The Innate Anti-HIV-1 Activity of Human Seminal Plasma

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THE INNATE ANTI-HIV-1 ACTIVITY OF
HUMAN SEMINAL PLASMA

by

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A dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Biomedical Sciences
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Major Professor: Alexander M. Cole
ABSTRACT

Human immunodeficiency virus (HIV) has become a global pandemic over the past few decades, with new infections and related deaths in the millions each year. There is no cure in sight for HIV-1 infection, and there has been little progress in developing an efficacious vaccine. Heterosexual transmission of HIV-1 remains the principal mode of transmission throughout the world and thus measures, such as topical vaginal microbicides, to prevent infection of the female reproductive tract are actively being explored. Recent trials of topical vaginal microbicides have shown that their interaction with the mucosal surfaces of the female reproductive tract as well as semen can hinder microbicide effectiveness against HIV-1 infection. Therefore, understanding the role these fluids play in HIV transmission would be critical towards developing effective antiviral prophylaxes.

A recent study from our group demonstrated that human cervicovaginal secretions contained numerous cationic antimicrobial peptides and proteins, which collectively inhibited HIV-1 infection of target cells and tissues. To ascertain if human seminal plasma (SP), the main vector responsible for transmitting HIV-1, exhibited antiviral activity we utilized several anti-HIV assays in the presence or absence of minimally manipulated SP. The majority of the intrinsic anti-HIV-1 activity of SP resided in the cationic polypeptide fraction. Antiviral assays utilizing luciferase reporter cells and lymphocytic cells revealed the ability of whole SP to prevent HIV-1 infection, even when SP was diluted 3200-fold. Subsequent fractionation by continuous flow acid-urea (AU)-PAGE and antiviral testing revealed that cationic polypeptides within SP were responsible for the majority of anti-HIV-1 activity. A proteomic approach was utilized to resolve and identify 52 individual cationic polypeptides that contribute to the
aggregate anti-HIV-1 activity of SP. One peptide fragment of semenogelin I, termed SG-1, was purified from SP by a multi-step chromatographic approach, protein sequenced, and determined to exhibit anti-HIV-1 activity against HIV-1. Anti-HIV-1 activity was transient, as whole SP incubated for prolonged time intervals exhibited a proportional decrease in anti-HIV-1 activity that was directly attributed to the degradation of semenogelin I peptides. Collectively, these results indicate that the cationic polypeptide fraction of SP is active against HIV-1, and that semenogelin-derived peptides contribute to the intrinsic anti-HIV-1 activity of SP.

Conversely, naturally occurring peptidic fragments from the SP-derived prostatic acid phosphatase (PAP) have been reported to form amyloid fibrils called “SEVI” capable of enhancing HIV-1 infection in vitro. In order to understand the biological consequence of this proviral effect, we extended these studies in the presence of human SP. PAP-derived peptides were agitated to form SEVI and incubated in the presence or absence of SP. While PAP-derived peptides and SEVI alone were proviral, the presence of 1% SP ablated their proviral activity in several different anti-HIV-1 assays. The anti-HIV-1 activity of SP was concentration dependent and was reduced following filtration. Supraphysiological concentrations of PAP peptides and SEVI incubated with diluted SP were degraded within hours, with SP exhibiting proteolytic activity at dilutions as high as 1:200. Sub-physiological concentrations of two prominent proteases of SP, prostate-specific antigen (PSA) and matriptase, could degrade physiological and supraphysiological concentrations of PAP peptides and SEVI. While human SP is a complex biological fluid, containing both antiviral and proviral factors, our results suggest that PAP peptides and SEVI may be subject to naturally occurring proteolytic components capable of reducing their proviral activity.
Our studies demonstrate the overall antiviral activity of human SP, but there is still a critical need for effective topical vaginal microbicides that can prevent HIV-1 transmission. The synthetic human retrocyclins are cyclic antimicrobial peptides that are remarkably active against HIV-1, and are being developed as topical vaginal microbicides. Herein, we assessed whether the putative proviral SEVI was able to adversely affect the anti-HIV-1 activity of the retrocyclin analog RC-101. While SEVI alone enhanced viral infection, this effect was completely negated in the presence of RC-101. Retrocyclins such as RC-101 are inhibitors of HIV-1 entry, by preventing gp41-mediated viral fusion. Interestingly, using an HIV-1 reverse transcriptase (RT) specific assay, we also determined that RC-101 directly inhibited the activity of RT in a dose dependent manner, suggesting a secondary mechanism of viral inhibition. Our group has determined that RC-101 induces only a modest level of resistance in HIV, which may be due in part to RC-101’s dual mechanisms of viral inhibition.
I dedicate this dissertation to my amazing families, the Martellinis and the Moores

Especially to my loving parents, for all of their support and sacrifice that allowed me the opportunities to follow my passions

&

To my loving husband, whose patience and understanding has kept me balanced through this long and arduous journey
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2D-PAGE</td>
<td>two-dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>AU</td>
<td>acid urea</td>
</tr>
<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix-assisted laser desorption/ionization</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PAP</td>
<td>prostatic acid phosphatase</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate specific antigen</td>
</tr>
<tr>
<td>RC-101</td>
<td>retrocyclin analog 101</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEVI</td>
<td>semen-mediated enhancer of virus infection</td>
</tr>
<tr>
<td>SG I</td>
<td>semenogelin I</td>
</tr>
<tr>
<td>SP</td>
<td>seminal plasma</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>VF</td>
<td>vaginal fluid</td>
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</table>
CHAPTER 1 BACKGROUND INFORMATION

1.1 Heterosexual transmission of HIV-1 is the most prevalent route of transmission worldwide

The HIV-1 epidemic is worldwide, with 2.6 million newly infected individuals in 2009, and 33.3 million living with HIV-1. Studies show the majority of new HIV-1 infections are transmitted sexually (UNAIDS 2010 report). Preventing the spread of this disease is a critical priority, especially in developing countries that harbor the majority of infected people. The risk of infection is subject to myriad factors including the viral load of the infected partner, the type and frequency of sexual encounter, underlying infections including sexually transmitted infections (STI), and the effectiveness of the viral strain [1]. While these factors can adjust the risk of infection, the normal transmission rate has been estimated to be about 3 per 1000 coital acts [2]. Various strategies have been developed in the attempt to prevent sexual transmission of HIV-1, with the major thrusts being in vaccine and topical microbicide development. Vaccines have been largely unsuccessful, likely due to the ability of HIV-1 to evade the adaptive immune response [3]. Topical microbicides are drugs that have the ability to prevent HIV-1 infection at mucosal surfaces [4]. Topical microbicides are relatively new members of the anti-HIV armamentarium, and would be applied vaginally or rectally to prevent or reduce sexual transmission of HIV-1 in men and women. Indeed, the recent CAPRISA004 phase II microbicide trial revealed that a 1% tenofovir vaginal gel reduced HIV-1 infections by 54% in high-risk women, when used as instructed [5].
The continued development of topical intravaginal microbicides will require a comprehensive understanding of the interacting biological components within SP, the main vector of HIV-1 transmission. Even with the success of a single clinical trial [5], many other reported topical microbicide trials have yielded discouraging results [6]. Evidence suggests that semen and seminal plasma proteins are able to hinder the activity of certain microbicides, thus reducing the potency of these topical agents. In order to fully elucidate the innate host defense against HIV-1 infection, the mucosal immunity of seminal plasma and the intrinsic antimicrobial and proviral factors therein must be thoroughly investigated.

1.2 The composition and post-coital processing of human seminal plasma

The primary function of human semen is to transport spermatozoa, the major cellular component of semen and the male contribution to reproduction. While semen has been a subject of study for over a century now, only recently has evidence of the complexity of this vital fluid emerged. Semen is comprised of 90% seminal plasma, and 10% cells including spermatozoa, epithelial, and immune cells [7]. Male accessory sex organs synthesize and secrete the different components of the ejaculate, while the testes contribute the spermatozoa. Upon leaving the testes, spermatozoa enter the epididymis. Here, maturation of spermatozoa involves the acquisition of motility and alterations of the sperm plasma membrane. Strong muscular contractions from the epididymis and vas deferens transport the sperm-containing fluid to the accessory organs [8]. Prostate secretions are first, and then seminal vesicle secretions are added. Within the seminal plasma medium, the prostate secretion represents about 60% of the volume at
an acidic 6.6 pH, followed by the seminal vesicles slightly alkaline pH 7.8 representing 30% of the volume, resulting in an overall slightly alkaline pH of 7.2-7.6 in semen [7]. Seminal vesicles secrete high flavin and fructose concentrations, which are responsible for providing the energy for spermatozoa function, as well as the bulk of the seminal plasma proteins, namely semenogelins. Also, prostaglandins originally thought to have come from the prostate, are contributed by the seminal vesicles. The prostate contributes citric acid, zinc, and a variety of proteases.

Ejaculation results in the immediate formation of a non-covalently linked sperm-entrapping coagulum comprised of the most abundant proteins in seminal plasma: semenogelin I (SG I), and semenogelin II (SG II). There is one cysteine at residue 216 of SG I for disulfide linkage to other SP proteins, and the mature protein does not undergo post-translational modification [9]. The immediate coagulation of ejaculate serves as a protectiveencasement for the delicate spermatozoa while the buffering capacity of the seminal fluid increases the harsh acidic pH of the vagina. The coagulum is structurally supported by disulfide linkages and divalent zinc ions derived from the prostate [10-12]. Once ejaculated, hydrolysis of the semenogelin coagulum leads to liquefaction and processing into fragments by prostate derived proteins, mainly the kalikrein-like protease prostate specific antigen (PSA), and prostatic acid phosphatase (PAP) [13]. While PSA has optimal activity at neutral pH, the phosphatase activity of PAP requires an acidic environment, indicating activity specific to the acidic environment of the female genital tract [14].

Semenogelin is rich in zinc binding histidine residues, and once cleaved, the fragments remain zinc ligands. This holds significance due to the fact that divalent cations reduce the
binding of Sg II to PSA. Zinc is stored in the prostate at concentrations up to 7 mM, where it inhibits the activity of prostatic enzymes. After ejaculation, zinc is chelated by the coagulum proteins to form the gelatinous network. SG I is present at 0.4-0.6 mM concentration, but each molecule will bind several ions of zinc via histidine residues. Free from the inhibitory action of zinc, the activated prostate enzymes proteolytic activity continues to cleave the coagulum network [11,15,16].

Figure 1-1: Schematic illustration of the male reproductive tract, and post-ejaculatory events.
1.3 Sexual transmission of HIV-1

Semen is the main vector for HIV-1 transmission. HIV-1 is transmitted as cell-free virions, infected leukocytes, or spermatozoa-associated. The spread of HIV-1 most commonly occurs via mucosal surfaces, the majority of which occur through the vaginal or rectal mucosa. The infectiousness of semen is dependent on several factors, including the stage of the disease of the infected person. Viral loads are highest at the earliest stages, and end-stage of the disease, with the viral load peaking at 4.5 +/- 0.4 log10 copies/ml [17,18]. Seminal virus may originate from different compartments of the male reproductive tract, due to the immune privileged environment of the testis, seminal vesicles and prostate. Evidence suggests seminal plasma virions originate from compartments distal to the vas deferens, since vasectomized subjects are capable of transmitting the virus [19].

The primary mode of HIV-1 infection in the postcoital environment is not completely understood. HIV-1 virions have been shown to infiltrate into the gaps between squamous epithelial cells [20]. It is also possible that common microabrasions from consensual coitus allow HIV-1 to reach subepithelial target cells [21,22]. Subepithelial stromal tissues possess dense populations of immune cells, including macrophages, dendritic, and T cells: the cells expressing the CD4 HIV-1 receptor, and CXCR4 or CCR5 coreceptors. If the virus reaches the lamina propria, it can progressively infect the immune cells expressing the necessary receptors/coreceptors and transport to the lymph nodes where they robustly replicate and spread [20].

In order to infect a target cell, HIV-1 enters via binding HIV-1 receptors and fusing with the host cell. The virion gp120 surface protein must first bind the primary host cell surface
receptor CD4, a glycoprotein expressed by immune cells, including T-cells, macrophages, and dendritic cells [23]. The HIV transmembrane domain fusion protein gp41 in turn binds to the host chemokine coreceptor CXCR4, or CCR5. The type of coreceptor the virion binds to depends on the tropism: T-cell tropic (T-tropic), syncytium-inducing isolates (X4 viruses) bind to CXCR4, while macrophage-tropic (M-tropic), nonsyncytium-inducing isolates (R5 viruses) bind CCR5. It has been realized that R5 viruses are transmittable through mucosal or intravenous exposure, while X4 viruses are commonly seen during the late stages of disease, or once a patient advances into AIDS [3,24]. With the coreceptor bound, a structural rearrangement is triggered in the gp41 fusion protein, leading to viral-host membrane fusion. The most widely accepted viral fusion model involves a triple-stranded coiled-coil that facilitates the gp41 hydrophobic amino terminus harpooning the host cell membrane. The coiled-coil then bends back to form a six helix bundle that has the gp41 protein and transmembrane domain on the same end [24]. This hairpin formation finalizes viral and host membrane fusion, enabling HIV-1 to release its core contents into the target cell cytoplasm.

