *C. trachomatis* or *N. gonorrhoeae*. The mean age of the volunteers that completed the study was 31 ± 8 years; the age distribution is displayed in Figure 4-1. The average pH (n = 62) was 4.5 ± 0.6, the pH distribution is shown in Figure 4-2. The majority of the volunteers were Caucasian; and the race distribution is shown in Figure 4-3. A large percentage of the volunteers (49.4%) did not use any type of contraceptive (Figure 4-4); and when used, condoms were the most often contraceptive method used. The day of the menstrual cycle on which the samples were collected, was calculated based on the information reported in the questionnaire by the volunteer about the first day of their last menstrual period (Figure 4-5). The bacterial vaginosis (BV) score for each individual is presented in Figure 4-6. All other information obtained from the questionnaire was complied and is presented in Table 4-2.

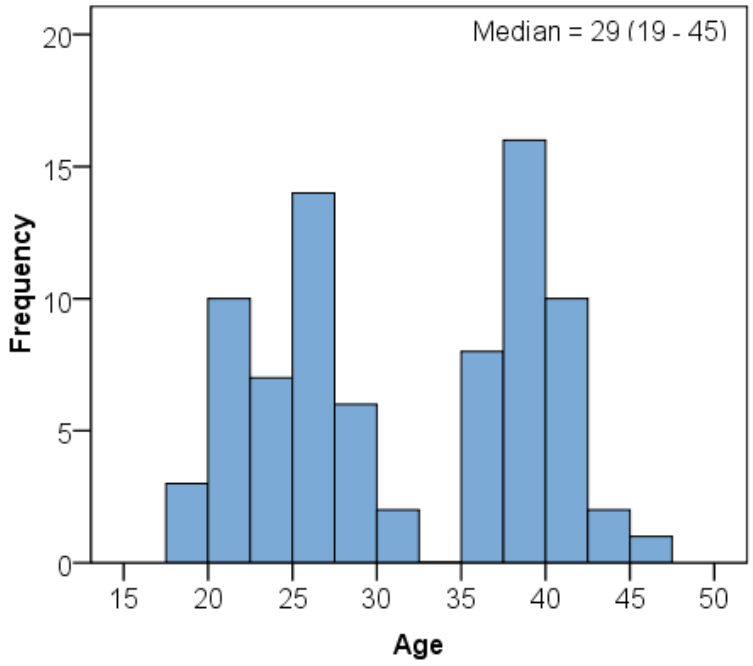

Figure 4-1. Age distribution frequency of volunteers enrolled in the study. based on the answers provided in the questionnaire.

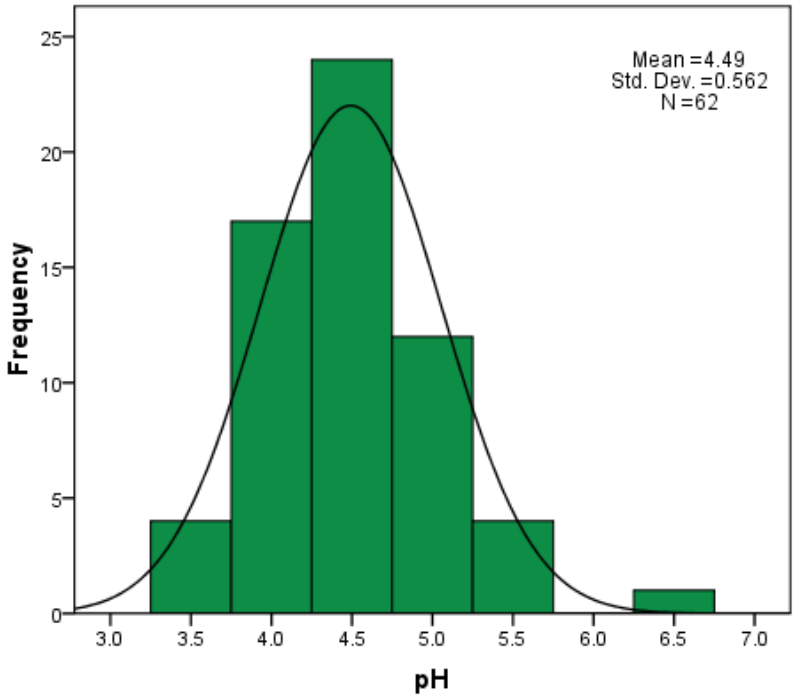

Figure 4-2. Distribution frequency of individual pH values determined for each fluid sample collected for the studies.

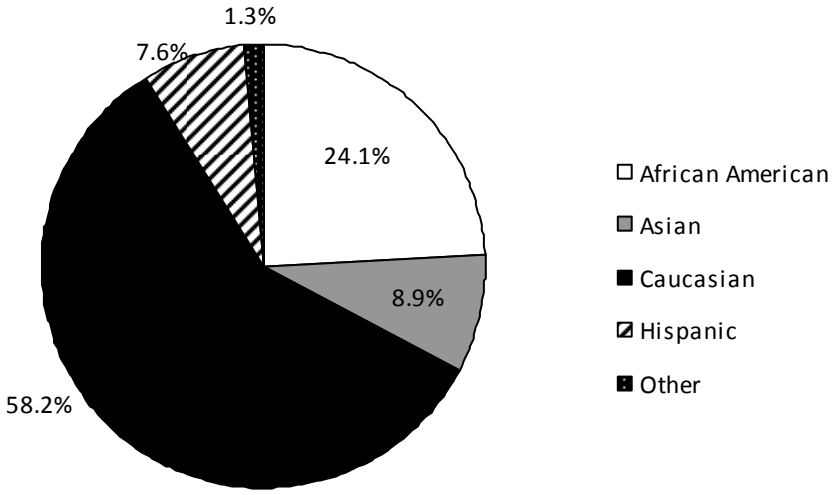

Figure 4-3. Distribution (%) of the volunteers by race.
Based on the answers provided in the questionnaire.

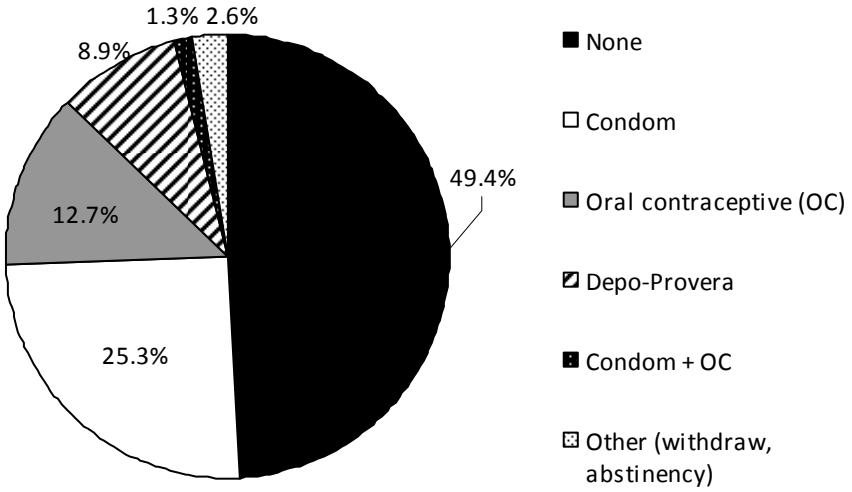

Figure 4-4. Distribution (%) of the volunteers by contraceptive method used.
Based on the answers provided in the questionnaire.

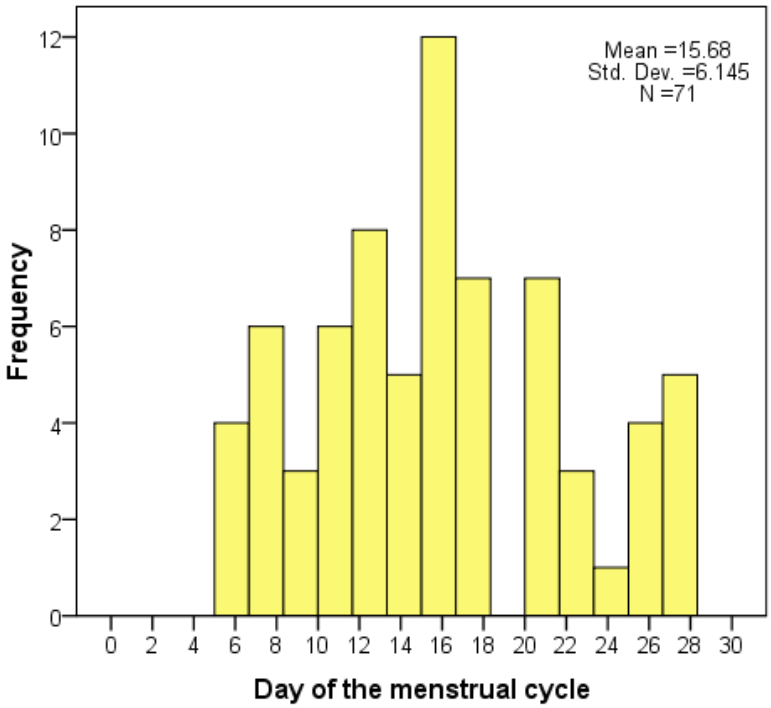

Figure 4-5. Day of the menstrual cycle at the collection day.

Calculated for each volunteer based on the answers provided in the questionnaire.

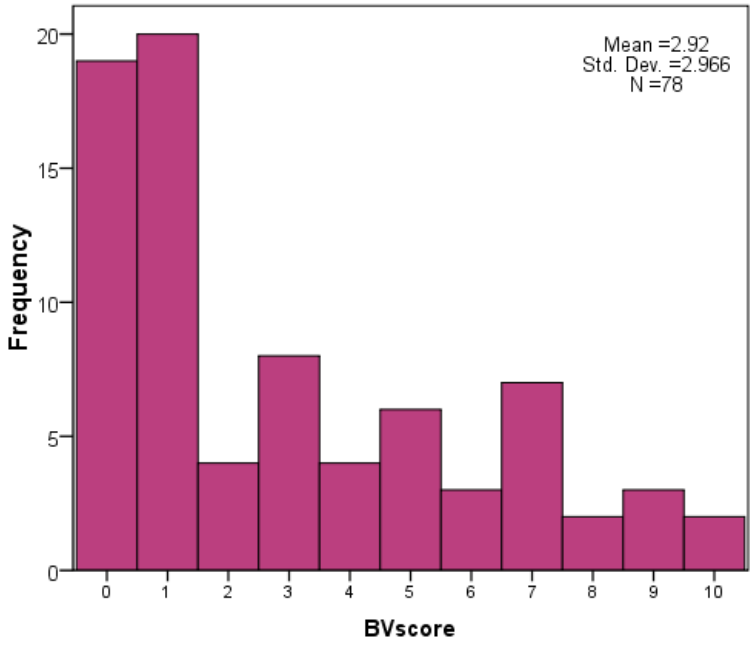

Figure 4-6. Distribution of Bacterial Vaginosis (BV) Nugent score.

Determined by Gram stain testing for each individual sample collected.

Table 4-2. Compiled data from answers provided in the questionnaire for each volunteer.

Characteristic	No (%)
Last alcohol consumption	
Within 2 days	10 (12.7)
Between 2 and 7 days	25 (31.6)
More than 7 days prior	37 (46.8)
Never	4 (5.1)
Did not answer the question	3 (3.8)
Smoking status	
Never	39 (49.4)
Smoking	15 (19.0)
Quit less than 1 year ago	2 (2.5)
Quit more than 1 year ago	23 (29.1)
Sexually active	
Yes	61 (77.2)
No	18 (22.8)
Last sexual intercourse	
Between 2 and 5 days prior	23 (29.1)
6 or more days prior	40 (50.6)
Not sexually active or did not answer the question	16 (20.3)
Age of the first menstrual period	
8 – 10	10 (12.7)
11 – 13	53 (67.1)
14 – 16	16 (20.2)
Duration of the period	
1 – 3	18 (22.8)
4 – 5	49 (62.0)
6 – 7	9 (11.4)
Continuous pill or Depo-Provera	3 (3.8)
Duration between periods	
15 – 20	5 (6.3)
21 – 26	12 (15.2)
27 – 32	56 (70.9)
33 – 35	1 (1.3)
Continuous pill or Depo-Provera	5 (6.3)

Characteristic (Continuation)	No (%)
Currently using vaginal products	
Yes (more than 2 days prior)	3 (3.8)
No	76 (96.2)
Gynecological problems	
None	73 (93.6)
Ovarian cysts or polycystic ovary	4 (5.1)
Uterine Fibroid	1 (1.3)
Did not answer the question	1 (1.3)
Gynecological procedures	
None	72 (91.1)
Removed one ovary	2 (2.6)
Tubal ligation	2 (2.6)
Did not answer the question	3 (3.8)
Previous pregnancies	
Never	50 (63.3)
Once	6 (7.6)
Twice	16 (20.3)
3 or more	7 (8.9)
Medical conditions	
No	61 (77.2)
Yes*	18 (22.8)
Current medications	
None	42 (53.2)
OTC**	27 (34.2)
Prescription drugs***	16 (20.2)

***Medical conditions reported: anemia, anxiety, bipolar-disorder, blood clot, depression, high cholesterol, hypertension, and pain.**

****OTC drugs reported: acetaminophen, allergy medicine, fish oil, ibuprofen, multivitamin and mineral supplements, naproxen sodium, and nasal spray.**

*****Prescription drugs reported: amlodipine, atenolol, atorvastatin, almotriptan malate, bupropion, escitalopram, metformin, sertraline. simvastatin, trazodone, varenicline (Chantix), and warfarin.**

Initial samples collected from healthy volunteers were used for method development. Several challenges were encountered during analytical method development. The presence of interfering peaks from the fluid eluting at the same time in the HPLC with the RC-101 peak made it impossible for analysis using HPLC assay. Several approaches for sample purification prior to HPLC analysis were evaluated using different conditions for solid phase extraction, ion exchange microcentrifuge columns, and molecular weigh microcentrifuge filters (data not shown). After development of the method for LC-MS/MS, samples from volunteers were pooled on the day of collection generating 3 pools (Pool 1, 2 and 3) for normal HVF, and one pool (BV pool) for HVF positive for BV. Samples were used as detailed in Table 4-1. All the other data obtained from the questionnaire was compiled for each pool and is presented in Table 4-3.

Table 4-3. Compiled data from answers provided in the questionnaire for each volunteer enrolled in the study, per sample pool.

Characteristic	Pool 1	Pool 2	Pool 3	BV Pool
Age				
Mean ± SD	28.7 ± 6.7	30.6 ± 8.9	33.2 ± 7.8	35.2 ± 5.2
pH	4.1 ± 0.3	4.3 ± 0.3	4.1 ± 0.4	5.8 ± 0.6
BV score				
Between 0 and 3	3 (75.0)	5 (71.4)	3 (50.0)	0
Between 4 and 6	1 (25.0)	2 (28.6)	1 (16.7)	1 (25.0)
Between 7 and 10	0	0	2 (33.3)	3 (75.0)
Race				
African American	0	1 (14.3)	0	4 (100.0)
Asian	0	1 (14.3)	1 (16.7)	0
Caucasian	4 (100.0)	4 (57.1)	5 (83.3)	0
Hispanic	0	1 (14.3)	0	0
Other	0	0	0	0

Characteristic (continuation)	Pool 1	Pool 2	Pool 3	BV Pool
Last alcohol consumption				
Within 2 days	1 (25.0)	1 (14.3)	0	0
Between 2 and 7 days	1 (25.0)	3 (42.9)	0	1 (25.0)
More than 7 days prior	2 (50.0)	2 (28.5)	5 (83.3)	2 (50.0)
Never	0	1 (14.3)	1 (16.7)	1 (25.0)
Smoking status				
Never	0	4 (57.1)	3 (50.0)	3 (75.0)
Smoking	1 (25.0)	0	0	1 (25.0)
Quit less than 1 year ago	1 (25.0)	0	0	0
Quit more than 1 year ago	2 (50.0)	3 (42.9)	3 (50.0)	0
Sexually active				
Yes	4 (100.0)	4 (57.1)	5 (83.3)	4 (100.0)
No	0	3 (42.9)	1 (16.7)	0
Last sexual intercourse				
Between 2 and 5 days prior	1 (25.0)	1 (14.2)	2 (33.3)	2 (50.0)
6 or more days prior	3 (75.0)	3 (42.8)	3 (50.0)	2 (50.0)
Not sexually active	0	3 (42.8)	1 (16.7)	0
Age of the first menstrual period				
8 – 10	1 (25.0)	1 (14.3)	1 (16.7)	1 (25.0)
11 – 13	3 (75.0)	5 (71.4)	5 (83.3)	2 (50.0)
14 – 16	0	1 (14.3)	0	1 (25.0)
Duration of the period				
1 – 3	0	2 (28.6)	1 (16.7)	0
4 – 5	3 (75.0)	4 (57.1)	4 (66.7)	3 (75.0)
6 – 7	1 (25.0)	1 (14.3)	1 (16.6)	0
Continuous pill or Depo-Provera	0	0	0	1 (25.0)
Duration between periods				
15 – 20	0	2 (28.6)	1 (16.6)	1 (25.0)
21 – 26	0	0	0	0
27 – 32	4 (100.0)	4 (57.1)	4 (66.7)	2 (50.0)
33 – 35	0	0	1 (16.7)	0
Continuous pill or Depo-Provera	0	1 (14.3)	0	1 (25.0)

Characteristic (continuation)	Pool 1	Pool 2	Pool 3	BV Pool
Currently using vaginal products				
Yes (more than 2 days prior)	0	0	0	0
No	4 (100.0)	7 (100.0)	6 (100.0)	4 (100.0)
Gynecological problems				
None	4 (100.0)	7 (100.0)	5 (83.3)	4 (100.0)
Ovarian cysts or polycystic ovary	0	0	1 (16.7)	0
Gynecological procedures				
None	4 (100.0)	6 (85.7)	5 (83.3)	2 (50.0)
Tubal ligation	0	0	0	2 (50.0)
Did not answer the question	0	1 (14.3)	1 (16.7)	0
Previous pregnancies				
Never	3 (75.0)	6 (85.7)	4 (66.7)	0
Once	0	0	0	1 (25.0)
Twice	1 (25.0)	1 (14.3)	2 (33.3)	1 (25.0)
Thrice	0	0	0	2 (50.0)
Medical conditions				
No	4 (100.0)	7 (100.0)	6 (100.0)	3 (75.0)
Yes*	0	0	0	1 (25.0)
Current medications				
None	2 (50.0)	4 (57.1)	4 (66.7)	4 (100.0)
OTC**	2 (50.0)	2 (28.6)	1 (16.7)	0
Prescription drugs***	0	1 (14.3)	1 (16.7)	0

*** Medical condition reported: hypertension**

****OTC drugs reported: acetaminophen, allergy medicine, fish oil, ibuprofen, multivitamin and mineral supplements, naproxen sodium, and nasal spray.**

*****Prescription drugs reported: amlodipine, atenolol, atorvastatin, almotriptan malate, bupropion, escitalopram, metformin, sertraline. simvastatin, trazodone, varenicline (Chantix), and warfarin.**

The distribution of the day of the menstrual cycle for pools 1, 2, 3, and BV pool are shown in Figure 4-7, Figure 4-8, Figure 4-9, and Figure 4-10, respectively. Pool 1 and 3 showed that the majority of women were in the secretory phase of their menstrual cycle, while pool 2 and BV pool were mostly in the proliferative phase.

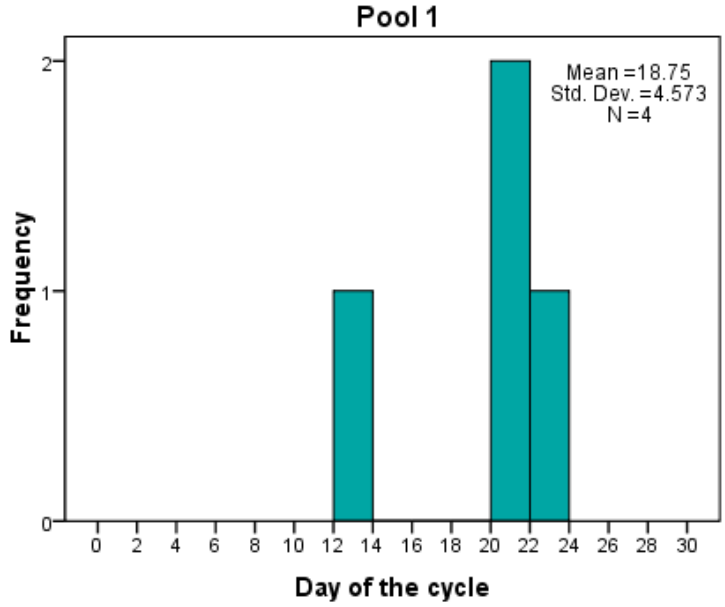

Figure 4-7. Distribution frequency (%) of the day of the menstrual cycle for Pool 1.

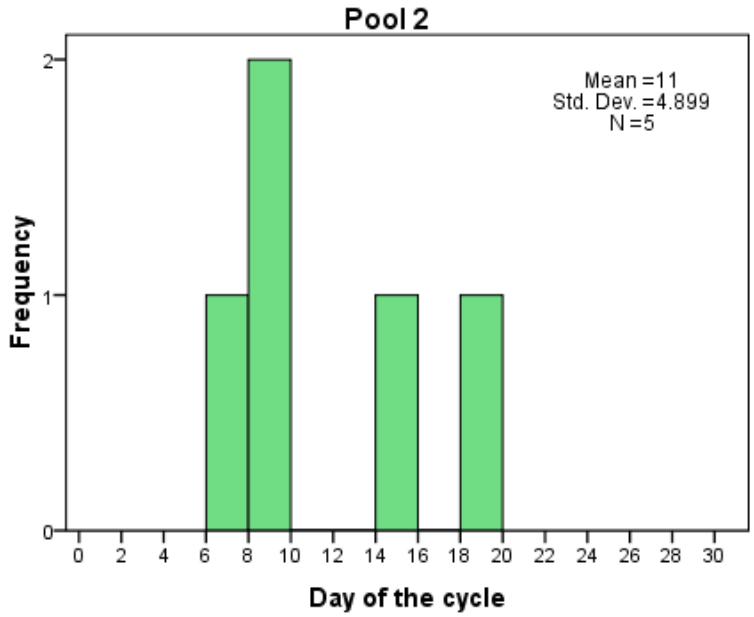

Figure 4-8. Distribution frequency (%) of the day of the menstrual cycle for Pool 2.

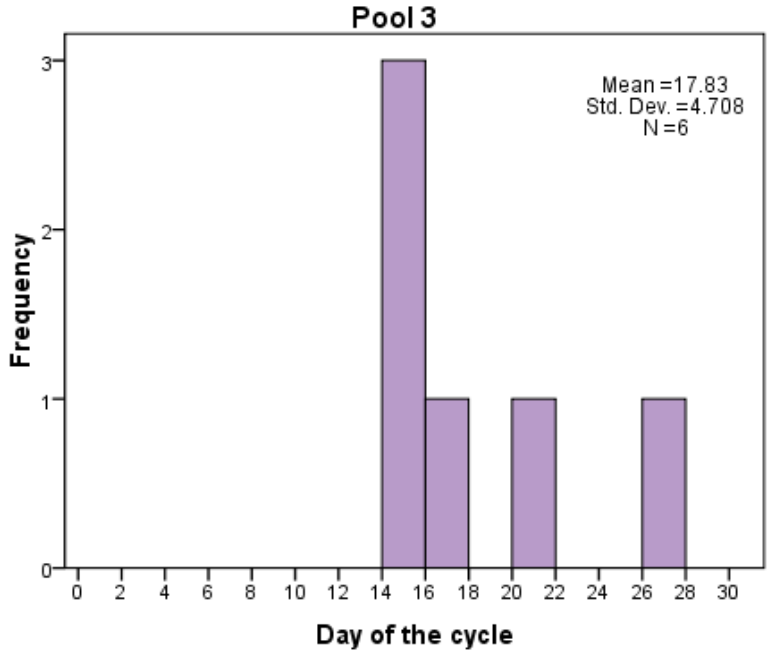

Figure 4-9. Distribution frequency (%) of the day of the menstrual cycle for Pool 3.

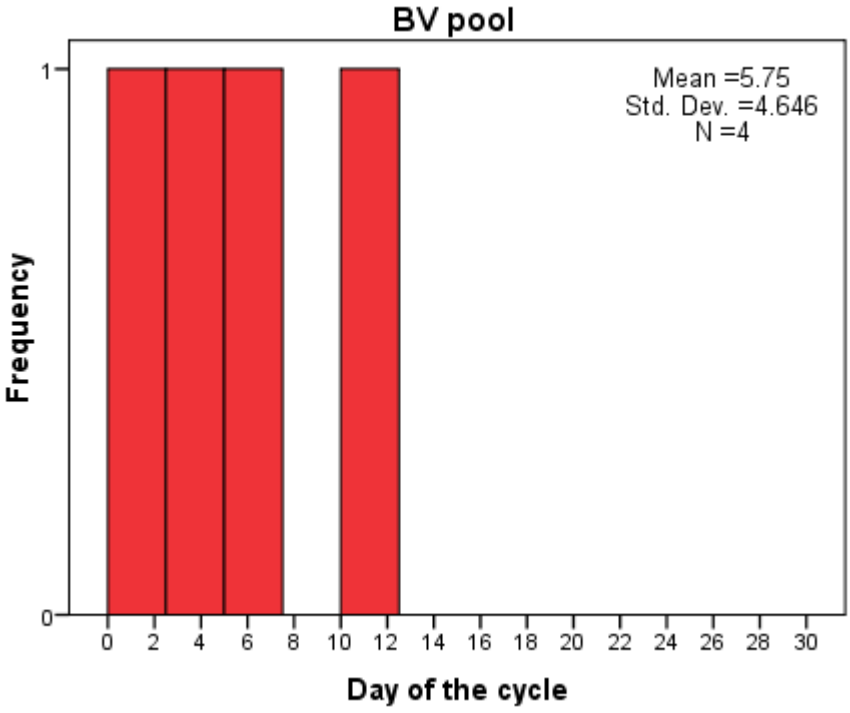

Figure 4-10. Distribution frequency (%) of the day of the menstrual cycle for BV pool.

Nanoflow Liquid Chromatography Selected Reaction Monitoring (SRM) Mass Spectrometry

RC-101 peptide abundance was measured by selected reaction monitoring (SRM). Initially, confirmation of the peptide detection was obtained on a high resolution Orbitrap mass spectrometer (Thermo Scientific). The initial base peak chromatogram with a representative mass spectrum of the $[M + 4H]^{4+}$ RC-101 molecular ion is shown in Figure 4-11.

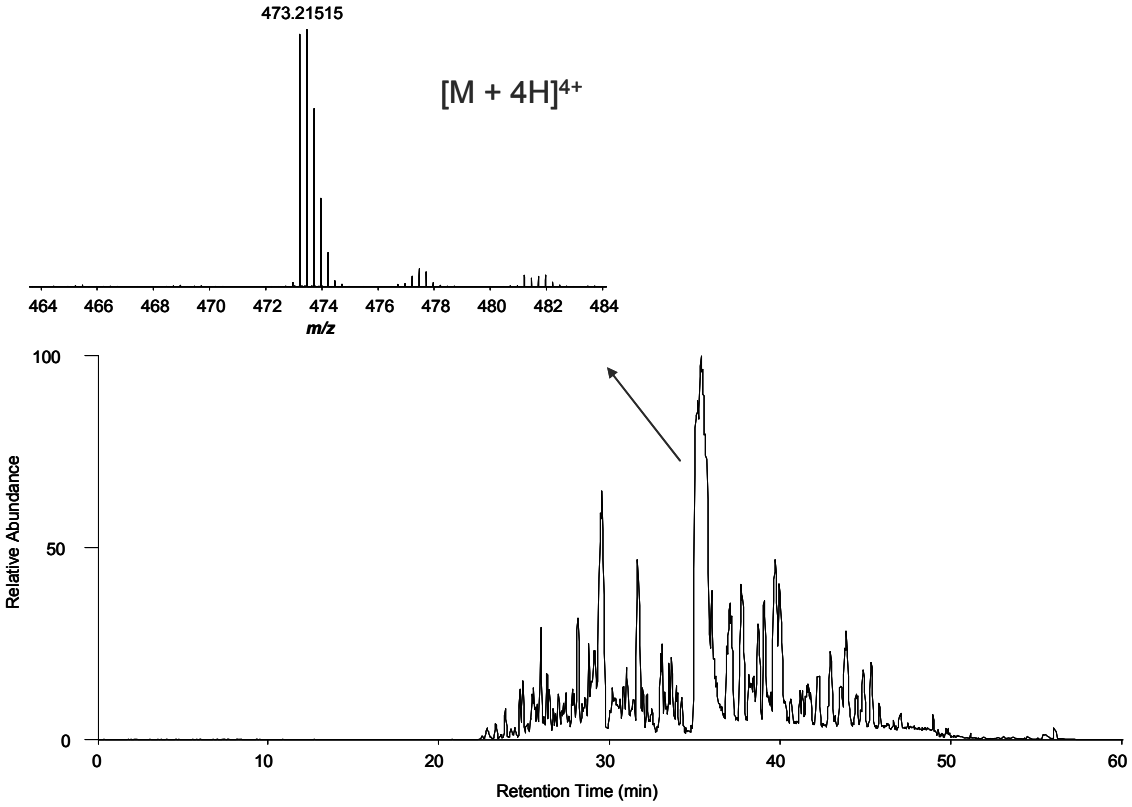

Figure 4-11. LC-MS/MS of RC-101 (1 µg/mL) solution in water.

After the incubation period of RC-101 combined with HVF, each sample (described in Table 4-1) was removed from the incubation chamber and processed for LC-MS/MS analysis as described in Section 4.2.4. For each condition analyzed, supernatant and cells, the LC-MS/MS chromatogram was obtained. Representative LC-MS/MS chromatograms at time 0 are shown

for Sample A supernatant (blank HVF), Sample B supernatant (RC-101 solution + HVF), Sample C supernatant (RC-101 solution control), and Sample D (RC-101 film + HVF) in Figure 4-12, Figure 4-13, Figure 4-14, and Figure 4-15, respectively. RC-101 was detected at a retention time of 20.8 to 22.8 min. Variation in retention time is attributed to the use of a capillary column and pre-column. Sample A (HVF control) showed the presence of several peaks; however, no interference peaks were detected, indicating that the method was suitable for detection of RC-101. For all other chromatograms, the *m/z* was confirmed for RC-101 detection. Representative LC-MS/MS chromatograms at time 0 are shown for Sample E supernatant (RC-101 film control), and Sample G (Placebo film control) in Figure 4-16 and Figure 4-17, respectively. No interference peaks were detected in the placebo film.

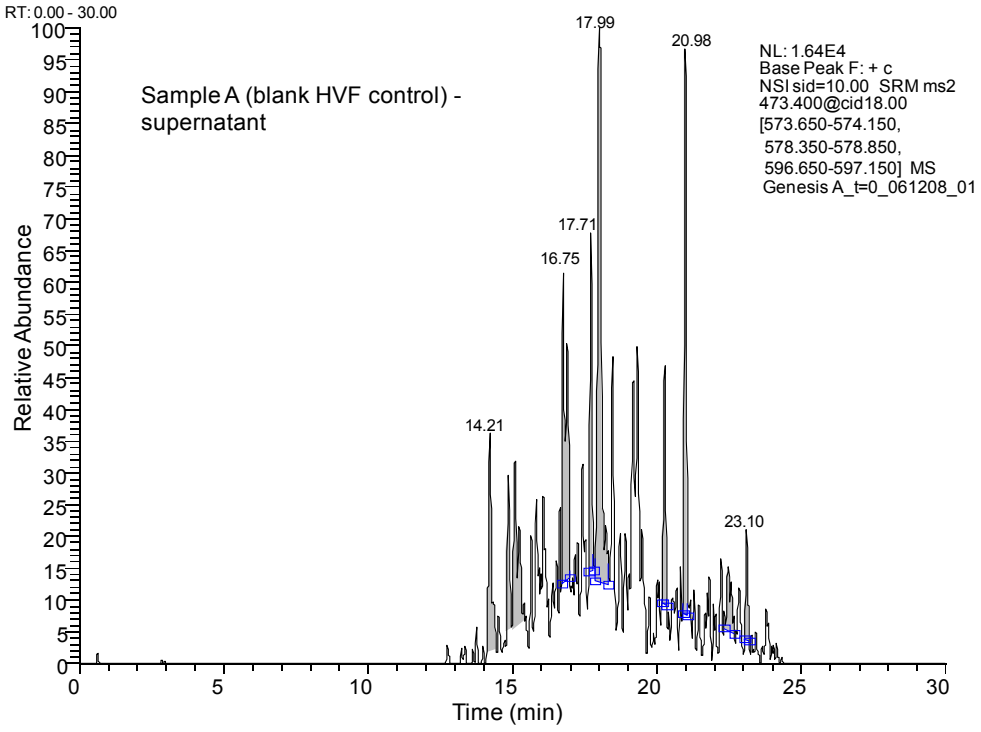

Figure 4-12. Sample A (blank HVF control) representative LC-MS/MS chromatogram of supernatant solution, at time 0.

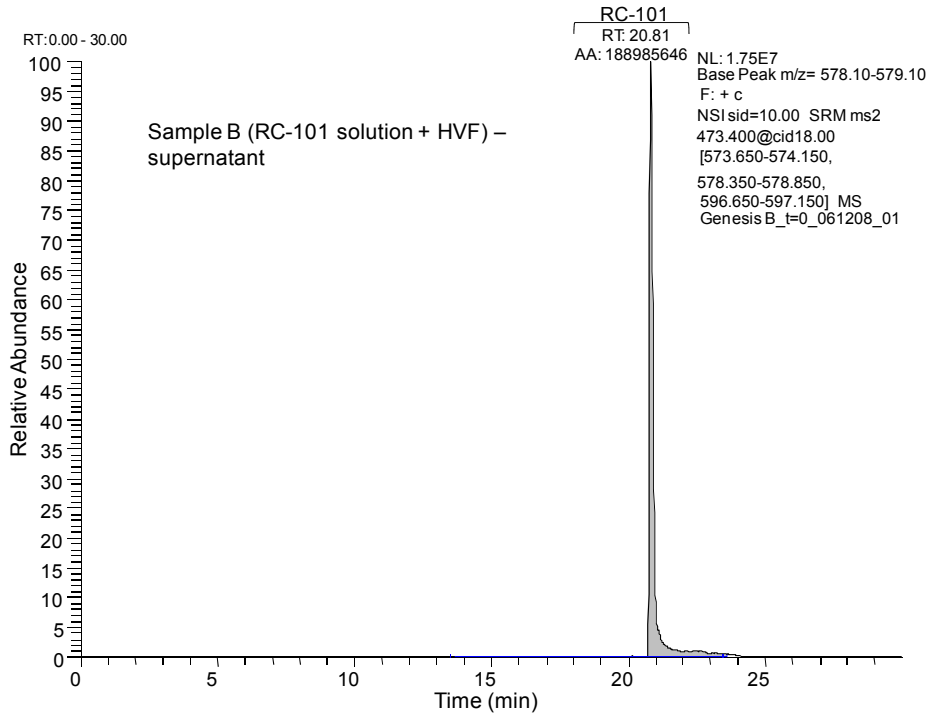

Figure 4-13. Sample B (RC-101 solution + HVF) representative LC-MS/MS chromatogram of supernatant solution at time 0.