Inside the cell, the virion undergoes uncoating. Free of the capsid, the viral reverse transcription complex generates, and is liberated from the inner plasma membrane. Docking with actin microfilaments, the complex proceeds with viral DNA synthesis, in turn developing the HIV-1 preintegration complex (PIC); which, contains the double-stranded viral cDNA. The PIC migrates toward the cell nucleus, docking with nuclear-pore complex components. Within the nucleus, the integration of the double-stranded viral DNA into the host cell chromosome is conducted by viral proteins. Long Terminal Repeats (LTRs) flank both ends of the viral DNA, promoting transcriptional initiation. Spliced viral transcripts encode structural enzymatic
accessory proteins that, once transported to the cytoplasm, will assemble new virions. Once all components are recruited to the plasma membrane, the virion is constructed, and budding transpires [25,26]. The HIV-1 virion life-cycle is a complex process; however, the fundamental steps of viral replication are the targets for researchers to find broad spectrum antiviral drugs.

1.4 The intrinsic antimicrobial components of human seminal plasma

The first line of defense against most invading microbes is the innate immune system. The main effectors of this defense mechanism are antimicrobial peptides, possessing a broad range of actions against gram-negative and gram-positive bacteria, fungi, and viruses. Protection of the host develops via antimicrobial substances within inflammatory cells and epithelial cells. Once antimicrobial components are released into the surrounding fluid, their results are likely quick and effective. However, few studies have determined total antimicrobial activity of different tissue and mucosal fluids [27].

The antibacterial activity of seminal plasma was established little over 30 years ago. Recent studies have started focusing on the cationic peptides found in seminal plasma, such as members of the beta-defensin family, and the human cathelicidin hCAP-18. One very potent antimicrobial family is the defensins, which are small cationic peptides that harbor extensive antiviral mechanisms. It has been determined that defensins block viral infection via direct inhibition by disrupting virion envelope, interacting with viral glycoproteins by functioning as lectins, or interfering with viral infection indirectly by altering the host cell in some way [28]. The Human β-Defensin-1 (HBD-1) peptide is expressed in the testis, as well as ejaculated
spermatozoa, and seminal plasma, where multiple soluble forms are found. Transcripts for the \( \alpha \) -defensins human neutrophil peptide 1-3 (HNP1-3), human defensin-5 (HD-5), and HD-6 are detected in the testis as well. It is found that the moderate salt concentration normally found in semen is highly favorable to the activities of defensins [29]. Humans possess only one cathelicidin, hCAP-18, which is cleaved into the active peptide LL-37. The highest concentration of hCAP-18 production is found in the epididymis, and therefore is secreted in the seminal plasma [30]. hCAP-18 resides in the granules of neutrophils, and epithelial cells of the epididymis as an inactive proprotein [31]. The acidic pH in the female reproductive tract activates the prostate derived protease gastricsin, which in turn activates hCAP-18 into an alternative active peptide, ALL-38.

While some SP components are common innate elements, others are specific to this biological fluid and the tissues that contribute to the generation of SP. One group of small, cationic secretory peptides are found within the defensin gene cluster, and are expressed exclusively in the epididymis. The human epididymis gene, otherwise known as \( HE2 \), expresses multiple mRNAs, with the two isoforms HE2a and HE2b1 having confirmed antimicrobial activity. After mRNA processing, proteolytic cleavage activates the peptides. Matrilysin, a matrix metalloprotease, is secreted luminally in the male reproductive tract. This protease is implicated in \( \alpha \)-defensin activation, and may cleave HE2 prosegments, increasing the number of bioactive HE2 peptides in ejaculate [32]. Negatively charged phospholipids found on the membranes of invading microbes will bind to the positively charged peptides, leading to their eradication [33].
Antimicrobial peptides are a structurally diverse group, but possess common properties such as a net positive charge, and an amphipathic spatial arrangement. Mucosal surfaces and secretions usually present an array of antimicrobial peptides, allowing the broadest defense possible. Only a handful of studies have determined the mechanisms with which antimicrobial peptides inhibit viral infections. For example, it was determined that the polypeptides lysozyme, Secretory Leukocyte Protease Inhibitor (SLPI), and lactoferrin, found abundantly in most mucosal secretions, only exhibit modest antiviral activity against HIV-1 [27]. However, it is not the work of one individual antimicrobial peptide that protects the host from invading microbes, but the aggregation of the various components.

Evidence pertaining to the role of the synergistic activity of antimicrobial polypeptides found in vaginal fluid determined that the mucosal secretions retain intrinsic anti-HIV-1 properties directly attributed to the aggregate of cationic polypeptides [34]. Evidence suggests human SP not only possesses intrinsic antimicrobial components, but also generates them post-ejaculation. For example, the natural processing of the semenogelin coagulum by PSA and PAP not only leads to protein fragmentation and subsequent release of spermatozoa; but, it also generates a number of SG I cleavage products possessing antimicrobial activity [35,36]. We investigated the cationic polypeptide portion of human SP, and significant anti-HIV-1 activity was expressed by the whole fluid, the most cationic-dense fractions, and a single cationic SG I fragment. These studies are further elaborated in Chapter 2.
1.5 HIV-1 enhancing components derived from human seminal plasma

There are multiple components and properties of human semen that promote the possibility of HIV-1 infection. A prominent innate defense mechanism is the naturally acidic environment of vaginal fluid due to colonization with lactic acid producing strains of *Lactobacillus* [37]. While the pH of vaginal fluid does vary with age of a female, a woman of reproductive age has a pH of 4-5 [38]. However, the post-coital female reproductive tract undergoes neutralization post-ejaculation due to the alkaline buffering of semen. Semen also stimulates inflammatory cytokines [39], and promotes an influx of HIV-1 target cells [40]. The prostate serves as the source for the powerful immunosuppressor, transforming growth factor – beta (TGF-β), observed in vast amounts, both free and latent [41]. Activation occurs via a kalikrein-related peptidase cascade within seminal plasma, that results in the inhibition of lymphocyte and monocyte activation [42]. Though it acts as a form of necessary protection for spermatozoa in the harsh environment of the female genital tract, it also neutralizes the natural defenses of the female immune system from acting against invading pathogens [43].

Immunosuppressive activity in seminal plasma is also attributed to the E series prostaglandins (PGE), which inhibit the functioning of NK cells and clonal proliferation of T lymphocytes [44,45]. The concentration of prostaglandins is higher in semen than in any other part of the body. Also, the PGE 19-hydroxy is exclusively found in semen. Prostaglandins induce tolerance of non-self components inhibiting the antigenic stimulation cytokine IL-12 and stimulating the anti-inflammatory cytokine IL-10 [46,47]. Spermatozoa serve to benefit from this tolerance, but so do invading microbes.
Studies have recently found that specific fragments of the semen marker prostatic acid phosphatase (PAP) have the ability to form amyloid fibrils that enhance HIV-1 infection [48]. The fibrils were termed semen-derived enhancer of virus (SEVI), which expressed similar HIV-1 enhancing activity compared to amyloid fibrils associated with Alzheimer’s disease [49]. PAP originates from the prostate, and is secreted abundantly in seminal plasma [48]. Post-ejaculation, PAP can be detected in the female reproductive tract for up to 24 hours [50]. When in amyloid fibril form, SEVI captures the HIV-1 virions, resulting in enhanced attachment to the target cells, and increased viral infection. The 39-residue PAP fragment consists of eight basic residues, causing it to be very cationic (pI = 10.21) [48]. This positive charge allows SEVI to decrease the negative-negative repulsion between the HIV-1 virions and the target cells, and increases fusion of the two entities during infection [51]. SEVI enhances the infection of target cells by HIV-1 envelopes in a dose-dependent manner, however the enhancement of infection tends to be donor specific [52]. R5-, X4- and dual-tropic clones of HIV-1 were enhanced by SEVI, and the greatest proviral activity was observed at lower concentrations of virus [48]. Our data demonstrated the natural degradation of SP proteins over time, due to an array of intrinsic proteases. We expanded the SEVI studies in the presence of SP, and determined that PAP-derived fragments and fully formed SEVI underwent degradation by SP proteases that negated their proviral activity. These studies are explained further in Chapter 3.
1.6 Retrocyclins: effective antiretroviral peptides

To curtail the spread of the HIV-1 pandemic, preventative therapies need to be developed. Topically applied microbicides serve to inhibit viral transmission when innate defenses are eluded. They act by disrupting the viral membrane or disabling the route of infection. Due to the genetic variability of HIV-1, an effective microbicide will need to possess broad spectrum activity, and the ability to act in the presence of native mucosa at the sites of infection [25,53].

Defensins are a group of antimicrobial peptides that are small and cationic. Secreted by leukocytes and epithelial cells, there are three types of defensins, namely alpha-, beta- and the circular theta-defensins. All forms are cysteine-rich, resulting in very stable structure, secured by intramolecular disulfide bonds. One group of theta-defensins in particular, retrocyclins has a circular structure, with 18 residues and 3 disulfide bonds. While they are produced by many nonhuman primates, it has been determined that humans express a theta-defensin pseudogene for retrocyclins, which contains a premature stop codon in the signal sequence thus precluding its translation [54,55].

Synthetic Retrocyclins, recreated based on the available genetic information in the corresponding human pseudogenes, are very effective HIV-1 inhibitors [55]. Evidence suggests retrocyclins inhibit viral entry during infection by binding to carbohydrate containing surface biomolecules used by HIV-1 for entry into the target cells, namely gp41 [56]. Retrocyclin prevents infections by both M-tropic and T-tropic strains of HIV-1, while remaining noncytotoxic to the host cells. For an antiviral peptide to effectively prevent viral infections, it must possess a broad range antiviral activity, a characteristic shared by cationic peptides of the
innate immune system, including retrocyclins. We decided to test the effectiveness of the retrocyclin analog, RC-101 in the presence of SEVI. RC-101 maintained antiviral activity, and expressed an additional facet of HIV-1 inhibition by directly inhibiting reverse transcriptase activity. These studies are further explained in Chapter 4.
CHAPTER 2 CATIONIC POLYPEPTIDES CONTRIBUTE TO THE ANTI-
HIV-1 ACTIVITY OF HUMAN SEMINAL PLASMA

2.1 Introduction

HIV-1 has become a pandemic over the past few decades, newly infecting an estimated 2.7 million people in 2007 alone. Due to the rapid mutation rate of HIV-1, and unforeseen problems in the clinic, vaccine development has gained insignificant headway over viral spread. Even in the absence of preventative measures, natural mucosal host defenses are thought to play a role in preventing or suppressing HIV-1 transmission. Principal molecular effectors of mucosal host defense include antimicrobial polypeptides, which exhibit a broad range of actions against gram-negative and gram-positive bacteria, fungi, and viruses [28,57,58]. Mucosal tissues throughout the human body elaborate a number of antimicrobial polypeptides that, once released into the overlying fluid, quickly and effectively eradicate invading pathogens. While biologic evidence for antibacterial and antifungal host defense has been established [59], only a comparably few studies have focused on the antiviral activities and mechanisms of these polypeptides [60].

The main antimicrobial peptides and proteins found on mucosal surfaces are cationic in nature and include lysozyme, secretory leukocyte protease inhibitor (SLPI), lactoferrin, defensins, and the cathelicidin LL-37. Alone they exhibit only modest antiviral activity against HIV-1 yet in concert with other peptides, for example in human vaginal fluid, they can exert significant anti-HIV-1 activity [34]. While human seminal plasma contains several antimicrobial
polypeptides including hCAP18, the precursor protein of LL-37, and defensins [10,29] there are a number of other cationic polypeptides whose roles in antiviral host defense have not yet been studied. The most abundant proteins in seminal plasma are semenogelin I and semenogelin II, secreted from the seminal vesicles. These proteins immediately form a non-covalently linked coagulum, which is subsequently liquefied by proteases that hydrolyze the gel proteins into soluble fragments [9,10,12,61]. Studies have reported antibacterial activity of semenogelin derived peptides [35,62], which our group has recently confirmed and extended [36].

While studies have determined the antibacterial effects of individual cationic polypeptides found in seminal plasma, the antiviral activity of the cationic component of seminal plasma has not been investigated. Moreover, histidine-rich peptides have not been reported to play a major role in anti-HIV-1 host defense. In the current study, the anti-HIV-1 activity of seminal plasma was found to be contained in the cationic polypeptide fraction, revealing a significant host defense function against HIV-1. Further, electrophoretic and chromatographic fractionation followed by proteomic analyses identified 52 separate cationic polypeptides, many of which contributed to the aggregate anti-HIV-1 activity of SP. Notably, a recent study reported semen-mediated enhancement of HIV-1 transmission, due to prostatic acid phosphatase (PAP)-derived amyloid fibrils [48]. While certain methods differ between this report and our current study, taken together it is clear that both pro- and antiviral factors are present within SP. Our results provide biological evidence that SP is naturally antiviral against HIV-1, suggesting an innate defense mechanism that prevents HIV-1 transmission.
2.2 Materials & Methods

2.2.1 Collection and processing of human seminal plasma

Samples of human semen were collected by the Center for Reproductive Medicine for routine seminal analyses, and the discarded de-identified seminal plasma component was used for this study. Patients were asked to refrain from ejaculation for 2-3 days, but no more than 5 days, prior to collection and semen was obtained using dry masturbation into a sterile polypropylene cup. All samples were allowed to liquefy for 30 min at room temperature, and centrifuged for 30 min at 1500 x g in order to remove any cellular debris. A total of 103 seminal plasma samples were collected, with volumes ranging from 0.5 ml to 6 ml, and equal volumes of all donor samples were pooled, aliquotted, and stored at -80 °C until needed. The average protein concentration of SP was acquired using a Micro BCA Protein Assay using diluted bovine serum albumin (BSA) as a standard. The assay was repeated three times in triplicate, revealing an average protein concentration of 161.3 mg/ml. Average pH of whole undiluted seminal plasma was determined with colorpHast pH indicator strips, and observed at a pH of 7.75 (n=3). Whole seminal plasma utilized for antiviral cell culture assays was not manipulated further.

2.2.2 Cell lines & viruses

The TZM-bl and PM1 cell lines were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD, USA). TZM-bl cells are a
stable line of HeLa-derived epithelia. They contain Tat-regulated reporter genes, and neutralize virus after single round infections. When used with Env-pseudotyped laboratory strains of HIV-1, TZM-bl cells can undergo a luciferase (Luc) reporter gene assay that performs a sensitive and rapid report of the extent of viral infection. TZM-bl cells were grown in D10 medium: high glucose DMEM (Mediatech, Manassas, VA, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) fetal bovine serum. PM1 cell cultures were maintained at a density of 0.4 – 0.8 x 10^6 cells/ml in R20 medium: RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 100 mM HEPES, and 20% (v/v) fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA). The HIV-1 strain BaL (R5) was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. HIV-1 BaL was propagated in PM1 cells over 16 days, and virus containing supernatant was collected every other day starting on day 5 of infection. Supernatant was filtered through a 0.45-µm pore size filter, and stored in 0.5 ml or 1 ml aliquots at -80 °C until needed. Viral quantification was achieved via a sensitive commercial ELISA for p24gag (PerkinElmer, Waltham, MA, USA).

2.2.3 Experimental determination of anti-HIV-1 activity & cytotoxicity effects

For antiviral experiments with whole seminal plasma, fractionated seminal plasma, synthetic, and purified SG-1, Tzm-bl cells were trypsinized, counted via hemocytometer, and seeded to 6 x 10^4 cells/ml (6000 cells/well) in a 96 well microtiter plate. After 24 h, cells were treated in triplicate with 50 µl of D10 containing treatment sample. Immediately following treatment, 50 µl of virus diluted to a constant final concentration (5 ng p24/ml) in D10 was
added to each well, and incubated at 37 °C in 5% CO₂. After a 24 h infection period, cells were lysed using a Bright Glo luciferase system (Promega, Madison, WI, USA), and luciferase was quantified with a Spectramax luminometer with an integration time of 5 s/well [63]. Ability to prevent HIV-1 infection was measured as a percent reduction in luciferase (relative light units) compared to the positive viral control (media and virus only), while the negative control (media only, no virus) acted as the baseline comparison. Metabolic activity and cytotoxicity of the cells were verified by a tetrazolium-based (MTT) assay according to manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA) and a standard Trypan blue dye exclusion assay [64], performed in parallel with the HIV-treated cells.

PM1 cells (1.5 x 10⁵/0.1 ml) were treated with whole seminal plasma diluted in R20, infected with HIV-1 BaL (2 ng p24/ml), and incubated for 3 h at (37 °C/5% CO₂) with agitation of the cell suspension every 30 min. The cell suspension was then diluted with 2 ml of R20, pelleted, resuspended in 0.5 ml of diluted whole SP in R20 medium, or vehicle only, and incubated 37 °C/5% CO₂. 3 days post-infection, the supernatants were collected and stored at -20 °C, and the remaining cells in culture were resuspended in 0.5 ml of diluted whole seminal plasma or R20 alone. On day 5, supernatants were collected and stored, and a Trypan blue dye assay was performed on the cells as previously described. To quantify the viral inhibition of SP, day 5 supernatants underwent an ELISA for p24gag (PerkinElmer, Waltham, MA, USA), and were compared to vehicle-only control.
2.2.4 Resolution of seminal plasma via 2-D PAGE

Whole seminal plasma was electrophoresed on 2D Acid-Urea (AU)/Tricine-SDS-PAGE as previously described [34]. Briefly, seminal plasma was prepared in a 1:1 ratio with 0.2% cetyltrimethyl ammonium bromide (CETAB), and then admixed 1:1 with 3x AU loading dye (9 M urea, 5% acetic acid, and methyl green). A 12.5% native acid urea-polyacrylamide gel (AU-PAGE) was used as the first dimension to separate peptides based on their overall cationic charge density. The gel was electrophoresed at 65 V, for 20 – 22 h, stained with 10% (v/v) Amido Black, and each lane was excised and prepared for the second dimension by soaking in equilibrium buffer containing 10 mg/ml DTT. Gel strips were inserted horizontally and electrophoresed on a 16% Tricine-SDS-PAGE (2nd dimension) at 35 mA, for 27 – 29 h. Individual protein spots were visualized using SYPRO Ruby gel stain (Bio-Rad, Hercules, CA, USA), excised from the gel, and stored in 0.01% acetic acid at -20 ºC until analyzed by mass spectrometry.

2.2.5 Identification of individual cationic components in human seminal plasma

Excised 2D-PAGE spots underwent trypsin digestion and mass spectrometric (MALDI-TOF/TOF MS/MS) analyses as described previously [34]. The resulting digested peptide fragments were identified utilizing GPS Explorer 2.0 software (Applied Biosystems, Foster City, CA, USA) and a MASCOT (www.matrixscience.com) search engine. The NCBInr database and Homo sapien taxonomy were used for the searches.
2.2.6 Fractionation of seminal plasma based on charge-density

Whole seminal plasma was prepared for fractionation by adding 3X AU loading dye (1:2 with 70 µl SP-1). The Mini-prep Cell Fractionation and Continuous-Elution Electrophoresis System (Bio-Rad) with a 12.5% AU-PAGE column gel was utilized to collect larger amounts of cationic polypeptides. Elution buffer was 5% acetic acid using an isocratic gradient collecting at 0.1 ml/min. The column gel was electrophoresed at 70 V overnight, and fractions were collected every 15 min (1.5 ml/fraction). 40 fractions were collected for each run and either processed for purification immediately or stored at 4 ºC overnight.

2.2.7 Processing of fractionated seminal plasma

Fractions collected from the AU-PAGE column gel were dried under vacuum (Speedvac; ThermoSavant, Holbrook, NY, USA) with no heat. Fractions were then resuspended in HPLC-grade H₂O, and electrophoresed in order of elution (#1-40) on mini-16% Tricine-SDS gels (4 µl sample + 5 µl Laemli buffer + 3 µl SDS-loading buffer). These single-dimension electropherograms were compared with the 2D-PAGE gels to identify bands.

Fractions (#1-40) then underwent solid phase extraction to desalt and concentrate each sample in preparation for cell culture. Sep-Pak C18 polypropylene syringe-barrel cartridges (1cc, 50 mg) were used according to manufacturer’s instructions (Waters Corp., Milford, MA, USA). Cartridges were preconditioned with ddH₂O/0.1% trifluoroacetic acid (TFA), and eluted at a rate of ~0.25 ml/min. To ensure maximum binding, the flow-through was reapplied to the column bed thrice, washed with ddH₂O/0.1%TFA, and eluted with 60% ACN in HPLC-grade.
H$_2$O, followed by 100% methanol. All elutions were collected, dried under vacuum, and resuspended in HPLC-grade H$_2$O. Mini-16% Tricine-SDS gels were used to confirm that the protein banding patterns were identical to pre-desalted fractions.

### 2.2.8 Purification of SG-1 utilizing reverse-phase high pressure liquid chromatography

Fractions #5 – 9 (out of 40) containing a cationic peptide fragment of semenogelin I, which we termed “SG-1”, were pooled and subjected to reversed-phase (RP)-HPLC. For RP-HPLC, a Waters 2795 separations module and dual wavelength absorbance detector with a Waters XTerra analytical column (silica, C18, 4.6 mm x 250 mm, 5.0 µm particle size) were used. The aqueous mobile phase used was HPLC-grade H$_2$O with 0.1% TFA, against a 1ml/min gradient from 0-60% ACN/0.08%TFA. Peak fractions (280nm absorbance) were vacuum-dried and resuspended in 0.1% TFA and HPLC-grade H$_2$O. All fractions containing the semi-purified peptide were pooled together, and were subjected to microbore RP-HPLC (silica C18, 1.0 mm x 150 mm, 3.5 µm bead size, XTerra column; Waters Corp.) under a similar ACN gradient. Eluted fractions corresponding to the specific SG-1 peak on the chromatogram were vacuum-dried and resuspended in HPLC-grade H$_2$O. A Micro BCA Protein Assay, using diluted BSA as a standard, was performed to quantify the concentration of purified SG-1 in the pooled sample. Purified SG-1 was analyzed by MALDI-TOF-MS and N-terminal Edman degradation to determine exact mass of the polypeptide sequence.
2.2.9 Semenogelin specific Western blot analysis

Samples were electrophoresed in either 16% mini-Tricine-SDS gels, or 12.5% mini-AU gels, as described previously [65,66]. Gels were either stained with SYPRO Ruby gel stain (Bio-Rad) or transferred onto an Immobilon-P (Millipore, Billerica, MA, USA) membrane activated with methanol and Tris-buffered saline (TBS) at 180mA for 45min using the Mighty Small Transphor vertical electrophoresis system (Amersham Biosciences, Pscataway, NJ, USA). Membranes were fixed with 0.05% glutaraldehyde in TBS for 20 min, and blocked for 30 min at 37°C with Superblock (Pierce, Rockford, IL). Membranes were incubated overnight with a mouse polyclonal anti-semenogelin I (SGI) antibody (NOVUS Biologicals, Littleton, CO, USA), immunoreactive against the full-length human protein, diluted 1:1000 in antibody buffer (Superblock in TBS+0.05% Tween-20). Once washed, blocked at 37°C for 15min, and incubated with peroxidase conjugated anti-mouse immunoglobulin-G for 2 h, the membranes were again washed and developed using the Immun-Star HRP (Bio-Rad). Membranes were imaged and analyzed using ChemiDoc XRS with Quantity One software (Bio-Rad).

2.2.10 Statistical analyses

The antiviral and cytotoxicity assays were performed in triplicate for each treatment of each experimental condition. For the TZM-bl antiviral assays, luciferase was measured as relative light units (RLU), and the infected vehicle-only controls were set as 100% infection. For the PM1 antiviral assays, p24 ELISA quantification established the infected, vehicle-only control as 100% infection. Metabolic and cytotoxicity assays compared results to the untreated, vehicle-
only control, calculating variations as a percentage of the baseline. Individual treatments were analyzed by either one-way ANOVA with Tukey’s multiple comparisons post-test, or two-tailed unpaired t-test. Mass spectrometric analysis of excised spots from the 2D-PAGE were performed, and identified proteins with a confidence index >85% and ion scores >40 were considered positive.

2.3 Results

2.3.1 Human seminal plasma (SP) exhibits significant anti-HIV-1 activity

In order to determine the intrinsic anti-HIV-1 activity of SP, we assayed whole SP against the R5 strain HIV-1 BaL in TZM-bl cells. An R5 phenotype was chosen since it is primarily responsible for sexually transmitted HIV-1. After 24 h of infection the cells were lysed, and luciferase activity was measured (Figure 2.1A, P<0.001, n=3). Whole SP revealed significant antiviral activity: at 1% concentration (v/v, 1:100) viral inhibition remained >95%, and SP-1 dilutions greater than 1:100 reduced the rates of infection in a dose dependent manner in the absence of cytotoxicity (Figure 2.1B, n=3) or adverse effects on cellular metabolism (Figure 2.1C, n=3). Because SP diluted up to 1:3200 retained anti-HIV-1 activity, these data suggest that the SP component of semen is potently active against HIV-1.
Figure 2-1: Whole seminal plasma inhibits HIV-1 infection

(A) TZM-bl cell cultures were treated with serial dilutions of SP or a PBS vehicle control, and infected with the BaL laboratory strain of HIV-1 (5 ng/ml p24) for 24 hours. Inhibition of viral infection was measured as a percent reduction in luciferase activity (Relative Light Units – RLU) compared to an infected, vehicle-only control. All
dilutions compared to the control were significantly different, (P<0.0001).  (B) Trypan blue assays were performed at 24 h to measure the cytotoxicity of SP, and were expressed as the percent of nonviable cells over the total number of cells.  (C) An MTT assay was also performed in the absence of infection, and measured at 24 h post-treatment. Reduction in cellular metabolic was measured as a percent of decreased tetrazolium production in comparison to a vehicle-only control. All dilutions compared, except for 1:10 (P<0.0001), did not statistically reduce metabolic activity as compared to the control.  n = 3; and error bars represent SEM.

To further validate the anti-HIV activity of SP, the antiviral activity was confirmed by infecting PM1 cells with HIV-1 BaL and measuring viral p24 release. Virus in the presence or absence of vehicle or serial dilutions of SP was incubated with PM1 cells for 5 days.  As compared to a vehicle-only control, the lowest dilution (1:50) of SP exhibited complete viral inhibition, while serial dilutions displayed dose-dependent antiviral activity (Figure 2.2A, P<0.0001, n=3), in the absence of appreciable cytotoxicity (Figure 2.2B).  Taken together, these results confirm the anti-HIV-1 activity of SP.
Figure 2-2: Whole SP inhibits HIV-1 release
(A) PM1 cells were infected with HIV-1 BaL (2 ng/ml p24) in the presence or absence of serially diluted SP or vehicle-only control. Five days post-infection supernatant was collected to quantify p24 release by ELISA. Data are expressed as a percent inhibition of infection in relation to the infected vehicle-only control (for all dilutions, P<0.0001). (B) PM1 cells were subjected to a Trypan blue assay, and the measurement of cytotoxicity was expressed as the percentage of cells over the total number of cells in the infected vehicle-only control. Except for the 1:50 dilution (P<0.05), no other dilutions were cytotoxic. n=3-6; error bars represent SEM.