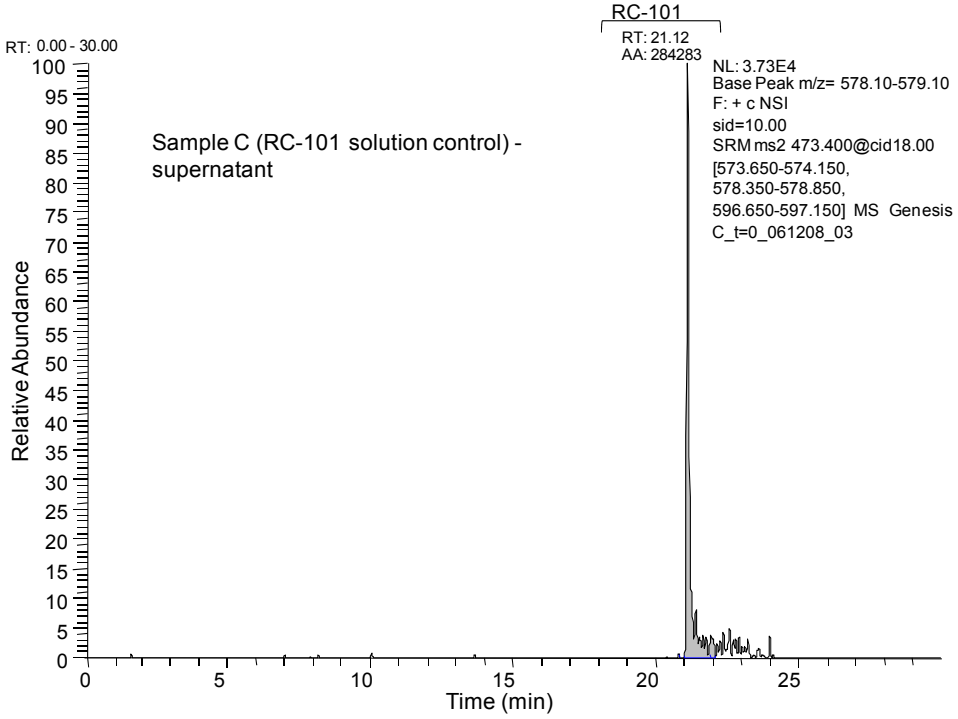

Figure 4-14. Sample C (RC-101 control) representative LC-MS/MS chromatogram of solution at time 0.

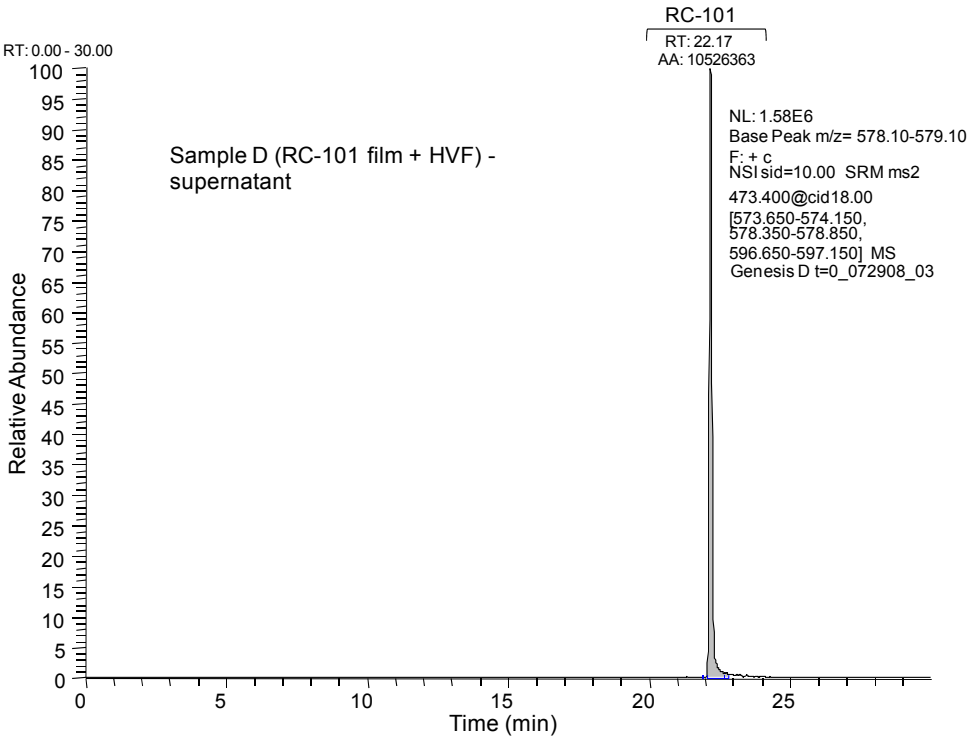

Figure 4-15. Sample D (RC-101 film + HVF) representative LC-MS/MS chromatogram of supernatant solution at time 0.

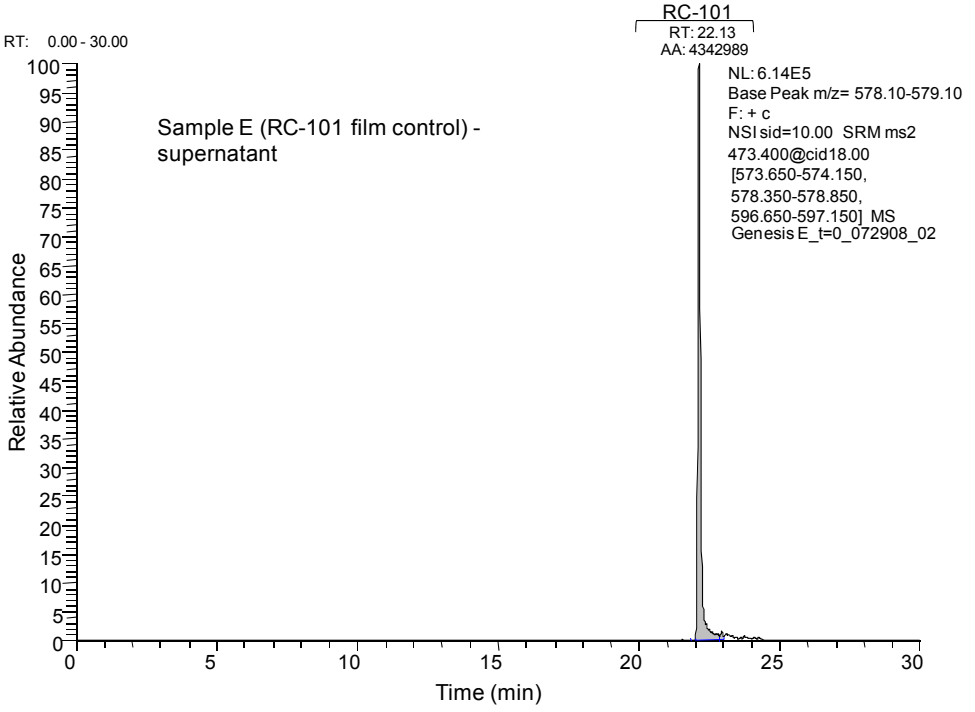

Figure 4-16. Sample E (RC-101 film control) representative LC-MS/MS chromatogram of solution at time0.

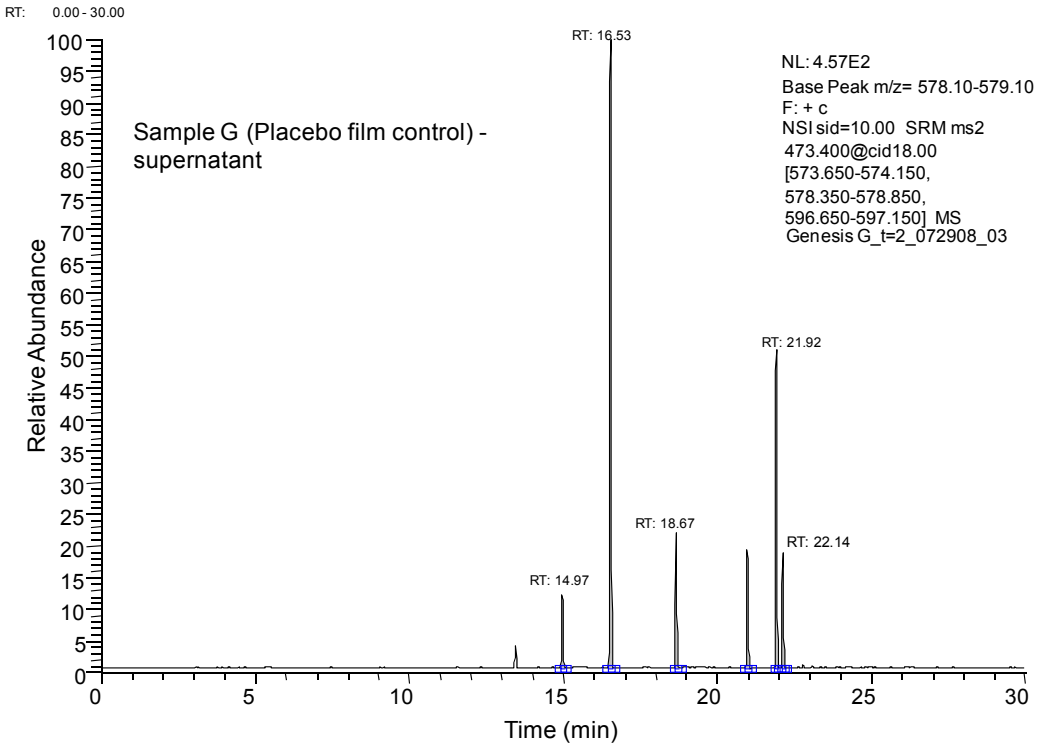

Figure 4-17. Sample G (Placebo film control) representative LC-MS/MS chromatogram of solution at time 0.

Representative chromatograms of solutions obtained from cell lysate for the initial time point (time 0) as well as the chromatograms at the end point of the experiment for all conditions tested are shown in Appendix C. The area from the RC-101 peak was obtained for each time point and plotted in Figure 4-18 for the Sample B (RC-101 + HVF). The area represents the total area of the peak for RC-101 obtained from the supernatant and cells samples. Each pool represents the fluid from one day of collection. Since the LC-MS/MS method developed is not a quantitative method, the amount of RC-101 was not obtained. Overall, RC-101 was detected for 48 h in two pools tested and up to 72 h in another pool tested. To better illustrate the detection of RC-101 over time, representative LC-MS/MS chromatograms for Sample B supernatant solution are shown in Figure 4-19, Figure 4-20, and Figure 4-21, for 24, 48, and 72 h after exposure to HVF at 37 °C.

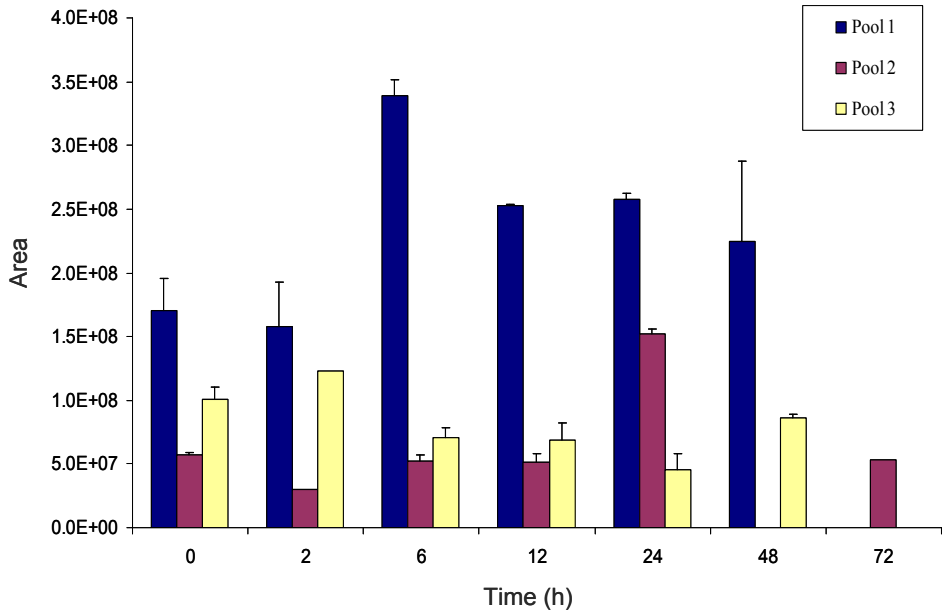

Figure 4-18. Area of RC-101 (supernatant + cells) obtained from Samples B. (RC-101 solution + HVF) for each pooled samples, at each time point. Average area of three injections + S.D.

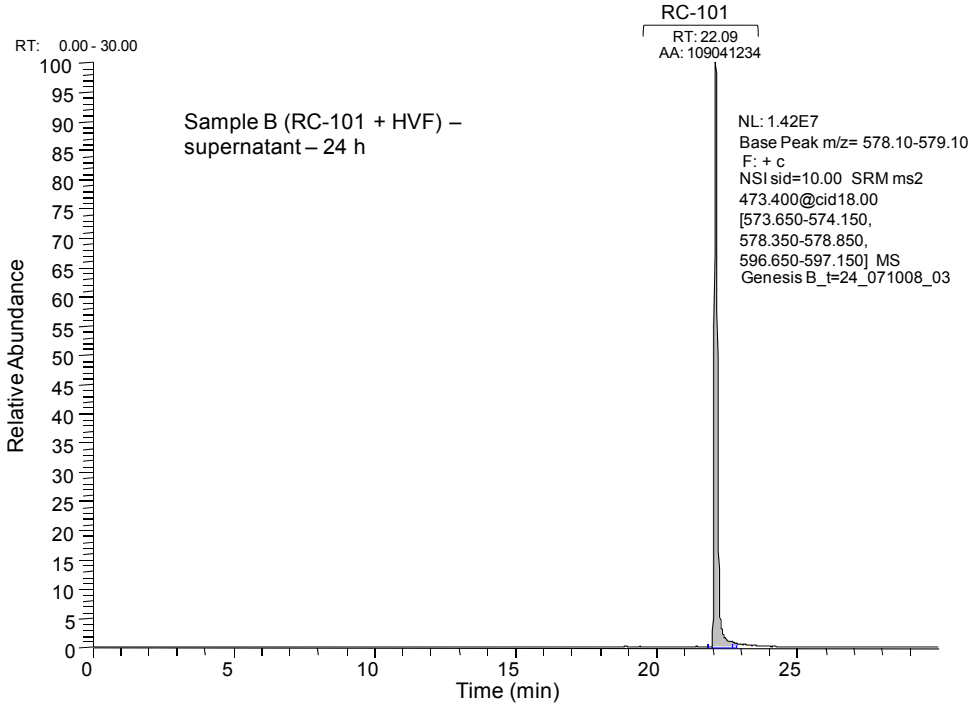

Figure 4-19. Sample B (RC-101 + HVF) representative LC-MS/MS chromatogram of supernatant solution after 24 h incubation.

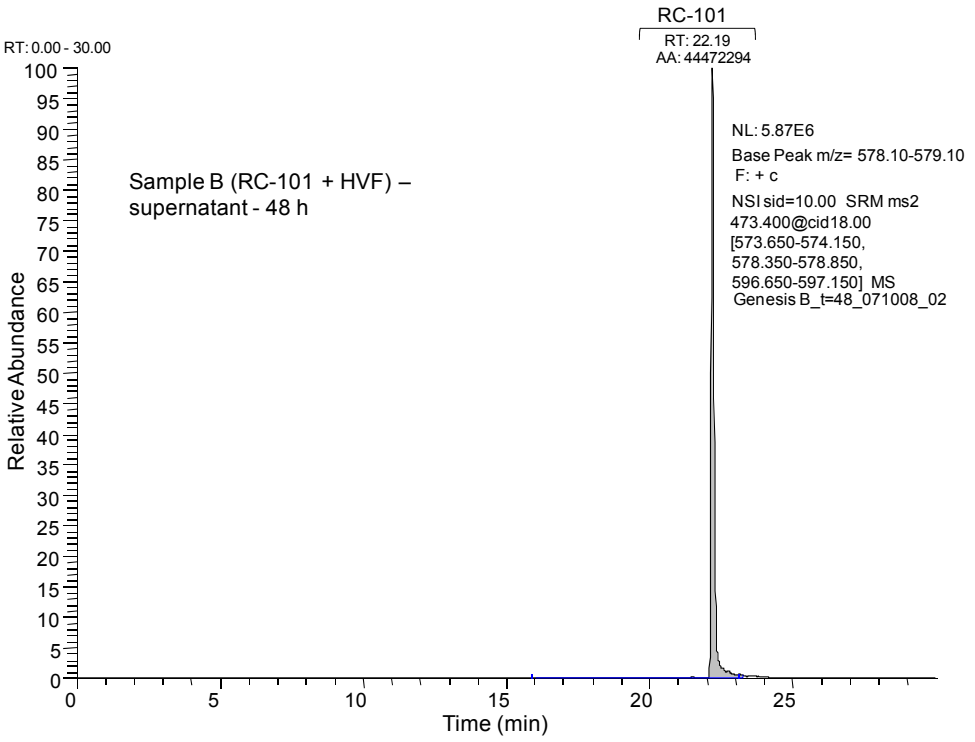

Figure 4-20. Sample B (RC-101 + HVF) representative LC-MS/MS chromatogram of supernatant solution after 48 h incubation.

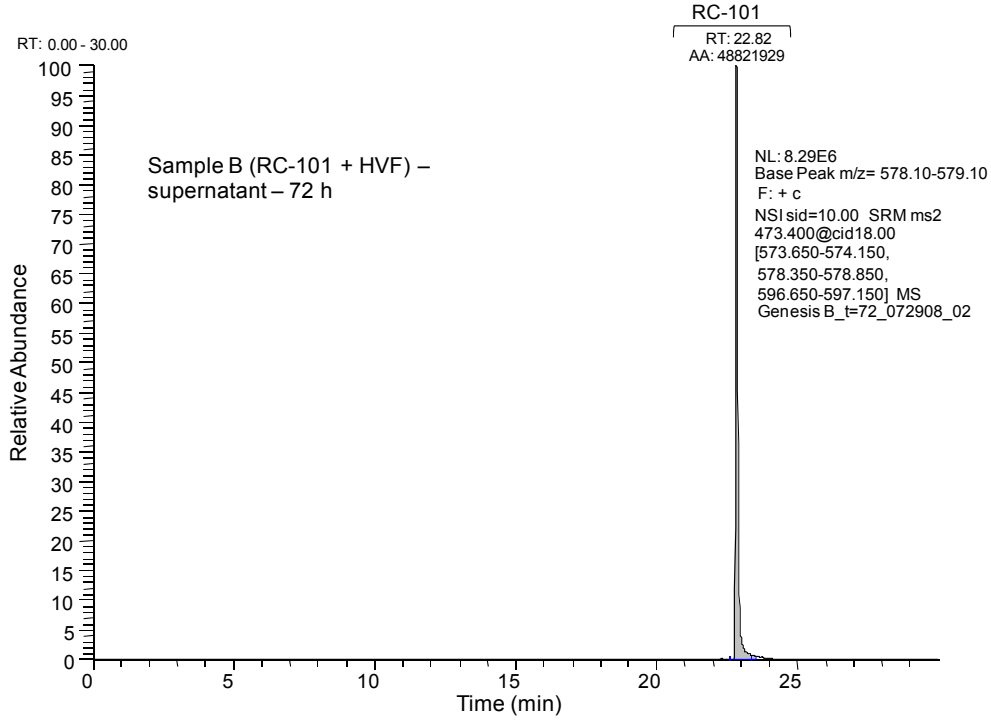

Figure 4-21. Sample B (RC-101 + HVF) representative LC-MS/MS chromatogram of supernatant solution after 72 h incubation.

Detection of RC-101 was also observed when the RC-101 film was incubated over time with HVF. The area from the RC-101 peak was obtained for each time point and plotted in Figure 4-22 for the Sample D (RC-101 film + HVF). The area represents the total area of the peak for RC-101 obtained from the supernatant and cells samples. Each pool represents the fluid from one day of collection. As previously mentioned, LC-MS/MS method developed is not a quantitative method, so the amount of RC-101 was not obtained. Overall, RC-101 from a film formulation was detected for 48 h in one pool tested and up to 72 h in another pool tested. To better illustrate the detection of RC-101 over time, representative LC-MS/MS chromatograms for Sample D supernatant solution are shown in Figure 4-23, Figure 4-24, and Figure 4-25, for 24, 48, and 72 h after exposure to HVF at 37 °C.

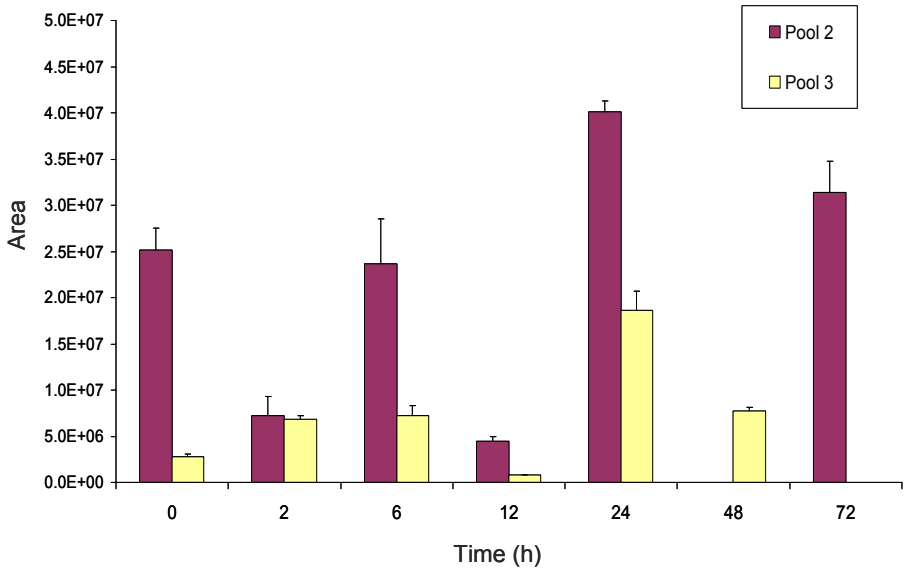

Figure 4-22. Area of RC-101 (supernatant + cells) obtained from Samples D. (RC-101 film + HVF) for each pooled samples, at each time point. Average area of three injections + S.D.

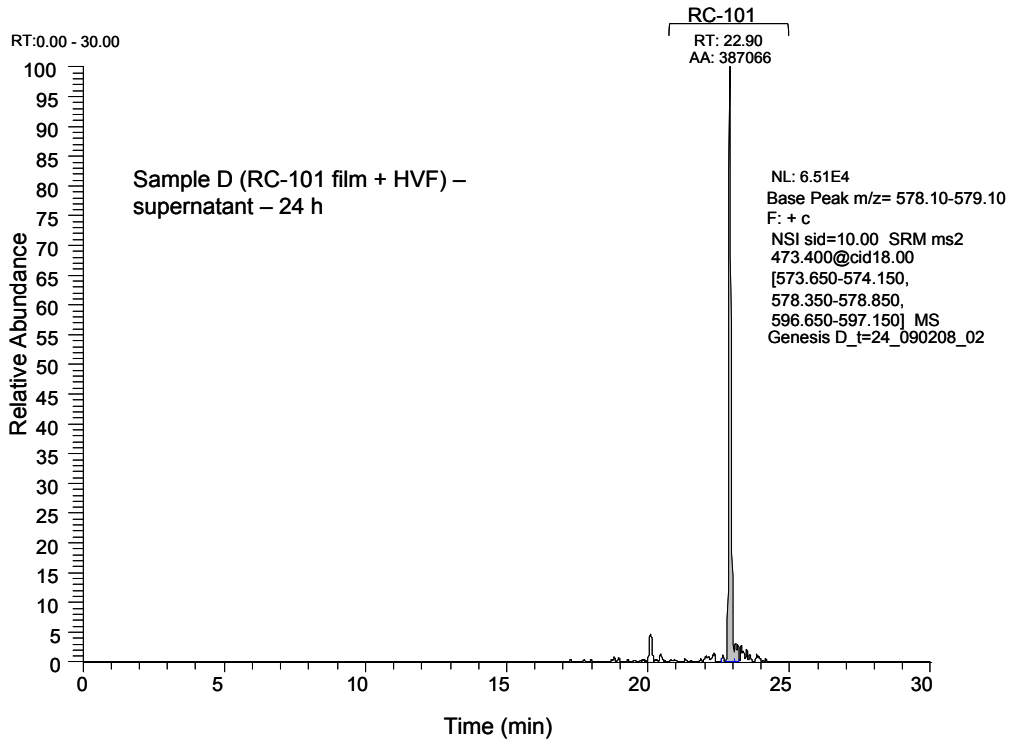

Figure 4-23. Sample D (RC-101 film + HVF) representative LC-MS/MS chromatogram of supernatant solution after 24 h incubation.

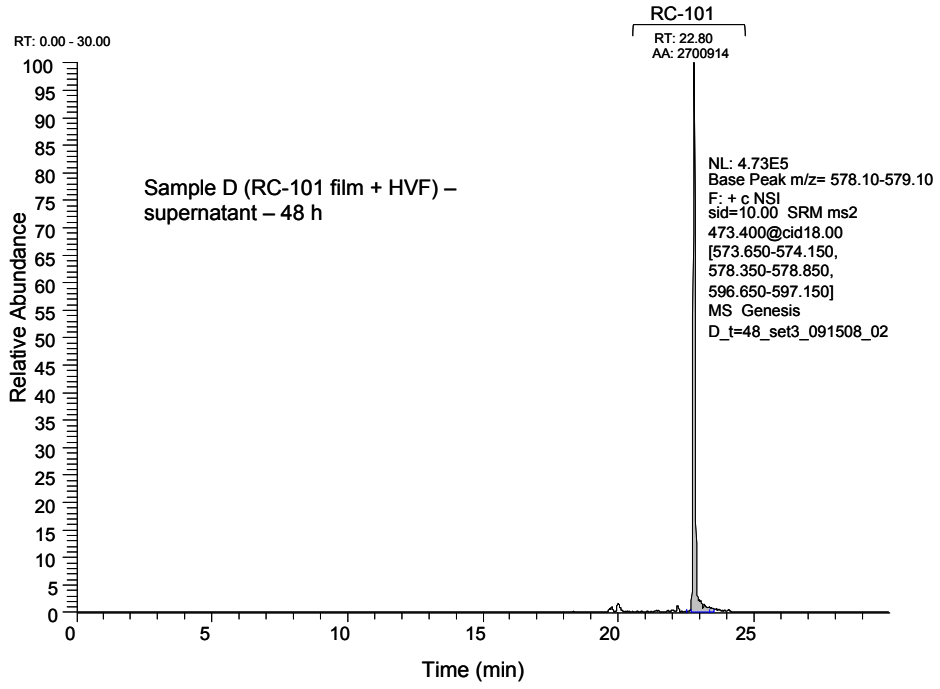

Figure 4-24. Sample D (RC-101 film + HVF) representative LC-MS/MS chromatogram of supernatant solution after 48 h incubation.

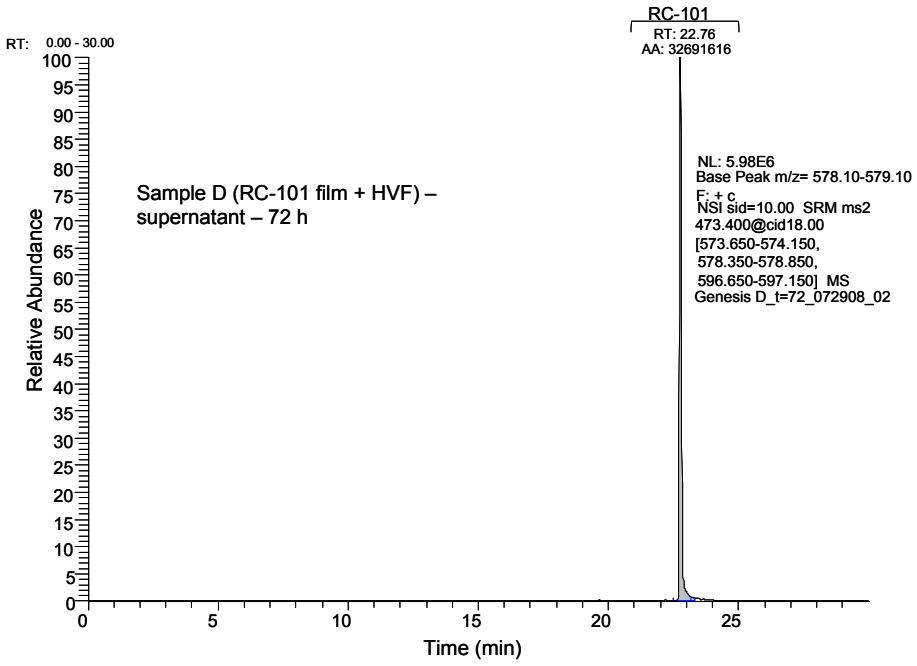

Figure 4-25. Sample D (RC-101 film + HVF) representative LC-MS/MS chromatogram of supernatant solution after 72 h incubation.

The incubation period of two pooled samples (Pool 1 and Pool 3) differ from Pool 2. The total incubation period of Pool 2 was 72 h, instead of 48 h as conducted for Pools 1 and 3, due to scheduling conflicts with volunteers and sample analysis. Detection of RC-101 by LC-MS/MS was observed in duplicated pools for up to 48 h and in one pool for 72 h, indicating the stability of the peptide for at least 48 h. Formulation of RC-101 into the film still maintained the stability of RC-101 over the same time period. Overall, RC-101 was detected after exposure to HVF at least for 48 h, and no difference was observed for RC-101 in a solution or a film formulation.

To verify if the freezing process would interfere with the stability of RC-101 in the fluid, frozen HVF was used for incubation with RC-101 solution (Sample B_F). Representative LC-MS/MS chromatograms are shown in Figure 4-26 for the sample B_F (RC-101 solution + HVF frozen) after 48 h exposure to HVF at 37 °C. It was expected that RC-101 would be detected at a higher concentration when using frozen HVF, due to the decrease in enzymatic activity of the

fluid upon freezing. Since the LC-MS/MS method is not quantitative, it was not possible to determine this difference in concentration. Figure 4-26 shows that RC-101 is detected in frozen HVF, suggesting that no detectable differences were observed in the peptide after incubation with frozen fluid. The total area (supernatant + cells) of each sample was calculated and compared to the RC-101 using fresh HVF. Frozen HVF combined with RC-101 solution utilized in the study (Sample B_F) showed a slight difference in the stability of RC-101. However this variability is within the variability observed in the LC-MS/MS method and may be an artifact of the analysis rather than a difference in the fresh versus frozen fluid. The results show that RC-101 was detected up to 48 h when frozen HVF was used.

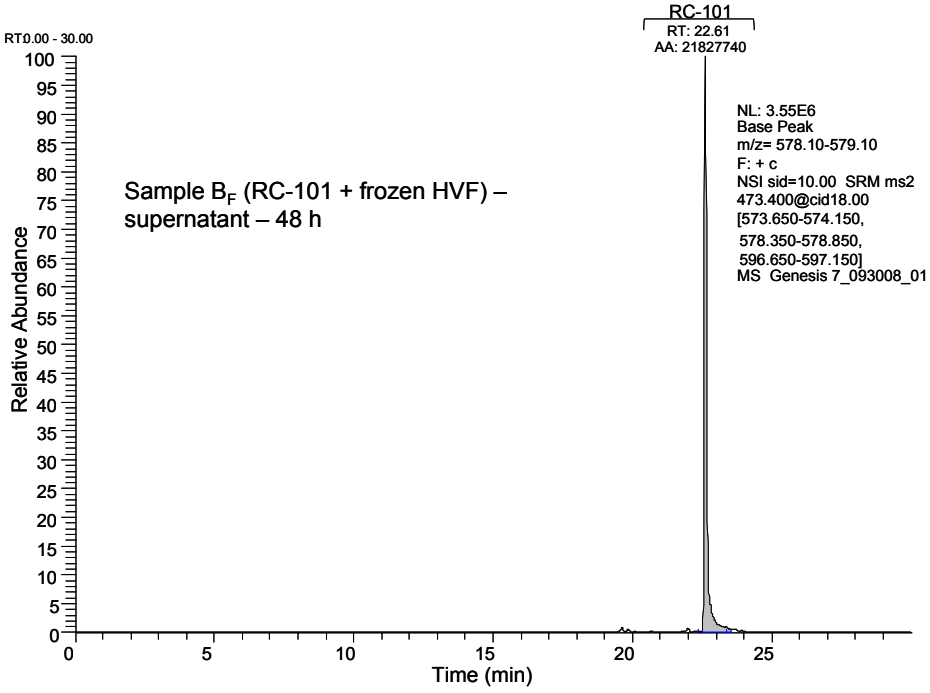

Figure 4-26. Sample B_F (RC-101 film + frozen HVF) representative LC-MS/MS chromatogram of supernatant solution after 48 h incubation.

Stability of RC-101 over time was also investigated in bacterial vaginosis (BV) fluid obtained from volunteers (HVF BV^+). These samples were collected and stored at -80 °C, prior

to incubation with RC-101. When RC-101 was combined with HVF BV^+ Pool (samples B_{BV+}), RC-101 was undetectable in the LC-MS/MS analysis at any time point studied, demonstrating that RC-101 was not stable in those fluids. Representative LC-MS/MS chromatograms of supernatant solution from Sample A_{BV+} (blank HVF BV^+), and sample B_{BV+} (RC-101 solution + HVF BV^+) at time 0 are shown in Figure 4-27 and Figure 4-28, respectively. No RC-101 was detected at any time point in the Samples B_{BV+} in either supernatant or cells. Since this study was only conducted in one pool of BV samples, more studies are recommended to evaluate a larger pool of HVF BV^+ samples to confirm this finding.

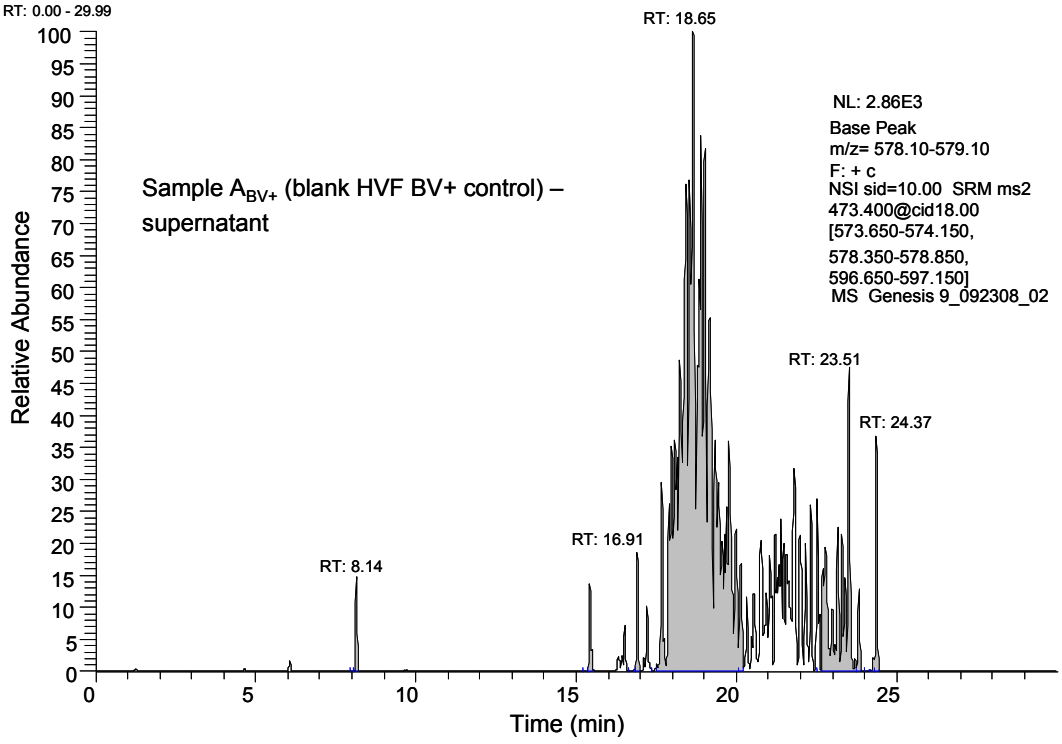

Figure 4-27. Sample A_{BV+} (blank HVF BV^+ control) representative LC-MS/MS chromatogram of supernatant solution at time 0.