2.3.2 Human SP contains numerous cationic polypeptides

We have previously reported that the majority of cationic polypeptides within human vaginal fluid collectively inhibit HIV-1 infection [34]. Herein, we sought to determine if human
seminal plasma (SP) contains cationic polypeptides, whether these polypeptides are similar to those of human vaginal fluid, and whether the polypeptides exert activity against HIV-1. A unique 2D AU/Tricine-SDS-PAGE approach was utilized to resolve the cationic polypeptide fraction of SP. Figure 3 reveals a SYPRO Ruby-stained 2D electropherogram of whole SP (6.5 µl) electrophoresed in an AU-PAGE gel in the first dimension (horizontal) followed by Tricine-SDS-PAGE in the second dimension (vertical), with each spot representing an individual (poly)peptide. Spots were excised from the gel, digested with trypsin, and 52 individual polypeptides were identified by tandem mass spectrometric analysis (MALDI-TOF/TOF MS/MS). The identity of each of the 52 polypeptides in Figure 2.3 is provided in Table I. Interestingly, 33 of the spots were found to represent fragments of eight parental proteins commonly found in seminal plasma, several of which have putative roles in innate host defense: semenogelin I, semenogelin II, prolactin-induced protein, prostatic acid phosphatase, lactoferrin, prostatic-specific antigen, beta-microseminoprotein, and cystatin C.
Figure 2-3: Resolution and identification of 52 individual cationic polypeptides in human SP. Whole seminal plasma (6.5 µl SP-1) was electrophoresed by AU-PAGE in the first dimension, followed by a Tricine-SDS-PAGE in the second dimension, and stained with SYPRO Ruby. Note that polypeptides with the highest cationic charge density migrated towards the lower right side of the gel. Protein spots were excised and identified by MALDI-TOF/TOF MS/MS analysis. All labeled spots (#1-52) correspond to proteins described in Table I.
Table 1: Identified cationic polypeptides from 2D-PAGE (Figure 3) and MALDI-TOF/TOF MS/MS analysis.

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<th>Ion Score</th>
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### 2.3.3 Cationic polypeptides are responsible for the anti-HIV-1 activity of SP

We utilized a continuous-elution semi-preparative AU-PAGE column gel to isolate each cationic polypeptide fraction from whole seminal plasma for downstream analysis of anti-HIV-1 activity and cytotoxicity. Forty SP fractions, which corresponded to the majority of cationic polypeptides present in Figure 3, were collected and individually subjected to Tricine-SDS-PAGE. Note that the combination of using a fractionated AU column-gel and Tricine-SDS gels is equivalent to the first and second dimensions, respectively, of the analytical 2D AU/Tricine-SDS-PAGE. Referencing the protein bands in the Tricine-SDS gels (Figure 2.4A) to the corresponding spots of a 2D PAGE electropherogram of SP (Figure 2.4A and Figure 2.3) afforded the identification of each protein band.
Each of the 40 SP fractions was subsequently prepared for antiviral assays by undergoing lyophilization to remove volatile acetic acid, followed by solid phase extraction to desalt the samples. Samples were diluted 1:10 in ddH₂O, and subjected to antiviral assays against HIV-1 BaL as described above. Fractions #1-10, which contained polypeptides with the highest
Figure 2-4: Cationic polypeptide fractions of SP exhibit significant anti-HIV-1 activity.
(A) After column-gel AU-fractionation of whole SP, 40 fractions were collected and electrophoresed on mini-
Tricine-SDS gels. Silver staining revealed peptide bands retained in each individual fraction. To reference the
fractions to the corresponding spots previously identified on the 2D-PAGE, the 2D SYPRO Ruby-stained gel image
was inverted and displayed as a negative digital effect, and flipped in the horizontal axis as compared with Figure 1. In this orientation, the most cationic polypeptides are to the left of the gel. (B) Cells were treated in a TZM-bl antiviral assay with the fractionated SP (fractions 1-40) at a dilution of 1:10. Fractions #5-9 were the most active, and contained the identified semenogelin I peptide termed SG-1 (spot #35 in Figure 1). (C) An MTT assay conducted in parallel to the antiviral measured metabolic reduction as the percent reduction of the media-only negative control. n = 4; error bars represent SEM.

cationic charge density, exhibited the greatest activity against HIV-1 (Figure 2.4B, n=4) as well as negligible cytotoxicity and reduction of cellular metabolic activity (Figure 2.4C, n=4).

2.3.4 SG-1, a 52 residue semenogelin I-derived peptide is active against HIV-1

Next, we assessed whether individual cationic polypeptides in SP were directly active against HIV-1. The most abundant, highly cationic protein on SYPRO Ruby-stained 2D-PAGE gels (Spot #35, Figure 2.3) was chosen since the corresponding fractions, eluted from an AU-PAGE column gel, exhibited appreciable activity against HIV-1 (Figure 2.4B). Fractions #5-9 from an AU-PAGE column gel (Figure 2.4A) were pooled, and subjected to a two-step RP-HPLC purification (Figure 2.5).
Figure 2-5: Purification of SG-1 by analytical and microbore RP-HPLC. SG-1 was purified by a two-step RP-HPLC, with fractionation using the organic solvent acetonitrile and 0.08% TFA as an ion pairing agent. The solvent gradient is shown as a percentage (dotted line), while the eluted peptides are represented as the intensity of the total ion current (TIC; solid line). (A) The analytical column was loaded with the fractionated SP-1 pool (fractions #5-9 from Figure 4) the semi-pure peak fraction representing SG-1 was loaded onto a microbore column (B) and SG-1 eluted at 17.25% of the ACN solvent.

The resulting single protein band was visualized (Figure 2.6) and sequenced by a combination of N-terminal Edman peptide sequencing and MALDI-TOF mass spectrometry (Figure 2.7A). The peptide, termed “SG-1”, was identified as a 5750.638 Da peptide whose mass and N-terminal sequence (HNKQEGRDHDKSKGH-), corresponded to the theoretical mass of 5750.837 Da from the following 52 amino acid sequence: NH2-
HNKQEGRDHDKSKGKHFRVVIHHKGKAHRGTQNPSQDQGNSPSGKGISSQY-COOH, which is a naturally cleaved fragment of the mature 462 amino acid semenogelin I polypeptide [12]. The SG-1 peptide had been identified previously as one of several basic proteins in liquefied SP [67].

Figure 2-6: Stepwise purification of the SG-1 peptide.
Purification of SG-1 from whole SP to isolated pure peptide was represented by electrophoresing samples from each step of purification on a mini-Tricine-SDS gel. A 1:100 dilution of SP, a sample of fractionated SP (fractions #5-9 pool), a sample of the semi-purified SG-1 from analytical RP-HPLC, and the purified SG-1 from the microbore RP-HPLC column (4 µl each) were visualized with (A) Coomassie blue staining, and (B) silver staining. Native SG-1 was utilized in the remaining assays.
To determine if SG-1 exhibited antiviral activity, the purified peptide was subjected to anti-HIV-1 assays. SG-1 maintained anti-HIV-1 activity in both a TZM-bl cell assay (Figure 2.7B, n=3), and a PM1 cell antiviral assay (Figure 2.7C, n=3), and displayed negligible cytotoxicity (Figures 2.7B-C). As the concentration of SG-1 active against HIV-1 in vitro is far less than the estimated physiological concentration (approximately 80 µg of SG-1 per ml of whole SP, data not shown), it is likely that the SG-1 peptide contributes significantly to the antiviral host defense of SP.

Figure 2-7: SG-1 peptide is naturally active against HIV-1. SG-1 was sequenced and identified by Edman degradation and mass spectrometric analysis. Mature semenogelin I, lacking the 23 residue signal peptide sequence is represented in (A), and the 52 amino acid SG-1 sequence is identified. (B) A TZM-bl antiviral assay against HIV-1 BaL (5ng/ml p24) was performed with the natural SG-1 peptide (5 µg/ml). Inhibition of viral infection was measured as a percent reduction in luciferase activity, compared
to the vehicle-only control. A Trypan blue assay was performed in parallel to measure cytotoxicity and expressed as a percent of nonviable cells from the total number of cells. (C) PM1 cells were infected with HIV-1 BaL (2 ng/ml p24) in the presence or absence of natural SG-1 peptide (15 ug/ml) or vehicle-only control. Supernatant was collected five days post-infection to quantify p24 release by ELISA. Data are expressed as a percent inhibition of infection in relation to the infected vehicle-only control. On day 5, post-infection PM1 cells were subjected to a Trypan blue assay, and the measurement of cytotoxicity was expressed as the percentage of cells over the total number of cells in the infected vehicle-only control. n = 3; error bars represent SEM.

2.3.5 Prolonged incubation of whole SP reduces anti-HIV-1 activity and degrades the majority of semenogelin I peptides

Since semenogelin-derived peptides continue to degrade overtime, we wanted to determine if proteolytic cleavage affects the anti-HIV-1 activity of human SP, the SP samples were incubated at timed intervals at 37 °C with constant mild agitation (250 rpm). Incubated SP was tested for antiviral activity in comparison to whole SP without incubation in a TZM-bl cell assay (Figure 2.8A, n=3). SP incubated up to 24 h retained significant anti-HIV-1 activity in the absence of cytotoxicity (Figure 2.8B), while incubation intervals longer than 24 h exhibited a reduction in antiviral activity. These data suggest that the anti-HIV-1 activity of incubated SP decreases proportionally with increased incubation time.
Post-ejaculation, the SP coagulum liquefies due to proteolytic processing; however, this proteolytic cleavage does not cease once the coagulum is liquefied [11]. To determine if the effects of prolonged SP incubation included degradation of cationic polypeptides, the samples
were subjected to AU-PAGE (Figure 2.9A), and Tricine-SDS-PAGE (Figure 2.9C). Protein band patterns visibly changed at 6 h of incubation and progressively dispersed into protein bands with higher cationic charges and smaller molecular weight. To track the effects of prolonged incubation on the antimicrobial semenogelin I proteins, samples were subjected to Western analysis (Figures 2.9B and 2.9D) with an anti-semenogelin I polyclonal antibody. It has been shown that semenogelins are selectively degraded overtime [36]. The effects of incubation on semenogelin I proteins were visible after 6 h, exhibiting an increasingly degraded protein band pattern in samples incubated for 24 h or longer. These data suggest that the decreased anti-HIV-1 activity of incubated SP is directly correlated with the increased degradation of semenogelin I proteins. Due to the abundance of semenogelin derived peptides in comparison to other identified peptides observed in our study (Table 1), and the activity exhibited by semenogelin derived peptides, this study confirms the importance of semenogelins to the intrinsic antiviral activity of SP.
Figure 2-9: Prolonged incubation of SP results in increasingly degraded Semenogelin I. Incubated SP samples (1 µl/lane) were electrophoresed on mini-polyacrylamide gels. Samples were electrophoresed on 12.5% mini-AU gels and stained with, (A) SYPRO ruby protein stain, or (B) Western blotting with a polyclonal anti-semenogelin I antibody. Degradation of cationic semenogelin I peptides was evident with prolonged incubation time. SP samples were also electrophoresed on 16% mini-Tricine-SDS gel, and stained with, (C) SYPRO ruby protein stain, or (D) Western blotting with the anti-semenogelin I antibody. A similar pattern of peptide degradation was shown with prolonged incubation.

2.4 Discussion

Mucosal secretions are thought to play a fundamental role in host defense, due in large part to intrinsic antimicrobial peptides and proteins elaborated in the fluid. In the current study, we revealed that SP exhibited significant anti-HIV-1 activity, and that the activity resided mainly
in the cationic polypeptide fraction. Furthermore, polypeptide fractions with greater cationic charge density demonstrated increased anti-HIV-1 activity as compared to less cationic polypeptide fractions, suggesting that charge was an essential determinant of antiviral activity. Upon prolonged incubation of SP, antiviral activity decreased concomitant with the degradation of semenogelin I peptides, further emphasizing the role of cationic antimicrobial polypeptides in anti-HIV-1 host defense. While our study is the first to associate the majority of cationic polypeptides found in human SP with the fluid’s intrinsic anti-HIV-1 activity, this finding was not altogether surprising: the vast majority of polypeptides with reported activity against microbes and viruses are cationic in nature [68].

The 2D AU/Tricine-SDS-PAGE system was chosen for its unique ability to separate cationic polypeptides. Indeed we have utilized this technique to identify cationic polypeptides that contribute to the antimicrobial and antiviral host defenses of human nasal fluid and vaginal fluids [34,54]. In the current study, 33 of the identified polypeptides were relevant to host defense. Many polypeptides were cleavage products of semenogelin I and semenogelin II, the most abundant proteins in SP [9,69,70], both of which have been reported to exhibit antibacterial activity [36,62]. In addition, the protease prostatic-acid phosphatase (PAP) is involved in the processing and degradation of semenogelins, and likely plays an important role during postcoital coagulum processing to liberate active anti-HIV-1 peptides [14]. The seminal vesicle derived prolactin-induced protein (PIP), also known as gp17 glycoprotein, is a high affinity CD4 ligand that has been shown to inhibit HIV-1 gp120-mediated syncytium formation [71]. Lactoferrin, a broad spectrum antimicrobial protein, can prevent the early stages of HIV-1 infection by preventing the virus from entering host cells [72-75]. Beta-microseminoprotein is one of the
predominant proteins secreted from the prostate; and, while there are no definitive biological functions ascribed to this protein, it has been shown to decrease tumor growth in the prostate, and has purported roles in host defense [76,77]. The cysteine proteinase inhibitor cystatin C reportedly prevents viral replication of HSV and coronaviruses [78-80]. Collectively, the identity and function of these polypeptides strongly suggest a function for SP in antiviral host defense. As most of these polypeptides are not components of vaginal fluid, it remains to be determined whether the antiviral polypeptides from SP work in concert with polypeptides from vaginal fluid to prevent sexual transmission of HIV-1.

SP is a complex biological fluid, containing both antiviral and proviral factors [81]. Importantly a recent study revealed that naturally occurring PAP fragments in semen form amyloid fibrils which enhance HIV-1 infection by capturing virions and promoting attachment to target cells [48]. There are several differences between their approach and our current study that might explain these contrasting findings. Methodological differences in our study include the initial liquefaction of SP, the use of FBS in HIV-1 infection assays, and extended incubation of target cells with SP. While their study defends the washing of cell monolayers after 3h due to concerns of cytotoxicity, our study suggests that at the low concentrations of SP needed to observe antiviral activity (less than 2% v/v of SP) cellular metabolism was normal and insignificant cytotoxicity was observed. Our 2D analysis displayed PAP fragments at ~16.5kDa, but we did not observe any at the designated 4-4.5 kDa range previously published as HIV enhancers. While these differences prevent a truly paralleled comparison, both studies should be taken together as they suggest that pro- and antiviral forces may be in direct competition in SP in vivo. In fact, we have also shown that vaginal fluid elaborates components (e.g., calgranulin A)
that are permissive for viral infection [34]. Whether the relative concentrations of pro- and 
antiviral components in an individual’s seminal or vaginal fluid are predictive of susceptibility to 
HIV-1 infection is a subject that warrants further investigation.

Our study demonstrated that human SP exhibits significant intrinsic anti-HIV-1 activity and characterized a cationic polypeptide (SG-1) as an anti-HIV-1 component of SP. Future 
studies, which we are actively pursuing, will further analyze the mode of viral inhibition SG-1 
employs. While previously established as antibacterial, no other studies have determined that 
semenogelins exhibit significant antiviral activity. These findings set the stage for future studies 
to determine the host defense role of the myriad other cationic polypeptides we identified in SP. 
The presence of such an anti-HIV-1 mechanism in SP may be one reason why the chance of 
infection is so low (approximately 3 in 1000 coital acts) in healthy populations [82].
CHAPTER 3 HIV-1 ENHANCING EFFECT OF PROSTATIC ACID PHOSPHATASE PEPTIDES IS REDUCED IN HUMAN SEMINAL PLASMA

3.1 Introduction

Mucosal tissues express a number of antimicrobial peptides and proteins that exert broad spectrum activity against fungi, bacteria, and viruses such as HIV-1 [28,53,58]. Many are cationic in nature, and owe their ability to prevent microbial and viral infections in part to electrostatic interactions with membrane surfaces [59,83]. The antimicrobial activity of human seminal plasma (SP) has been established for decades [84,85]. Several reports have studied the individual ubiquitous innate immune components present in SP, including lactoferrin, lysozyme, HBD-1, and antimicrobial chemokines [29,35], as well as SP specific antimicrobial peptides, including HE2α C-terminal fragments [32], and semenogelin-derived peptides [36,62]. While the antibacterial properties of seminal fluid have been established, only recently have the anti-HIV-1 activities of human SP been described [86].

While human SP contains various antimicrobial factors, a number of proviral factors have also been identified [46,81]. One recent study has reported the ability of a natural proteolytic fragment of the protein prostatic acid phosphatase (PAP\textsuperscript{286}; residues 248-286) to form amyloid fibrils that are capable of enhancing HIV-1 infection. Amyloid fibrils from the PAP-derived peptide were generated \textit{in vitro} through long periods of agitation, and deemed Semen-derived Enhancer of VIRus (SEVI) [48]. Interestingly, the positive charge (pI = 10.2) of SEVI reportedly
decreases the electrostatic repulsion between the negative charge of the HIV virions and the negative overall charge of the target cell membrane, leading to enhanced virion attachment [51].

Whole PAP is stored in the prostate, along with various other enzymes and a large zinc ion reservoir that maintains prostate-derived enzymes in an inactive state [13,87]. Upon ejaculation, semen forms a gelatinous meshwork and is subsequently liquefied by activated prostatic enzymes, principally the kallikrein-like serine protease prostate-specific antigen (PSA) [11,12]. At neutral pH, PAP reportedly exhibits amidolytic activity on semenogelins, the major components of the seminal coagulum [14]. We have recently reported that SP continues to degrade most of its intrinsic proteins after liquefaction [86]; however, it has yet to be determined how PAP undergoes cleavage into the PAP$^{286}$ fragments.

In the current study, we sought to elucidate the biological role of PAP$^{286}$ with respect to its ability to form amyloid fibrils and promote HIV-1 infection in the presence of human SP. While we could confirm that PAP-derived amyloid fibril formation exhibited HIV-1 enhancing activity, we found that this proviral activity was neutralized by human SP. Moreover, SP retained significant anti-HIV-1 activity in the presence of supra-physiological concentrations of PAP amyloid fibrils. PAP peptides were degraded into fragments that were not proviral within 3 h of incubation with diluted SP, and this proteolytic degradation was due to SP enzymes, including prostate derived PSA and prostasin [12,88], and epithelial derived matriptase [89]. Together, these results confirm the ability of PAP-derived amyloid fibrils to enhance HIV-1 infection alone; however, in the physiological milieu these peptides and their resulting fibrils might be susceptible to proteolytic degradation that could inhibit their proviral activity.
3.2 Materials & Methods

3.2.1 Ethics Statement

Samples of human semen were collected by the Center for Reproductive Medicine as described previously [86]. As these samples were discarded from routine testing, and not linked to any identifiers, the University of Central Florida IRB has deemed them exempt human subjects; therefore obtaining informed consent was not applicable for this study.

3.2.2 Processing of human seminal plasma and PAP peptides

Semen was collected by the Center for Reproductive Medicine as described previously [86]. Briefly, semen was collected from patients who were asked to refrain from ejaculation for 2-5 days prior. Semen was obtained via dry masturbation into a sterile polypropylene cup, and allowed to liquefy for 30 min at room temperature. A total of 103 individual seminal plasma (SP) samples were centrifuged for 30 min at 1500 x g, and the supernatants were pooled and stored at -80 °C or used for subsequent analyses. An aliquot of the SP pool, referred to as “SP+(Ab)” hereafter, was filtered through a sterile, nylon, 45 μm syringe filter (Fisher Scientific, Pittsburgh, PA USA) and supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin as described previously [48]. An additional pool of eight semen samples was collected, allowed to liquefy at room temperature for 30 min, and portioned into three fractions, “Pre-SP” (SP obtained from semen prior to freezing at -20 °C), “Post-SP” (SP
obtained from semen after freezing) and whole semen. All semen and SP samples were then stored at -80 °C until analyzed.

The synthetic PAP peptides corresponding to fragments PAP 248-266 (PAP^{266}) and PAP 248-286 (PAP^{286}) were synthesized as previously described [48], via standard Fmoc solid phase chemistry using a model 433A peptide synthesizer (Applied Biosystems, Foster City, CA, USA). Crude peptides were purified by preparative reversed-phase HPLC to >95% purity, and were lyophilized. Masses were confirmed by MALDI-TOF MS. The purified synthetic peptides were resuspended in sterile PBS and stored at -20 °C. To generate SEVI, peptides were agitated in a thermomixer at 1400 rpm for 18 h at 37 °C as described previously [48]. For visual documentation, PAP peptides (1 – 5 mg/ml) with or without SP (1%) were agitated in a thermomixer at 1400 rpm for 36 h at 37 °C to form observable amyloid fibrils as described in [48,90], and were pulsed for 20 s at 10,000 rpm in a microcentrifuge to sediment amyloid fibrils. Amyloid fibril formation was monitored with Congo red staining as previously described [90]. Fibril formation was measured at OD_{490}nm using a spectrophotometer. Stained fibrils were visualized using phase contrast microscopy (Axiovert 200M microscope, and Axiovision 4.5 software, Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

3.2.3 Cell lines and viruses

The HeLa-derived epithelia TZM-bl cell line and the lymphocytic PM1 cell line were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD, USA), and Peripheral Blood Mononuclear Cells (PBMCs) were obtained
from healthy donors by AllCells, LLC (Emeryville, CA, USA). TZM-bl cells were cultured in high glucose DMEM (Mediatech, Manassas, VA, USA) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% (v/v) Fetal Bovine Serum (FBS) (Gemini Bio-Products, West Sacramento, CA, USA). PM1 cell cultures were maintained at a density of 0.4 – 0.8 x 10^6 cells/ml in RPMI 1640 supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, and 20% (v/v) FBS. PBMCs were stimulated with phytohemagglutinin (PHA) (5 µg/ml) and 50 units/ml IL-2 for the first 3 days, and then maintained at a density of 0.75 - 1.5 x 10^6 cells/ml in R10 medium (RPMI 1640 with 10% FBS) supplemented with 25 units/ml IL-2 (Roche Applied Science, Indianapolis, IN, USA). HIV-1 BaL, an R5-tropic strain, was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD, USA). HIV-1 BaL was propagated in PM1 cells, and supernatant was filtered and stored at -80 ºC until needed. Viral quantification was achieved via a sensitive commercial ELISA for p24gag (PerkinElmer, Waltham, MA, USA).

3.2.4 Antiviral and cytotoxicity assays

Antiviral assays were performed utilizing TZM-bl cells as previously described [86]. Briefly, plated cells (6 x 103 cells/well incubated for 48 h, 4 x 103 cells/well incubated for 24 h, or 8 x 103 cells/well incubated for 24 h, 100 µl/well, 96 well plates) were treated in triplicate. Original media was removed, and 50 µl treatments were added to cells, which included vehicle only (PBS) or a dilution of SP, semen, SEVI (agitated PAP286), agitated PAP266, or a combination. Within 5 min of treatment being supplemented, 50 µl of HIV-1 BaL (4 ng p24/ml
or 200 pg p24/well) or control media were added to cells. Cells were either treated for 24 h, 3 d, or washed 3 h post-infection as previously described [48].

A repeat of the methodology used to determine the effect of seminal fluid on HIV-1 in infection [48] was carried out as follows. TZM-bl cells were plated (1.4 x 10⁴ cells/ml incubated 24 h, 280 µl/well, 96 well plate), and treated in triplicate. 40 µl treatments of SP diluted in PBS with or without SEVI or media only controls were pre-incubated with 40 µl of HIV-1 BaL (120 ng /ml or 4.8 ng p24) for 10 m at room temperature. Cells were infected by adding 20 µl of the pre-incubated treatment to the 280 µl of media, thus diluting the treatment and/or virus 15-fold. After 3 h of incubation the treatment media was removed, cells received 200 µl fresh media, and they were incubated for 3 days.

All treated cells were lysed using a Bright Glo luciferase system (Promega, Madison, WI, USA), and the ability to prevent HIV-1 infection was measured as a percent reduction in luciferase (relative light units or RLU) compared to the positive viral control (media and virus only). Metabolic activity of the cells was verified by a tetrazolium-based (MTT) assay according to manufacturer’s instructions (R&D Systems, Minneapolis, MN, USA), while cytotoxicity was measured using the standard trypan blue dye exclusion assay.

PM1 cells (1.5 x 10⁵/0.1 ml) and PBMCs (5 x 10⁵/0.1 ml) were treated with SEVI with or without SP, and infected with HIV-1 BaL (200 pg p24/0.1 ml) for 2 h. Cells were then washed, and resuspended in fresh media with sample treatments for 5-7 days. Supernatants were collected on alternate days, and cells in culture were resuspended in sample or media alone. To ensure cell viability, standard trypan blue dye exclusion assays were performed. To quantify
viral inhibition, the amount of p24gag was measured in cell supernatants with an ELISA (PerkinElmer, Waltham, MA, USA).