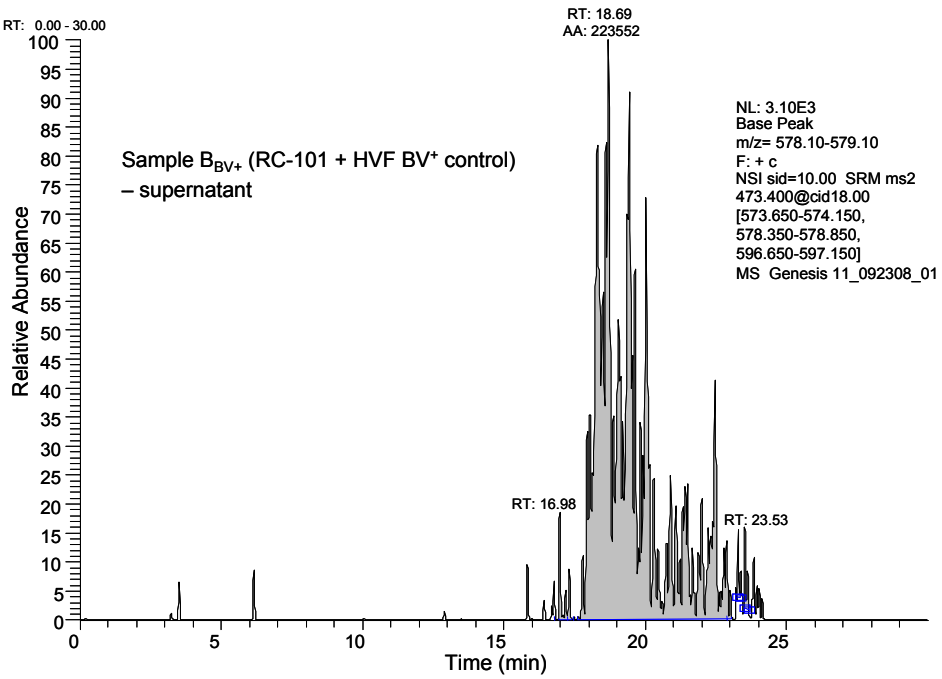

Figure 4-28. Sample B_{BV+} (RC-101 + HVF BV^+) representative LC-MS/MS chromatogram of supernatant solution at time 0.

Table 4-4. Summarized results for detection of RC-101 by LC-MS/MS

		Time (h)						
Sample Code	**Sample Description**	**0**	**2**	**6**	**12**	**24**	**48**	**72***
A	Blank HVF	-	-	-	-	-	-	-
B	RC-101 solution combined with HVF	+	+	+	+	+	+	+
C	RC-101 solution control	+	+	+	+	+	+	+
D	RC-101 film combined with HVF	+	+	+	+	+	+	+
E	RC-101 film control	+	+	+	+	+	+	+
G	Placebo film control	-	-	-	-	-	-	-
A_F	Blank HVF frozen	-	-	-	-	-	-	NA
B_F	RC-101 solution with frozen HVF	+	+	+	+	+	+	NA
A_{BV+}	Blank HVF BV positive	-	-	-	-	-	-	NA
B_{BV+}	RC-101 solution with HVF BV^+	-	-	-	-	-	-	NA

(-) represents absence of RC-101 peak; (+) represents presence of RC-101 peak detected by LC-MS/MS.

***only one pool of sample analyzed up to 72 h. NA = not analyzed samples at this time point.**

Overall, RC-101 detection by LC-MS/MS is summarized in Table 4-4, showing the detection of RC-101 after incubation with HVF for each time point.

4.4 DISCUSSION AND CONCLUSIONS

Few studies have investigated the interaction of simulated vaginal fluid and cervical mucus with microbicide candidates (161, 165-168). In those studies, the physical and chemical properties of the vaginal microbicide product, as well as the bioactivity of the microbicide drug have been investigated. However, only a few studies report the interaction of human and animal vaginal fluids with microbicide agents (109, 153, 169). In addition, most of those studies use lavages or otherwise extensively diluted fluid, decreasing the concentration of enzymes present, and thereby underestimating the potential for degradation due to enzymatic activity.

Several reports that involve the collection of human vaginal fluid and cervical mucus have been published (170-178). The collection methods varied and reflected the objective of each respective study, and included: rotating a Dacron-tipped plastic applicator into the posterior vaginal fornix for 15 sec (171), holding sterile double-tipped swab into the posterior vaginal fornix for 20 sec until saturation (173), inserting a 4 mL syringe and rinsing with 0.9% sodium chloride (174), aspirating fluid from the endocervix with a long tuberculin syringe for tentative collection of pure cervical mucus (175), applying a Rovumeter vaginal aspirator in the posterior fornix (176), inserting a sterile gauze vaginally for 1 h (177), and inserting a menstrual collection device (Instead Softcup®) (178). From the methods available in the literature, Instead Softcup® appeared to be the device most suitable for our study purpose. In the study reported (178), among 27 samples, an average of 0.5 g of secretions (ranging from 0.1 to 1.5 g) was collected.

The Instead Softcup® enables a rapid and convenient collection of large volumes of undiluted cervicovaginal secretions.

To understand the impact of vaginal fluids on the stability of RC-101, fresh human vaginal fluid was collected and the interaction between fluid and peptide was investigated. An appropriate method to collect human vaginal fluid (HVF) was identified. The vaginal microbicide product will encounter both vaginal fluid and cervical mucus. Because it is difficult to collect those fluids separately, a method for the collection of both fluids combined was selected. In these studies, HVF was collected by using an Instead Softcup®.

Several factors such as menstrual status, oral contraceptive use, and age will affect the amount and characteristics of vaginal fluids. A questionnaire was applied to all participant volunteers to characterize the demographics of the population, including: day of the menstrual cycle, drinking status, and smoking status. Increase in estrogen levels is associated with increase in the amount of vaginal fluid and cervical mucus. During ovulation, the mucus volume increases 10 to 20-fold, becomes less viscous, and has a higher oligosaccharide content. Some studies have also reported an increase in the enzymatic activity during ovulation (48, 50, 179). However, Andersch-Bjorkman *et al.* studied the protein pattern of cervical mucosal secretions over the menstrual cycle and no correlative protein pattern was found (175). Oral contraceptives (OC) may also play a role in the vaginal mucosa and fluid status. However, a study examined thirty women before and after the use of oral contraceptive (for 2 months) to verify the effect of OC in the vaginal discharge, epithelium and flora (180). It was found that OC did not change the gross colposcopic or histologic appearance of the vaginal epithelium or characteristics of vaginal or cervical discharge. Vaginal flora essentially remained unchanged after 2 months of OC use (180). Information about OC use was collected for our studies; however, due to the number of

volunteers used and the necessity to pool samples together to obtain a significant volume for the analysis, we were unable to make any conclusions regarding the OC use and the stability of RC-101 in the fluids.

Volunteers were not allowed to participate if they had had sexual intercourse in less than 48 h before the time for collection. This is a regular practice in collection of human vaginal fluid to avoid any contamination with semen in the fluids. Macaluso *et al.* reported the identification of a marker in vaginal fluid, prostate-specific antigen (PSA), to determine condom failure (181). It was shown that in 24 h, mean PSA values were close to baseline levels, and in 48 h, no PSA was detected, suggesting that in 48 h there is no presence of semen in the vaginal fluid (181).

Drinking and smoking may affect the amount of vaginal fluid and its characteristics. Mendelson *et al*. found that acute ingestion of vodka punch (0.7 g/kg ethanol) raised circulating estradiol levels by more than 50% in women in the follicular phase. However, no significant changes in estrogen were observed when the same amount of alcohol was given in the luteal phase (182). Increase in estrogen is associated with increase in the amount of vaginal fluid and cervical mucus. In another study, Reichman *et al*. studied 30 premenopausal women randomized to receive either 30 g of alcohol or placebo beverage, nightly for 3 months. After the chronic alcohol consumption, midcycle estradiol levels increased by 28%, plasma estrone by 21%, and urinary estradiol by 32% (183).

The influence of smoking status on vaginal fluid has been investigated, and some epidemiologic studies suggest that female smokers have lower endogenous estrogen than nonsmokers (184-186). It has also been suggested that the irritant and carcinogenic chemicals in cigarette smoke are toxic to normal vaginal flora. These chemicals inhibit the synthesis of cytoprotective eicosanoids and alter the immune defense mechanism of vagina (186). In

addition, smoking was found to induce accelerated exfoliation of cervicovaginal epithelial cells (184). In our study, the sample size was insufficient to correlate any differences in vaginal fluids with behavioral status (drinking and smoking). However, any future larger studies should take these factors into consideration.

Enzymatic activity of vaginal fluids has still not been fully characterized. Aminopeptidases are the most abundant type of enzyme present in human vaginal mucosa, and a major contributor to protein and peptide degradation. The aminopeptidase activity is located mainly in the cytoplasm, and the concentration/activity in the vaginal mucosa ranges from 0.5 to 1.4 μmol substrate hydrolyzed/min/mg protein (187). The identification and quantification of enzymes present are not very well established but lysozyme has also been identified in human cervical mucus (60). Other enzymes, including aminopeptidases, are present in both vaginal and cervical secretions and their activity may also vary with the menstrual cycle (61, 62). Recently, with the advances in proteomic analysis, determination of proteins and enzymes present in human cervical-vaginal fluids have been conducted. In one study, most of the proteins identified in vaginal fluid were plasma components, such as: albumin, transferrin, apolipoprotein I, calgranulins A and B, and alpha-1-acid glycoprotein 1 (174). Other studies have found similar results (171, 173, 174, 177, 188). In the preformulation studies conducted in Chapter 3, Section 3.3, we have shown that RC-101 was stable after incubation for 1 h at 37 °C to aminopeptidase, lysozyme, and proteinase K, providing an indication of stability of the peptide for a short time period in biological fluids.

One of the caveats of all the studies for identification and quantification of enzymatic activity in HVF is that it was necessary to freeze the fluid collected before beginning analysis. The impact of the freezing process leads to a change in the enzymatic activity of the fluid,

contributing to the data obtained from these studies. To evaluate the impact of freezing on the degradation pattern of RC-101, fluid pooled from one day of collection was analyzed prior and after freezing process. Results suggest that RC-101 possess the same stability in HVF after freezing process as in fresh HVF. No indication that freezing process alters the enzymatic activity of the HVF was obtained from this study. However, it is recommended that further studies with fresh versus frozen fluid be evaluated in replicates to confirm this result.

This is the first study in the microbicide field to evaluate a microbicide candidate using fresh HVF. RC-101 was detected by LC-MS/MS assay at least 48 h after incubation with HVF at 37 °C. In one of the pooled samples studied, RC-101 was detected up to 72 h after incubation with HVF. No difference was observed between RC-101 in solution and RC-101 formulated into a film. Both incubation samples showed the presence of RC-101 for at least 48 h, indicating presence of the peptide for this time period in HVF. The results obtained in this study will promote a better understanding of the impact of human vaginal fluid on the microbicide product development and efficacy. It is still uncertain how long a microbicide drug should be active in the vaginal lumen. However, it has been suggested that the HIV stays active in the vaginal lumen for a period of 48 h (66, 158, 159). This implies that any microbicide drug delivered in the vaginal lumen should be stable for at least this time period. Other important points are the time required for HIV to bind to the receptors in the host cell and the time to infect immune host cells. It has been observed by Gallo *et al.* (189) and Dimitrov *et al.* (190) that conformational changes observed between gp41 and CD4 takes around 15 to 20 min. Another study by Hu *et al.* (158) have shown that SIV enters the vaginal mucosa within 60 min of intravaginal exposure, infecting primarily intraepithelial dendritic cells. It is known that RC-101 binds with high affinity to glycoproteins preventing six-helix bundle formation of gp41 in a reversible process

(107). If RC-101 can be detected in HVF for at least 48 h, it is suggested that RC-101 will be available for binding to gp120 during that time period, conferring protection against HIV. The prolonged stability of RC-101 in HVF indicates that this molecule is a promising candidate to be delivered vaginally and survive the enzymatic activity present in the normal vaginal fluid. However, further studies *in vivo* are recommended to confirm the results obtained. Another advantage of the stability of RC-101 for at least 48 h in HVF is the dose regimen selected for the microbicide. The stability suggests that the final RC-101 microbicide product could be applied once every two days or once a day, without being coital-dependent. This would increase patient adherence to the product, and would be more favorable to a successful product. As a future recommendation, the RC-101 detected after incubation with HVF should be tested for bioactivity against HIV. This bioactivity test is already planned to be conducted in the laboratory of Dr. Cole (University of Central Florida).

The impact of HVF positive for bacterial vaginosis (BV) has also been investigated. It has been shown that RC-101 was completely unstable in fluid positive for BV evidenced by the undetectable levels of RC-101 after exposure to HVF positive for BV at all time points. Some studies have evaluated the difference between normal HVF and HVF positive for BV, and a difference in the enzymatic activity between a normal fluid and a BV positive fluid has been demonstrated (145, 191, 192). BV is characterized by a reduction in vaginal colonization by Lactobacillus and an overgrowth of anaerobic gram-negative bacteria. Intensive production of hydrolytic enzymes in BV may lead to a decreased mucosal barrier in the vaginal and cervical mucosa. Glycosidases and proteases, produced by anaerobic gram-negative bacteria, are associated to the degradation of mucins in the HVF (192). In addition to those enzymes, it has been shown that an increase in lactate dehydrogenase activity (191), and glycosulfatase (193) in

HVF is associated with BV patients. The higher enzymatic activity found in BV can explain the immediate degradation of RC-101 in the presence of HVF positive for BV. This finding is extremely important as a future study for the development of biopharmaceuticals and other molecules as microbicides. Bacterial vaginosis is a highly prevalent condition, affecting almost one third of women between the ages of 14 and 49 years old in the United States, according to the 2001 - 2004 National Health and Nutrition Examination Survey (194). Considering the high prevalence of BV, further studies should investigate the effects of HVF positive for BV on the stability of microbicide drug candidates.

Overall, this study has shown that RC-101 is present in HVF for at least 48 h. Formulation of RC-101 into a film maintains the stability of RC-101 in HVF for the same time period, freezing may not affect the study of RC-101 stability, and HVF positive for BV will considerably affect the stability of RC-101. As a continuation of this study, the *in vitro* activity of RC-101 against HIV-1 after exposure to HVF will be conducted to verify if activity is maintained. The important findings from this project have shown that RC-101 is a promising microbicide candidate. Further studies for RC-101 formulation development, permeability and tissue localization in human and monkey reproductive tracts were conducted and are discussed in Chapters 5 and 6.

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5.0 RC-101 FORMULATION IN A QUICK-DISSOLVE POLYMERIC FILM

5.1 INTRODUCTION

As discussed in Chapter 1, current therapeutic strategies to prevent transmission of HIV have not been completely successful. Considering that many women are unable to negotiate condom use with their partners and are more susceptible to HIV, strategies to prevent heterosexual transmission of HIV must include female-controlled methods (11, 25). One approach is the development of a vaginal topical microbicide product to be used before intercourse to prevent transmission and acquisition of HIV.

RC-101 is a circular 18-residue peptide microbicide candidate. Throughout this dissertation work, it has been shown that RC-101 is stable under most of the conditions evaluated in the preformulation studies conducted (Chapter 3) and is stable in the presence of human vaginal fluid for a period of at least 48 h (Chapter 4), making this peptide a viable candidate as a topical vaginal microbicide. Further development of RC-101 as a vaginal microbicide product requires its formulation into a viable dosage form that is suitable for the intended route of administration, acceptable to the patient, and able to maintain the stability of the peptide in the product during its shelf-life and in the biological environment.

Most of the vaginal microbicides in development are formulated as hydrogels (120, 195). More recently, new dosage forms have been considered such as vaginal rings, tablets, ovules, and polymeric films (122-125). The choice of dosage form is directly dependent on the physical and chemical characteristics of the microbicide drug, on patient acceptability, and on maintaining drug efficacy and targeting (123).

The successful formulation of biopharmaceuticals (proteins and peptides) is often challenging. Due to their high susceptibility to degradation, protective strategies applied in their formulation may prevent peptide degradation and, consequently, preserve the activity of the product during its shelf-life and in the biological environment. Semi-solids are the most frequently used vaginal dosage form. However, a semi-solid product would not be suitable as a dosage form for RC-101. If an aqueous semi-solid dosage form is developed, it would expose RC-101 to a significant amount of water accelerating the degradation of the peptide. In Chapter 3, it has been shown that the major degradation pathway of RC-101 is oxidation, which can be facilitated in the presence of water. In addition, it was shown that the peptide is stable for 2 weeks over a range of pH from 3 to 7. This short-term stability indicates that RC-101 is stable; however, long-term stability storage in the final product formulation still needs to be evaluated. As an alternative, a non-aqueous based semi-solid product could be formulated. However, the product would have very low patient acceptability and difficult miscibility in the fluids encountered in the vaginal lumen.

Based on the characteristics of RC-101, an alternative dosage form was investigated. Polymeric films contain lower water content when compared to traditional gel formulations, and can provide rapid release of the drug from the delivery system in contact with cervicovaginal fluids. In addition, polymeric films have several advantages: convenience for women to use without the need of an applicator, lower cost per dose, absence of messiness relative to current vaginal gel products, and increased stability of the drug.

In this study, RC-101 was formulated into a quick dissolve polymeric film. Film assessment, *ex vivo* safety and *in vitro* efficacy studies using human ectocervical tissue, and *in*

vivo non-human primate safety studies were conducted. Film characterization and stability were also assessed.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Retrocyclin-1 (RC-101) was synthesized by the Peptide Synthesis Facility at the University of Pittsburgh (Pittsburgh, PA). Polyvinyl alcohol (PVA) was obtained from Kuraray America Inc. (New York, NY). Glycerin was purchased from Dow Chemical Company (Midland, MI). Hydroxypropylmethylcellulose (HPMC) 6 cps was purchased from Sigma (St. Louis, MO). Ethylene diamine tetraacetic acid disodium salt (EDTA) was purchased from Spectrum Laboratory Inc. (Gardena, CA). All other materials were obtained from Fisher Scientific (Fair Lawn, NJ) unless specified otherwise.

5.2.2 Quantitative assay for RC-101 in film product

An HPLC system (Waters Corporation, Milford, MA) was used for the analysis of RC-101 in the formulation as described in Section 3.2.2. Briefly, the HPLC Waters system was equipped with an autosampler model 717 (Waters), a quaternary pump model 600 (Waters), and an ultraviolet (UV) detector model 2487 (Waters). Separation of the RC-101 was achieved by using a Jupiter 5 μ C5 column 4.6 x 250 mm, 300 Å (Phenomenex, Torrance, CA) protected by a guard

cartridge Widepore C5 4 x 3.0 mm (Phenomenex). Empower PRO, Empower 2 software (Waters Corporation) was used to control the HPLC system.

5.2.3 Formulation development

RC-101 polymeric vaginal film manufacturing

A quick dissolve polymeric vaginal film formulation containing RC-101 was developed. Vaginal films were prepared by casting a polymeric film solution into an 8-well-plate (dimensions: 27.5 x 33.5 mm/well). Three film formulations were prepared; an initial film formulation (**RC_F+LA**) containing 0.8% (w/w) lactic acid to maintain an acidic pH to simulate the conditions found in the vaginal environment; a second formulation without lactic acid (**RC_F**); and a final optimized formulation (**RC_F+EDTA**) containing 0.05% (w/w) EDTA in an effort to protect RC-101 against oxidation. Incorporation of EDTA in the RC-101 film was based on the results found in the preformulation studies. It has been shown in Chapter 3 (Section 3.3), that only 10% loss of RC-101 in 30 days is observed when in solution containing EDTA in the presence of hydrogen peroxide as compared to a 40% loss of RC-101 in 6 days without addition of EDTA.

All three formulations contained a basic polymeric solution base prepared as described. The polymeric film solution base was prepared by adding 40 ml of Milli Q water into a beaker. PVA (3.0 g) was slowly added to water with mixing, using a magnetic stir bar; followed by the addition of 60 mg of HPMC. The solution was heated for 20 min at 95 °C for complete dissolution of the polymers. After cooling the solution to room temperature under mixing, 1.5 g of glycerin was added. For **RC_F+LA**, 0.8% lactic acid was added and the films were prepared at a dose of 100 µg RC-101/film. The second formulation, without lactic acid, was prepared at

two dose levels: 100 µg/film (**RC_F100**) and 2000 µg/film (**RC_F2000**). The optimized formulation (**RC_F+EDTA**) was prepared by adding 0.05% EDTA, and RC-101 at a dose of 100 µg RC-101/film. The completed formulation solution (2.4 g) containing RC-101 was poured into each well of the 8-well plate. The plate was placed into a vacuum oven at 30 ± 2 °C for 20 ± 4 h for drying. After this period of time, the films were removed from the plate. Placebo films were prepared without the addition of RC-101. All films were removed from the plates, weighed, and stored in PET/Aluminum foil pouches (Amcor Flexibles Healthcare Inc, Mundelein, IL) until further analysis.

5.2.4 Formulation assessment

5.2.4.1 Physical and chemical characterization

Dimensions of the film (thickness, length and width) were determined using a digital micrometer caliper (Fisher Scientific, Fair Lawn, NJ).

Disintegration was determined by placing the film into 3 mL of Milli Q water in a crystallization dish (50 x 35 mm). The crystallization dish was rotated on an orbital shaker Rotomix Type 48200 (Thermolyne, Dubuque, IA) at 90 rpm, at room temperature, and the time to visual disintegration of the film was recorded.

Determination of water content of the film was obtained by Karl Fisher titration using a 758 KFD Titrino Metrohm (Brinkmann Instruments Inc., Westbury, NY). Films were initially dissolved in 3 ml of dimethyl sulfoxide (DMSO) for a period of 20 h. An aliquot (0.5 mL) of the dissolved film solution was weighed and added to the Karl Fisher titration vessel for moisture determination. The water content of the DMSO was determined as a blank and the result was subtracted from the sample's results. Water content was expressed in percent (w/w).

Tensile strength of the films was evaluated using a texture analyzer TA.XT2 (Texture Technologies, Scarsdale, NY). Films were held between two clamps positioned at a distance of 4.85 mm. During measurement, the films were pulled by the top clamp at a rate of 3 mm/sec until break point. The force at the break point was recorded. Tensile strength was calculated as:

Tensile strength (N/mm^2) = breaking force (N)/ cross-sectional area of sample (mm^2)

Stability studies were conducted to evaluate RC-101 content throughout the manufacturing process and storage. Storage conditions used were: room temperature (25 ± 2 °C/ 60 ± 5 % relative humidity (RH) and 40 ± 2 °C/ 75 ± 5% RH). At each time point, films (n = 3) were removed from the packaging container, cut into six pieces, and dissolved into 0.5 mL of Milli Q water. Each solution was analyzed in duplicate by the HPLC-UV method described in Section 5.2.2. Results were expressed as percent (%) of RC-101 compared to amount of RC-101 added per film.

5.2.4.2 Efficacy studies

In vitro efficacy studies using TZM-bl cells

Each excipient of the film formulation as well as the film products were tested for bioactivity against HIV-1. Experiments were conducted in the laboratory of Alexander Cole, Ph.D. (University of Central Florida, Orlando, FL). Excipients were prepared and dissolved in 1 mL of water to obtain the final concentration equivalent to that contained in one film dissolved in 1 mL of water. Each sample was further diluted with growth medium (GM) at the ratios of 1:10, 1:20, and 1:40 (v:v). Activity of RC-101 against HIV-1_{BaL} was determined using TZM-bl reporter cells and quantified for luciferase expression as previously described (196). Briefly, cells (5000/well; 96-well plates) were treated with 50 μL of either plain GM or GM containing the

samples. To each well, 50 µL of GM containing HIV-1_{BaL} (2 ng of p24/ml) was added. Supernatants were then removed, and cells were lysed with 100 µL of 1x Glo Lysis Buffer (Promega Corporation, Madison, WI). Luciferase activity was measured using Bright Glo luciferase assay buffer (Promega). Protection from infection was measured as the percentage of reduction in luciferase activity, relative light units (RLU), compared with the HIV-1_{BaL}-infected control (vehicle only, no peptide). To confirm that the observed reductions in luciferase activity were not due to toxic effects of RC-101 on the cells, the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were performed on identically treated TZM-bl cells. For placebo and RC-101 2000 µg films (**RC_F2000**), films were dissolved in 1 mL of GM and then processed as described above.

Ex vivo efficacy studies using human ectocervical tissue

Ex vivo efficacy studies were performed in the laboratory of Phalguni Gupta, Ph.D. (University of Pittsburgh, Pittsburgh, PA). Human ectocervical tissues were obtained from premenopausal women undergoing hysterectomy. The *ex vivo* efficacy studies were set up using an organ culture system previously described (67, 197). Briefly, cells (5 x 10^5) were placed directly in the bottom chamber of the Transwell system (Corning Inc, Corning, NY). A Transwell with agarose only in the top chamber served as a negative control, while Transwell with the membrane only served as a positive control. To study the transmission of virus, a defined amount of pre-titered cell-free HIV-1 was added to the top chambers of the tissue and control wells and incubated at 37 °C for 3 to 4 days. After incubation, the top chamber of the well was removed and culture of $CD8^-$ depleted cells in the bottom chamber was continued for an additional 10 days. Viral growth was monitored by measuring HIV-1 p24 antigen levels in the culture supernatant in the bottom chamber of the tissue well. A 3-fold or greater increase in HIV-1 p24 during the 2-week

period was taken as positive virus infection of the $CD8^-$ depleted cells. Suppression of viral transmission was measured by monitoring viral infectivity of transmitted HIV-1 in the bottom well, determined at day 14 of culture by measuring HIV-1 p24 in the culture supernatant of the bottom well.

5.2.4.3 *Ex vivo* and *in vivo* safety and toxicity studies

Excised macaque reproductive tract

Reproductive tracts from sexually mature female *Macaca nemestrina* were obtained from a colony of animals at the Washington National Primate Research Center. Prior approval for use of monkeys in this protocol was obtained from the Institutional Animal Care and Use Committee at the University of Washington. Animals were handled humanely, and experiments were performed within the National Institutes of Health's laboratory animal use guidelines. Animals were sacrificed and the whole reproductive tract was removed and transported to Magee-Womens Research Institute (Pittsburgh, PA) in ice within 24 h. Upon arrival, the tissue was warmed to 37 °C and immediately used for the experiments.

Ex vivo safety studies in full monkey reproductive tract tissue

Safety of placebo films and RC-101 (100 μg) films were evaluated in full excised monkey reproductive tract. The protocol was designed to simulate the experimental conditions used in an *in vivo* setting. During an *in vivo* experiment, the animals are sedated and stay in a horizontal position for about 30 min. For that reason, freshly excised monkey reproductive tissue was maintained in a horizontal orientation for the first 30 min after insertion of the film. After that time, the tissue was oriented vertically for another 90 min. Excised monkey reproductive tract was warmed to 37 °C, and placed in a 15 mm Petri dish containing 2 mL of Milli Q water. The

film product was inserted into the vaginal cavity with a glass rod. Full reproductive tract tissue was incubated for a total period of 2 h at 37 °C. The tissue was then removed from the incubator and washed (4 mL of water, 10 times) by inserting a 5 mL syringe in the vaginal opening. A cytobrush was inserted and gently rotated 6 times into the cervical os. The cytobrush was then placed in 4 mL of water and vortexed. The tissue was cut open and 6 mm (n = 3) biopsy samples were collected from each part of the tissue (lower vagina, middle vagina, upper vagina, transformation zone, endocervix, ectocervix, and uterus), and stained for histology.

Excised human ectocervical tissue

Freshly excised human ectocervical tissue was obtained from the Tissue Procurement Facility at Magee-Womens Hospital (Pittsburgh, PA), according to approved IRB protocol #0503103. Tissue was obtained from premenopausal women undergoing hysterectomy for benign conditions. No specimens were used when there was evidence of tissue abnormality that could influence the state of the mucosa. All tissue specimens were obtained within 1 h of surgical excision, and held at 4 °C in Dulbecco's Modification of Eagles Medium (DMEM) (Mediatech Inc, Herndon, VA) during transfer from surgery to the laboratory. Tissue samples were retained for histological evaluation for comparison before and after the experiment.

Ex vivo safety studies in human tissue

Ex vivo safety studies were conducted using a Franz-cell system, a two-compartment system consisting of an upper chamber (donor compartment) and a lower chamber (receptor compartment). The Franz-cells were water-jacketed and the temperature was maintained at 37 °C throughout the experiment via a circulating water bath. DMEM was used in the receptor

chamber, continuously stirred by a magnetic stir bar. Human ectocervical tissue was sandwiched between the two compartments with the epithelial side facing the donor solution. The tissue was equilibrated with DMEM in the donor compartment for 5 min prior to the initiation of the safety study. After the equilibration period, DMEM was removed from the donor compartment and replaced with 100 µL of vaginal fluid simulant (VFS) (198) and a 6 mm punch biopsy of a RC-101 containing film (**RC_F+LA**, **RC_F100**, or **RC_F+EDTA**). After a 2 h period, tissue samples were removed and processed for histological evaluations.

Histological evaluations

Histological evaluations were conducted on all tissue specimens tested. Retained tissue from each test specimen was fixed for histology and processed to compare with post-experimental histology. Tissue was fixed in Clark's solution (ethanol-acetic acid (75:25)) for 24 h, transferred to ethanol and incubated for 24 h, and subsequently embedded in paraffin. Tissue sections of 5 µm were cut and stained with hematoxylin and eosin (H & E). Histology was conducted to evaluate gross alterations in tissue morphology caused by exposure to the film product.

In vivo safety evaluation

Safety evaluations in animals were conducted by the laboratory of Dorothy Patton, Ph.D. (University of Washington, Seattle, WA). A total of five sexually mature female *Macaca nemestrina* were obtained from a colony of animals at the Washington National Primate Research Center. Prior approval for use of monkeys in this protocol was obtained from the Institutional Animal Care and Use Committee at the University of Washington. Animals were

handled humanely, and experiments were performed within the National Institutes of Health's laboratory animal use guidelines.

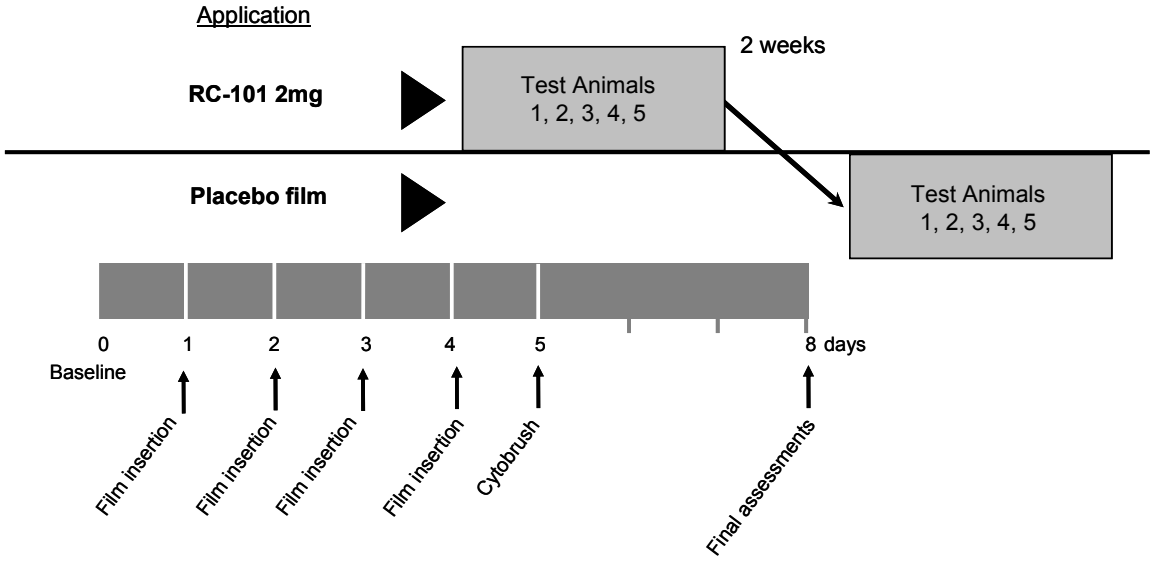


Figure 5-1. Study design for *in vivo* safety protocol.
Application of RC-101 2 mg film in 5 animals, wash-out period of two weeks, application of placebo film. Four consecutive (one per day) applications of film for each animal. Cytobrush was conducted only on day 5. Final assessments (cytobrush, pH, colposcopy, microbiology) conducted on day 8.

Films were applied to the animals following the experimental design shown in Figure 5-1. Films of RC-101 2000 μg/film (**RC_F2000**) were assessed for safety with multiple vaginal applications and compared to a placebo film. Each animal controls for itself by completing both arms of the study (first RC-101 and then placebo film). Two experimental runs, each lasting 8 days and evaluating five animals per arm, were conducted with a 2-week recovery time scheduled between runs. Each animal received one film/day for a period of 4 days. On study days 1 to 4, colposcopy assessments, swabs for vaginal pH, and microflora were obtained. Immediately following specimen collections, an intravaginal application of 1 film (RC-101 or

placebo) was administered to each animal. Colposcopy, and vaginal swabs for pH assessment were collected 30 min after film application. Colposcopy was conducted every day after 30 min of application and before the next day application. On study days 5 and 8, colposcopy, and swabs for vaginal pH, and microflora were collected to document recovery. Standardized colposcopic assessments were conducted by a team of three cross-trained individuals. In order to document the appearance of the cervicovaginal tissues prior to potential tissue perturbation caused by sampling, colposcopy took place immediately after speculum placement before any swab collections. Vaginal pH was determined by rolling a swab of vaginal secretions onto a pH indicator strip with a resolution of 0.5 pH unit. A second vaginal swab was collected and immersed in a transport tube (Port-a-Cul; Becton Dickinson Microbiology Systems, Cockeysville, MD) for quantitative microbiologic characterization. Microbiologic characterization was conducted in the laboratory of Sharon Hillier, Ph.D. (Magee-Womens Research Institute, Pittsburgh, PA).

5.2.5 Statistical analysis

HPLC data obtained from the stability studies were expressed as the average percentage of the RC-101 concentration ± standard deviation (n = 3). Results were analyzed by one-way analysis of variance (ANOVA) with comparisons of individual time points to time 0 by using Dunnett correction to detect significant differences. P-values ≤ 0.05 were considered to be statistically significant, unless specified otherwise. Bioactivity data was expressed as average percentage suppression of the virus (n = 2).

5.3 RESULTS

Formulation assessment

The appearance of all films (placebo, **RC_F+LA**, **RC_F100** and **RC_F2000**) was clear, without bubbles, flexible, no sharp edges, and with a smooth surface. A summary of results of the film assessment tests for placebo and **RC_F100** are presented in Table 5-1.

Table 5-1. Summary of physical and chemical assessment of film formulation

TEST	Placebo film	RC_F100
Dimensions (mm)	27.5 x 33.5	27.5 x 33.5
Thickness (mm)	0.192 ± 0.003	0.208 ± 0.005
Weight (mg)	238.2 ± 9.3	240.2 ± 3.7
Disintegration (min)	3.1 ± 0.5	4.8 ± 1.1
Water content (%)	0.30 ± 0.04	0.46 ± 0.06
Tensile strength (N/mm^2)	5.22 ± 1.88	6.38 ± 1.30
Amount RC-101 (%)	N/A	96.2 ± 3.8

The stability of RC-101 content in the films, determined by HPLC, when stored at 25 °C and at 40 °C/ 75% RH was evaluated by HPLC analysis. Figure 5-2, Figure 5-3, and Figure 5-4 show the RC-101 content (%) (average ± standard deviation) results of the stability study conducted for **RC_F+LA**, **RC_F100**, and **RC_F+EDTA** films, respectively. In **RC_F+LA** and **RC_F100,** when stored at 40 °C/75% RH, RC-101 quickly degraded within the first month. In the films containing lactic acid (**RC_F+LA**), 15% loss was observed after 90 days when stored at room temperature; and 50% loss of RC-101 was observed after 30 days when stored at 40 °C/ 75% RH. No RC-101 was detected in the films stored for more than 30 days at 40 °C/75% RH (Figure 5-2). In the film formulation without lactic acid (**RC_F100**), 25% loss was observed

after 90 days when stored at room temperature; and 40% loss of RC-101 was observed after 30 days stored at 40 °C/ 75% RH. No RC-101 was detected in the films stored for more than 30 days at 40 °C/ 75% RH (Figure 5-3). Addition of EDTA to the RC-101 films (**RC_F+EDTA)** did not show a significant improvement in the stability of the drug, contradicting the results obtained in the preformulation studies. In the film formulation containing EDTA (**RC_F+EDTA**), 35% loss was observed after 45 days when stored at room temperature; and 33% loss of RC-101 was observed after 30 days stored at 40 °C/ 75% RH. No RC-101 was detected in the films stored at analysis at 90 days at 40 °C/ 75% RH (Figure 5-4). It should be noted that although loss of parent peak (RC-101) occurred over time, no secondary peaks were generated in the HPLC. Preliminary studies have shown that the bioactivity of RC-101 films after storage for 6 months, retain their activity against HIV-1 *in vitro* in more than 90% suppression of the virus (personal communication – Dr. Alexander Cole, University of Central Florida). This result indicates that loss of RC-101 can be attributed to a mechanism of degradation different than oxidation or thermal degradation, and does not affect bioactivity.

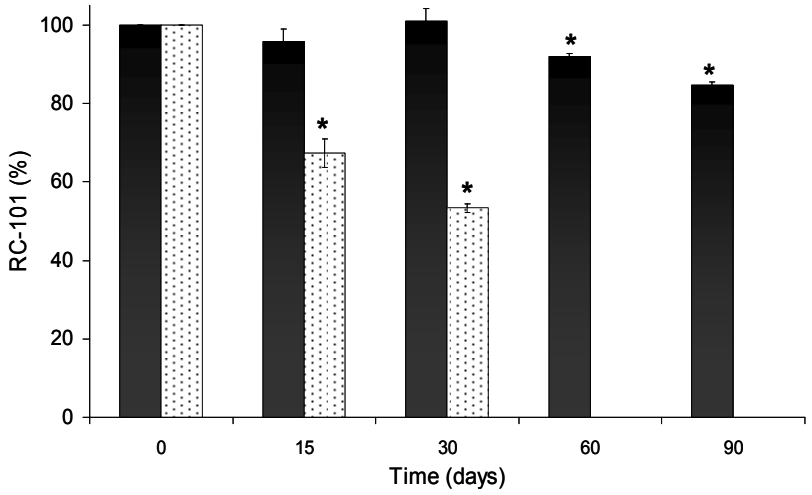


Figure 5-2. Stability of RC-101 100 µg film containing 0.8% lactic acid (RC_F+LA).
Analyzed by HPLC, n = 3, mean ± S.D. (■) 25 °C and (▨) 40 °C/ 75% RH. No RC-101 was detected at 60 and 90 days stored at 40 °C/ 75% RH. *p < 0.002, Dunnett analysis for multiple comparisons.

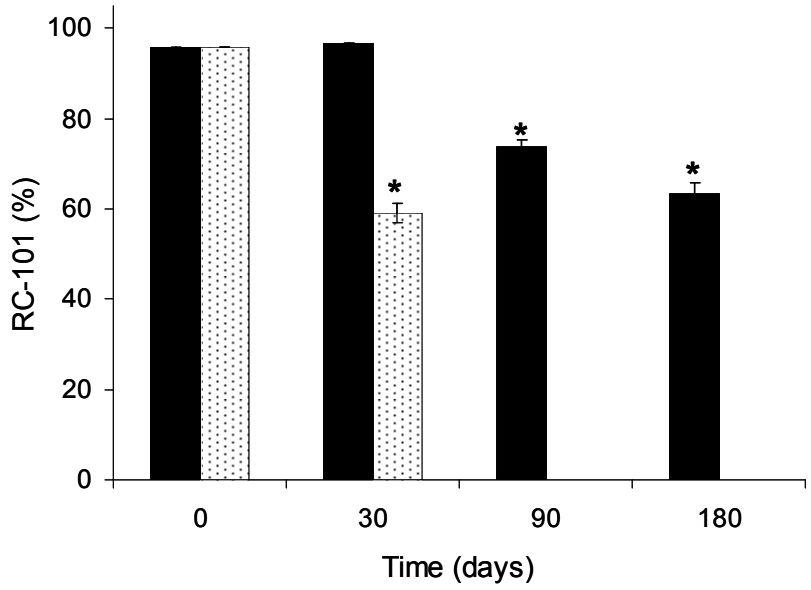


Figure 5-3. Stability of RC-101 100 µg films (RC_F100).

Analyzed by HPLC, n = 3, mean ± S.D. (■) 25 °C and (⚄) 40 °C/ 75% RH. No RC-101 was detected at 90 or 180 days stored at 40 °C/ 75% RH. *p < 0.001, Dunnett analysis for multiple comparisons.

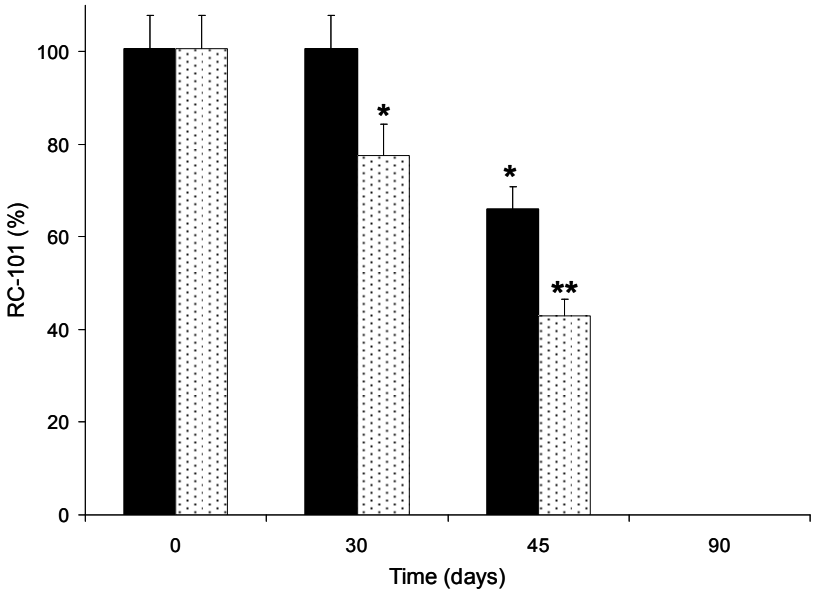


Figure 5-4. Stability of RC-101 100 µg films + EDTA (RC_F+EDTA).

Analyzed by HPLC, n = 3, mean ± S.D. (■) 25 °C and (⚄) 40 °C/ 75% RH. No RC-101 was detected at 90 days stored at 40 °C/ 75% RH. *p < 0.05, **p < 0.001, Dunnett analysis for multiple comparisons.

In vitro efficacy evaluations

The toxicity and bioactivity of each excipient used in the film formulation was tested against TZM-bl cells. MTT results are expressed as percentage viability (%) and are shown in Figure 5-5 for each excipient used in the film formulation. Figure 5-5 represents the average of two MTT determinations (n = 2). Duplicates did not differ by more than 20%. A media control (growth medium) was used for those studies. Most of the excipients showed no difference in viability when compared to the blank vehicle (water) used to dissolve the excipients. However, lactic acid showed toxicity as identified by the MTT assay, when compared to control (viability less than 30%).

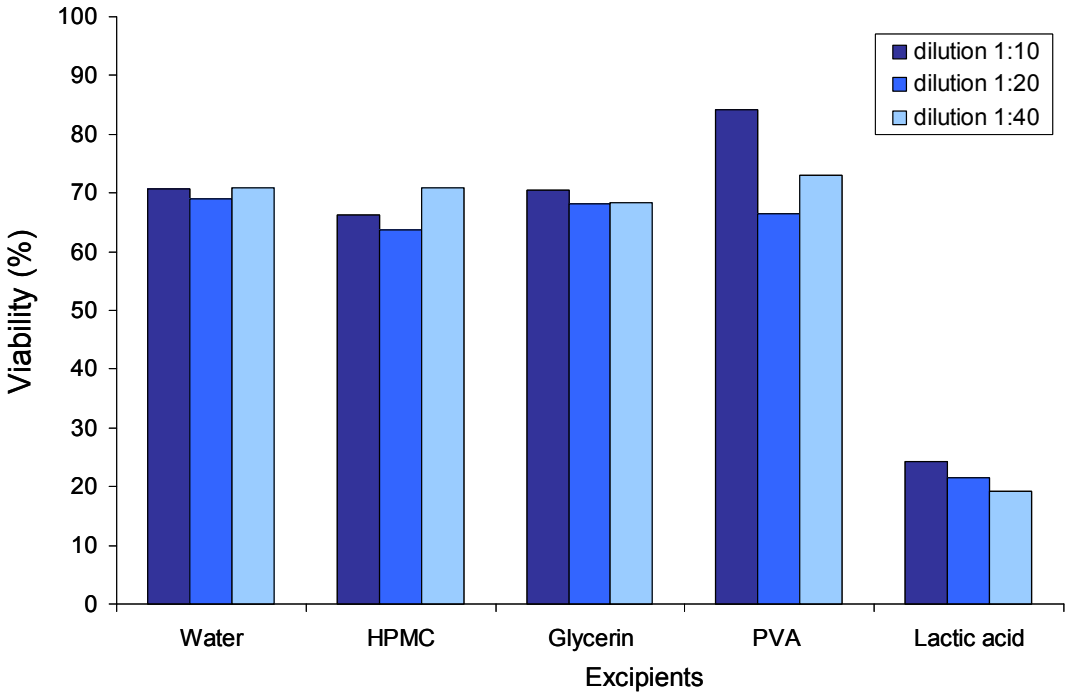


Figure 5-5. MTT results for each excipient used in the film formulation.
3 dilutions (1:10, 1:20, and 1:40), n = 2 for each dilution were tested.

The *in vitro* bioactivity of each excipient, demonstrated by the percent of HIV-1 suppression is shown in Figure 5-6. PVA and lactic acid showed activity against HIV, by showing HIV-1 suppression higher than 70% for PVA, and higher than 95% for lactic acid for the initial dilution. Figure 5-6 represents the average of two bioactivity determinations (n = 2). Duplicates did not differ more than 10%. Bioactivity observed for lactic acid, evidenced by the high HIV suppression, is most likely due its toxicity to the cells used in the test. As shown in Figure 5-5, viability of cells after exposure to lactic acid is lower than 30%. For this reason, lactic acid was removed from the formulation.

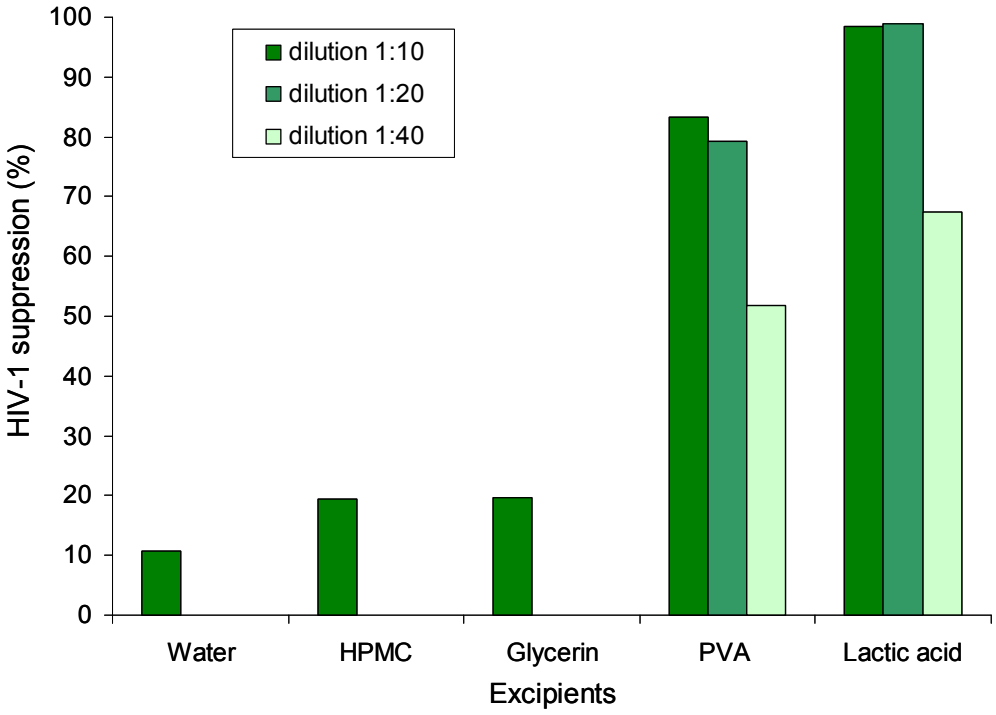


Figure 5-6. Suppression of HIV-1 (%) for each excipient used in the film formulation. 3 dilutions (1:10, 1:20, and 1:40) were tested, n = 2 for each dilution.

RC_F2000 and placebo films were tested for bioactivity using an *in vitro* and an *ex vivo* method. For the *in vitro* method, **RC_F2000** and placebo films were tested against HIV-1 using

the TZM-bl cell model to verify efficacy of these two formulations. **RC_F2000** films were further used in safety studies *in vivo*, as described in Section 5.3 (*In vivo* safety studies). Results of HIV suppression (%) are shown in Figure 5-7. A second graph was plotted using the log of the final RC-101 concentration (µM) of each dilution versus the suppression of HIV (%) (Figure 5-8). The IC_{90} and IC_{50} were determined from Figure 5-8 as the concentration that suppressed 90% and 50% of HIV-1, respectively. Log calculations estimated an IC_{90} of 5.0 µM, and an IC_{50} of 2.2 µM. Calculations of IC_{90} and IC_{50} are commonly used in determination of efficacious concentrations of a compound.

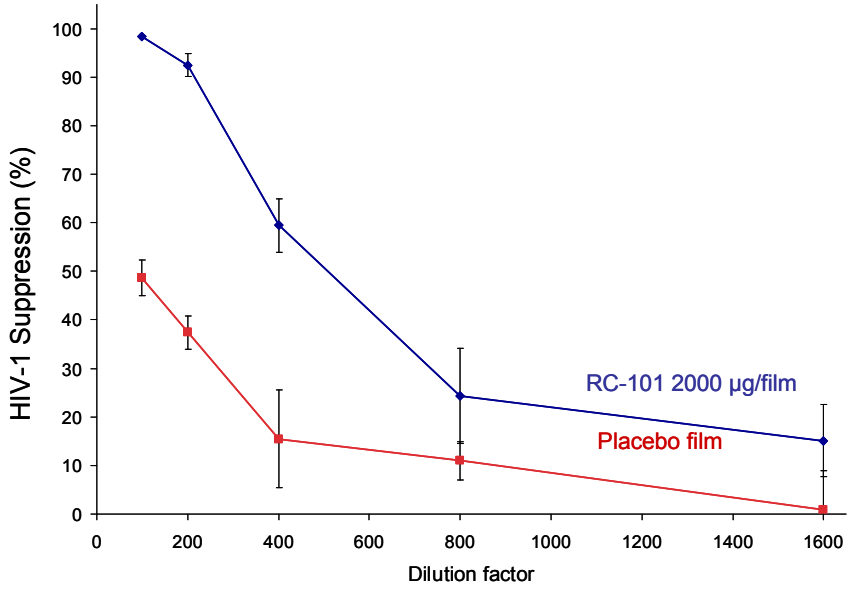


Figure 5-7. Suppression (%) of HIV-1 using TZM-bl cell *in vitro* model for RC_F2000 (RC-101 2000 µg/film) and placebo film. Dilution factor represents how many times the sample was diluted in growth medium.

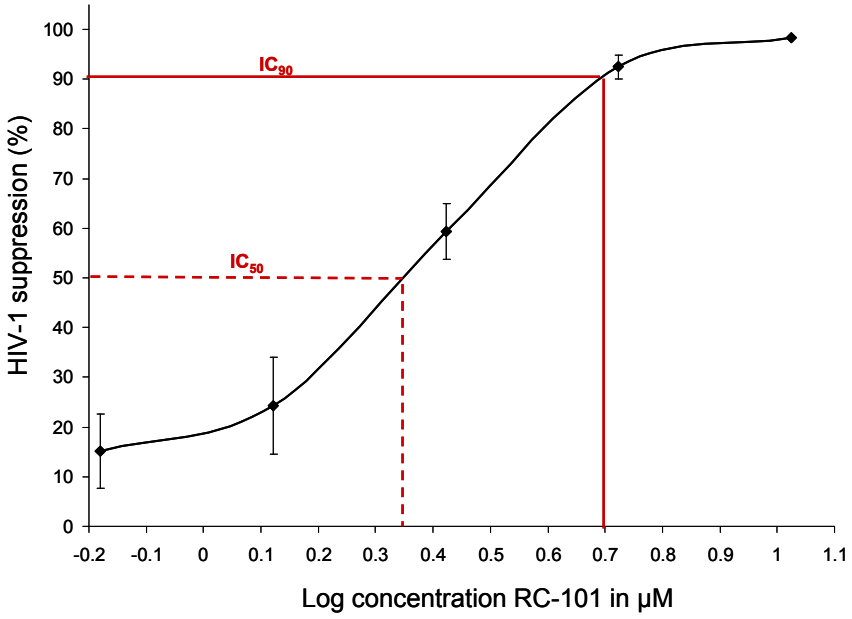


Figure 5-8. Suppression (%) of HIV-1 versus log RC-101 concentration (µM).
RC_F2000 using TZM-bl cell *in vitro* model. Continuous line shows IC_{90} at 90% HIV-1 suppression. Dotted line shows the IC_{50} at 50% HIV-1 suppression.

RC_F2000 films were tested using the e*x vivo* explant model of human ectocervical tissue, and the results are shown in Figure 5-9. Activity of **RC_F2000** was observed up to a 20 fold dilution of the films. **RC_F2000** films upon dilution of 100 times still demonstrated some effectiveness against HIV-1. However, the suppression after 100 times dilution was around 60%, which is below the minimal suppression required to be considered active against HIV-1 when using this *ex vivo* explant method (89, 161). A second graph was constructed by plotting the suppression of HIV-1 (%) versus the log of the final RC-101 concentration (µM) of each dilution (Figure 5-10). Since the suppression values were greater than 60%, it was not possible to estimate the IC_{50}. In this case, only IC_{90} was calculated and it was estimated to be 57.5 µM. The IC_{90} result for **RC_F2000** film using *ex vivo* model represents a 10 fold increase over the **RC_F2000** film tested using an *in vitro* model (IC_{90} = 5.0 µM). A decrease in IC_{90} observed between the models is expected. In a cell-based assay (*in vitro*), target receptors for RC-101 are

readily available, requiring a lower concentration of the peptide for the same inhibition effect. In contrast, in the *ex vivo* model, RC-101 must penetrate the tissue to attach to $CD4^+$ cells in addition to being available to attach to the glycoproteins in the virus, requiring a higher concentration to achieve the same virus inhibition of 90%.

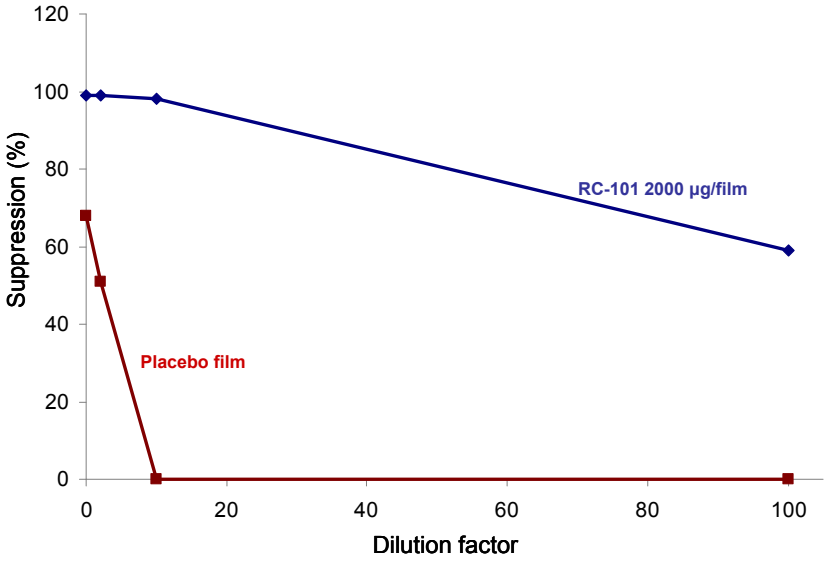


Figure 5-9. Suppression (%) of HIV-1 using human ectocervical tissue *ex vivo* model for determination of activity of RC_F2000 and placebo film.

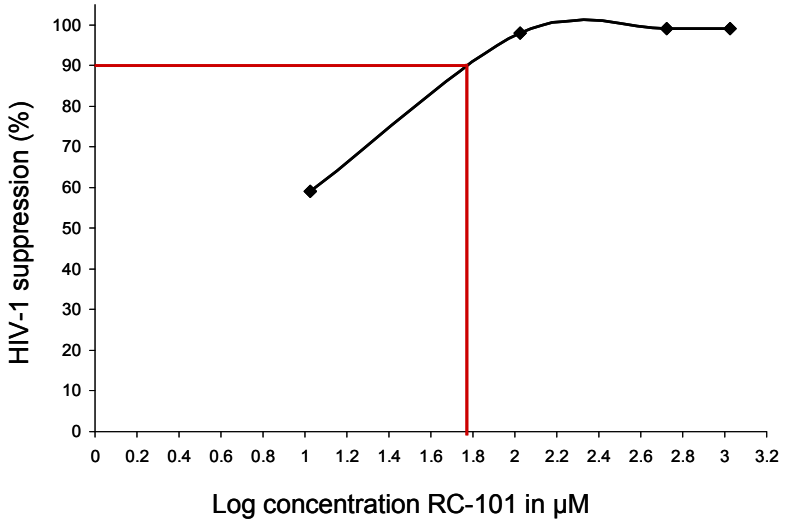


Figure 5-10. Suppression (%) of HIV-1 versus log RC-101 concentration (µM). RC_F2000 using an *ex vivo* ectocervical tissue model. Red line represents the IC_{90} at 90% suppression.

Ex vivo safety studies in human tissue

Results presented in the efficacy section show that RC-101 is effective against HIV using *in vitro* and *ex vivo* models. The next step is to determine if the formulated films are safe for female reproductive tract tissues. RC-101 films were exposed to human ectocervical tissue using a Franz-cell model. After 2 h exposure, tissues were removed and stained for histological evaluations. Hematoxylin and eosin (H & E) staining conducted for tissues exposed to placebo film with lactic acid, or **RC_F100,** or **RC_F+EDTA** are shown in Figure 5-11, Figure 5-12, and Figure 5-13, respectively. No gross changes in morphology were observed for all three samples when compared to the pre-exposure tissue or DMEM exposure, indicating that the RC-101 formulations and placebo are safe to human ectocervical tissue. Most importantly, when aliquots of the receptor phase of the Franz-cell system were analyzed by HPLC, no RC-101 was detected. This finding indicates that systemic uptake of RC-101 is not likely to occur. Further experiments conducted with RC-101 films with human and monkey reproductive tissues to verify permeability of RC-101 through these tissues are detailed and discussed in Chapter 6.

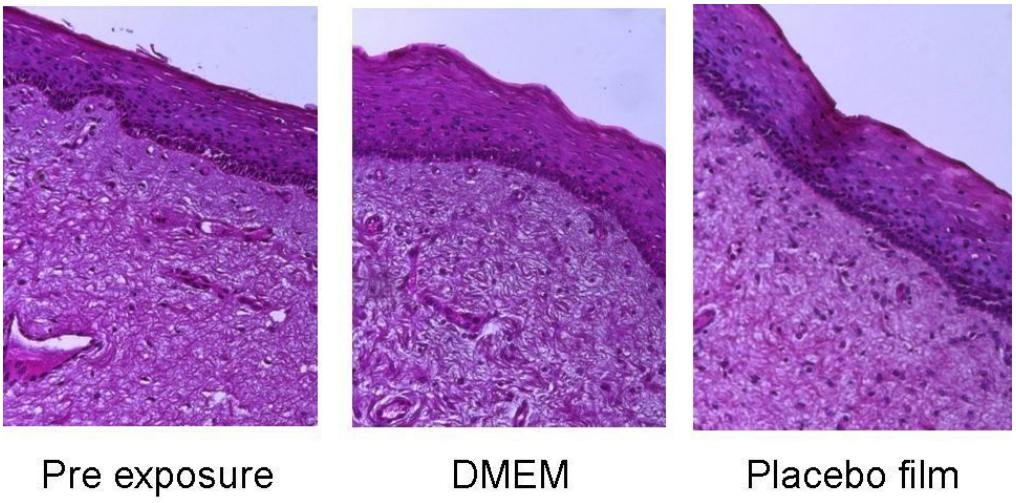


Figure 5-11. Histology results for Placebo film with lactic acid.
Pre exposure tissue, post exposure (2 h) to DMEM, and post exposure (2 h) to placebo film containing 0.8% lactic acid.

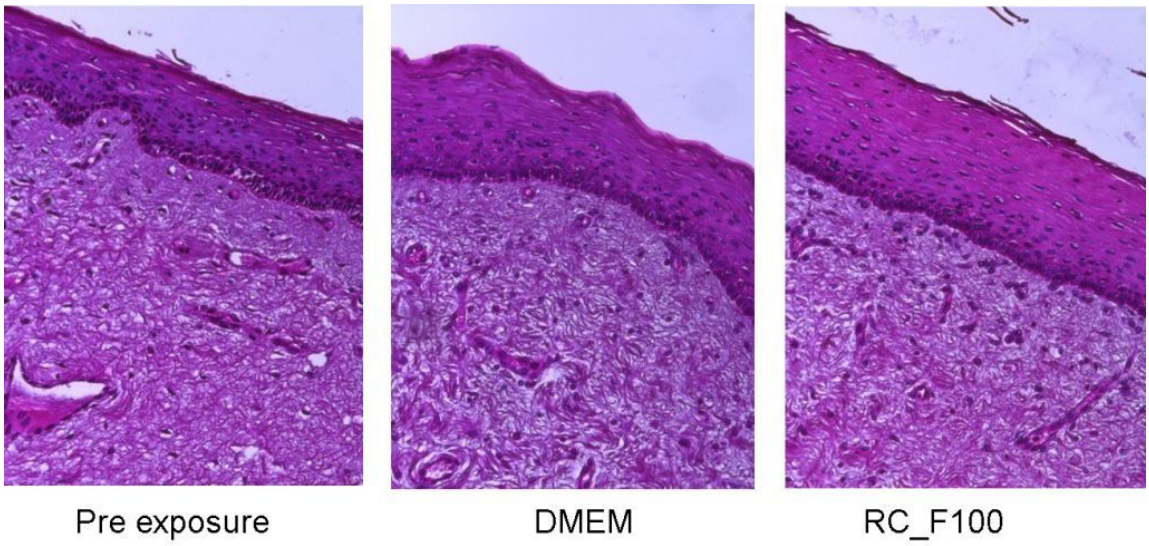


Figure 5-12. Histology results for RC_F100.

Pre exposure tissue, post exposure (2 h) to DMEM, and post exposure (2 h) to RC_F100 film.

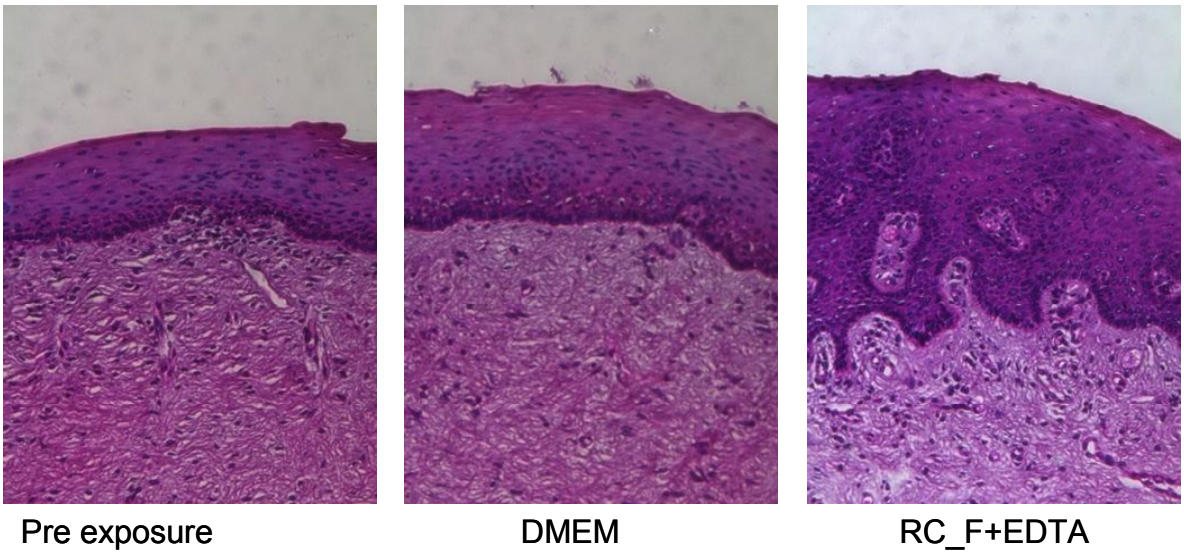
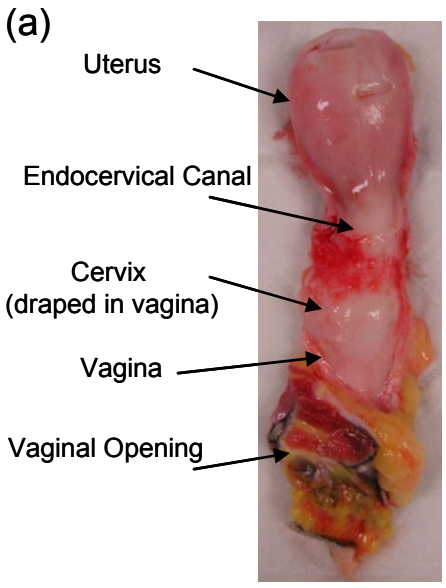


Figure 5-13. Histology results for RC_F+EDTA

Pre exposure tissue, post exposure (2 h) to DMEM, and post exposure (2 h) to RC_F+EDTA film.

Ex vivo safety studies in full monkey reproductive tract

Safety studies for RC-101 films were conducted in the *Macaca nemestrina ex vivo* model using full excised reproductive tract. Figure 5-14 (a) shows the full reproductive tract of the excised monkey tissue. Figure 5-14 (b) shows the histology (H & E staining) of the biopsies obtained from the monkey tissue after exposure to the **RC_F+LA**. No gross morphological changes were observed throughout the whole reproductive tract after exposure to the films, suggesting that RC-101 and placebo formulations are safe to full monkey reproductive tract.



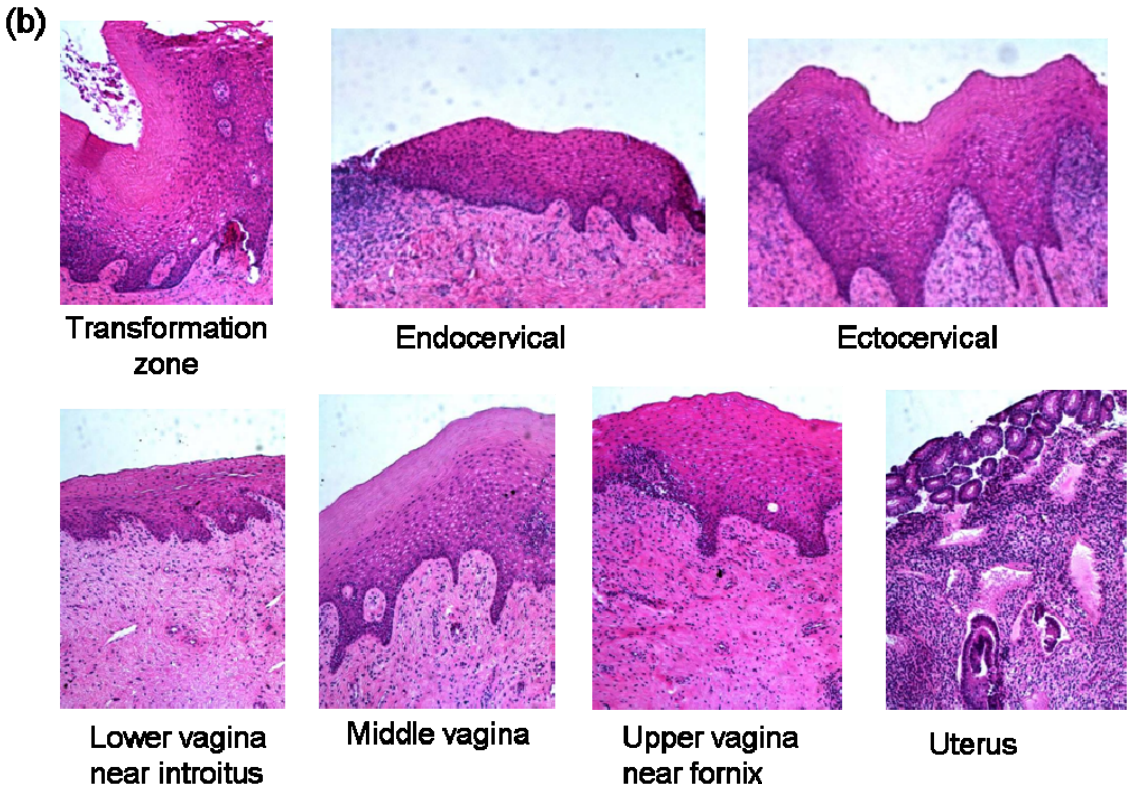


Figure 5-14. Safety studies in excised *Macaca nemestrina* reproductive tract tissue.
(a) Picture of a full reproductive tract of *Macaca nemestrina* before experiment was conducted.
(b) Histological evaluations of excised *Macaca nemestrina* reproductive tract tissue after exposure for 2 h with RC_F+LA. Stained with H & E.

In vivo safety studies

Evaluation of RC-101 films in an *in vivo* animal model was conducted using a film containing RC-101 at a concentration of 2 mg/film. The higher dose of RC-101 in the films used in this study was selected for two reasons. First, evaluation of safety of a product *in vivo* should be conducted at a higher dose to best verify the toxic effects, if any, of the product. Second, the RC-101 dose selected to be used in *in vitro* studies may not reflect the final dose of the product that will be selected when used *in vivo*. It is known that doses required for efficacy *in vivo* are 5 to 10 times higher than doses required for efficacy *in vitro*. For biopharmaceuticals, this increase

in dose can reach levels of 10^2 to 10^6 fold. In our studies, the dose selected for RC-101 film safety study *in vitro* was 1000 times higher than the IC_{50} of RC-101 *in vitro* (196).

In vivo safety evaluations were conducted as described in Figure 5-1, and Section 5.2.4.3. Colposcopy, pH and vaginal microflora determinations were the parameters used to evaluate safety of RC-101 films. The colposcopy of the cervix of representative animals before film insertion (**RC_F2000** or placebo film), at time 0, 30 min, and 24 h after the film insertion is shown in Figure 5-15. Complete disintegration of the film was observed. No visual changes were seen in the cervix following film exposure, indicating safety of the **RC_F2000** film in *in vivo* animal models. This procedure was repeated after each film application (total of 4 films) and no visual changes were observed in any of the colposcopic evaluations.

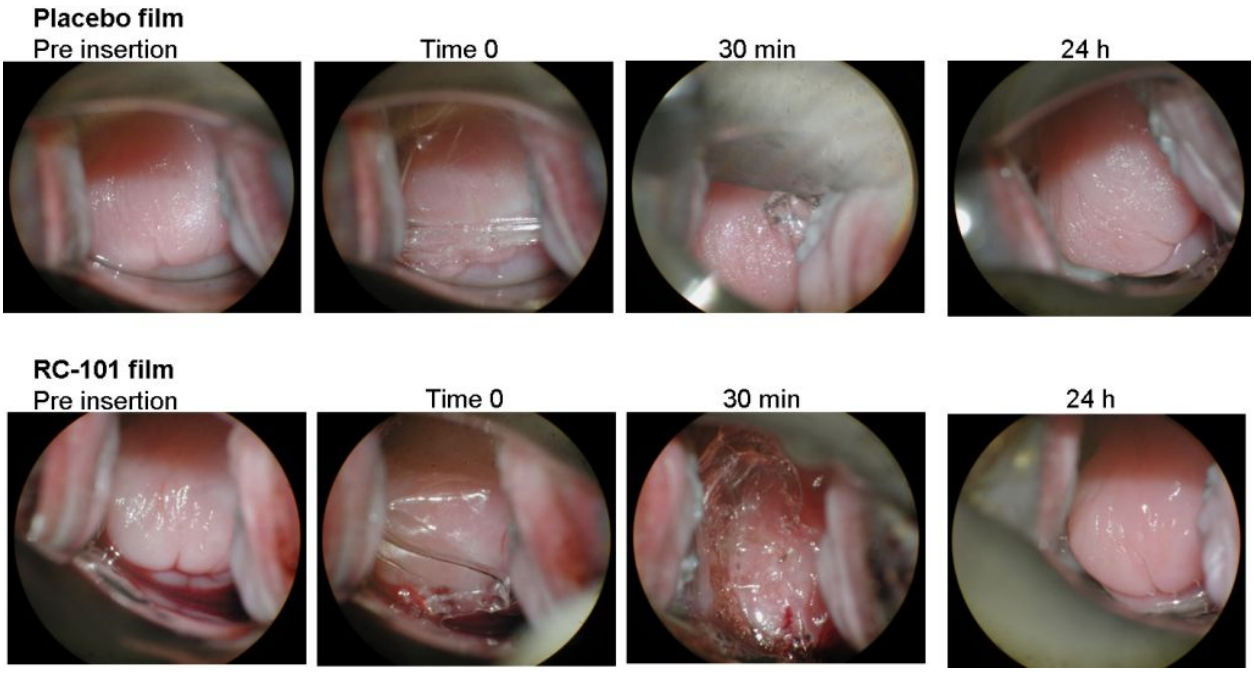


Figure 5-15. Colposcopy results for placebo and RC-101 2mg/film (RC_F2000).
Images from colposcopy examination of cervical and vaginal mucosa of non-human primates prior to insertion of films (placebo or RC_F2000), at time 0 after insertion, 30 min and 24 h after insertion.