3.2.5 PAP peptide incubations with SP and proteases

SP with or without a broad spectrum Protease Inhibitor (PI) cocktail containing, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin (Sigma) was incubated with PAP\textsuperscript{286} or PAP\textsuperscript{266} for 1 – 24 h at 300 rpm and 37 °C. Serial dilutions of whole SP (1% – 1:3200) were incubated with PAP\textsuperscript{286} for 24 h at 1400 rpm and 37 °C. Semen derived PSA and PAP were obtained from Sigma, trypsin was obtained from Difco Laboratories (Detroit, MI, USA), and prostasin and matriptase were produced as previously described [91,92]. The proteases PSA, prostasin, trypsin, and matriptase were diluted with sterile PBS to a final concentration of [1 µM] and incubated with PAP\textsuperscript{286} [54 µM] for 24 h at 300 rpm and 37 °C. A series of PSA concentrations (1.5 µM – 0.125 µM) were incubated with PAP\textsuperscript{286} (54 µM), and were agitated at 300 rpm for 24 h at 37 °C. Whole PAP was resuspended in sterile PBS to [10 µM]. 1% SP was incubated with PAP at a final concentration of [2 µM] at 300 rpm and 37 °C for timed intervals of 3, 6, 12 & 24 h. All incubation sample tubes were pulsed briefly in a microcentrifuge, and immediately stored at -20 °C. Samples were electrophoresed on mini-16% Tricine-SDS gels, and stained.
3.2.6 Identification of protease cleavage products

Digested peptide fragments from protease incubations underwent MALDI-TOF/TOF MS/MS analysis using the Model Ultraflex III mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with LIFT capability. Samples were desalted using C18 ZipTips (Millipore, Bedford, MA, USA) and were analyzed in positive reflector mode. External calibration was performed using a mass standards kit for proteomics analyzer (Applied Biosystems). Samples also underwent analysis via nano-LC ESI-TOF MS/MS using the maXis ESI-Q-TOF (Bruker Daltonics) mass spectrometer online with the Dionex model U3000 nanobore HPLC [93]. Data were analyzed with the Sprot database using the Bruker ProteinScape program version 2.1 and Mascot program version 2.2 with enzyme setting to semiTrypsin.

3.2.7 Statistical analyses

The antiviral, metabolic, and cytotoxicity experiments were performed at least three times, and each of the assays were performed in triplicate or quadruplicate. For the TZM-bl antiviral assays the infected vehicle-only controls were averaged and set as 100% infection. For the PM1 and PBMC antiviral assays, p24 ELISA quantification established the infected vehicle-only control as 100% infection. Metabolic and cytotoxicity assays compared results to the vehicle-only control, calculating variations as a percentage of the baseline. Individual treatments were analyzed by either one-way ANOVA with Tukey’s multiple comparisons post-test or two-tailed unpaired t-test.
3.3 Results

3.3.1 PAP$^{286}$ amyloid fibril formation is inhibited by seminal plasma

To confirm our synthetic PAP peptides would form detectable amyloid fibrils in vitro, the PAP$^{286}$ and PAP$^{266}$ peptides were agitated separately at either 1 mg/ml or 5 mg/ml according to the established protocol for fibril formation [48]. Only agitated PAP$^{286}$ (“SEVI”) generated turbid solutions and formed an observable precipitate after centrifugation that was concentration-dependent (Figure 1A). In the presence of 1% SP (v/v), generation of precipitate was either totally (1 mg/ml SEVI) or partially (5 mg/ml SEVI) inhibited (Figures 1A). To visualize the amyloid fibrils, samples were homogenized or stained with Congo red, wet mounted on a slide, and viewed under phase contrast at 40x magnification. Several random field images were taken, and SEVI alone (5 mg/ml) (Figures 3.1B & 3.1E) revealed numerous fibrillar clusters, while the addition of 1% SP (Figures 1C & 1F) substantially reduced fibril formation. The fibril formation comparisons among SEVI, SEVI with 1% SP, and agitated PAP$^{266}$ are demonstrated with the OD values from the Congo red stained samples (Figure 3.1D). To confirm that SEVI fibrils expressed HIV-1 enhancing activity, SEVI was added at various concentrations to TZM-bl cells with R5 strain HIV-1 BaL for 24 h (Figure 3.1G). SEVI demonstrated enhancement of HIV-1 in a dose-dependent manner. To ensure cell viability, MTT assays were run in parallel to the infection assays, and showed no adverse effects to cell metabolism (Figure 3.1H). These studies confirmed that our synthetic PAP peptides form active amyloid fibrils in vitro, and that diluted human SP can inhibit amyloid fibril formation.
Figure 3-1: SEVI amyloid fibril formation is inhibited by SP.
The synthetic PAP286 peptide was agitated at concentrations of 1 mg/ml and 5 mg/ml with or without whole SP (1%) for 36 h at 1400 rpm at 37 °C. (A) Sample tubes were centrifuged briefly at 10,000 rpm, and each photographed at the same angle. The 5 mg/ml samples without SP (B) and with SP (C) were vortexed briefly and 20 µl was wet mounted on a slide. (D) Agitated 5 mg/ml PAP266, 5 mg/ml SEVI, or SEVI+1% SP were stained with Congo red and measured at 490 nm with a spectrophotometer. Images of stained 5 mg/ml SEVI without SP (E) or with 1% SP (F), were captured at 40x magnification with phase contrast filters under white light using the Axiovert 200M microscope and Axiovision 4.5 software. (G) TZM-bl cells (6 x 103 cells/well incubated for 48 h) were treated with serial dilutions of SEVI and infected with HIV-1 BaL (200 pg p24/well). Results are presented as a percent enhancement of viral infection compared to an infected, vehicle-only control. (H) Identically treated cells were also subjected to MTT metabolic assays, presented as percent reduction in cellular metabolism when compared to cells
3.3.2 The HIV-1 enhancing activity of SEVI is inhibited by seminal plasma

In order to determine if the HIV-1 enhancing activity of SEVI is affected by SP, TZM-bl cells (plated at 6 x 103 cells/well and incubated for 48 h) were infected with HIV-1 BaL for 24 h in the absence or presence of agitated PAP\textsuperscript{286} (Figures 3.2A, C, E & G) and agitated PAP\textsuperscript{266} (Figures 3.2B, D, F & H). Note that since fibrils formed only with PAP\textsuperscript{286} peptides, but not PAP\textsuperscript{266} peptides, the term “SEVI” applies only to agitated PAP\textsuperscript{286} peptides. In specified conditions, whole SP was also agitated to determine if SEVI fibrils would form and exhibit HIV-1 enhancing activity. SP was utilized at a final concentration of 1% (v/v) to mitigate cytotoxic effects. Treatments with SEVI (Figure 3.2A) and to a lesser extent agitated PAP\textsuperscript{266} (Figure 3.2B) revealed HIV-1 enhancing activity at physiological concentrations (35 µg/ml), but these enhancing effects were negated when combined with agitated or non-manipulated SP. We then repeated these antiviral assays, but instead washed the cells with PBS and replaces with media alone 3 h post-infection as reported in [48]. As shown in Figures 2C & D, the trends remained as compared to the 24 h treatment (Figures 3.2A & 2B). Moreover, we performed certain experiments in the absence of FBS as reported in [48], which also revealed that SP could reduce the proviral activity of SEVI and PAP\textsuperscript{286} (data not shown). To verify cell viability, MTT assays were run in parallel to all antiviral assays, and revealed no significant adverse effects to metabolic activity of cells treated for 24 h (Figures 3.2E & 3.2F) or washed after 3 h (data not
shown). For further confirmation, trypan blue assays were also performed in parallel to the infection assays at 24 h of treatment (Figures 3.2G & 3.2H) and revealed a lack of cytotoxicity.

Figure 3-2: Agitated PAP peptides do not significantly inhibit the anti-HIV-1 activity of SP. Whole SP, synthetic PAP$^{286}$, or synthetic PAP$^{266}$ were agitated at 37 °C to promote fibril formation. TZM-bl cells (6 x 103 cells/well incubated for 48 h) were then treated with either PBS, unagitated SP, agitated SP, agitated PAP$^{286}$ (SEVI, Panel A), agitated PAP$^{266}$ (Panel B), or combinations of agitated peptides and SP, and infected with HIV-1 BaL (200 pg p24/0.1 ml) for 24 h (A & B). SP preparations were administered at a final concentration of 1% (v/v) when diluted with cell media and virus. PAP peptides were administered to cells at a final concentration of 35
µg/ml. (C & D) TZM-bl assays were performed as in A-B except that the cells were washed 3 h post-infection. Results for A-D are presented as % inhibition or enhancement of viral infection compared to the cells infected in the presence of PBS alone. Identically treated TZM-bl cells were also subjected to MTT metabolic assays (E & F) as described in Fig. 1, and trypan blue cytotoxicity assays (G & H) in which % cytotoxicity was calculated from non-viable versus viable cell counts for each treatment condition. All experiments were performed at least 3 times. Error bars represent SEM.

In the results above, SP exhibited antiviral activity as previously seen in our work [86], but in contrast to the results demonstrated by others using subtle differences in methodology [48]. Therefore, to determine if the difference in SP activity was influenced by methodology of treatment, the methods used to determine the effect of seminal fluid on HIV-1 infection were followed precisely as described previously [48]. Minimally manipulated SP (0.4%, 2%, 10%) was tested in parallel with antibiotic supplemented and filtered SP “SP+(Ab)”, with or without SEVI, such that the final concentrations of SP were (0.026%, 0.113%, 0.66%) (Figure 3.3). While there was no significant reduction in metabolic activity, viral infection was still inhibited by all treatments containing SP at the final concentration of 0.66%, similar to the 1% final concentration of SP used in the current manuscript and in our report [86]. Of note, minimally manipulated SP alone exhibited antiviral activity at every tested concentration, while filtered SP+(Ab) and filtered SP+(Ab) containing SEVI both exhibited HIV-1 enhancing activity. Importantly, these results suggest that viral enhancing and inhibitory activities are concentration-dependent, and that the process of filtration may interfere with certain antiviral components of SP.
Figure 3-3: SP manipulation, as well as different infection methods, reveals a contrast in SP antiviral activity. TZM-bl cells were plated at 1.4 x 10^4 cells/ml with 280 µl/well, and incubated for 24 h. Treatments of 10%, 2% or 0.4% of SP, or SP+(Ab) with or without SEVI (35 µg/ml) or media only controls were pre-incubated with HIV-1 BaL (4.8 ng p24) for 10 min at room temperature. Cells were infected by diluting the treatment and/or virus 15-fold in adding it to the cell media (final 0.66%, 0.113%, and 0.026% SP). After 3 h of incubation the treatment media was removed, cells received fresh media, and they were incubated for 3 d. Inhibition of viral infection is presented as a percent reduction in luciferase activity compared to an infected, vehicle-only control (A). Cells were also subject to MTT metabolic assays (B), presented as the percent metabolic reduction as compared to the negative control. For graphs, n = 3; and error bars represent SEM.

We next explored the effect of cell density and SP preparation on antiviral activity. For a 24 h infection period (Figure 3.4), cells seeded at 4 x 10^3 cells/well and 8 x 10^3 cells/well were
treated with either Pre-SP, Post-SP or Semen at 0.4%, 2%, or 10% (final concentrations as in [48]), and infected with a final concentration of 2 ng/ml HIV-1 BaL. The overall antiviral activity of the Pre-SP, Post-SP, and Semen treatments exhibited similar trends between the differing cell densities. However, when the same experiment was extended to a 3 d infection period (Figure 3.5), as performed in reference [52], a significant increase in cytotoxicity was observed.

Figure 3-4: Infection of two cell densities for 24 h reveals similar activity.
TZM-bl cells seeded at 4 x 103 cells/well (A&B) and 8 x 103 cells/well (C&D) were incubated for 24 h. Cells were treated with a final concentration of 10%, 2% and 0.4% of Pre-SP, Post-SP and semen, and then immediately infected with the BaL laboratory strain of HIV-1 (200 pg p24) for 24 h. Due to limited amount of sample, whole semen was not tested at 10%, and deemed as Not Determined (ND). Inhibition of viral infection was measured as a percent reduction in luciferase activity compared to an infected, vehicle-only control (A&C). Cells were subject to MTT metabolic assays (B&D), given as the percent metabolic reduction as compared to the negative control. For graphs, n = 3; and error bars represent SEM.
Figure 3-5: Cell density influences the antiviral and cytotoxicity of a 3 d infection.
TZM-bl cells seeded at 4 x 10^3 cells/well (A&B) and 8 x 10^3 cells/well (C&D) were incubated for 24 h. Cells were treated with a final concentration of 10%, 2% and 0.4% of Pre-SP, Post-SP and semen, and then immediately infected with the BaL laboratory strain of HIV-1 (200 pg p24) for 3 d. Due to limited amount of sample, whole semen was not tested at 10%, and deemed as Not Determined (ND). Inhibition of viral infection was measured as a percent reduction in luciferase activity compared to an infected, vehicle-only control (A&C). Cells were subject to MTT metabolic assays (B&D), given as the percent metabolic reduction as compared to the negative control. For graphs, n = 3; and error bars represent SEM.

In order to validate our findings shown in Figure 2, infection assays were performed on PM1 cells (Figures 3.6A & 3.6C) and PBMCs (Figures 3.6B & 3.6D) by measuring viral propagation over a multi-day time course. Agitated PAP and SEVI were added to cells at a final concentration of 35 µg/ml with or without 1% SP. PBMC infections confirmed HIV-1 enhancing activity of SEVI alone, while both assays confirmed significant antiviral activity of SEVI spiked with SP. No appreciable cytotoxicity to the cells was detected by trypan blue.
assays (Figure 3.6C & 3.6D). Collectively, these results indicate that antiviral activity of SP is retained in the presence of SEVI.

Figure 3-6: Agitated PAP peptides do not significantly alter SP inhibition of HIV-1 release. (A) PM1 cells and (B) PBMCs were infected with HIV-1 BaL (200 pg p24/0.1 ml) in the presence of whole SP (1%), SEVI, agitated PAP266 (35 µg/ml), or SP (1%) and agitated PAP peptides (35 µg/ml) combined, as well as a vehicle-only control. Five days (PM1 cells) and seven days (PBMC’s) post-infection, supernatant was collected to quantify p24 release by ELISA. Data are presented as a percent inhibition of infection in relation to the infected vehicle-only control. Treated PM1 cells (C) and PBMCs (D) were also analyzed by trypan blue exclusion for assessment of cytotoxicity. All graphs represent, n=3.