Vaginal pH was monitored 30 min after film application and at days 5 and 8 after the last film application for continuous follow-up. No significant changes in pH were observed after application of RC-101 film or placebo; the pH remained within the normal range of vaginal pH.

Vaginal microflora examined showed detection of hydrogen peroxide-producing *Lactobacilli*, other *Lactobacilli* species, hydrogen peroxide *Viridians*, and black anaerobic gram negative rods. Identification of the species was not performed. A small decrease in lactobacillus was observed on days 2 and 3 of the study; however, full recovery to baseline levels was detected in the follow-up assessments. *Staphylococcus aureus* was detected in 3 animals after exposure to RC-101 films, and in one animal after exposure to placebo films. Microbiological evaluation of vaginal microflora after application of RC-101 and placebo films (days 1 to 4, 5 and 8) revealed that no significant changes were observed in the flora composition evaluated prior to film application.

5.4 DISCUSSION AND CONCLUSIONS

Film formulation for vaginal drug delivery has several advantages over alternative formulations. When compared to semi-solid gels, film products are easy to apply, quickly release the drug, and can improve the stability of drugs that are sensitive to water. In contrast, conventional vaginal gel dosage forms are often associated with limitations of poor retention in the vaginal lumen, leakage, and messiness. In addition, there may be incompatibilities of peptide drugs with some conventional dosage forms. Based on the advantages of vaginal films as a drug delivery system for microbicides, films were selected as the final dosage form of RC-101.

RC-101 was formulated into a quick dissolving polyvinyl alcohol (PVA) film. PVA is a polymer prepared from polyvinyl acetate, primarily used in topical pharmaceutical formulations. It is generally regarded as a nontoxic material and it is approved by the Food and Drug Administration (FDA) for vaginal use.

RC-101 film formulation was optimized to obtain the most suitable formulation that would assure RC-101 stability during the shelf-life. Initial film formulations (**RC_F+LA**) contained 0.8% lactic acid in an attempt to maintain an acidic pH and thereby to simulate the conditions found in a vaginal environment. There is a certain limitation for a polymeric film dosage form to maintain the vaginal pH. In contrast to vaginal gels, the distribution and final volume of a film is dependent on the vaginal fluids present in the vaginal lumen. Initial development of a vaginal film included lactic acid to maintain the acidic pH in the vagina; however no tests were conducted to confirm this idea. Evaluation of stability of RC-101 in the film upon storage at 25 °C and 40 °C/ 75% RH showed loss of RC-101 content over time, indicating a short-term stability of this formulation. Despite the fact that the film formulation was safe to be used in human ectocervical tissue, films proved to be toxic when used with TZM-bl cells for determination of bioactivity. When decomposition of the gel was conducted and individual excipients were analyzed, it was found that lactic acid was the component that was cytotoxic. Lactic acid was removed from the formulation to avoid any toxicity to the cells during evaluation of *in vitro* activity against HIV-1. Since RC-101 was shown to be stable over a range of pH 3 to 7 (Chapter 3), there were no detrimental effects on drug stability in the formulation without lactic acid. The second formulation prepared without lactic acid (**RC_F100**) also demonstrated short-term stability of RC-101 over time, at 25 °C and at 40 °C/ 75% RH. A third formulation was prepared with the addition of EDTA as a chelating agent to

delay the oxidation process of RC-101. As shown in the preformulation studies (Chapter 3), RC-101 was stable for 30 days in the presence of hydrogen peroxide when EDTA was added to the solution. EDTA is a common pharmaceutical excipient that sequesters metal ions with its carboxylate and amine groups. Metal ions act as a catalyzer for cysteine oxidation, which are present in the peptide RC-101. Films prepared in this study contained a concentration of 0.05% EDTA and displayed no toxicity when exposed to human ectocervical tissue using single exposure in an *ex vivo* model. However, no chronic exposure to human ectocervical tissue was investigated and depending on the dose regimen for this product (coital-dependence), chronic exposure to the films should be evaluated. Preliminary studies in our laboratory have shown that EDTA is toxic to the human ectocervical tissue *in vitro* and may affect normal vaginal flora at a concentration of 0.1% in solution.

Films prepared with EDTA (**RC_F+EDTA**) did not show a significant improvement on stability results, as evidenced by a loss of RC-101 observed by HPLC over time. About 30% loss of RC-101 was observed after films were stored for 45 days at room temperature, similar to what was observed in the films without EDTA. The loss of RC-101 observed over time must be further investigated. In the HPLC analysis of all three film formulations, no additional peaks have been observed that would indicate the mechanism of degradation of RC-101. The HPLC method developed in Chapter 3 and used for stability studies in this chapter, is a stability indicating assay for RC-101 degradation by oxidative pathways and thermal degradation. Interestingly, preliminary studies have shown that the bioactivity of RC-101 films after storage for 6 months is maintained. RC-101 films provided more than 90% suppression of the HIV-1 *in vitro* (personal communication – Dr. Alexander Cole, University of Central Florida). This result indicates that disappearance of the RC-101 HPLC peak can be attributed to a mechanism of

degradation different than oxidation or thermal degradation, that does not affect bioactivity. RC-101 may be aggregating with the PVA polymer, decreasing detection of the parent peak by HPLC. Further investigations of RC-101 stability in films are being conducted to: 1) confirm bioactivity of RC-101 films against HIV-1 over time to understand if modification of the drug compromises bioactivity; 2) evaluate interaction of RC-101 with other polymers to verify compatibility; and 3) identify other analytical methods that will facilitate extraction of RC-101 from the polymer.

In vitro HIV-1 efficacy studies have been used extensively to screen microbicide candidates. Most of the *in vitro* methods published are cell-based assays to determine the suppression of HIV-1 by an investigational compound (15, 89, 199). In our studies, we have investigated the efficacy of RC-101 films against HIV using an *in vitro* cell assay and an *ex vivo* model. Results for *in vitro* assays show an IC_{90} of 5.0 μM, and an IC_{50} of 2.2 μM. In one study reported to evaluate inhibitory concentration of RC-101 tested against 27 primary isolates of HIV-1, it was shown that the IC_{50} was less than 1.25 μg/mL (0.66 μM) (107). Our results suggest that formulation of RC-101 into films required a concentration three times higher than RC-101 in solution for a 50% viral inhibition. This increase in the IC_{50} may be attributed to the lower availability of RC-101 in the formulation as compared to solutions.

More recently, *ex vivo* explant models using human ectocervical tissue (67, 81) have been investigated to assess efficacy of microbicide candidates. In a study by Cole *et al*., it was demonstrated that RC-101 (10 μM) prevented HIV-1 infection by more than 90%, in a biologically relevant explant model using cervicovaginal engineered epithelial tissue (109). In our studies, RC-101 formulated into film evaluated in an *ex vivo* model displayed an IC_{90} of 57.5 μM. Results suggest that formulation of RC-101 into films required a concentration about 5

times higher than RC-101 in solution for 90% viral inhibition. Again, the increase in the IC_{90} may be attributed to the lower availability of RC-101 in the formulation when compared to solutions.

An important objective of pharmaceutical drug development is to correlate *in vitro* with *in vivo* results. Experiments conducted in this study have yielded the effective concentrations of RC-101 in the formulation to inactivate 90% of HIV-1 in an *in vitro* and in an *ex vivo* model. Values obtained in the *ex vivo* model were 10 fold greater than what was observed in the *in vitro* model. An increase in inhibitory concentrations going from the *in vitro* to the *ex vivo* model is expected. In the drug development process, efficacy and toxicity studies are conducted starting with less complex systems and progressing to systems of greater complexity, finally ending with human studies. *In vitro* assays are used as a screening tool for new pharmaceutical compounds, followed by studies in small animals, larger animals, and ultimately clinical trials in humans. *Ex vivo* models represent a higher degree of complexity than cell-based assays, but do not represent an ideal *in vivo* situation. In microbicide development, *ex vivo* studies represent an alternative to investigate protection against HIV, and provide an additional screening tool. A cell-based assay is constituted of a monolayer of cells and does not represent the squamous epithelial structure of ectocervical and vaginal mucosa. Specifically for RC-101, in a cell-based assay (*in vitro*), target receptors for RC-101 are readily available. RC-101 binds to gp120 of HIV and CD4 receptors in immune cells. In contrast, in the *ex vivo* model, RC-101 must penetrate tissue before it can attach to the majority of CD4 receptors on immune cells, requiring a higher concentration to achieve the same virus inhibition of 90%. In addition, non-specific binding of RC-101 to other proteins or to biological matrices in an *ex vivo* experiment may decrease the efficacy of the peptide. As presented in Chapter 4, when RC-101 is combined with human vaginal fluids, the

peptide is found to be present in the supernatant of the fluid and also in the epithelial cells sloughed from the vaginal walls. The binding of RC-101 to epithelial cells can decrease amount of RC-101 available to bind to glycoproteins. On the other hand, the binding to epithelial cells may serve as a depot for RC-101 to be released later, prolonging the anti-HIV activity. In addition, it can offer protection from HIV infection for epithelial cells, in case the theories that HIV can infect epithelial cells are correct, as discussed in Section 1.5. Investigation of a microbicide candidate alone also results in different efficacy data when compared to a microbicide formulation. Non-specific binding of RC-101 to the polymers of the formulation may require a higher concentration to achieve the same IC_{90}. Further studies should be conducted to evaluate the efficacy of RC-101 in animals. An animal efficacy study is being planned and will provide valuable information for estimation of the *in vivo* dose.

Evaluation of tissue (vaginal and cervical mucosa) damage caused by product exposure is part of the pre-clinical development of any successful vaginal formulation. Preclinical activity and toxicity data are typically generated very early in the drug development process. According to the FDA, this information then serves as the basis for a preliminary risk/benefit assessment to determine product suitability for Phase I/II clinical testing. It is also recommended that toxicology studies be conducted in at least two animal species (one of which a non-rodent) and should use the intended route of exposure (i.e., vaginal administration), to assess acute, sub chronic and chronic toxicity. The rabbit is typically the non-rodent species of choice for vaginal irritation studies (200-202) because of the similarity of the rabbit and human menstrual cycle and tissue morphology (41). However, this model failed to predict the vaginal toxicity caused by nonoxynol-9 in humans (203, 204), which moved the microbicide field towards the development

of new models to assess toxicity. Other animals studied are mice (205-207), and non-human primates (208).

Before the use of animal models, an *in vitro* or *ex vivo* model is necessary to assess the safety of a formulation. Excised human vaginal and ectocervical tissue has been extensively used in our laboratory to assess the changes in morphology after exposure to the vaginal microbicide solution or the final product formulation. In the research presented in this chapter, none of the film formulations induced gross changes in morphology in human ectocervical tissue, indicating the relative safety of the formulation to human tissue.

The pig-tailed macaque, *Macaca nemestrina*, has been used as a model for human vaginal and cervical physiology, safety, and for study of transmission of sexually transmitted infections. Recently, this model has been applied to evaluate topical vaginal and rectal microbicides (209, 210). It has been demonstrated that the vaginal microflora found in the *M. nemestrina* is very similar to the vaginal flora in humans (210). However, some limitations to the model apply. In contrast to the human vagina, the pig-tailed macaque has a smaller vaginal depth (4 cm), and a cervical diameter of 1.5 cm (209). Human vagina has a depth of 7 cm, and a cervical diameter of 3.25 cm. Another limitation of this model is the vaginal pH range, which is 4.0 to 8.5 in the macaque compared to 3.5 to 5.0 in humans (209). Even with these limitations, the macaque model is still the closest model to humans available for safety studies. When the animal safety protocol for RC-101 films was being developed, a pilot study to determine the appropriate size of the film was conducted. Placebo films were prepared in three different sizes and sent to Dr. Patton (University of Washington) for evaluation in monkeys. Films were inserted vaginally and disintegration time was determined. Small films were very difficult to manipulate and started to form a gel prior to complete insertion. In this study it was shown that

the smallest sized film (7 x 11 mm) dissolved in 30 min, the medium sized film (11 x 16 mm) dissolved in 45 min and the largest sized film (21 x 32 mm) dissolved in 60 min. Even though the largest film took the longest to dissolve, films continued to be formulated at the largest size, because of the difficulty in manipulating smaller film sizes in the macaque model.

Our *in vivo* studies conducted in the *M. nemestrina* demonstrated that RC-101 films at a dose of 2000 µg/ film are safe when applied daily for 4 consecutive days. There was no evidence of changes in pH, microbiology, and colposcopy when comparing either untreated animals with placebo films or films containing RC-101. Since the early 1990s in the development of vaginal products, colposcopy is the established method to assess vaginal irritation and detect epithelial changes that may increase the likelihood of HIV or acquisition of other sexually transmitted diseases assessing (211).

In this Chapter, we have described RC-101 formulation development, efficacy from a cell based assay to an *ex vivo* model, and safety from an *ex vivo* model to an animal study. Major results show that RC-101 was successfully formulated in a quick-dissolve polymeric film. This film demonstrated to be efficacious and safe in an *in vitro* method used. The safety of RC-101 films in animals after multiple exposures to a high concentration RC-101 film further validates this peptide as a promising microbicide. Stability of RC-101 films over time still requires further investigation prior to completion of the formulation development. While efficacy studies in animals still needs to be conducted, important findings from this research provide a significant contribution to the further development of RC-101 into a successful microbicide product. In addition, the rational process of microbicide development presented here may be considered as a template to be used for the systematic development of other microbicide molecules.

Investigation of penetration of RC-101 in human and monkey female reproductive tracts are presented in Chapter 6.

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6.0 PERMEABILITY AND TISSUE LOCALIZATION OF RC-101

6.1 INTRODUCTION

More than 25 years have passed since the discovery of the human immunodeficiency virus (HIV), and the numbers of infected people have reached pandemic levels. A feminization of the epidemic has been observed, which is attributed to the difficulty for women to negotiate condom use and the higher transmission and infection rate from male to female relative to female to male (11). Considering that many men are often unwilling to use condom and women are more susceptible to HIV, strategies to prevent heterosexual transmission of HIV must include female-controlled methods (25).

In this scenario, the idea of a topical microbicide product appears to be one of the best options for HIV prevention. A microbicide product would be applied vaginally or rectally before intercourse, to prevent transmission and acquisition of HIV (26-28). During male to female transmission, virus in semen penetrates the stratified squamous epithelium of the vagina and ectocervix or the columnar epithelium of the endocervix to infect immune cells within and below the epithelium (79, 80). It is still uncertain if the virus must cross the epithelium or if the Langerhans cells (LC) will reach out for the virus. However, studies have indicated that HIV is present in the epithelial mucosa and penetrates the mucosa in a gradient distribution (212).

RC-101 is among several microbicide drugs that have been investigated. It is a circular, 18-residue peptide, with three cysteine disulfide bonds. Activity of RC-101 against HIV *in vitro* has been shown against X4 and R5 strains of HIV-1 (102), making this peptide a potential microbicide agent to prevent mucosal transmission of HIV-1 (102, 106). The mechanism of

action of RC-101 against HIV occurs by high affinity binding to CD4, galactosylceramide (alternative cell surface receptor for $CD4^-$ cells), and gp120. RC-101 operates by preventing six-helix bundle formation of gp41, thereby conferring protection against HIV-1 (106).

The dual mechanism of action observed for RC-101 (binding to the glycoprotein of the virus and binding to the receptor of the host cell) offers a strong incentive to develop RC-101 as a topical vaginal microbicide product. In order to take advantage of this dual mechanism, RC-101 must be targeted to both the epithelium and vaginal lumen.

Important findings described throughout this dissertation work have validated RC-101 as a promising microbicide candidate, encouraging further investigation of the molecule. Preformulation studies (Chapter 3), compatibility with human vaginal fluids (Chapter 4), and formulation and safety (Chapter 5) of RC-101 were investigated. The key observations indicate that RC-101 is stable in biological fluids, possess activity against HIV, and is safe. In this chapter we further investigate the ability of RC-101 to penetrate into human ectocervical and endometrial tissue and monkey vaginal and endometrial tissue. The purposes of this study were to first investigate the permeability of human and monkey female reproductive tract tissues to RC-101 using an *ex vivo* system to characterize systemic absorption. And second, to analyze the distribution of RC-101 in human and monkey reproductive tracts tissue to verify targeting to immune cells when delivered in a solution or a formulated state. Quantification of RC-101 in solutions was conducted by HPLC and immuno-dotblot. Detection of RC-101 in human and animal tissue was accomplished by immunohistochemistry, an immunostaining method, using a polyclonal antibody of RC-101.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Retrocyclin-1 (RC-101) was synthesized by the Peptide Synthesis Facility at the University of Pittsburgh (Pittsburgh, PA). Polyvinyl alcohol (PVA) was obtained from Kuraray America Inc. (New York, NY). Glycerin was purchased from Dow Chemical Company (Midland, MI). Hydroxypropylmethylcellulose (HPMC) 6 cps was purchased from Sigma (St. Louis, MO). All other materials were obtained from Fisher Scientific (Fair Lawn, NJ) unless specified otherwise.

6.2.2 Quantification and detection of RC-101

Quantification by High Performance Liquid Chromatography (HPLC)

An HPLC system (Waters Corporation, Milford, MA) was used for the analysis of RC-101 in the formulation as previously described (Section 3.2.2). Briefly, the HPLC system was equipped with an autosampler model 717 (Waters), a quaternary pump model 600 (Waters), and an ultraviolet (UV) detector model 2487 (Waters). Chromatographic separation of RC-101 was achieved by using a Jupiter 5 μ C5 column 4.6 x 250 mm, 300 Å (Phenomenex, Torrance, CA) protected by a guard cartridge Widepore C5 4 x 3.0 mm (Phenomenex). Empower PRO, Empower 2 software (Waters) was used to control the HPLC system.

RC-101 quantification by quantitative immuno-dotblot

Aliquots from permeability studies were frozen at -80 °C and sent to the laboratory of Alexander Cole, Ph.D. (University of Central Florida, Orlando, FL). Samples were processed as previously

described by Cole *et al*. (109). Briefly, a known volume of each sample was dried using a ThermoSavant speed vacuum (ThermoSavant, Waltham, MA), and resuspended to the original volume in 0.1% acetic acid. Extracts (4 µl/dot) were dotted onto a polyvinylidene fluoride (Immobilon-P) membrane. Results were expressed as a percent of the RC-101 in the donor compartment at the start of the experiment.

RC-101 detection by immunohistochemistry

Detection of RC-101 in the tissue was conducted as previously described by Cole *et al*. (109). Briefly, unstained tissue sections were deparaffinized, washed in tris-buffered saline (TBS), and incubated overnight with anti-RC-101 or preimmune serum, each diluted in TBS containing 1% gelatin, 0.05% Tween 20 and 0.01% thimerosal (TBS-T). Following three 20 min washes in TBS-T, an alkaline phosphatase-conjugated secondary antibody was applied at 1:2000 dilution and the sections were left overnight at room temperature. The next day, the slides were washed three times for 20 min in TBS-T, developed with Fast Red (Sigma, St. Louis, MO), and counterstained with Harris hematoxylin.

6.2.3 Permeability and tissue localization protocol

6.2.3.1 Excised human tissue and monkey tissue

Human cervical and endometrium tissue

Freshly excised human ectocervical and endometrium tissue were obtained from the Tissue Procurement Facility at Magee-Womens Hospital (Pittsburgh, PA), according to approved IRB protocol #0503103. Tissue was obtained from 18 premenopausal women, 30 to 50 years old, undergoing hysterectomy for benign conditions. No specimens were used when there was

evidence of tissue abnormality that might have influenced the state of the mucosa. All tissue specimens were obtained within 1 h of surgical excision, and held at 4 °C in Dulbecco's Modification of Eagles Medium (DMEM) (Mediatech Inc, Herndon, VA) during transfer from surgery to the laboratory.

Excised macaque reproductive tract

Reproductive tracts from sexually mature female *Macaca nemestrina* were obtained from a colony at the Washington National Primate Research Center (Seattle, WA) as previously described in Chapter 5. Animals were euthanized and the whole reproductive tract was removed and transported to Magee-Womens Research Institute (Pittsburgh, PA) in ice within 24 h. Upon arrival, the tissue was warmed to 37 °C and immediately used for the experiments.

6.2.3.2 Experimental set up

RC-101 film and solution samples

A quick-dissolving polymeric vaginal film formulation containing RC-101 was used in these studies and compared to unformulated RC-101 in a solution of 100 mM phosphate buffered saline (PBS), pH 7.4. RC-101 and placebo films were formulated as described in Chapter 5. Briefly, vaginal films were prepared by casting a polymeric film solution containing PVA, glycerin, and HPMC. Two formulations have been used for the permeability studies and tissue localization tests: an initial film formulation (**RC_F+LA**) containing 0.8% of lactic acid to maintain an acidic pH to simulate the conditions found in a vaginal environment; and a second formulation without lactic acid (**RC_F100**). Both films were prepared at a dose of 100 µg RC-101 per film. Placebo films were prepared identically without the addition of RC-101. All films

were removed from the plates, weighed, and stored in PET/Aluminum foil pouches (Amcor Flexibles Healthcare Inc, Mundelein, IL) until further analysis. Prior to the experiments, each film was dissolved in 1 mL of PBS, pH 7.4. RC-101 100 µg/mL solution in phosphate buffered saline (PBS), pH 7.4 was used as a control (**RC_SOL**).

RC-101 tissue localization in excised full monkey reproductive tract tissue

RC_F+LA (n = 2) was evaluated in whole excised monkey reproductive tract as described in Chapter 5. Briefly, the excised monkey reproductive tract was warmed to 37 °C, and placed in a 15 mm Petri dish containing 2 mL of Milli Q water. The film product was inserted into the vaginal cavity using a glass rod. The reproductive tract was kept in a horizontal orientation for the first 30 min after insertion of the film. After that time, the tissue was oriented vertically for another 90 min. The protocol was designed to simulate the experimental conditions used in an *in vivo* setting as described in Section 5.2.4.3. The tissue was then removed from the incubator and rinsed (4 mL of water, 10 times) by inserting a 5 mL syringe in the vaginal opening. Lavage samples were collected, centrifuged at 10,000 rpm for 10 min, and the supernatant was separated for further analysis by HPLC and immuno-dotblot. A cytobrush was inserted and gently rotated 6 times into the cervical os. The cytobrush was then placed in 4 mL of water and vortexed. The tissue was cut open and 6 mm diameter (n = 3) biopsy samples were collected from each part of the tissue (lower vagina, middle vagina, upper vagina, transformation zone, cervix, and uterus), and stained for histology. Tissue biopsy samples were fixed using two procedures: one specific for evaluation of histological changes and the second one for RC-101 localization. Histological changes were evaluated by Hematoxylin and Eosin (H & E) staining following the procedure described in Chapter 5. Tissue fixation for localization of RC-101 was conducted by placing the

tissue in 4% paraformaldehyde solution and storing at 4 °C overnight. Tissue was then transferred to a solution of 75% (v/v) ethanol for 24 h, and subsequently embedded in paraffin. Immunohistochemistry (RC-101 localization) analyses were performed as described in section 6.2.2.

Permeability studies and tissue localization using Franz-cell apparatus

Permeability studies were conducted using a Franz-cell system previously described for the safety studies in Chapter 5, with a few modifications. For permeability studies, 4.5 mL phosphate buffered saline (PBS) pH 7.4 solution was used in the receptor chamber, which was continuously stirred by a magnetic stir bar. Permeability studies were conducted using excised monkey reproductive tract (vaginal and endometrium tissue), and excised human (ectocervical and endometrium) tissue. In the Franz-cell system, the tissue is sandwiched between the two compartments with the epithelial side facing the donor solution, with the apical side of the tissue exposed to the solution to be tested. The tissue was equilibrated with PBS in the donor compartment for 5 min prior to the initiation of the permeability study. After the equilibration period, the PBS solution was removed from the donor compartment and replaced with 400 µl of either RC-101 100 µg/mL solution in PBS (**RC_SOL**) or RC-101 100 µg/film (**RC_F+LA** or **RC_F100**) dissolved in 1 mL of PBS. An aliquot of 50 µL was removed from the donor compartment at time 0 for quantitative analysis of RC-101. Aliquots of 200 µl were removed from the receptor compartment at various time intervals for a total period of 6 h. Aliquots were replaced with 200 µL PBS (pH 7.4) to maintain sink conditions. All solutions (donor and receptor) were analyzed by HPLC for quantification of RC-101. Since the limit of quantification for RC-101 in the HPLC assay is 7.9 µg/mL, the amount of RC-101 in the receptor phase would

be below the limit of quantification in the receptor compartment, but it is still above the limit of detection of the method. Therefore, any leaks in the tissue would be identified by the HPLC method. For quantification purposes, all receptor solutions were additionally analyzed by immuno-dotblot. The limit of detection for the immuno-dotblot method is 0.25 µg/mL (109). Tissue samples after the experiment were processed and stained with H & E, embedded in paraffin, and cut into slides to be sent to Alexander Cole, Ph.D. (University of Central Florida) for staining with anti-RC-101, to determine RC-101 tissue localization as described in Section 6.2.2.

6.2.4 Statistical analysis

Data obtained from the permeability studies were expressed as concentration of RC-101 in µg/mL (mean ± S.D.). Results were analyzed with Mann-Whitney test for comparisons of each condition investigated in the permeability studies, without corrections for multiple comparisons. P-values ≤ 0.05 were considered to be statistically significant, unless specified otherwise.

6.3 RESULTS

<u>RC-101 tissue localization in excised monkey reproductive tract</u>

Full excised monkey reproductive tract studies were conducted using tissues from two animals exposed to **RC_F+LA** (RC-101 100 µg/film with lactic acid). Lavage samples obtained after a 2 h exposure to RC-101 film were centrifuged and supernatant was separated from the cells. Supernatant lavage samples were analyzed by HPLC for quantification of RC-101; however, no

RC-101 was detected in any lavage sample analyzed. The same samples (supernatant and cells) were sent for testing by immuno-dotblot. Results are presented (Table 6-1) as the percent of RC-101 recovered from the original amount of RC-101 introduced in the monkey reproductive tract, for supernatant, cells, and total amount. A low concentration of RC-101 was detected in the recovered lavage. RC-101 was found in the epithelial cells sloughed from the vaginal walls during lavage and in the supernatant. In tissue 1, about 80% of the amount of RC-101 found was present in the cells. In tissue 2, about 40% of RC-101 was present in the cells.

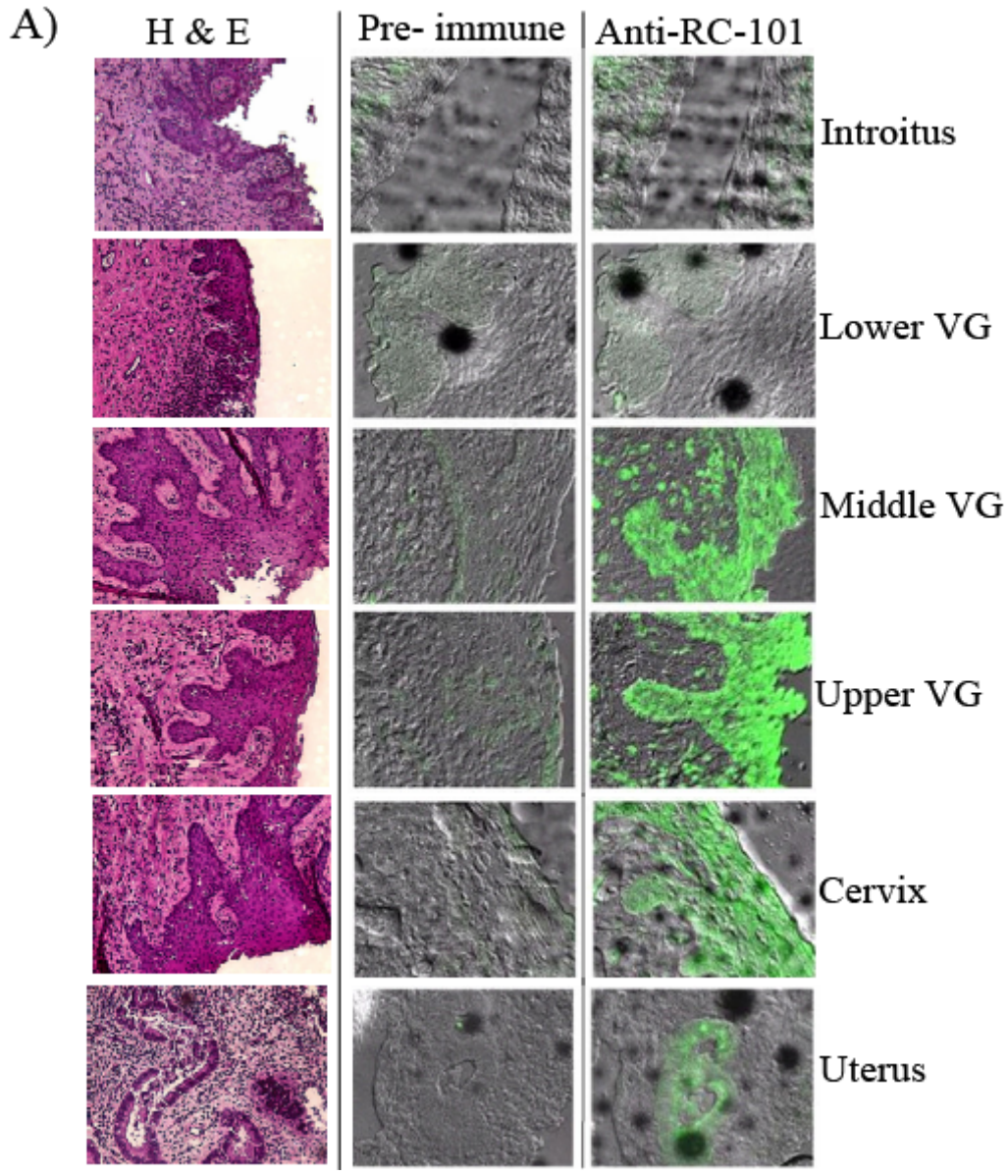
Table 6-1. RC-101 in lavage samples expressed as percentage of the amount introduced.

Description	Supernatant RC-101 (%)	Cells – RC-101 (%)	Total RC-101 (%)
Tissue # 1	1.91	8.14	10.04
Tissue # 2	1.94	1.57	3.51

Immuno-dotblot analysis of the first lavage samples obtained from full monkey reproductive tract exposed to RC_F+LA.

After exposure for 2 h with RC-101 vaginal film (**RC_F+LA**), full reproductive tract tissue was cut and biopsies were used for determination of RC-101 localization in the tissue. RC-101 staining for each tissue is shown in Figure 6-1. The figure shows the staining by H & E to demonstrate that there were no gross morphological changes that would provide evidence of damage to the epithelial layer, the tissue pre-immuno staining, and after the RC-101 staining (green fluorescent). In the tissue from both animals, the staining (green fluorescent) shows that RC-101 is present throughout the entire reproductive tract and is more concentrated in the cervix, and middle and upper vagina. When RC-101 films are placed vaginally in the full reproductive tract, the films are placed near the cervix and covered the upper and middle vaginal wall.

Physical localization of the film explains the higher presence of RC-101 in the cervix, middle and upper vagina. Evidence of localization of RC-101 in the tissue can be shown by the presence of RC-101 in the epithelial layer suggesting its penetration into the mucosa.



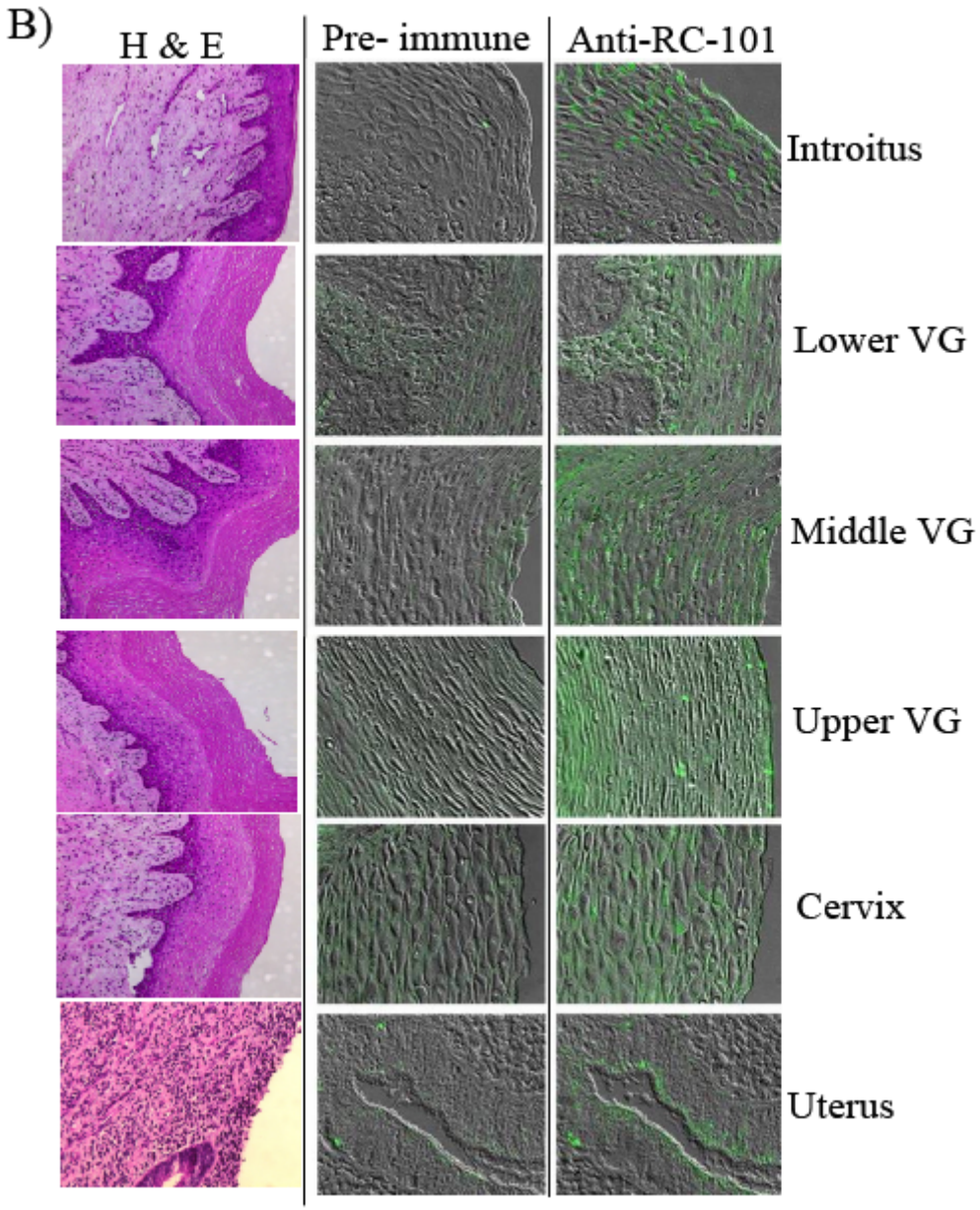


Figure 6-1. RC-101 tissue localization in excised monkey whole reproductive tract.

H & E staining, pre-immune and after anti-RC-101 immunohistochemistry staining of excised *Macaca nemestrina* reproductive tract tissue after exposure for 2 h with RC-101 100 µg/ film (RC_F+LA). Pre-immune staining and anti-RC-101 staining show the overlayed images of FITC (green fluorescence) and DIC (differential interference contrast). A) Monkey tissue 1, and B) Monkey tissue 2.

Permeability studies and tissue localization using the Franz-cell apparatus

After finding evidence that showed localization of RC-101 in the full reproductive tract and suggestion of RC-101 penetration into the epithelial mucosa, further investigations using excised monkey and human tissue were conducted. To evaluate permeability and tissue localization of RC-101 in these tissues, a Franz-cell system was utilized.

Monkey vaginal (VG) and endometrium (UT) tissue were exposed to RC 100 µg/mL solution (**RC_SOL**), RC-101 100 µg/film with lactic acid (**RC_F+LA**), and RC-101 100 µg/film without lactic acid (**RC_F**). The concentration of RC-101 in the receptor compartment was analyzed by both HPLC and immuno-dotblot. No RC-101 was detected in the receptor solution at any time point by HPLC assay. However, immuno-dotblot results showed no more than 10% of RC-101 was detected in the receptor compartment at the end of the experiment, indicating that low permeability of RC-101 through the tissue occurred. The solution from the donor compartment was analyzed by HPLC at time 0 and at the end of the experiment. The concentration (µg/ml) of RC-101 in the donor compartment at the end of the experiment (6 h) was determined and plotted for each tissue obtained (each subject) and for each exposure condition (**RC_SOL**, **RC_F+LA**, and **RC_F**). Results for monkey tissues are shown in Figure 6-2. Closed symbols represent monkey vaginal tissue and open symbols represent monkey endometrium tissue. Circled symbols represent tissue obtained from one animal that presented a high level of parakeratosis and extensive inflammation, with some ulceration of the tissue. Due to the small number of tissues analyzed, no statistical calculations were conducted. Results from the permeability study presented in Figure 6-2, suggest that RC-101 penetrates the tissue as evidenced by the decrease in the amount of RC-101 detected in the donor compartment at the end of the experiment. In some tissues, no decrease in the donor compartment was observed. To

normalize the data, and eliminate variability in the thickness of the tissue caused by hand-microtome, a graph with diffusion of RC-101 in monkey reproductive tissues was calculated and plotted (Figure 6-3).

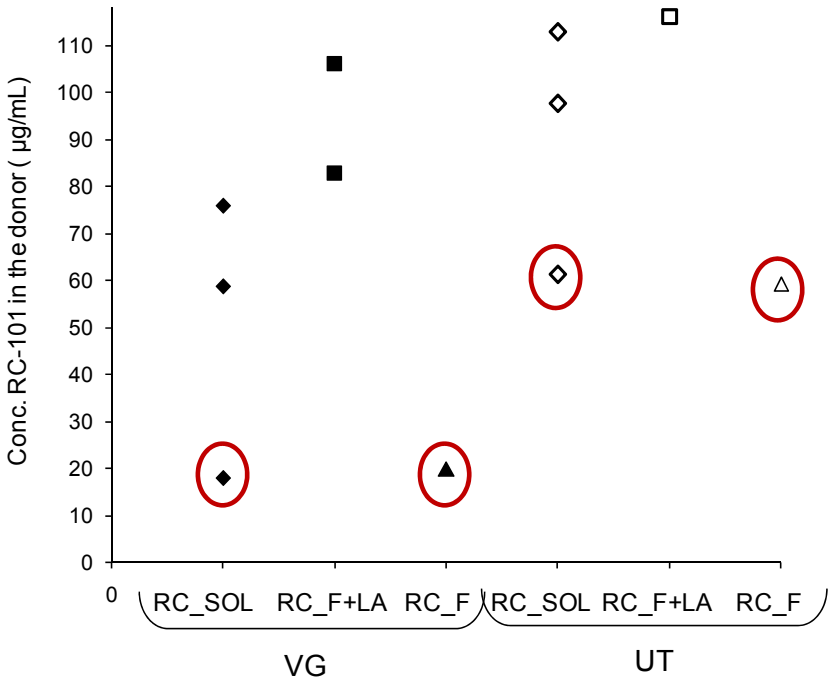


Figure 6-2. RC-101 permeability in monkey female reproductive tract tissue using the Franz-cell system for 6 h. Circled symbols represent tissue obtained from one animal that exhibited a high level of parakeratosis and extensive inflammation, with some ulceration of the tissue. Closed symbols represent monkey vaginal tissue (VG). Open symbols represent monkey endometrium tissue (UT).

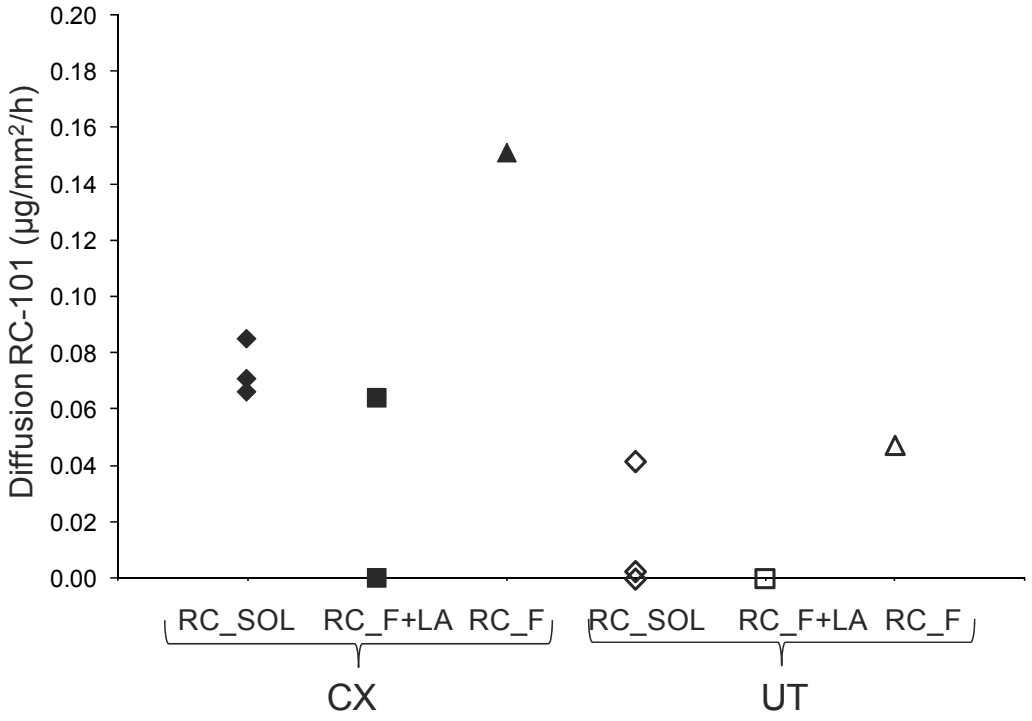


Figure 6-3. RC-101 diffusion (µg/mm^2/h) into monkey female reproductive tract tissue using the Franz-cell system for 6 h. Closed symbols represent monkey vaginal tissue (VG). Open symbols represent monkey endometrium tissue (UT).

With the evidence presented that RC-101 penetrates into monkey tissue, a more extended study was performed using human ectocervical and endometrium tissue in a Franz-cell system. Human ectocervical (CX) and endometrium (UT) tissue were exposed to RC 100 µg/mL solution (**RC_SOL**), RC-101 100 µg/film with lactic acid (**RC_F+LA**), or RC-101 100 µg/film without lactic acid (**RC_F**). The concentration of RC-101 in the receptor compartment was analyzed over time; however, no RC-101 was detected in the receptor solution by HPLC assay at any time point. When immuno-dotblot was used, no more than 10% was detected in the receptor compartment at the end of the experiment, indicating that low permeability of RC-101 through the tissue occurred. The solution from the donor compartment was analyzed by HPLC at time 0

and at the end of the experiment. The concentration (µg/ml) of RC-101 in the donor compartment at the end of the experiment (6 h) was determined and plotted for each tissue obtained (each subject) and for each exposure condition (**RC_SOL**, **RC_F+LA**, or **RC_F**). Results for human tissues are shown in Figure 6-4. Closed symbols represent human ectocervical tissue and open symbols represent human endometrium tissue. Results from the permeability study conducted presented in Figure 6-4 suggest that RC-101 penetrates into the tissue as evidenced by the decrease in the amount of RC-101 detected in the donor compartment at the end of the experiment for most of the tissue samples studied.

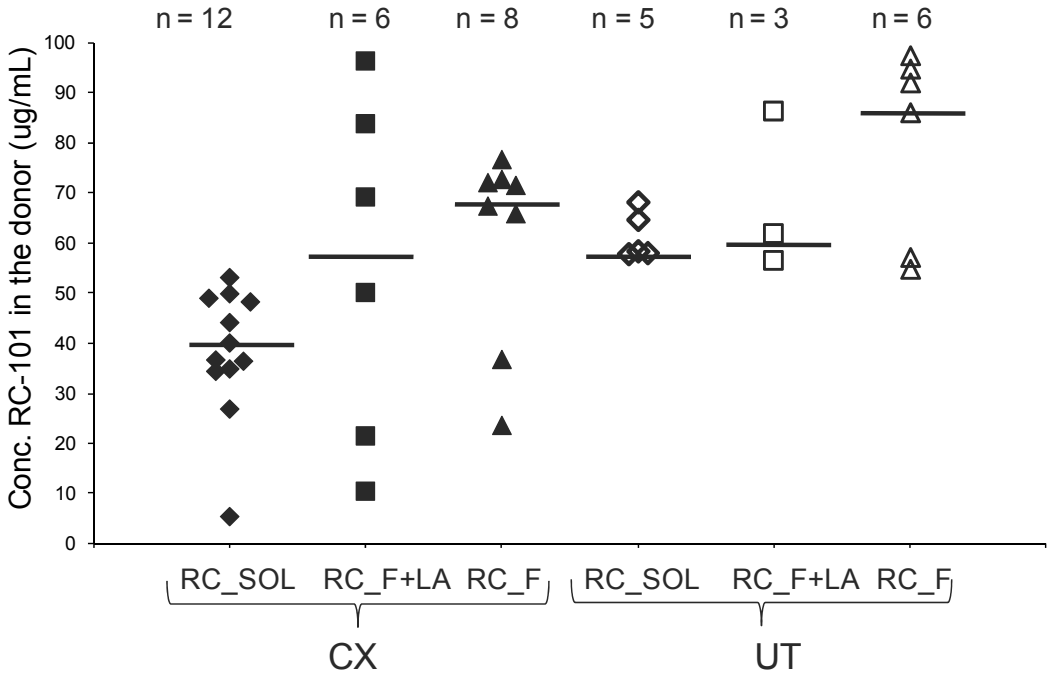


Figure 6-4. RC-101 concentration (µg/mL) in the donor compartment in human tissues using the Franz-cell system, after 6 h incubation. Closed symbols represent human ectocervical tissue (CX). Open symbols represent human endometrium tissue (UT). Bars represent the median for each condition.

In an attempt to understand the variability observed for each condition, the pathology reports were evaluated and patient demographic data identified by means of an honest broker. No pathological conditions could be correlated with the differences in the results obtained for permeability studies. As a representative example, some demographic data was identified and shown in Figure 6-5, Figure 6-6, and Figure 6-7. The phase of the menstrual cycle is shown in Figure 6-5, where the circles represent women in the secretory phase of their menstrual cycle. Women between the ages of 45 and 50 years old (circled data) are identified in Figure 6-6, and race is represented in Figure 6-7, where the circled data represents African American women.

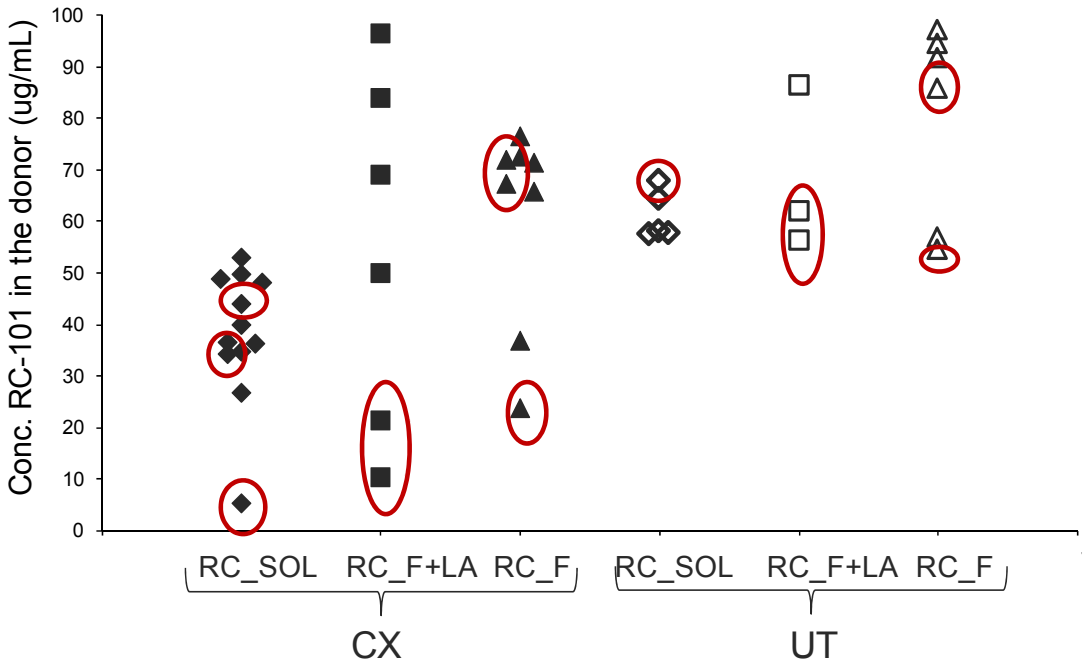


Figure 6-5. Evaluation of menstrual cycle phase for RC-101 concentrations (µg/mL) in the donor compartment in human tissues using the Franz-cell system, after 6 h incubation. Closed symbols represent human ectocervical tissue (CX). Open symbols represent human endometrium tissue (UT). Circles represent women during the secretory phase of their menstrual cycle.

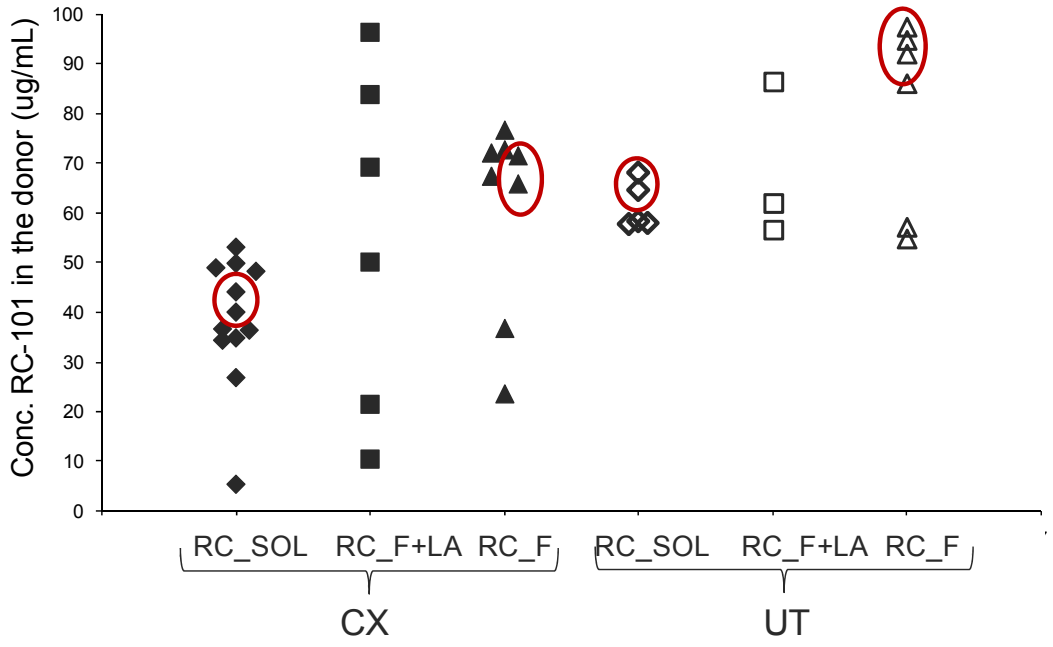


Figure 6-6. Evaluation of age for the RC-101 concentrations (µg/mL) in the donor compartment in human tissues using the Franz-cell system, after 6 h incubation. Closed symbols represent human ectocervical tissue (CX). Open symbols represent human endometrium tissue (UT). Circles represent women in the ages between 45 and 50 years old.

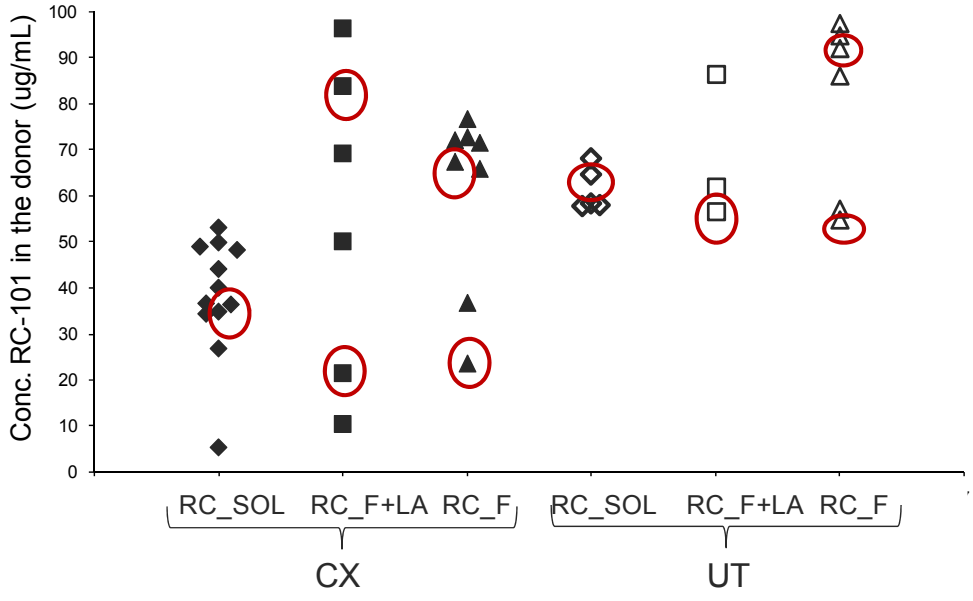


Figure 6-7. Evaluation of race for the RC-101 concentrations (µg/mL) in the donor compartment in human tissues using the Franz-cell system, after 6 h incubation. Closed symbols represent human ectocervical tissue (CX). Open symbols represent human endometrium tissue (UT). Circles represent African American women.

No correlation was observed between the results obtained from permeability studies and any of the demographic variables analyzed. Overall, there is a statistically significant difference in the concentration of RC-101 obtained in the donor compartment between **RC_SOL** and **RC_F** when used in human ectocervical tissue (Mann-Whitney, $p = 0.02$), and no statistically significant difference between **RC_SOL** and **RC_F** when used in human endometrium tissue ($p = 0.36$). When the same samples were statistically compared to evaluate the difference in the type of tissue, it was observed that a significant statistically difference was observed between ectocervical and endometrium tissue for **RC_SOL** ($p = 0.02$), and no statistical difference was observed for **RC_F** ($p = 0.12$). Two major findings are important in these studies. First, both tissue types (ectocervical and endometrium) showed penetration of RC-101 by evidence of decrease in the donor compartment concentration of RC-101. Second, no difference in penetration was observed between RC-101 solution (**RC_SOL**) and RC-101 film (**RC_F**). However, a difference was observed when the permeability results were corrected for the thickness of the tissue as discussed below.

To diminish the tissue thickness variability due to the experimental method used to isolate the epithelial layer, calculations of the diffusion of RC-101 through the tissue were made. In these calculations, the amount of RC-101 that diffused into a cross-sectional area of the tissue over time was plotted for each subject and each condition. Results are shown in Figure 6-8. A statistically significant difference ($p < 0.05$, Mann-Whitney test) was observed in all conditions showing that there is a difference between **RC_SOL** and **RC_F**, and a difference between tissue types (ectocervical and endometrium). There is a lower diffusion of RC-101 through the endometrium when compared to human ectocervical tissue.

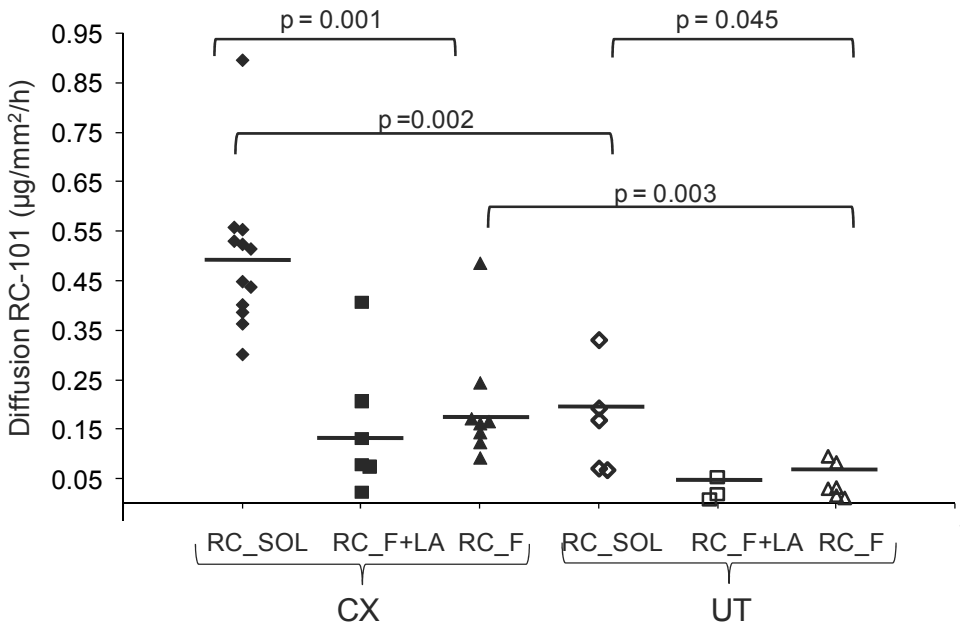


Figure 6-8. RC-101 diffusion (µg/mm^2/h) in human female reproductive tract tissue using the Franz-cell system. Closed symbols represent human ectocervical tissue (CX). Open symbols represent human endometrium tissue (UT). Bars represent the median for each condition. P values were calculated using Mann-Whitney test.

After permeability studies were conducted for RC-101 in the Franz-cell system, tissues were processed and stained for localization of RC-101 by immunostaining with a polyclonal RC-101 antibody. Results for the monkey vaginal and endometrium tissues are shown in Figure 6-9 A and Figure 6-9 B, respectively. Both figures show three pictures of each tissue. In the first picture, the staining by hematoxylin and eosin (H & E) demonstrates that there were no gross morphological changes after exposure to the sample that would evidence damage to the epithelial layer. The second picture shows the negative control for the RC-101 immunostaining, the tissue pre-immune staining (PRE). And the third picture shows the tissue after the RC-101 staining (green fluorescent) (POST). RC-101 in monkey tissue was mostly detected in the upper epithelial layer of the mucosa. No differences were observed between tissues exposed to **RC_SOL** or **RC_F+LA**.

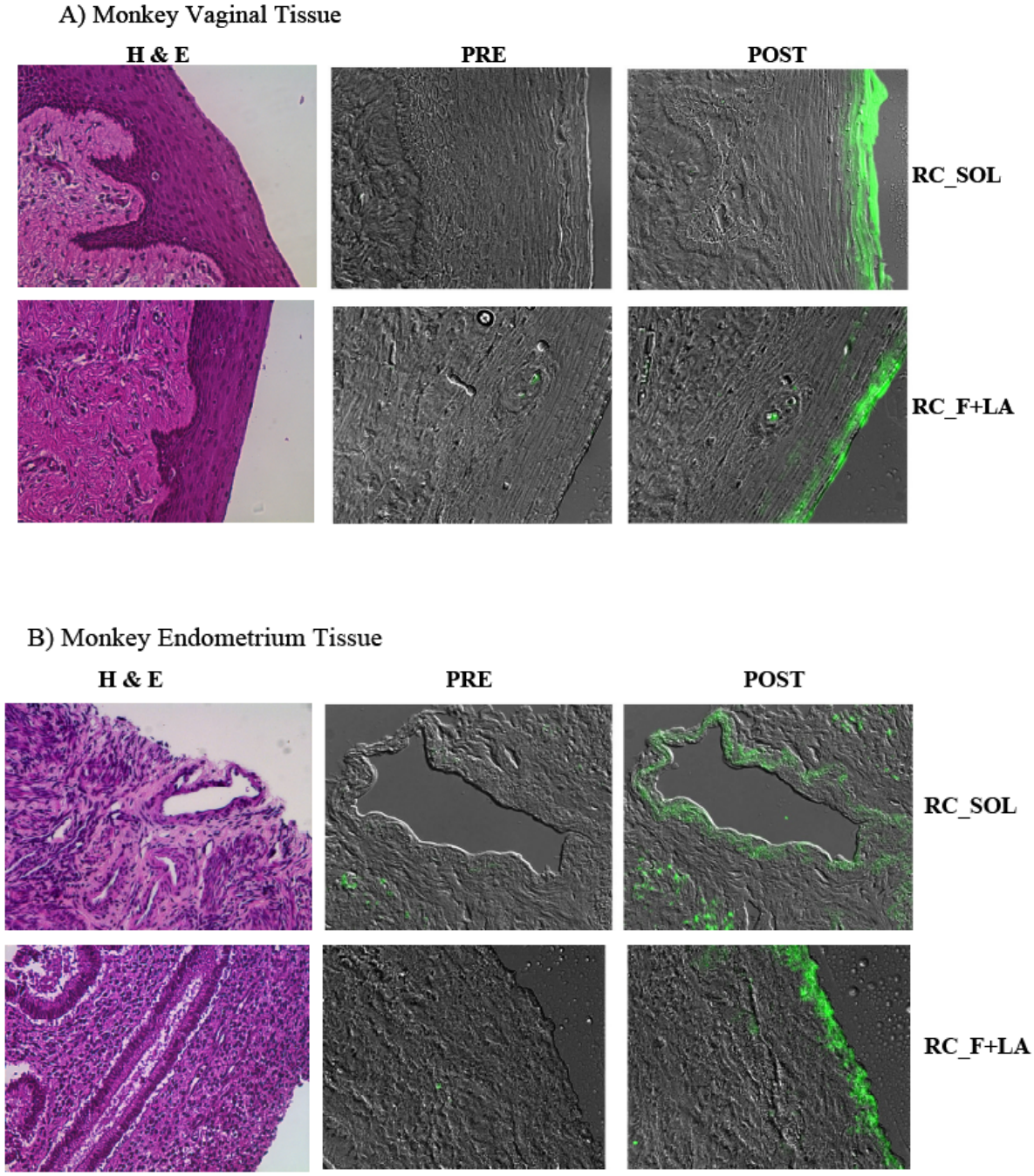


Figure 6-9. RC-101 tissue localization in monkey tissue.

Exposure of RC-101 solution and film to monkey vaginal and endometrium tissue. Hematoxylin and Eosin (H & E) staining of tissue post experiments; pre-immune staining (PRE); and after anti-RC-101 staining after exposure (POST) to RC-101 solution (RC_SOL) or RC-101 film with lactic acid (RC_F+LA). A) vaginal tissue; B) endometrium tissue.

Localization of RC-101 for human ectocervical tissue in the proliferative and secretory phase of the menstrual cycle are shown in Figure 6-10 A and Figure 6-10 B, respectively. Localization of RC-101 for human endometrium tissue in the proliferative and secretory phase of the menstrual cycle are shown in Figure 6-11 A and Figure 6-11 B, respectively. Both figures show three pictures of each of the tissues in the same way as was presented for the monkey tissue. In the first picture, the staining by hematoxylin and eosin (H & E) demonstrates that there were no gross morphological changes after exposure to the sample, providing no evidence of damage to the epithelial layer. The second picture shows the negative control for the RC-101 staining, the tissue pre-immune staining (PRE). And the third picture shows the tissue after the RC-101 staining (green fluorescent) (POST). RC-101 was mostly detected in the upper epithelial layer of the human ectocervical and endometrium mucosa. No differences were observed in tissues exposed to **RC_SOL** (RC-101 solution), **RC_F+LA** (RC-101 film with lactic acid) or **RC_F** (RC-101 film). Similar results were obtained in the other replicates. Only one tissue showed a slight penetration of RC-101 into the deeper layers of the epithelial mucosa. Data obtained from the RC-101 localization studies in monkey and human excised tissues indicate that RC-101 localizes in the upper epithelial layers of the mucosa.

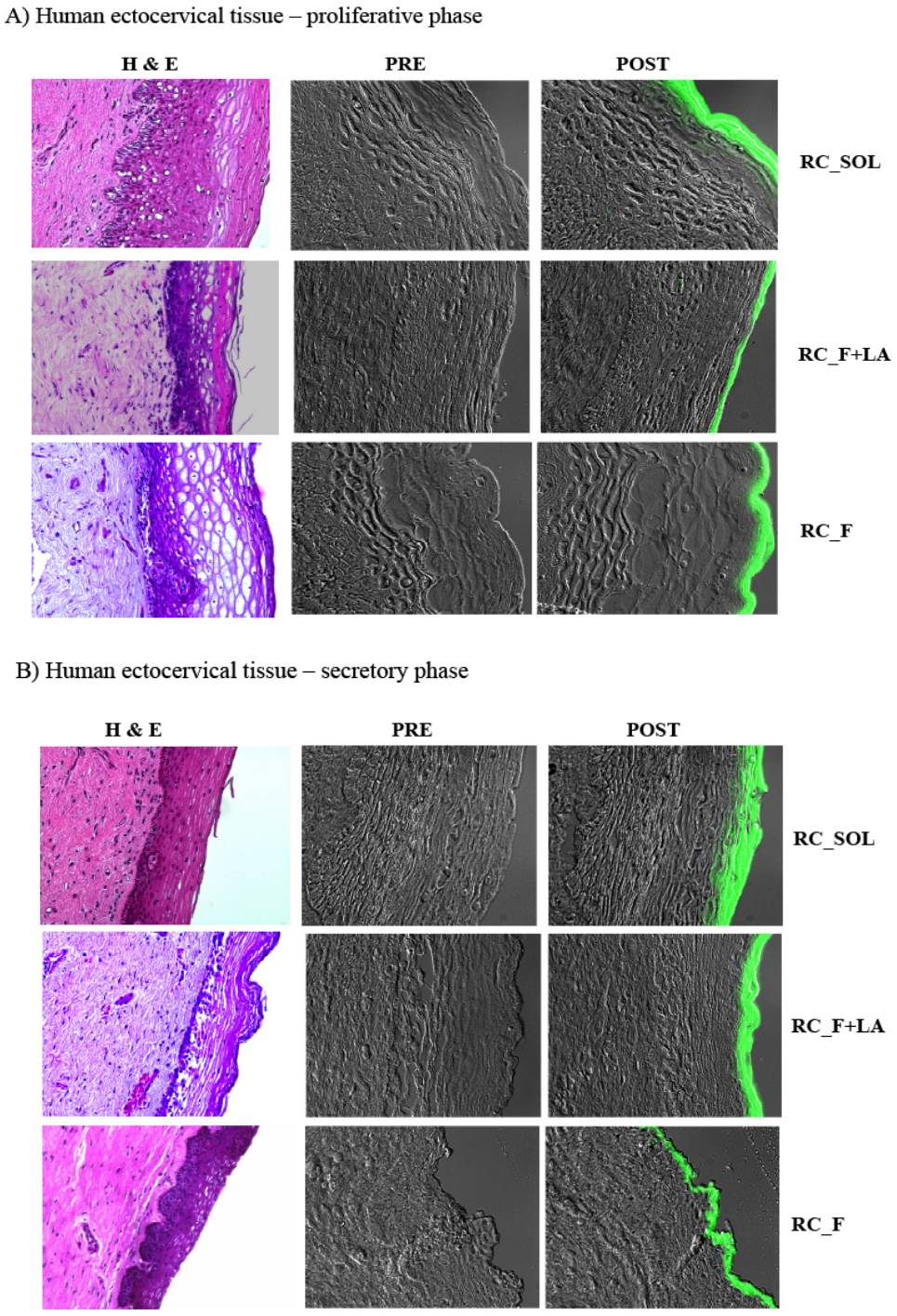


Figure 6-10. RC-101 tissue localization in human ectocervical tissue.

Exposure of RC-101 solution and film to human ectocervical tissue. Hematoxylin and Eosin (H & E) staining of tissue post experiments; pre-immune staining (PRE); and after anti-RC-101 staining after exposure (POST) to RC-101 solution (RC_SOL), RC-101 film with lactic acid (RC_F+LA), or RC-101 film (RC_F). A) proliferative phase; B) secretory phase.

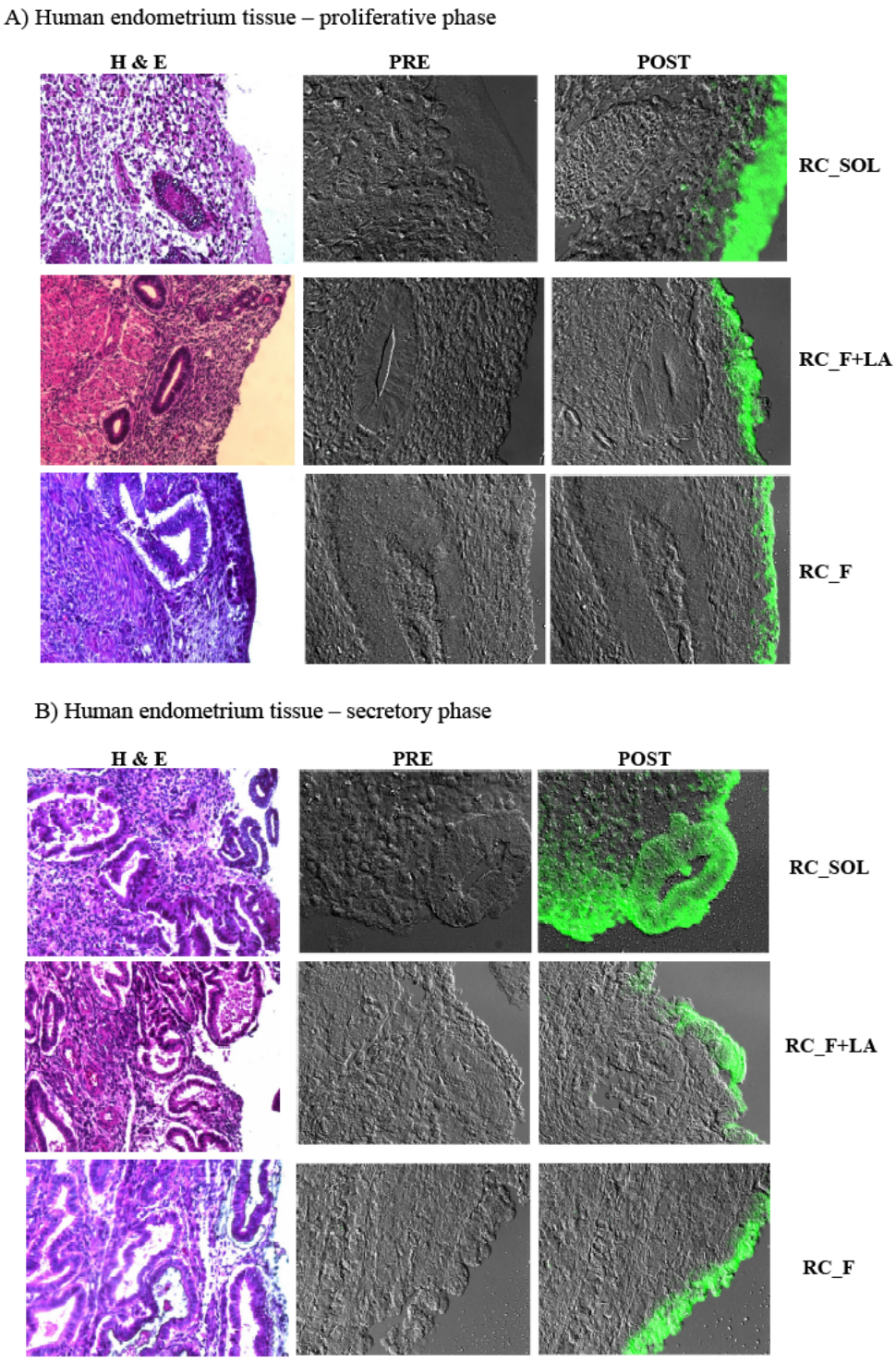


Figure 6-11. RC-101 tissue localization in human endometrium tissue.