3.3.3 Seminal plasma naturally degrades PAP peptides over time

We previously demonstrated that SP naturally degrades native proteins over extended periods of time [86]. Since the proviral activity of SEVI and agitated PAP266 were significantly altered by SP, we aimed to determine if PAP was susceptible to intrinsic degradation. Whole
PAP [2 μM] was incubated with 1% SP for varying time periods at 300 rpm and 37 ºC, electrophoresed on a Tricine-SDS polyacrylamide gel, and silver-stained (Figure 3.7). The majority of the PAP protein was degraded within 12 h of incubation, indicating the susceptibility of whole PAP to SP proteases. Note that the PAP protein alone did not demonstrate any observable self-cleavage (data not shown).

![Figure 3-7: Whole PAP is proteolytically degraded by SP over time.](image)

Whole PAP protein [2 μM] was incubated with whole SP diluted 1:100 at 300 rpm at 37 ºC for timed periods. Sample tubes were immediately stored at -20 ºC when incubations times were ended. 4 µl of each sample were electrophoresed on a mini-Tricine-SDS-gel, and silver stained.

Next, SP with or without a protease inhibitor (PI) cocktail was incubated with either PAP<sup>286</sup> (Figure 3.8A) or PAP<sup>266</sup> (Figure 3.8B) for set increments of time at 300 rpm and 37 ºC. Tricine-SDS electrophoresis revealed that PAP<sup>286</sup> and PAP<sup>266</sup> underwent partial degradation within 1h, and complete degradation within 3 h. Samples containing the PI exhibited preservation of the PAP peptides over time, indicating that SP proteases were capable of degrading PAP.
Figure 3-8: PAP peptides undergo degradation by SP within hours.

Purified PAP peptides were incubated with either whole SP, SP spiked with a broad spectrum protease inhibitor (PI), or PBS, and incubated at 300 rpm, at 37 ºC, for the indicated durations. (A) Three volumes of PAP$^{286}$ (1 mg/ml) were incubated with one volume of whole SP, and 2 µl of samples were added to gels. (B) PAP$^{266}$ (1 mg/ml) was similarly incubated with whole SP, and 4 µl of samples were added to the gels. All samples were electrophoresed on Tricine-SDS-gels, and stained with Coomassie blue.

Since 1% SP neutralized the activity of SEVI, the catalytic concentration of SP necessary for PAP peptide degradation was investigated. Serial dilutions of whole SP were incubated with PAP$^{286}$ and samples were electrophoresed on Tricine-SDS polyacrylamide gels, and stained with Coomassie (Figure 3.9A) and then silver stain (Figure 3.9B). Partial degradation of PAP$^{286}$
occurred at dilutions of SP as high as 1:3200. Complete degradation of PAP$_{286}$ was observed at 1:200 dilutions of SP, and lower. These results indicate that SP contains fast-acting, PAP-degrading proteases in excess.

Figure 3-9: SP can degrade PAP$_{286}$.
Freshly resuspended PAP$_{286}$ (1 mg/ml) was incubated with serial dilutions of whole SP at 1400 rpm for 24 h at 37°C. Samples were electrophoresed on mini-Tricine-SDS-gels, and visualized with Coomassie blue (A) and then silver stain (B).
3.3.4 Proteases within SP are capable of degrading PAP$^{286}$

Prediction of the protease cleavage sites (Expasy – peptidecutter) revealed trypsin- and chymotrypsin-like cleavage sites within the PAP$^{286}$ peptide, giving rise to the possibility of proteolytic degradation of PAP$^{286}$ by these classes of proteases. In order to determine the specific PAP-degrading proteases in SP, PAP$^{286}$ was subjected to incubation with the SP proteases prostate-specific antigen (PSA), prostasin, and matriptase, and with trypsin as a positive control. PSA exhibits chymotrypsin-like activity, while prostasin and matriptase exhibit trypsin-like activity. The samples were electrophoresed using Tricine-SDS gels to reveal any resulting cleavage products (Figure 3.10A). PSA and matriptase treatments resulted in degradation of the PAP peptide with visible cleavage products, while prostasin did not effectively degrade PAP$^{286}$. Trypsin completely degraded the peptide, with no cleavage products visualized. PAP$^{286}$ was incubated with serial dilutions of PSA (Figure 3.10B), which demonstrated complete PAP$^{286}$ degradation at 0.75 µM. This corresponded to a 1:72 molar ratio of PSA: PAP$^{286}$ necessary for complete cleavage. Note that even the lowest concentrations of PSA tested (0.125 µM) promoted partial PAP$^{286}$ degradation.
Figure 3-10: Proteases within SP degrade PAP<sup>286</sup>.

(A) PAP<sup>286</sup> (54 µM; 250 µg/ml) was incubated with various SP proteases (1 µM) at 300 rpm for 24 h at 37 ºC. 3 µl of each sample were electrophoresed on Tricine-SDS-gels, and silver stained. (B) The predominant SP protease PSA was incubated with PAP<sup>286</sup> (54 µM) in serial molar dilutions. For each sample, 3 µl were electrophoresed on mini-Tricine-SDS-gels, and silver stained.

SP was analyzed in triplicate with a PSA-specific ELISA to quantitate the intrinsic amount of this protease, which measured 311 µg/ml (11 µM) in whole SP, consistent with
previous reports [67,85]. Cleavage products from the protease incubations were analyzed via MALDI TOF/TOF MS/MS and nano-LC-MS/MS analysis. All proteases generated detectable cleavage products from incubation with PAP$^{286}$ (Figure 3.11) with some overlap in cleavage sites among the different proteases. Taken together, these results demonstrate the ability of multiple SP proteases to cleave PAP$^{286}$ to various degrees, while the complete degradation of PAP$^{286}$ was demonstrated by the most abundant SP protease, PSA.

**Figure 3-11: Cleavage products of PAP$^{286}$, following incubation with SP proteases.**
The incubation products from the SP protease incubations were analyzed via MALDI TOF/TOF MS/MS and nano-LC-MS/MS analysis. The cleavage products for each individual protease were marked under the whole PAP$^{286}$ sequence. Underlined asparagine (N) residues could undergo deamidation, while methionine (M) residues marked with an asterisk (*) could undergo oxidation.
3.4 DISCUSSION

Our studies confirmed previous reports that SEVI alone was capable of enhancing HIV-1 infection [48], and additionally revealed that the native PAP\textsuperscript{286} peptides and truncated forms (PAP\textsuperscript{266}) were also proviral. This may suggest additional situations \textit{in vivo} in which PAP derived peptides could exert activity in the absence of fully elongated amyloid fibrils. However, we also revealed that proteolytic mechanisms within SP could reduce the proviral effects of SEVI and PAP peptides under certain conditions. Moreover, differences in treatment of SP and semen might also affect concentrations of the antiviral cationic peptide components that we have reported [86]. \textit{In vivo}, both pro- and antiviral situations could easily be explained by heretofore unknown donor-to-donor differences in PAP peptide concentration, protease concentration, and other factors that might affect the pro- and antiviral activity of SP directly or indirectly.

It is interesting to note that the ability of PAP-derived peptides to form amyloid fibrils is a common characteristic for many peptides and proteins given the correct conditions and time [94]. Fibril formation follows a model nucleation-dependent elongation mechanism, initiated by a lag phase for nucleus seeding [95]. When tested at a concentration 57-fold higher than the 35 µg/ml physiological concentration, SEVI exhibited a lag phase of ~10 h [96]. Since concentration of the purified peptide plays a significant role in fibril formation, the spontaneous formation of SEVI from purified PAP\textsuperscript{286} observed in previous studies may be a prime example of this, due to supraphysiological stock concentrations (i.e. 10 mg/ml) used for fibril formation [48]. Without agitation, it was found that fibril formation at lower concentrations of PAP\textsuperscript{286} may not occur or would require an exponentially longer lag phase time [96]. Considering the lack of
intense agitation post-ejaculation in vivo and the significantly lower physiological concentration of PAP$^{286}$, the lag phase of SEVI formation might afford ample time for intrinsic inhibitors of SEVI to act.

As we observed, native proteases were responsible for the degradation of whole PAP as well as PAP peptides in the presence of SP. It is important to note that several protease incubation studies we conducted contained a significant excess of PAP or PAP peptides compared to SP or the protease of interest, suggesting that catalytic amounts of proteases in SP are responsible for PAP degradation in vitro. In addition, our SP samples contained greater than a 100-fold excess of PSA than would be necessary to degrade PAP$^{286}$. One conclusion might be that the physiological concentration of SP would be sufficient to degrade PAP and PAP peptides in vivo. Conversely, it is plausible that an unknown promoter or stabilizer of SEVI formation might exist, which induces the formation of fibrils more rapidly in vivo. Notably, mechanisms behind the in vivo formation of SEVI warrant additional investigation, given that in vivo-formed SEVI fibrils themselves have not yet been reported.

It must be noted that while PSA is the primary candidate for the majority of PAP degradation, we also revealed other SP proteases that could proteolyze PAP$^{286}$. Likewise, proteases in human vaginal fluid, or mucosal proteases activated by the low pH in vaginal fluid might also degrade PAP$^{286}$ or SEVI in the post-coital environment [30]. While it is highly suggestive from our studies that PAP and PAP peptide degradation can occur, the level to which this occurs in vivo may vary widely and be one of several reasons why the proviral effect has been reported to vary between individuals [52].
In our study, we have assessed the pro- and antiviral activity of PAP peptides and SEVI, under multiple conditions, many of which reproduced methods and techniques utilized by other groups [48,52,90]. In short, the various testing conditions all had minor effects on the pro- and antiviral activities, yet the major finding that SP can abrogate part or all of the \textit{in vitro} proviral activity of SEVI was still substantiated. Syringe-filtered SP could confer HIV-1 enhancing activity under the right conditions, perhaps due to the loss of cationic peptides and proteins as a result of the filtration process. Both the concentration and duration of treatment influenced the overall activity of SP. Variable cell density led to differences in pro- and antiviral activity of SP and semen, as well as differences in cytotoxicity. While methodological nuances may help explain the disparity in data given between research groups, it is unclear which more closely represents the \textit{in vivo} environment of HIV-1 infection.

Previous studies have demonstrated the broad spectrum antimicrobial and antiviral activity of human seminal plasma [36,86]. The heterosexual transmission of HIV is not efficient, occurring as infrequently as 1 in every 1000 coital acts [2], which might be rationalized in part by the observed antiviral activity of human SP and the cationic antimicrobial and antiviral peptides therein. Still, there are factors in SP that exhibit proviral activity \textit{in vitro}. PAP peptides have been confirmed in semen, and the extent of their HIV-1 enhancing activity varies on an individual donor basis [52]. Our current studies suggest that the formation and activity of SEVI \textit{in vivo} might be challenging in the presence of SP, due to the natural degradation of PAP peptides by several intrinsic proteases within SP. While to date there is a lack of evidence confirming the ability of SEVI amyloid fibrils to form naturally in non-manipulated human SP under physiological conditions \textit{in vivo}, it still remains possible that PAP peptides can generate
fibrils in vivo and exhibit HIV-1 enhancing activity under the right circumstances. Together, we anticipate that our findings will not only spark intense discussion, but will unlock avenues for continued research on the proviral and antiviral aspects of human SP.
CHAPTER 4 THE HIV-1 ENTRY INHIBITOR RC-101 ALSO EXHIBITS ANTI-REVERSE TRANSCRIPTASE ACTIVITY

4.1 Introduction

Antimicrobial peptides are some of the most effective and ancient inhibitors of microbial infection. As effectors of the innate immune system, antimicrobial peptides express broad spectrum activity against bacteria, viruses, and fungi [58,97]. One family of antimicrobial peptides is the small, cationic, and cysteine-rich defensins. There are three subtypes of defensins, which include the alpha-, beta- and theta-defensins. While alpha and beta defensins have been extensively studied, the theta defensin family has only recently been isolated from the leukocytes and bone marrow of rhesus macaques [59,98,99]. These 18-residue peptides are derived from two 9-residue precursors, each contributing 3 cysteines to form a stable, circular peptide [100].

It has been shown that nonhuman primates, sans chimpanzees and gorillas, are able to generate theta-defensin peptides [98]. However, human bone marrow expresses a homologous pseudogene of theta-defensins. The genetic sequence contains a premature stop codon, preventing the peptide from being translated naturally. [55]. Utilizing the genomic sequence, the theta-defensins and their analogues were produced via solid phase synthesis into what have been deemed retrocyclins [56]. The synthesized peptide was tested for antimicrobial activity, and it was found that endogenous retrocyclin (RC-100) exhibited antibacterial activity and significant
anti-HIV-1 activity. In fact, retrocyclin demonstrated antiviral capabilities against both X4 and R5 strains of HIV-1, as well as herpes simplex and influenza A viruses [55,56,101-103].

Several analogues of the RC-100 peptide have since been shown to effectively inhibit HIV-1 infection \textit{in vitro}. One such analogue, RC-101, possesses a single arginine-to-lysine mutation [101]. Having been tested with multiple human cell lines, this particular peptide expresses neither hemolytic activity towards red blood cells nor any cytotoxicity to cells at therapeutic concentrations. [34,55]. Also of note was the ability of RC-101 to prevent infection of a reporter cell line of cervical epithelia by 27 clinical HIV-1 isolates at low to sub-micromolar concentrations \textit{in vitro} [56,101].