Exposure of RC-101 solution and film to human endometrium tissue. Hematoxylin and Eosin (H & E) staining of tissue post experiments; pre-immune staining (PRE); and after anti-RC-101 staining after exposure (POST) to RC-101 solution (RC_SOL), RC-101 film with lactic acid (RC_F+LA), or RC-101 film (RC_F). A) proliferative phase; B) secretory phase.

6.4 DISCUSSION AND CONCLUSIONS

Topical vaginal microbicides are intended to prevent transmission of HIV and other sexually transmitted infections (STIs). The development of RC-101 into a vaginal microbicide film has been shown to be a promising HIV prevention method, as studied using *in vitro* and *ex vivo* models. This study focuses on the permeability and penetration of RC-101 into excised human and monkey reproductive tract as a means of offering a more targeted protection against HIV. This is the first study in the microbicide field performed to evaluate the presence of a microbicide candidate in full animal reproductive tract tissue as well as in excised human and monkey tissue.

During male to female transmission, virus in semen will penetrate the stratified squamous epithelium of the vagina and ectocervix or the columnar epithelium of the endocervix to infect target immune cells (macrophages, T cells, and dendritic cells), within and below the epithelium (79, 80). The majority of these cells are located in the subepithelial layers of vaginal and cervical mucosa. However, studies have confirmed the presence of Langerhans cells (LCs) in the epithelial mucosa (72, 81), and the ability of these cells to take up HIV-1. This uptake by LCs can extend near to, or into the mucosal lumen (85).

The mechanism of action of each microbicide agent will define the appropriate vaginal drug delivery system for those products. If a microbicide agent acts by blocking the receptor or co-receptor in the target host cells, it is imperative that the delivery system is able to deliver the drug to the site of action. Conversely, a microbicide agent that disrupts the viral membrane before attachment of the virus to the host cell can be delivered to the vaginal lumen without deeper penetration in the vaginal mucosa. Defining the specific site of action for a microbicide that targets immune cells has been the research focus of several groups (83, 212, 213). Studies

suggest that immune cells are more concentrated in the basal layers, but are present throughout the whole epithelial tissue (69-71, 83, 212, 213). As described in Section 1.3, normal cervical stratified squamous epithelium consists of several layers of epithelial cells. The layers can be subdivided into four different classes according to the stage of maturation: basal, parabasal, intermediate, and superficial. The presence of target cells throughout the whole epithelial mucosa makes it a challenge to effectively target the drug. Drug delivery systems should enable access to a broad distribution of target cells for the development of an effective and successful microbicide product.

In addition to several studies regarding the localization of immune cells, research groups have been focusing on understanding the mechanism of sexual transmission of HIV. As described in Section 1.5, several mechanisms have been proposed such as: direct infection of epithelial cells, transcytosis through epithelial cells, epithelial transmigration, uptake by intra-epithelial Langerhans cells, and circumvention of the epithelial barrier. However it is still uncertain if these mechanisms fully explain the HIV-1 transmission *in vivo*. Two schools of thought in the microbicide research field co-exist, explaining one of the HIV mechanisms of transmission. One of these schools suggest that HIV-1 could directly infect epithelial cells, through binding to alternative receptors of CD4 (82, 83), such as galactosylceramide. The other one has shown that HIV-1 is unable to infect epithelial cell lines, due to the lack of CD4 receptor and co-receptors in epithelial cells, even when galactosylceramide was detected (66, 84). However, it has also been suggested that epithelial cells can transfer HIV to activated immune cells, and may contribute to sexual transmission of HIV-1 (66). Both theories play a role and add to the complex matrix of HIV prevention strategies and to the identification of site of action and targets for microbicides.

Distribution of the vaginal microbicide product throughout the vaginal and cervical tissues may offer better protection against HIV. For semi-solid gel formulations, distribution of the vaginal gel may add additional mechanisms of protection against HIV by coating the mucosa and forming a mechanical barrier to the virus, and protecting against disruption of the epithelial mucosa by increasing lubrication. A few studies have assessed the distribution of microbicide product in the vaginal lumen in *in vivo* models (11, 121, 208). However, no study has analyzed the distribution of the drug in the tissue, either in an *in vitro* or an *in vivo* model. For semi-solid microbicides, spreadability of the gel product has been characterized by imaging the distribution of the product in the vaginal lumen (11, 121, 208). Polymeric vaginal films, such as the one developed for RC-101, are more dependent in the presence of vaginal fluids to disintegrate the film and distribute its drug contents throughout the vaginal canal. The quick dissolve film developed for RC-101 (Chapter 5) will rapidly release the drug upon disintegration of the film, to be readily bioactive.

Independent of the dosage form chosen, localization of the microbicide drug at the site of action is required for an efficacious microbicide product. According to the mechanism of action of RC-101, penetration of RC-101 into the tissue will provide an advantage to improve efficacy of the final microbicide product. We have conducted a RC-101 localization study using full excised reproductive tract monkey tissue. RC-101 films were vaginally inserted into excised full female monkey reproductive tract and immunostaining conducted after 2 h exposure to the film. Results have shown that RC-101 penetrated into the epithelial mucosa. Presence of RC-101 was evident throughout all epithelial layers, including submucosal and basal layers, as detected by immunostaining of RC-101. This result suggests that RC-101 penetrates the epithelial mucosa, being available for target immune cells. However, full excised tissues are obtained after

monkeys are euthanized, then the whole reproductive tract is removed, placed in media, and shipped on ice to our laboratory, taking up to 24 h to arrive. Upon arrival, the tissue is warmed to 37 °C and immediately used for the experiments. When a tissue is removed from the whole animal, it is immediately deprived of its physiological supportive and protective mechanisms. There is no supply of nutrients to the cells, no removal of waste products, and no maintenance of pH and osmotic pressure. Due to this fact, caution should be taken when interpreting these results. Future studies should include evaluation of RC-101 distribution in an *in vivo* animal model.

A number of experimental models for permeability studies have been reported in the literature (214-217). A simple *in vitro* method extensively used for intestinal permeability study utilizes Caco-2 cells. This method is easy to perform in large numbers. However, in this model there is a low paracellular permeability present and a lack of absorption barriers such as a mucus layer (214). More specific to vaginal and cervical permeability, studies using cultured human vaginal-cervical epithelial cells have been described (217-219). These methods utilizes human endocervical and ectocervical cell lines cultured on filters. The major disadvantage of using cell-based assays for permeability studies of vaginal and cervical tissues is that cell models are found in monolayers, which does not represent the multilayer squamous epithelium of vaginal and ectocervical tissues. More complex models, such as the Ussing chamber (220) and Franz-cell (221), have been investigated using excised human or animal tissues. The Ussing chamber consists of two half-chambers connected to electrodes, which measure the short-circuit current as an indicator of the transport taking place across an epithelium. The Franz-cell system also consists of two half-chambers. In both systems, the tissue is sandwiched between the two compartments, the whole apparatus is kept at 37 °C, and there is a possibility of sampling both

compartments over time. The Ussing chamber system allows for the monitoring of electrical resistance during the experiment and supplies oxygen in the donor compartment. In our studies, the Franz-cell system represented an advantage due to the fact that we could evaluate multiple pieces of tissue at the same time, and better investigate inter- and intra-patient variability.

Permeability of RC-101 was investigated using excised monkey and human tissues with a Franz-cell system. Monkey vaginal and endometrium tissues were exposed to RC-101 in solution and RC-101 in film formulation. None of the conditions tested showed significant concentrations of RC-101 in the receptor phase (less than 10% detected by immuno-dotblot), suggesting that systemic uptake of RC-101 will not be significant. Donor solutions from the Franz-cell were analyzed by HPLC to determine the amount of RC-101 that penetrated into the tissue during the exposure period. In the studies conducted for excised monkey tissue, penetration of RC-101 occurred for tissues exposed to RC-101 in solution (**RC_SOL**), RC-101 in film with lactic acid (**RC_F+LA**), or RC-101 film (**RC_F**). This penetration was observed for both tissue types: monkey vaginal and endometrium tissues. Due to the small number of tissues available for these studies, no statistical calculations were made.

Permeability of RC-101 into human excised ectocervical and endometrial tissue was also investigated using a Franz-cell system. The tissues were exposed to RC-101 in solution, or in film formulation. Similar to the results obtained in monkey tissue, none of the conditions tested showed significant concentration of RC-101 in the receptor phase (less than 10% detected by immuno-dotblot), suggesting that systemic uptake of RC-101 will not be significant. Donor solutions from the Franz-cell at time 0 and at the end of the experiment were analyzed by HPLC to determine the amount of RC-101 that penetrated the tissue. In the studies conducted for excised human tissue, penetration of RC-101 occurred in tissues exposed to RC-101 in solution

(**RC_SOL**), RC-101 in film with lactic acid (**RC_F+LA**), or RC-101 film (**RC_F**). Median values of RC-101 concentration in the donor compartment at the end of the experiment for human ectocervical tissue were 38.2, 59.6, and 36.8 μg/mL, for **RC_SOL**, **RC_F+LA**, and **RC_F**, respectively. Compared to the initial concentration of RC-101 (100 μg/mL) introduced to the Franz cell in the beginning of the experiment, permeability studies suggest that more than 60% of RC-101 penetrates into the tissue when **RC_SOL**, or **RC_F** is used, while more than 40% of RC-101 penetrates when **RC_F+LA** is used.

The RC-101 film formulation currently being pursued in the laboratory is the **RC_F**. Calculation of the median concentration of RC-101 in the donor compartment following exposure to **RC_F** shows that 63.2 μg/mL was lost from the donor compartment during the exposure period. Assuming that this concentration penetrated into the tissue and given the fact that the IC_{90} for the **RC_F2000** film described in Section 5.3 using an *ex vivo* model was estimated to be 57.5 μM (108.6 μg/mL), the results suggest that about 60% of the RC-101 necessary to suppress 90% of HIV is within the tissue. This concentration of RC-101 would be available to bind to CD4. The remaining 40% of RC-101 would remain in the vaginal lumen where it would be available to bind to glycoproteins of the virus, taking advantage of the dual mechanism of action of RC-101.

The variability observed in the individual values obtained for RC-101 concentration was investigated. No known demographic variables such as age, race, menstrual cycle or any other pathological condition observed in the histological sections of the tissue pre- and post-experiment could explain the variability. We have evaluated some of the characteristics of the tissue utilized in the study in order to understand the variability observed. However, each characteristic (race, age, and menstrual phase) analyzed by itself could not be correlated to the

varied results of RC-101 concentration obtained. The discrepancy in the results can be attributed to inter- and intra-patient variability, which is an intrinsic characteristic of vaginal and ectocervical tissues. Despite the variability observed, the major finding in this study is that penetration of RC-101 occurred over a 6-hour period in all 18 tissue samples analyzed including both tissue types (ectocervical and endometrium). This penetration is evidenced by the decrease in the donor compartment RC-101 concentration and the positive staining for RC-101 in the mucosal tissue.

In addition to patient variability, some experimental artifacts can contribute to the difference observed in the concentration values. Human tissues utilized in the Franz-cell system originate from fresh human tissue from women undergoing hysterectomy. This tissue is sectioned by the use of a hand-microtome, causing inconsistency in the thickness of the slices obtained. The thickness of the each section is measured prior to the experiment. To minimize the variability in the thickness encountered, permeability results were calculated as diffusion coefficient by calculating the amount of RC-101 (μg) per cross-section area of the tissue (mm^2) per time (h). Evaluation of diffusion coefficients obtained did not result in a reduction of the observed variability.

Correcting for differences in overall tissue thickness may not reduce variability due to the fact that differences in the number of epithelial layers may occur. One study has reported a difference in the number of cell layers according to the menstrual cycle (222). To evaluate the number of cell layers and epithelial immune cells, vaginal biopsies obtained from premenopausal women during 3 phases of the normal menstrual cycle (menstrual period, preovulatory, and postovulatory) were utilized. The mean number of epithelial cell layers underwent a small but statistically significant decrease from menstrual period to post ovulatory period, which can

contribute to the different drug permeability profiles being obtained. However, no significant changes were observed in the number of Langerhans cell, macrophage, CD4 or CD8 lymphocyte, and neutrophil cell populations during the three phases of the cycle (222), indicating that penetration of microbicide drugs into the tissue must occur to be able to protect against infection. This penetration is important in those microbicide drugs whose site of action requires targeting of the immune cells.

Because the transmission mechanism of HIV is not completely elucidated, it is important that the microbicide candidate be present throughout the epithelial mucosa with the objective of targeting the immune cells that would be available to the virus. Localization of RC-101 in the epithelial tissue after exposure to **RC_SOL** or **RC_F+LA** was evaluated after 6 h exposure using the Franz-cell system. When monkey excised vaginal and endometrium tissue was analyzed, immunostaining of RC-101 showed that the peptide is present in the upper epithelial layer of the tissue. No differences were observed in the penetration pattern of RC-101 in formulation or in a solution, or in different tissue types studied (vaginal and endometrium). Immunostaining of RC-101 after 2 h exposure to RC-101 film (**RC_F+LA**) into a full monkey reproductive tract showed that RC-101 penetrates into the epithelial mucosa, being evident throughout all epithelial layers, including submucosal and basal layers. Differences observed in the excised tissue versus the excised full reproductive tract can be explained by the complexity of each model used. In the same line of thought as the *in vitro* to an *ex vivo* model discussed in Section 5.4, the full reproductive tract represents a more complex system than the excised tissue in a Franz-cell system. Therefore, the results suggest that when the RC-101 product is applied *in vivo*, a deeper penetration of the drug may be observed. However, the limitations of the method should be taken into consideration, especially the fact that the full reproductive tract tissue had

been excised 24 h prior to the experiments conducted. Localization of RC-101 in the epithelial tissue was also observed when human ectocervical and endometrium tissue were investigated. Immunostaining of RC-101 showed that the peptide is present in the upper epithelial layer after exposure to **RC_SOL**, **RC_F+LA** or **RC_F** using the Franz-cell system.

In this Chapter, permeability and tissue localization of RC-101 were investigated using human and monkey tissue. Major findings from this study have shown that RC-101 formulated into a film, when inserted vaginally into an excised full monkey reproductive tract, results in the detection of RC-101 throughout all epithelial layers, including submucosal and basal layers. This result suggests that RC-101 will be present within the epithelial layer after administration of the film product, targeting the area rich in immune cells, offering protection against HIV. For future studies, we recommend investigating the potential of RC-101 to protect against HIV-1 after penetration into the tissue. Such studies can be conducted by challenging the tissues obtained from the full reproductive tract after exposure to the films, using the *ex vivo* model described in Section 5.2.4.2. Excised human and monkey studies using the Franz-cell system, also showed penetration of RC-101 into the tissue, evidenced by the decrease in the RC-101 donor concentration following tissue exposure to drug and detection of RC-101 by immunostaining in the upper epithelial layer. Again, we recommend as a future study to investigate the potential of RC-101 to protect against HIV-1 after penetration into the tissue in these experiments. Furthermore, to confirm the deeper penetration of RC-101 in the full excised reproductive tract, *in vivo* exposure to RC-101 should be conducted in animals such as monkeys. Biopsies of the vaginal mucosa should be obtained after exposure to the films, followed by challenging with the virus to verify if the peptide is still bioactive.

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7.0 MAJOR FINDINGS AND FUTURE DIRECTIONS

7.1 INTRODUCTION

The alarming pandemic levels of HIV and the lack of efficient prevention methods are contributing to the development of new strategies to prevent the spread of the virus. The idea of developing a microbicide product emerged in the 1990's mainly in response to the increasing number of HIV infections in women. The work presented contributes significantly to the microbicide research field, focusing on product development, preformulation strategies, stability in the biological environment, and drug targeting. We investigated two biopharmaceutical (protein/peptide) microbicide candidates: PSC-RANTES, a chemokine analog of RANTES; and RC-101, a circular θ-defensin analog.

In this dissertation project, we hypothesized that the use of a drug delivery system would protect a biopharmaceutical (protein or peptide) microbicide candidate against degradation before administration, and in biological fluids after administration, while maintaining drug activity. We further hypothesized that the interaction of biopharmaceutical microbicide candidates with human vaginal fluids could result in chemical modification of the drug. Our hypothesis was addressed by three specific aims that evaluated the degradation pathways for two microbicide drug candidates (PSC-RANTES and RC-101) (Chapters 2 and 3), assessed the stability of the most promising of the two (RC-101) in human vaginal fluid (Chapter 4), and designed a formulation for targeting of this microbicide drug candidate (Chapters 5 and 6).

The purpose of this final chapter is to present a summary of the major findings, with their implications and limitations, describe possible further development as a microbicide product, and discuss the contributions of the approaches we have followed to the development of other molecules as microbicide products.

7.2 SUMMARY OF MAJOR FINDINGS, IMPLICATIONS, AND LIMITATIONS

7.2.1 PSC-RANTES and RC-101 preformulation studies

The proposed study started with two potential biopharmaceutical microbicide candidates: PSC-RANTES and RC-101. Preformulation studies were conducted for both molecules in which their stabilities were characterized under various conditions including: temperature, pH, ionic strength, and presence of oxidizing agents. These studies are essential for the development of an effective microbicide product, providing information critical to the successful formulation and effective delivery of a drug.

PSC-RANTES, (N-α-(nonanoyl)-des-Ser1-[L-thioproline2, L-α-cyclohexyl-glycine3] RANTES), is chemically identical to the naturally occurring RANTES except for the substitution of a nonanoyl moiety, a thioproline, and a cyclohexyl glycine for the first three N-terminal amino acids of the endogenous protein. PSC-RANTES exhibits *in vitro* antiviral activity, for some HIV-1 isolates in the picomolar range (97). However, when challenged with HIV-1/SIV chimeric virus (SHIV), the PSC-RANTES concentration required for *in vivo* activity (1 mM) for vaginal transmission in Rhesus macaques was 10^6-fold greater than that needed *in vitro* (91). Reduced potency observed *in vivo* may be attributed to: 1) protein modification due to conformational changes, including aggregation; 2) protein modification due to covalent chemical changes; 3) proteolytic inactivation in the vaginal lumen, 4) low penetration of the drug into the mucosal tissue, and/or 5) drug binding (134). Preformulation studies revealed that: PSC-RANTES is unstable at pH 7 immediately upon exposure to the buffer; has short term stability at acidic pHs; forms insoluble aggregates under low and high ionic strength buffers; is prone to thermal and oxidative degradation; degrades in the presence of proteinase K; and may affect the

viability of normal, protective, vaginal flora. The physical and chemical instability of PSC-RANTES indicates that this microbicide candidate would not be stable in a traditional microbicide product formulation. For this reason, the molecule PSC-RANTES was not pursued for further development in this dissertation project. Protection of PSC-RANTES may be achieved by encapsulation of PSC-RANTES into nanoparticulated drug delivery systems, which would facilitate distribution of the microbicide agent and preserve the stability of the drug, as recently reported (150). This approach was not a part of this dissertation project; however, it is currently being pursued in our laboratory.

RC-101 is a retrocyclin analog θ-defensin peptide that contains 18 residues, is tetracyclic, and has three cystein disulfide bonds. Retrocyclins have the ability to bind with high affinity to CD4, galactosylceramide (alternative cell surface receptor for $CD4^-$ cells), and gp120; and thereby confers protection against HIV-1 (106). RC-101 possesses a single amino acid substitution from an arginine (R) to a lysine (K) on one of the β turns, which increases its potency. RC-101 operates by preventing six-helix bundle formation of gp41 (a 41,000 MW glycoprotein), and, thus far, is a promising antiretroviral drug. The dual mechanism of protection of RC-101 (binding to glycoprotein in the virus, as well as to receptors on the host cell) offers a significant advantage for a HIV prevention drug. In addition, based on the cyclic structure of the peptide and the presence of disulfide bonds, stability of RC-101 is expected to be higher than that of linear proteins (223). The majority of proteins are synthesized as a linear chain of aminoacids, with flexible ends that are targeted by peptidases, reducing the binding to receptors that mediate biological activity. Cyclic peptides, especially the ones containing disulfide bridges, have a more rigid structure and are less prone to deactivation by peptidases. Despite the predicted relative stability of the peptide, the results obtained from our

preformulation studies surpassed the expectations. RC-101 was stable over a range of pHs (3 to 7), stable at room and body temperatures, and did not form insoluble aggregates at pH 3 to 7 at low and high ionic strength. The only pathway of degradation identified was oxidation in the presence of hydrogen peroxide, but this degradation was decreased in the presence of EDTA, which functions as an antioxidant by chelating metal ions. In addition, RC-101 was stable in the presence of enzymes commonly present in human vaginal fluid and cervical mucus, suggesting that RC-101 has the potential to become a successful microbicide product. Based on the excellent results obtained in the preformulation studies for RC-101, it was a logical step to further develop this peptide into a microbicide product.

7.2.2 Interaction of RC-101 with human vaginal fluid

After verification of RC-101 stability under several conditions in preformulation studies (chemical and physical stability), analyses were conducted to verify its stability in a biological environment. To understand the impact of vaginal fluids on the stability of RC-101, fresh human vaginal fluid (HVF) was collected from healthy volunteers and the effect of the fluid on the peptide was investigated. This is the first study in the microbicide field to evaluate a microbicide candidate using fresh non-diluted HVF. We have shown that RC-101 is detected in human vaginal fluid for at least 48 h after exposure at 37 °C, indicating stability of the molecule in this biological fluid over time. In addition, formulation of RC-101 in a film delivery system maintained the stability of RC-101 in HVF for the same time period.

Another important finding resulted from studying the impact of HVF positive for bacterial vaginosis (BV) on RC-101 stability. It was shown that RC-101 was unstable in fluid positive for BV evidenced by the undetectable levels of RC-101 after exposure to HVF positive

for BV at all time points. The higher enzymatic activity reported in HVF positive for BV could explain the immediate degradation of RC-101. Given the fact that bacterial vaginosis is a highly prevalent condition (one third of women between the ages of 14 and 49 years old in the United States (194)), the efficacy of a microbicide product in the population may be greatly affected by the presence of BV. This finding is extremely important and should lead to a change in the way biopharmaceuticals and other molecules are developed as microbicides. In the future, the development of microbicides should include an evaluation of the effects of HVF positive for BV on the stability of microbicide candidates, as we have done with RC-101.

A considerable limitation of the LC-MS/MS method used for RC-101 detection is that the method was qualitative and not quantitative. This study showed the presence of RC-101 in HVF for at least 48 h, but absolute amounts of RC-101 could not be determined. The synthesis of a RC-101 stable isotope would be necessary for the quantitative determination of RC-101. However, due to funding limitations, we were unable to synthesize an isotopic peptide. Another approach to obtain a quantitative determination for RC-101 using LC-MS/MS is to construct a standard curve using RC-101. This approach was not followed due to the fact that RC-101 ionizes poorly when alone in solution, giving values with low reproducibility. Further development of a quantitative method is recommended to quantify the loss, if any, of RC-101 over time.

The RC-101 detection for at least 48 h in HVF gives a great indication of the stability of the molecule in this fluid, suggesting that it will maintain bioactivity. However, further studies should be conducted to evaluate if the RC-101 detected after exposure to HVF is still active against HIV. These studies are already being planned in collaboration with Dr. Alexander Cole (University of Central Florida). Based on the cyclic structure of RC-101, preformulation studies,

and LC-MS/MS detection in HVF, we hypothesize that RC-101 stability is preserved, maintaining bioactivity. If this is correct, RC-101 becomes an even stronger candidate as a microbicide for further studies in clinical trials.

So far, no microbicide product has been approved. Microbicide product development has been, as with any other pharmaceutical product, a long-term process. The incomplete understanding of HIV transmission and the lack of appropriate *in vitro* methods and animal models have contributed to the challenging process of microbicide product development. Recent setbacks in the microbicide field have raised concerns with methods used for preclinical evaluations and their correlation with human studies. In January 2007, the Contraceptive Research and Development Program (CONRAD) announced that two Phase 3 clinical trials with the candidate microbicide cellulose sulfate, a poly-anion, were stopped (33). Cellulose sulfate use was associated with a trend towards an increase in HIV infectivity. On February 2008 another unexpected setback occurred. The completion of a Carraguard Phase 3 clinical trial showed that no significant protection against HIV was observed when the product was compared to a placebo; however, the Carraguard product was shown to be safe for use in humans, and is still being investigated as a vehicle for other microbicide candidates (37, 38).

Taking into consideration the lack of suitable methods to predict the response of microbicides *in vivo*, the work presented in this dissertation attempts to provide new strategies to overcome this obstacle. One of the first barriers to the delivery of a microbicide product is the presence of biological fluids. The impact of human vaginal fluid and cervical mucus on the microbicide stability and bioactivity should be considered as a screening tool for the development of microbicides. The study conducted in this dissertation used freshly collected HVF. Certain limitations apply to this approach. It should be taken into consideration that *ex*

vivo use of biological fluid may underestimate the enzymatic activity present, therefore not accounting for all the potential degradation pathways for RC-101. Ideally, the results of this study should be correlated with findings in *in vivo* human clinical trials to validate the use of collected fluids as a predictive tool. It is also important to conduct more studies with biological fluids in the normal and pathological state, in view of the immediate loss of RC-101 in the presence of fluid positive for BV. Nevertheless, this is the methodology closest to an *in vivo* situation used to understand the impact of fluids on a microbicide available so far. The presence of RC-101 for at least 48 h is an important finding and provides a significant stimulus to pursue the further development of this peptide into a microbicide product.

Another aspect to be studied in future research projects is the stability of the microbicide candidate in seminal fluid. During male to female sexual transmission of HIV, the vaginally applied microbicide product will be in contact with semen. Some characteristics of the semen such as pH (pH = 7.8), strong buffering capacity, and high protein (224) content may inhibit the activity of a microbicide. It has been shown that the HIV-1 inhibitory activity of several polymeric candidate microbicides is diminished in the presence of seminal plasma (225). No studies have been conducted for RC-101 activity in the presence of semen up to now, and these studies are strongly recommended.

7.2.3 RC-101 formulation into a drug delivery system

Several surveys and research studies have centered on the user preferences for the dosage form of a microbicide product. In addition to women's preferences, the dosage form of choice must be such that it will deliver the microbicide candidate effectively and maintain its stability during shelf-life and in the biological environment. The use of a film formulation for vaginal drug

delivery has several advantages over alternative formulations. When compared to semi-solid gels, film products are easier to apply, quickly release the drug, eliminate the need for an applicator, and can improve stability of drugs that are sensitive to water. In contrast, conventional gel vaginal dosage forms are associated with limitations of poor retention in the vaginal lumen, leakage, messiness, in addition to being a poor vehicle of choice for peptide formulations. Due to the several advantages as a drug delivery system for microbicides, vaginal films were selected as the final dosage form for RC-101.

The RC-101 film product developed in this dissertation work is clear, flexible, and completely dissolves in water in less than 5 min. RC-101 films were effective against HIV (*in vitro* and *ex vivo*) and safe to human tissue (*ex vivo*) and animals (*ex vivo* and *in vivo*). However, stability of RC-101 in films over time was compromised. A decrease in RC-101 as determined by HPLC was observed over time, without the presence of secondary peaks, suggesting loss of the peptide by other mechanisms than those studied in the preformulation studies (Chapter 3). Despite these losses observed by HPLC, preliminary studies have shown that after storage for 6 months at room temperature, RC-101 films still maintain their bioactivity. *In vitro* suppression of more than 90% HIV was obtained with these RC-101 films stored over time (personal communication – Dr. Alexander Cole, University of Central Florida). This result indicates that the loss of RC-101 by HPLC involves a process different than oxidation or thermal degradation, and does not affect bioactivity. This loss can be attributed to reversible binding of RC-101 to the polymers in the formulation. Future studies should include evaluation of the aggregation of RC-101 with the polymer, and investigation of sample preparation methods that are able to extract the peptide from the formulation for HPLC analysis.

Several models exist to determine bioactivity of microbicide candidates; however, limitations of the bioactivity methods should be taken into consideration. *In vitro* HIV-1 efficacy studies have been used extensively to screen microbicide candidates. Most of the *in vitro* methods published are cell-based assays to determine the suppression of HIV-1 transfection by microbicide candidates (15, 89, 199). In our studies, we have investigated the efficacy of RC-101 films against HIV using an *in vitro* cell-based assay and an *ex vivo* model. Results from *in vitro* assays show an IC_{50} of 2.2 μM for RC-101 films, three times higher than unformulated RC-101 in solution (IC_{50} = 0.66 μM). This increase in the IC_{50} may be attributed to the lower availability of RC-101 in the formulation when compared to solutions. In studies with the *ex vivo* model, RC-101 formulated into film displayed an IC_{90} of 57.5 μM, at least 5 times higher than RC-101 in solution using the same model (IC_{90} = 10 μM). Again, the increase in the IC_{90} may be attributed to the lower availability of RC-101 in the formulation when compared to solutions. In addition to the differences observed using RC-101 solution and RC-101 film formulation, an increase (factor of 10) was observed from the *in vitro* to the *ex vivo* model. In the drug development process, efficacy and toxicity studies are conducted starting with less complex systems and progressing to systems of greater complexity, finally ending with human studies, as discussed in Section 5.4. An increase in IC_{50} or IC_{90} going from simple to more complex models is expected. A cell-based assay consists of a monolayer of cells and does not represent the squamous epithelial structure of ectocervical and vaginal mucosa. The *ex vivo* model, using excised human tissue, is more representative of the conditions found *in vivo*; however it still has limitations and is not an absolute predictor of concentrations necessary for *in vivo* efficacy. The lack of blood and oxygen, absence of other STIs, and lack of inflammatory response are some examples of the limitations of using an *ex vivo* model. Further studies should

be conducted to evaluate the efficacy of RC-101 in animals. Even when using animals for efficacy studies, there is still the question of which model would be the most suitable. This is a recent topic of discussion in the microbicide field. Initially, the monkey model using SHIV transmission had been used to predict doses for human clinical trials. However, the recent failures of microbicide products in Phase 2 and 3 have prompted the microbicide research community to re-evaluate the *in vivo* animal models used. Current methods to investigate efficacy *in vivo* include: SCID mice engrafted with human PBMCs, humanized mouse models, and non-human primates (226). The last model is the closest to the HIV transmission in humans, but still has been shown to fail in the case of cellulose sulfate (33, 227) and Carraguard (37) trials to predict results seen in humans. Based on these results, are non-human primates a valid model? There is no answer to this question yet. Until proof of concept is established for microbicide efficacy, there is no way of deciding which of these assays might best predict the physiological events in humans. There are no perfect models and caution should be taken when interpreting results from these models. In any case, *in vitro* and *ex vivo* models are helpful tools for microbicide screening during pre-clinical evaluation and provide valuable information for further studies.

The same discussion applies to the models used for safety evaluations. The classical example of safety studies conducted in animals not predictive of results in humans is the use of nonoxynol-9 (N-9). Nonoxynol-9, a nonionic surfactant, was initially tested for anti-HIV activity in clinical trials after being used in the market for over 25 years as a contraceptive. The product was safe in a rabbit vaginal irritation model, and safe to humans in Phase 1. However, when a larger study was conducted in humans, damage to the epithelial vaginal mucosa was observed especially in the frequent users of the product. It was also shown that the groups using

the product had a higher incidence of HIV when compared to the placebo group. Studies concluded that the therapeutic window for N-9 was very narrow, and toxicity included induction of inflammatory responses and cytotoxicity to epithelial cells (34-36). Most of the safety study (Phase 1) trials in humans are based on the colposcopy evaluations as a traditional endpoint of toxicity. However, investigations to identify biomarkers, inflammatory response, changes in pH, and changes in normal flora have been conducted in an effort to establish a more comprehensive toxicity evaluation (228). In our studies, *ex vivo* safety of RC-101 and RC-101 films were conducted by exposing human and monkey reproductive tract tissues to the solution or product. The product was considered safe when no gross morphological changes (H & E staining) such as loss of nuclei, sloughed epithelial layer, or changes in cytoplasm-to-nucleus ratio in the epithelial cells were observed. After the initial safety screening in excised tissue, *in vivo* safety studies were conducted using a monkey model. RC-101 film was shown to be safe when colposcopy, pH, and vaginal flora were investigated. Once again, within the limitations of the model, the safety studies show encouraging results and suggest safety of the product in humans, but only human clinical studies may be able to confirm these findings and better validate the use of monkey studies as a safety model.

7.2.4 Permeability and tissue localization of RC-101

Important findings described throughout this dissertation work have validated RC-101 as a promising microbicide candidate, encouraging further investigation of the molecule. The dual mechanism of action observed for RC-101 (binding to the glycoprotein of the virus and binding to the receptor of the host cell) offers a strong incentive to develop RC-101 as a topical vaginal microbicide product. In order to take advantage of this dual mechanism, RC-101 must be

targeted to both the epithelium and vaginal lumen. The ability of RC-101 to penetrate into human (ectocervical and endometrium) and monkey (vaginal and endometrium) tissues was assessed. In addition, the permeability of human and monkey female reproductive tract tissues to RC-101 was investigated. The distribution and permeability of RC-101 in a solution and a formulated state were analyzed using an *ex vivo* system.

Permeability studies conducted using a Franz-cell model showed that no more than 10% of RC-101 was found in the receptor compartment after 6 h exposure to human or monkey excised tissue, suggesting that only negligible systemic absorption would occur. To confirm this finding, pharmacokinetics studies are planned for the next safety evaluation in monkeys. These results have an essential implication for the safety of this microbicide. We are developing a topical vaginal microbicide that will offer protection against HIV. A limited penetration of RC-101 into the tissue is advantageous to target immune cells within the epithelial layer, but systemic absorption of RC-101 is not desirable. Systemic concentrations of RC-101 do not add to the mechanism of protection, and have not been studied regarding safety. A low permeability of RC-101 through tissues is beneficial to the development of RC-101 as a topical microbicide, keeping the peptide in its target tissue. From the experiments in the Franz-cell system, calculations of the median concentration of RC-101 in the donor compartment for RC-101 film represents that 63.2 μg/mL cannot be accounted for by mass balance and were not found in the donor or receptor compartment. Given the fact that the IC_{90} for RC-101 film described in Section 5.3 using an *ex vivo* model was estimated to be 57.5 μM (108.6 μg/mL), and assuming that the decrease in the RC-101 from the donor compartment in the Franz-cell represents penetration into the tissue, we can suggest that about 60% of the RC-101 necessary to suppress

90% of HIV is within the tissue, being available to bind to CD4 of the host cell. The remaining 40% of RC-101 is in the vaginal lumen being available to bind to glycoproteins of the virus.

Clearly, there are advantages and limitations to using an *ex vivo* model for permeability studies. The advantages are that human excised tissues are more comparable to *in vivo*, making it more suitable than cell-based models, as discussed in Section 6.4. Excised tissue resembles the structure of what will be encountered *in vivo*. However, results obtained from *ex vivo* models cannot be assumed to be identical to what would be obtained from whole animal or human studies. Excised tissues start to deteriorate from the moment they are removed during surgery, lacking a physiological blood and oxygen supply. Studies conducted in our laboratory have shown that the tissue is viable (as determined by MTT assay) for up to 8 h in a Franz-cell system, when maintained at body temperature. However, due to the lack of blood and oxygen supply, there is no guarantee that the permeability will be the same as in an *in vivo* model, and viability does not guarantee that regular physiological functions are intact. The concern with using an excised tissue model is similar to what has been discussed in Section 7.1.3 for use of *ex vivo* models for microbicide efficacy evaluations. Permeability studies conducted with RC-101 indicate that penetration of RC-101 occurs in an *ex vivo* model, and this represents further encouragement to conduct further studies with animal models followed by human clinical trials. Further characterization of the permeability of RC-101 in whole animal models is recommended.

In addition to the Franz-cell system, tissue localization of RC-101 was also evaluated in another *ex vivo* model: full excised monkey female reproductive tract. In the full female monkey reproductive tract, RC-101 films were vaginally inserted and immunostaining conducted after 2 h exposure to the film. Results have shown that RC-101 penetrated into the epithelial mucosa, being evident throughout all epithelial layers, including submucosal and basal layers, as detected

by immunostaining of RC-101. This result suggests that RC-101 penetrates the epithelial mucosa, being available to target immune cells. Contrary to the findings in the full reproductive tract, when monkey and human tissue were used in a Franz-cell system, tissue localization studies showed that RC-101 was predominantly found in the upper epithelial layer only. Differences between the two excised models must be elucidated in order to explain variation in the penetration of RC-101 observed. Full excised tissues and excised monkey tissue used in the Franz-cell experiments are obtained after the monkeys are euthanized, then the whole reproductive tract is removed, placed in media, and shipped on ice to our laboratory, taking up to 24 h to arrive. Upon arrival, the tissue is warmed to 37 °C and immediately used for the experiments. When a tissue is removed from the whole animal, it is immediately deprived of its physiological supportive and protective mechanisms. There is no supply of nutrients to the cells, no removal of waste products, and no maintenance of pH and osmotic pressure. When comparing the same type of tissue (monkey) in the two different models (full reproductive tract and Franz-cell), less penetration of RC-101 was observed when using the Franz-cell model. Considering that both tissues lack the physiological supporting and protective mechanisms, and have been excised at least 24 h prior to testing, we had expected to see similar results. Deeper penetration of RC-101 in the full reproductive tract tissue may be associated with a more complete organ structure that facilitates RC-101 penetration. We are unable to obtain full human reproductive tracts to repeat the experiments and verify if deeper penetration also takes place in human tissues.

Despite the limitations of the method, penetration of RC-101 into the deeper layers of the tissue was observed when using a full reproductive tract. This penetration may allow targeting of RC-101 to the immune host cells, increasing the activity of RC-101 against HIV. For future

studies, we recommend to test the tissues used in RC-101 *ex vivo* permeability/distribution studies and challenge against HIV-1. We have shown that RC-101 in film formulation protected against HIV using an *ex vivo* human ectocervical model (Section 5.3). Based on these results, we would anticipate that *in vivo* protection against HIV-1 infection will be achieved. In addition, when safety evaluations in animals are conducted, future studies should include biopsies obtained from the animal studies after exposure to RC-101 film to be challenged against HIV-1. These studies have already been planned by our laboratory and collaborators. Certainly, results obtained by *ex vivo* methods are only useful when they correlate with *in vivo* results. Our findings suggest that exposure to RC-101 *in vivo* will offer a deeper penetration of the drug into the tissue enhancing the protection against HIV. However, future studies are required to confirm this finding.

7.3 FURTHER DEVELOPMENT OF RC-101 AS A MICROBICIDE

Findings from this dissertation work show that: RC-101 formulated in a film drug delivery system protected the peptide from degradation prior to administration; anti-HIV activity of RC-101 was maintained in the formulation; RC-101 was stable for at least for 48 h in the presence of human vaginal fluid; and penetration of RC-101 into epithelial tissue was demonstrated. These results contribute significant information to the development of RC-101 into a successful microbicide product.

As discussed in Section 7.1, future studies are recommended to address the limitations of the methods used and progress the RC-101 film formulation forward into product development. Future studies are summarized below:

- Confirm results of degradation of RC-101 in the presence of HVF positive for BV.
- Develop a quantitative method to quantify the loss, if any, of RC-101 over time after exposure to HVF.
- Evaluate if the RC-101 detected after exposure to HVF is still active against HIV.
- Conduct *in vivo* human clinical studies to validate the use of collected fluids as a predictive tool for stability of RC-101 and other molecules.
- Study the stability of RC-101 in human seminal fluid.
- Identify the mechanism of loss of RC-101 observed in the dosage form, and re-formulate the product.
- Perform safety studies in animals and include biopsies for RC-101 distribution analysis, and efficacy evaluations.
- Include pharmacokinetic studies in animal models.
- Perform efficacy of RC-101 in animal models.

In addition, further development of the RC-101 film into a successful microbicide product should include strategies presented by the Alliance of Microbicide Development (143), and FDA (229) recommendations that have not been addressed in this dissertation project. These recommendations include: toxicology studies in at least two animal species; absorption, disposition, metabolism, and excretion (ADME) analysis; rabbit vaginal irritation tests; hypersensitivity and photosensitivity; genotoxicity effects; reproductive toxicology; carcinogenicity tests; condom compatibility tests; penile skin irritation tests; contraceptive effects; toxicity to normal human vaginal flora; and activity against other STDs.

Another important aspect in the development of a successful microbicide is the cost of the final product. A microbicide product containing a peptide might be expensive and unviable

for future commercialization. However, it should be considered that the production costs will be substantially reduced when the peptide is produced on a larger scale, as explained below. It is common knowledge that high production costs are associated with synthetic peptides, at approximately US$ 75 – 100 per gram per amino acid residue. These assumptions are based on a small scale synthesis. Based on a recent assessment of peptide production costs, if one scales-up the manufacturing process to approximately 100 kg, then the cost would drop to US$ 7.5 – 10 per gram per amino acid residue (230). Estimating the RC-101 film production costs, at a dose of 2 mg/film (used in the safety studies), the amount of RC-101 necessary for each film would cost US$ 0.36 (=10 x 18 x 0.002) if the peptide is produced at a scale of 100 kg batches. This cost comparison demonstrates that an economical product is possible. Additionally, the ability to manufacture adequate amounts of the drug substance is required for commercialization. If a woman uses RC-101 films every 2 days, each woman would receive a dose of 0.36 g/year. As a comparison, enfurvirtide, a 36-amino acid peptide used for HIV therapy, has been produced at a scale of 3 tons/year, and patients receive a dose of 80 g/year (230). This demonstrates that capacities required are easily achievable in commercialization. Another factor to reduce the cost of this peptide is the intensive efforts being conducted to produce RC-101 as a recombinant product. However, this strategy has not been successful so far.

Overall, it has been shown in this dissertation work that RC-101 developed in a film formulation is a promising candidate as a safe and effective microbicide product. The future development of RC-101 in greatly encouraged.

7.4 CONTRIBUTIONS TO THE MICROBICIDE FIELD

It is challenging, although fascinating, to be in a research field where no molecules have been approved yet. Limitations in the methods used are a constant concern, and until a successful microbicide is approved on the market, these limitations will not be defeated. This dissertation work not only provides encouraging results for RC-101 as a microbicide product, but also provides a systematic tool for the development of other microbicide molecules.

The dosage form selected for the delivery of RC-101 can be used for other microbicides. Polymeric films are not limited to the delivery of peptides and proteins only. In fact, this approach is being investigated in our laboratory for smaller molecules as well as larger proteins, and by other research groups such as the studies conducted with sodium polystyrene sulfonate (PSS) (231) and cellulose acetate 1,2-benzenedicarboxylate (CAP) (208). Polymeric films are also not limited to HIV protection only. This drug delivery system is currently in use in the market for the delivery of the contraceptive nonoxynol-9 (N-9). The product, Vaginal Contraceptive Film (VCF, Apothecus Pharmaceutical Corp., Oyster Bay, NY) is a PVA based film which contains 28% of N-9. The idea of a vaginal polymeric film can be expanded for use in other sexually transmitted diseases.

In conclusion, the approach used in this dissertation for the development of RC-101 as a microbicide product should be adopted as a tool for the development of other microbicide candidates. The impact of HVF (normal and pathological state) on the stability of RC-101 and the evaluation of RC-101 distribution in the epithelial mucosa are novel methodologies implemented to provide valuable information for the further development of RC-101. These methodologies can be utilized in development of other potential microbicide drugs and represent

a fundamental strategy for the evaluation of a microbicide candidate. In the future, human clinical studies will be able to validate this approach as a screening tool for microbicides.

Finally, a female-controlled microbicide RC-101 film (or any other microbicide product) is only one aspect in the fight against HIV-1. Any program to eradicate the HIV pandemic level needs to include a multi-disciplinary approach. Women and men should be educated on the proper use of preventive methods, in addition to the use of a microbicide. Although prevention of HIV infection is an overwhelming task, an ideal microbicide product will be achieved with focused and thorough scientific efforts. The work presented in this dissertation provides a significant contribution towards this goal.

APPENDIX A

MICROBICIDES IN THE PIPELINE

A.1 MICROBICIDE CANDIDATED IN ADVANCED PRE-CLINICAL STUDIES

ADVANCED PRE-CLINICAL STUDIES

Candidate	*Mechanism of Action*	*Developer/Researcher/Sponsor*
CAP	Fusion inhibitor	The New York Blood Center, NIAID
Cyanovirin-N	Entry/fusion inhibitor	NIH, Osel Inc., University of Pittsburgh
D-peptides	Entry/fusion inhibitor	NIH, University of Utah School of Medicine
DS001/L-860,167	Entry/fusion inhibitor	International Partnership for Microbicides (IPM)
Mapp66	Combination of antibodies, neutralization	Mapp Biopharmaceutical Inc.
Nisin	Combination	National Institute for Research on Reproductive Health
Octylglycerol	Surfactant	NY State Institute for Basic Research, Magee-Women's Research Institute, University of Pittsburgh

ADVANCED PRE-CLINICAL STUDIES (Continued)

Candidate	*Mechanism of Action*	*Developer/Researcher/Sponsor*
Opuntia spp	Entry/fusion inhibitor, Replication inhibitor	CIDEPLAN, SELADIS
PEHMB	Entry/fusion inhibitor	Drexel University College of Medicine, Novaflux, Biosciences, Inc.
Persulfated molecular umbrellas	Entry/fusion inhibitor	Albert Einstein College of Medicine, Lehigh University, Mount Sinai School of Medicine
Polycarboxylated aryl oligomer	Entry/fusion inhibitor	Albert Einstein College of Medicine TOPCAD/Rush University, YASO
PSC-RANTES	Entry/fusion inhibitor CCR5 inhibitor	La Jolla Foundation for Microbicide Research, Mintaka Foundation, NIAID, NIH, Scripps Research Institute, University of Pittsburgh
Retrocyclins	Entry/fusion inhibitor	NIH, UCLA, University of Central Florida, University of Pittsburgh, University of Washington
SJ-3991	Multiple mechanisms	ImQuest, IPM

Adapted from the Alliance for Microbicide Development website.
http://www.microbicide.org/cs/microbicide_pipeline - consulted on 10/24/2008.

A.2 MICROBICIDE CANDIDATES IN DISCOVERY AND EARLY PRECLINICAL STUDIES

DISCOVERY/EARLY PRECLINICAL

Candidate	Mechanism of Action	Developer/Researcher/Sponsor
C5A	Vaginal defense enhancer Mechanism ruptures the integrity of both the viral membrane and the mature core.	NIH, Scripps Research Institute, Viriome
Cyclotriazadisul fonamides (CADA)	Entry/fusion inhibitor, Uncharacterized mechanism(s)	Rega Institute, University of Nevada, EMPRO (D Schols, TW Bell)
CAP and combinations with NNRTIs and ZFIs	Combination	NIH
Diterpene	Combination Noncompetitive inhibitor of reverse transcriptase:	FAP, FIOCRUZ, UFF
DS003/BMS-599793	Entry/fusion inhibitor gp120 binder	IPM
DS004/L-860,872	Entry/fusion inhibitor CCR5 blocker,	IPM
DS005/L-860,882	Entry/fusion inhibitor CCR5 blocker	IPM
EBd peptides	Entry/fusion inhibitor	NIH, University of Wisconsin School of Medicine
Flavonoids (EGCG)	Entry/fusion inhibitor	NIAID, NY State Institute for Basic Research, University of Pittsburgh

DISCOVERY/EARLY PRECLINICAL (Continued)

Candidate	Mechanism of Action	Developer/Researcher/Sponsor
Fully synthetic 5P12-5P14- and 6P4-RANTES	Entry/fusion inhibitor	La Jolla Foundation for Microbicide Research, Mintaka Foundation, NIAID, NIH, Scripps Research Institute, University of Pittsburgh
Glycerol monolaurate (GML)	Uncharacterized mechanism(s)	NIAID, University of Minnesota
HHA, KRV2110, T20 Combinations	Combination	ANRS- Multi Micro Project
ISIS 5320	Entry/fusion inhibitor	ImQuest
K5-N, OS(H), K50SH	Entry/fusion inhibitor	San Raffaele Scientific Institute and Glycores 2000, EMPRO
KP1, KP17	Replication inhibitor, Combination	CONRAD
L'644 peptide	Entry/fusion inhibitor gp41 inhibitor	IPM
Maraviroc	Entry/fusion inhibitor CCR5 blocker	IPM
Nanobodies™	Entry/fusion inhibitor	University College London, University of Utrecht, Ablynx NV, EMPRO
NCp7 Thioesters (SAMTs)	Replication inhibitor	ImQuest
Novasomes	Combination, entry/fusion inhibitor, Uncharacterized mechanism(s)	Novavax

DISCOVERY/EARLY PRECLINICAL (Continued)

Candidate	Mechanism of Action	Developer/Researcher/Sponsor
Optimized dendrimers	Combination, entry/fusion inhibitor	NIH (DAIDS), Reprotect, Starpharma Pty Ltd.
PC-710	Combination Carraguard (entry/fusion inhibitor) and Zinc – appears highly effective against HSV-2	Population Council
Pyrimidindiones	Multiple mechanisms	ImQuest
Pyrimidindiones and ISIS 5320	Combination (2 products)	ImQuest
RANTES peptides	Entry inhibitor	San Raffaele Scientific Institute, Osel, Inc.
Recombinant lactobacillus (LAB)	Entry/fusion inhibitor	Aaron Diamond AIDS Research Center, NIH
REP 9C, REP 9AC	Entry inhibitor	REPLICor Inc., NIH/NIAID
sCD4-17b	Entry/fusion inhibitor	NIH
Single-chain ICAM	Entry inhibitor	Osel, Inc.
siRNA	Combination, Entry/fusion inhibitor	Immune Disease Institute, Harvard Medical School, NIH, IPM
Sodium Rutin Sulfate (SRS)	Entry/fusion inhibitor	Zhejiang CONBA Pharmaceutics Company
Soluble DC-SIGN	Entry/fusion inhibitor	Mintaka Foundation, Scripps Research Institute
Syndecan	Combination neutralize either the mucosal syndecans or the syndecan-binding of HIV-1, gp120;	Scripps Research Institute
Talactoferrin	Entry/fusion inhibitor, Uncharacterized mechanism	Aggenix, Inc.

DISCOVERY/EARLY PRECLINICAL (Continued)

Candidate	Mechanism of Action	Developer/Researcher/Sponsor
TATC-D peptides	Entry/fusion inhibitor	NIH, University of Wisconsin School of Medicine
Unipron	Vaginal defense enhancer	Institute of Primate Research – Dept Reproductive Health
x-REPLAB	Vaginal defense enhancer, Combination	Makerere College of Health Sciences, Restrizymes Canada Corporation, Restrizymes Biotherapeutics LTD
ZCM	Combination	Population Council
Zinc tetra-ascorbo-camphorate derivative "C14"	Combination	MGB Pharma

Adapted from the Alliance for Microbicide Development website.
http://www.microbicide.org/cs/microbicide_pipeline - consulted on 10/24/2008.

APPENDIX B

SCREENING FOR HUMAN SUBJECTS

B.1 PHONE SCREENING

Phone Screening Script

Thank you for calling to find out more about our research study. My name is Alex Sassi, and I am a researcher at the Magee-Womens Research Institute. The purpose of this research study is to evaluate the potential effects of cervical mucus and vaginal fluid on the activity of a vaginal product. This study will be towards the development of a vaginal product, a female controlled method to prevent transmission and acquisition of HIV. You will not be in contact with the virus or any drug during the course of this study. Your participation will only be to donate vaginal fluid.

We are recruiting pre-menopausal women from 18 to 45 years old. You will not be eligible to participate in this study if you have been recently exposed to any sexually transmitted disease, if you currently experience vaginal bleeding, if you are pregnant, if you delivered a baby in less than 90 days, if you are breast feeding, if you had a miscarriage or abortion in less than 90 days, or if you have a tilted uterus. If you qualify to take part in this research study, you will be

asked to sign a consent, take a pregnancy test, answer a questionnaire, and place flexible diaphragm like device (INSTEAD Softcup®) into your vagina. After 30 min, a physician will remove the cup. The same physician will conduct a test swab to verify presence of sexually transmitted diseases. No other visits are required. The total estimated time is 60 min, which includes reading and signing the consent form, answering the questionnaire, taking the pregnancy test, and placing and removing the device.

Do you think you might be interested in participating in that study?

If NO: Thank you very much for calling.

If YES: Before enrolling people in this study, we need to determine if they are eligible. And so what I would now like to do is to ask you a series of questions about your gynecologic history, including your menstrual cycle, age, and method of birth control used. There is a possibility that some of these questions may make you uncomfortable or distressed; if so, please let me know. You don't have to answer those questions if you don't want to. You also need to understand that all information that I receive from you by phone, including your name and any other identifying information, will be strictly confidential and will be kept under lock and key. The purpose of these questions is only to determine whether you are eligible for our larger study. Remember, your participation is voluntary; you do not have to complete these questions.

Do I have your permission to ask you these questions?

If NO: Thank you very much for calling.

If YES: How old are you? _____

Are you pregnant? _____

Do you use any type of Intra Uterine Device or Vaginal ring? _____

Are you currently using any vaginal products? (creams, gels, douches, foams, or lubricants) _____

What was the first day of your last menstrual period? _____

We can now schedule a time for you to come to the clinic to sign the consent form, answer the questionnaire, and collect vaginal fluid. It is important that you abstain from sexual intercourse and do not use any vaginal products for 48 hours before the test.

Phone screening conducted on: _____ By: _____

B.2 QUESTIONNAIRE

B.2.1 Questionnaire: Hormonal Status of premenopausal women

Please answer every question as best as you can; if something doesn't apply to you, please write N/A. Please do NOT leave any questions in blank.

Date form completed_____

1. Age range:

□ 18 – 20
□ 21 – 25
□ 26 – 30
□ 31 – 35

□ 36 – 40
□ 41 – 45

2. Race/ ethnicity
   _____ American Indian

   _____ Asian

   _____ African American

   _____ Hispanic

   _____ Caucasian

   _____ Other

3. Have you ever in your life consumed an alcoholic beverage?
   Yes ___ No _____

   If yes, when was the date of your last consumption? _____

   How often do you drink per week? _____

4. Smoking history:

   _____ Not currently smoking, but have in the past.

   How long ago did you quit? ____

   _____ Never smoked

   _____ Currently smoke. How many cigarettes do you smoke a day? _____

MEDICAL HISTORY

1. At what age did your menstrual period begin? _____

2. When was your last period? (day/month) _____

3. What is the duration of your period? _____

4. What is the duration (days) between your periods) _____

5. Have you ever undergone any of the following gynecological procedures:

Procedure	YES/ NO	Age	Month/ year
Hysterectomy (removal of the uterus)			
Both ovaries removed			
One ovary (or part of an ovary) removed			
Tubal ligation			

6. How many pregnancies have you had? _____

7. Are you pregnant? Yes _____ No _____

8. Have you ever had any problems with your uterus or ovaries such as ovarian cysts, fibroids, or endometriosis? Yes _____ No _____

If yes, please describe the problem and any associated surgeries_____

_____

9. Are you sexually active? Yes _____ No _____

10. When was your last sexual intercourse?_____

11. Are you currently taking any birth control pills? _____

If yes, please provide the name of the medication_____

12. What type of birth control do you currently use? _____

13. Do you use any type of Intra Uterine Device or Vaginal ring?

Yes _____ No _____

14. Are you currently using any vaginal products? (creams, gels, douches, foams, or lubricants) Yes _____ No _____

If yes, when you the last time you used it? _____

What was the name of the product? _____

15. Are you currently using any medications? Including over-the-counter products, allergy medicines and vitamins. Yes _____ No _____

If yes, please provide the names_____

_____

16. Do you have any medical conditions for which you are currently being treated?

Yes _____ No _____ If yes, please list the conditions:

You will now be asked to perform the procedure. Before you start it, please take a moment to read the instructions sheet provided to you.

APPENDIX C

LC-MS/MS CHROMATOGRAMS

Representative LC-MS/MS chromatograms of the samples prepared in Chapter 4 are presented here. Representative chromatograms include Samples A, B, D, B_F (described in Table 4-1) from the solution obtained from the cell lysate at time 0 and at the end of the experiment.

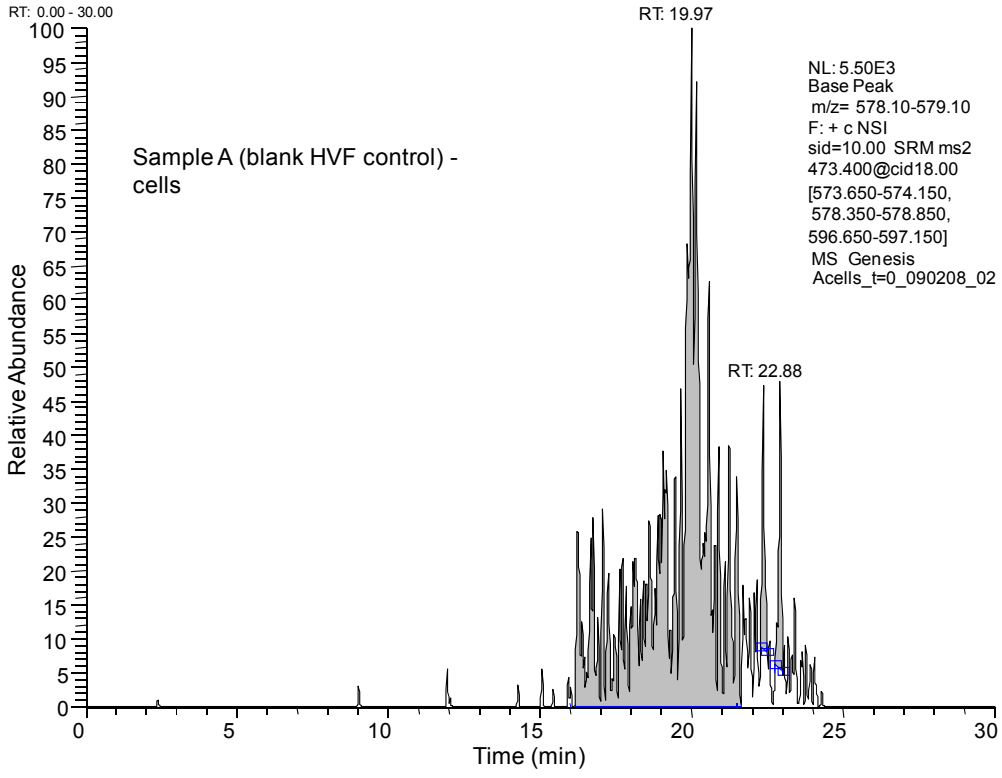

Figure Appendix C 1. Sample A (blank HVF control) representative LC-MS/MS chromatogram of solution obtained from cell lysate, at time 0.

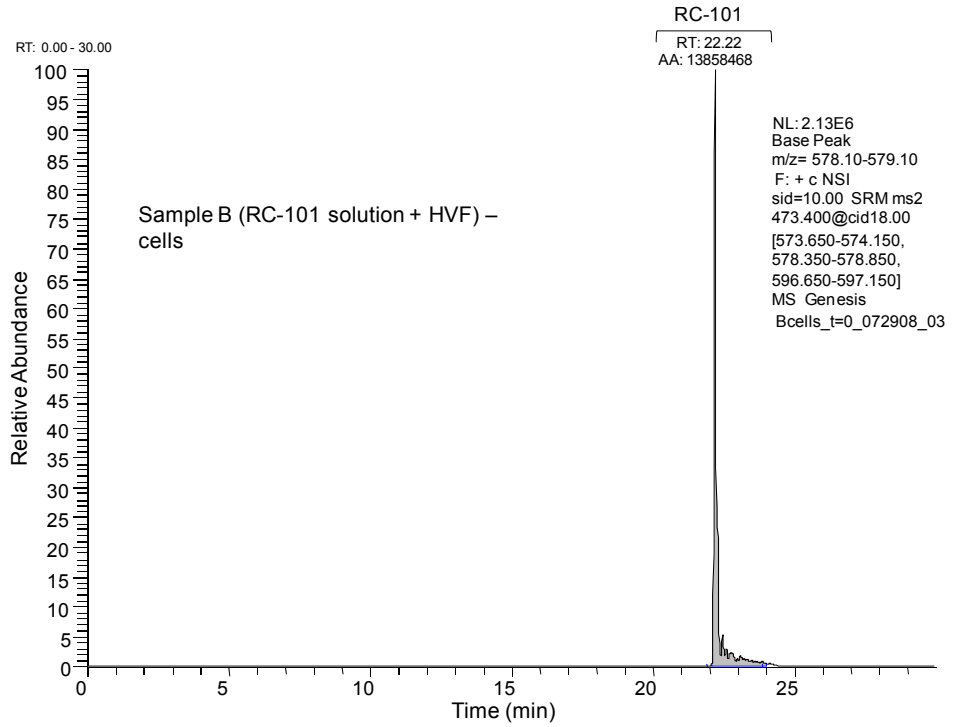

Figure Appendix C 2. Sample B (RC-101 + HVF) representative LC-MS/MS chromatogram of solution obtained from cell lysate, at time 0.

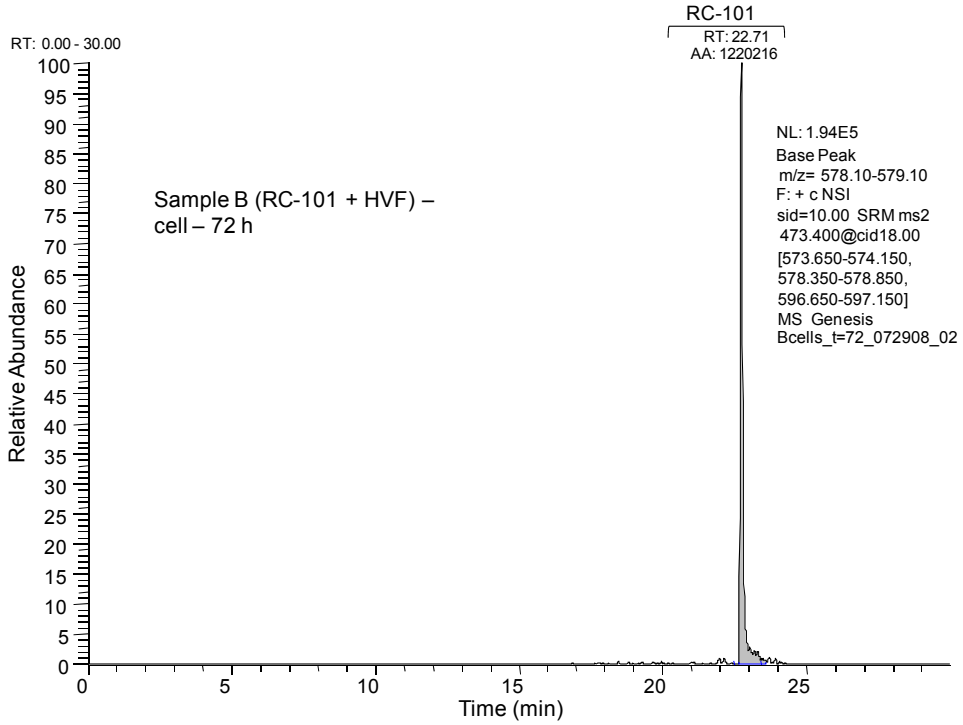

Figure Appendix C 3. Sample B (RC-101 + HVF) representative LC-MS/MS chromatogram of solution obtained from cell lysate, after 72 h exposure.

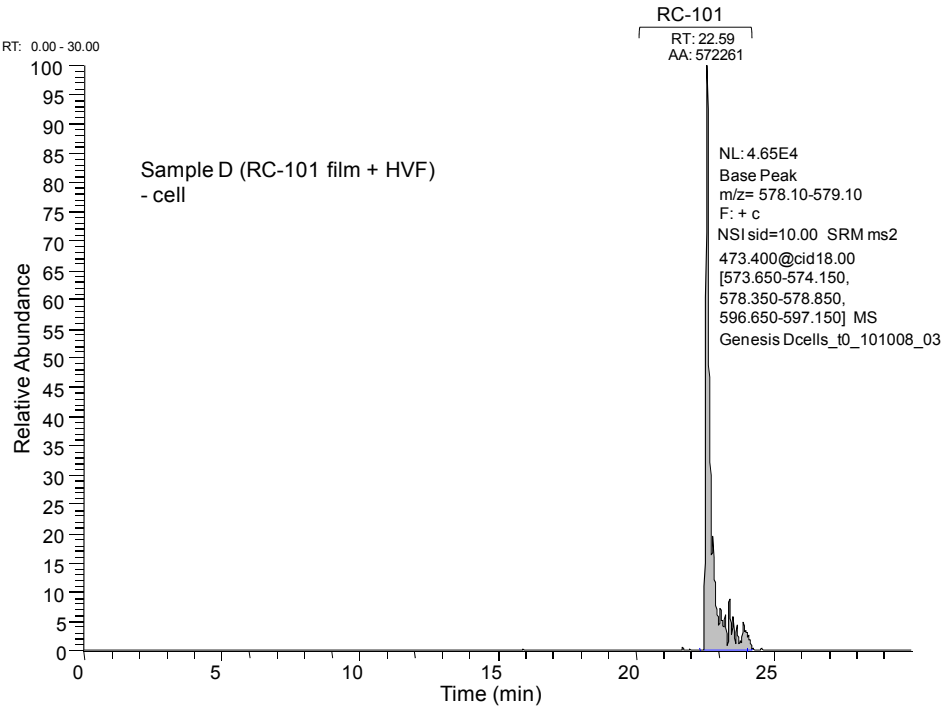

Figure Appendix C 4. Sample D (RC-101 film + HVF) representative LC-MS/MS chromatogram of solution obtained from cell lysate, at time 0.

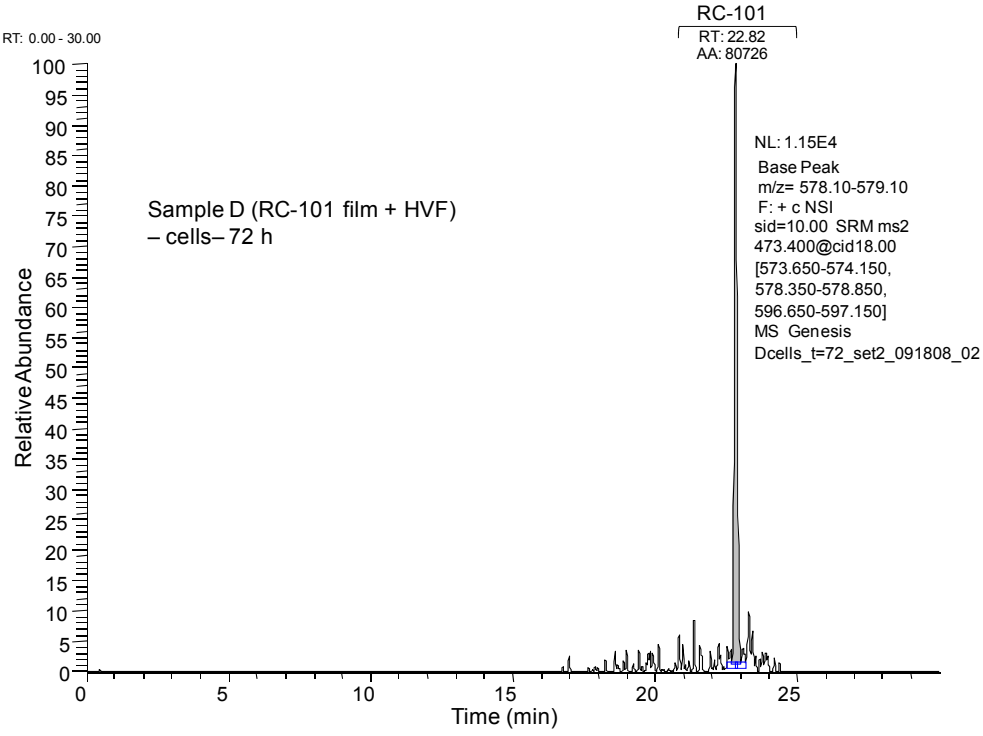

Figure Appendix C 5. Sample D (RC-101 film + HVF) representative LC-MS/MS chromatogram of solution obtained from cell lysate, after 72 h exposure.

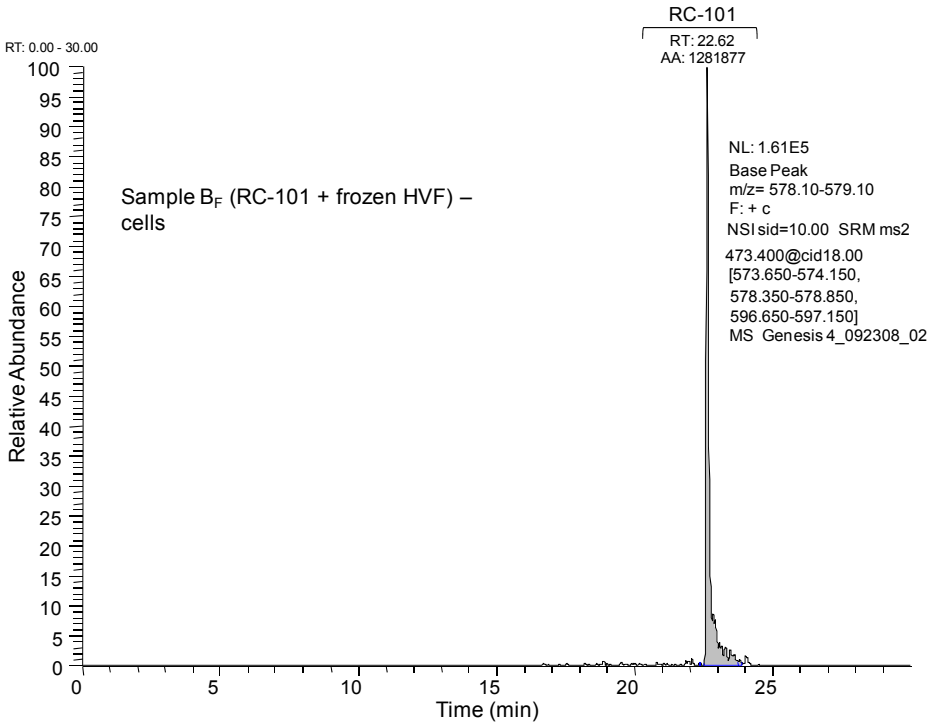

Figure Appendix C 6. Sample B_F (RC-101 + frozen HVF) representative LC-MS/MS chromatogram of solution obtained from cell lysate, at time 0.

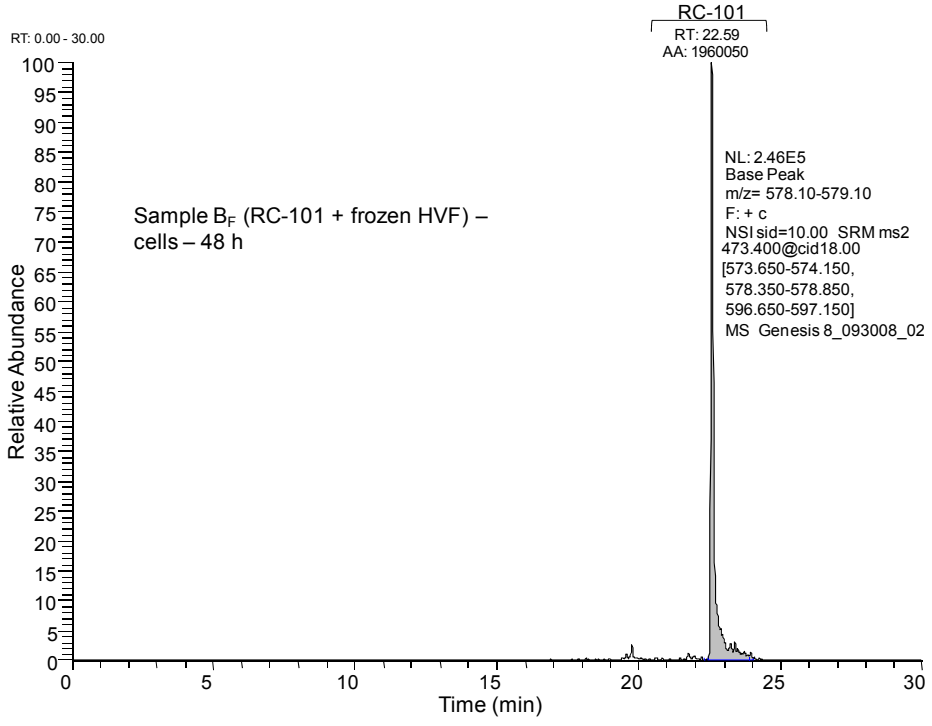

Figure Appendix C 7. Sample B_F (RC-101 + frozen HVF) representative LC-MS/MS chromatogram of solution obtained from cell lysate, after 48 h exposure.

APPENDIX D

LIST OF AMINO ACIDS AND ABBREVIATIONS

Amino acid	3-letter abbreviation	1 letter –abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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