While the majority of small cationic antimicrobial peptides recognize the anionic phospholipids on the membranes of invading microbes, it was demonstrated that retrocyclins are capable of recognizing and binding the sugar molecules on the membrane surface [56]. However, in HIV-1 studies, it was demonstrated that retrocyclins acted in a lectin-independent manner, and prevented HIV-1 entry into the target cell by binding to the gp41 glycoprotein and preventing viral fusion with the target cell [104,105]. Therefore, instead of disrupting the membrane itself, retrocyclins disrupt the normal functions of HIV-1 entry.

As previously stated, it is vital for microbicides to express broad spectrum activity in the milieu of viral transmission. While it has been shown \textit{in vitro} that retrocyclins, including RC-101, prevent HIV-1 infection by inhibiting viral entry, we wanted to confirm antiviral activity would be sustained in the presence of a naturally occurring proviral component. The SP protein-derived amyloid fibrils deemed SEVI boost HIV-1 infection \textit{in vitro} by enhancing virion fusion to target cells. Alone, SEVI demonstrated proviral activity, but in the presence of RC-101 the
infection was significantly inhibited, and antiviral activity was preserved. We also tested the ability of RC-101 to alter an enzyme responsible for a later stage of the viral life cycle, namely the activity of HIV-1 derived reverse transcriptase (RT). Interestingly, we have found that RC-101 exhibits direct RT inhibition. Without the activity of RT, the viral DNA cannot be synthesized, leading to an abrupt halt of viral infection. Although retrocyclin activity may rely primarily on entry inhibition, it is important to recognize that another facet of viral inhibition is possible.

4.2 Materials & Methods

4.2.1 Human SP samples and synthetic peptides

The pooled seminal plasma of 103 individual samples was prepared as previously described [86]. PAP peptides (248 – 286) were synthesized and agitated as previously described [48,106]. The RC-101 peptide was synthesized as previously described [55,98]. To synthesize the 18-residue peptide, the cyclic sequence GICRC ICGKG ICRCI CGR was generated via solid phase synthesis, and subjected to MALDI-TOF mass spectrometry analysis after each phase to confirm the mass was accurate. Final concentrations of the peptide were elucidated using quantitative peptide analysis, and a stock (1 mg/ml RC-101 in ddH₂O) was stored at -20 °C until needed.
4.2.2 Cell lines and viruses

The lymphocytic PM1 cell line was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD, USA). PM1 cell cultures were maintained at a density of 0.4 – 0.8 x 10⁶ cells/ml in RPMI 1640 supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, and 20% (v/v) FBS. HIV-1 BaL, an R5-tropic strain, was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Germantown, MD, USA). HIV-1 BaL was propagated in PM1 cells, and supernatant was filtered and stored at -80 ºC until needed. Viral quantification was achieved via a sensitive commercial ELISA for p24gag (PerkinElmer, Waltham, MA, USA).

4.2.3 Antiviral and cytotoxicity assays

PM1 cells (1.5 x 10⁵/0.1 ml) were treated with SP (1%), RC-101 (10µg/ml) or SEVI (35 µg/ml) and infected with HIV-1 BaL (200 pg p24/0.1 ml) for 2 h. Cells were then washed, and resuspended in fresh media with sample treatments for 5 days. Supernatants were collected on days 3 and 5, post-infection, and stored at -20 ºC. To ensure cell viability, standard trypan blue dye exclusion assays were performed.
4.2.4 PM1 – RT assay – ELISA

Supernatant from the 5 day PM1 antiviral assay was utilized for quantification of retroviruses. Virus particles were isolated by centrifugation according to the RT assay colorimetric kit instructions (Roche, Florence, SC, USA). Briefly, 0.2 ml supernatant was centrifuged at 250 x g for 10 min in a refrigerated centrifuge at 4 ºC, then transferred to a fresh centrifuge tube and incubated with 22 µl lysis buffer, for a final of 0.5% Triton X-100 for 30 min. RT supernatant was then centrifuged at 8,000 x g for 10 min in order to remove cellular debris. Supernatant containing the retroviral particles was transferred to a fresh tube and centrifuged at 22,000 x g for 2.5 h at 4 ºC. Supernatant was discarded, and pellets resuspended in 40 ul of lysis buffer. The suspension was transferred to a fresh tube and incubated at room temperature to solubilize the viral particles. HIV-1 reverse transcriptase was used to generate a standard curve. Reaction mixture including the template/primer hybrid poly (A) – oligo (dT)$_{15}$ (9 A$_{260}$ nm/ml), and biotin and digoxigenin labeled nucleotides was added to the viral lysates or HIV-1 RT standards and incubated for 3 h at 37 ºC. The complete mixture was applied to the microplate modules precoated with streptavidin, and incubated at 37 ºC for an hour. Plates were washed, and then the anti-DIG-POD solution was added to the plate, which was incubated for 1 h at 37 ºC. Modules on the plate were again washed and the ABTS peroxidase substrate solution added until color developed. Plates were read on a spectrophotometer at an absorbance of 405 nm.
4.2.5 Direct inhibition – RT assay

The RT-ELISA solutions were prepared as instructed by the manufacturer (Roche, Florence, SC, USA). Briefly, 6ng (3μL) of recombinant HIV-1 RT was added to lysis buffer (20μL) in an individual tube for each reaction. Negative controls included lysis buffer and inhibitor vehicle only. Each RT-inhibitor diluted in lysis buffer was then added (20 μL) to the RT-containing mixture. Positive control contained the inhibitor vehicle, lysis buffer, and HIV-1 RT. Finally, the reaction mixture including the template/primer hybrid, and labeled nucleotides was added (20μL) to the reaction tube and incubated for 3 h at 37 °C. The reaction mixture was added to the microplate modules and the kit protocol followed as stated above.

4.3 Results & Discussion

4.3.1 RC-101 inhibits viral proliferation in the presence of SEVI

Having recently determined the ability of SP to reduce the proviral activity of SEVI in vitro [106], we wanted to test if the potent HIV-1 inhibitor, RC-101, would retain anti-HIV-1 activity in the presence of SEVI. PM1 cells were treated with SP and the physiological concentration of SEVI, as previously reported [48]. RC-101 was added to cells at a previously established antiviral concentration [55,101]. Treated cells were infected with HIV-1 and incubated for 5 days post-infection. Supernatant was collected, and viral particles were isolated for quantification with a non-isotopic RT-assay (Figure 4.1).
Figure 4-1: RC-101 inhibits HIV-1 infection in the presence of SEVI.

PM1 cells were infected with HIV-1 BaL in the presence or absence of SP, RC-101, SEVI, or vehicle. Five days post-infection supernatant was collected, and centrifuged in order to isolate the virus particles. The isolated viral RNA underwent a reverse transcriptase reaction followed by an ELISA. Samples were tested in duplicate and RT activity quantified as compared to the RT calibration curve. Data are expressed as a percent inhibition of viral infection, in relation to the infected vehicle-only control. n=4; error bars represent SEM.

The antiviral activity of SP and RC-101, as well as the significant HIV-1 enhancing activity of SEVI alone were confirmed. It was also demonstrated that RC-101 retained the ability to inhibit HIV-1 infection in the presence of physiological concentrations of SEVI. SP possesses the ability to neutralize the HIV-1 enhancing activity of SEVI, but no known microbicide had been tested for the retention of their activity in the presence of SEVI. Our data suggests RC-101 remains a potent inhibitor of HIV-1 infection in the presence of proviral SEVI.
4.3.2 RC-101 direct inhibition of RT activity in a dose dependent manner

Retroviruses are dependent on reverse transcriptase for the completion of their life cycle. The uncoated retroviral RNA must be reverse transcribed into double-stranded DNA to then be integrated into the host DNA as a provirus [107,108]. This vital action makes RT a prime target for antiretroviral treatment. The ability of SP and/or RC-101 to inhibit RT activity during infection has never been explored. We wanted to quantify the direct effect of potential RT inhibitors. The non-nucleoside RT inhibitors (NNRTI) are not incorporated into the viral DNA, but instead inhibit RT by binding the enzyme [109]. The NNRTIs, Nevirapine and Etravirine were used individually as positive controls. SP was added, and RC-101 was tested at serial dilutions, all concentrations having previously been determined to inhibit HIV-1 infection [101]. All samples were tested for direct RT inhibition (Figure 4.2).

![Direct RT Inhibition Assay](image)

**Figure 4-2: RC-101 directly inhibits the RT enzyme in a dose dependent manner.**
Samples and established RT inhibitors were subjected to a reverse transcriptase reaction. The RT-inhibitors Etravirine (Etr) (100 uM) and Nevirapine (Nev) (100 uM) served as the negative control, while inhibitor vehicle-only samples served as the positive control. Diluted SP (10%) and serial dilutions of RC-101 were tested individually. The ELISA quantification established the vehicle-only control as 100% RT activity, and the data are

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represented as a percent inhibition of direct RT activity in relation to the vehicle-only control. n=3; error bars represent SEM.

Results demonstrated that one or even multiple SP-derived components could inhibit HIV-1 RT activity. Future studies with fractionated SP could help identify the specific anti-RT element(s). The entry inhibitor, RC-101 also demonstrated direct RT inhibition. At 5 – 10 ug/ml, RC-101 inhibits the RT reaction in a dose-dependent manner. However, below this threshold, at 2.5 ug/ml the outcome of the RT reaction is variable.

In short, if the established entry inhibitor RC-101 can also prevent another vital step in the retroviral life-cycle, it becomes a multi-faceted microbicide. More work must be done to determine if RC-101 is able to enter the cell, and to what extent. It will also be important to determine if RC-101 follows the NNRTI type of RT inhibition. Once these factors are understood, it would be possible to model other multi-faceted HIV-1 inhibitors after RC-101 as powerful microbicides.
Millions of men and women are newly infected with HIV-1 each year through sexual transmission of the virus [26]. Due to the ability of the virus to adapt via a rapid mutation rate, an effective vaccine has not been successfully developed [25,108]. The prevention of viral transmission using topical vaginal microbicides has been shown as a viable option [4,31]; however, in order for these prophylaxes to succeed they must retain antiviral activity in the presence of semen and vaginal mucosal secretions [1]. Due to the complexity of these reproductive biological fluids, extensive testing is required to determine the compatibility of the HIV-1 inhibitor and the milieu of viral transmission [1,38,110]

Our studies aimed to determine if human SP, the primary medium for the transmission of HIV-1 [26], expressed antiviral activity against HIV-1 in vitro. Previous studies in our lab demonstrated that the cationic polypeptides in human vaginal secretions were antiviral [34]; therefore, we examined the cationic portion of SP for similar activity. Using a proteomics approach, we identified 52 individual polypeptides, representing components of the cationic portion of SP. The overall anti-HIV-1 activity of SP was established, with the most significant activity having been expressed in the fractions possessing peptides with the highest cationic charge-density. However, it was also established that SP proteases degrade these cationic peptides over extended periods of time, leading to a reduction in antiviral activity. Still, even after days of proteolytic degradation, SP retained significant antiviral activity.

While SP contains potent antiviral peptides, it is understood that SP also has proviral components [81]. The HIV-1 enhancing activity of SEVI, amyloid fibrils formed from PAP-
derived fragments, has recently been reported [48]. We wanted to evaluate the activity of these fibrils in the presence of SP, using varying methodologies for sample preparation and *in vitro* infection assays. While it was confirmed that SEVI alone enhanced the infection of HIV-1, it was also determined that in the presence of SP the proviral activity of SEVI was neutralized and the antiviral activity of SP was retained. The intrinsic proteases in SP exhibited swift proteolytic degradation of both PAP peptides and fully formed SEVI. Multiple SP proteases were tested, and all of them exhibited catalytic activity against PAP peptides. The most abundant SP protease, PSA, exhibited significant catalytic activity against SEVI and PAP peptides at a fraction of the average physiological concentration of the protease. Thus, while we do not argue that SEVI by itself enhances HIV-1 infection, it is likely that *in vivo* the PAP peptides are subject to degradation by SP proteases that neutralize their proviral activity.

While SP counteracts the proviral activity of SEVI, it is also important to ensure that topical microbicides can also retain their activity in the presence of a viral enhancer. The microbicide our lab is developing is an analogue of retrocyclin, deemed RC-101, with potent antiviral activity [101]. Although the activity of this peptide is not affected by either SP or vaginal secretions, it had not been tested in the presence of a viral enhancer. We combined RC-101 with SEVI, and established that RC-101 impedes the proviral activity of SEVI while maintaining anti-HIV-1 activity. Although RC-101 may not directly interact with SEVI, the potent microbicide inhibits HIV-1 infections in the presence of SEVI. We also demonstrated that the confirmed entry inhibitor RC-101 directly inhibited RT activity in a dose-dependent manner. Future studies will determine whether this ability takes place inside target cells during
viral infection, because it will be important to determine if this potent microbicide possibly utilizes a multi-faceted antiviral mechanism.

The results of these studies have raised an opposing view of the activity of SP compared to recent studies [48,51,52]. Intrinsic SP proteases naturally degrade semenogelins, which generate an array of small, cationic peptides capable of significant antimicrobial activity. The same intrinsic SP proteases are capable of degrading PAP, PAP peptides and fully formed SEVI. While immunosuppressive and proviral components are present in SP, the overall antimicrobial activity of the innate components within SP maintain a significant defense against HIV-1.
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